Research Article

Hyperhomocysteinemia and C677T Polymorphism of MTHFR Gene in Patients with Venous Thromboembolism of the Algerian Population.

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ABSTRACT

The polymorphic mutation C677T in the methylenetetrahydrofolate reductase (MTHFR) gene and hyperhomocysteinemia remain controversial risk factors for venous thromboembolism (VTE). The aim of the study was to determine the total homocysteine (tHcy) levels, and the prevalence of MTHFR C677T polymorphism in patients with confirmed VTE, and healthy controls; then to investigate whether hyperhomocysteinemia and C677T polymorphism were associated with a risk of VTE. The present case control study involved 146 apparently healthy participants and 121cases. The MTHFR 677T polymorphism was genotyped by a PCR-RFLP and tHcy levels were determined by Immulite 2000. In the present study, the distribution of the MTHFR 677C/T polymorphism was not statistically different among cases and controls (p=0.29), and it was quite high with a TT genotype frequency of 15.1% among controls. Furthermore, this mutation tended to have higher plasma hcy levels among cases. Based on the 95th percentile of control values, hyperhomocysteinemia was observed in eight cases and six controls (11.1% versus 5.8%). Neither T/T homozygote (OR=1.7, 95% CI=0.8-3.4, p=0.15) nor hyperhomocysteinemia (OR=1.7, 95%IC=0.6-4.9, p=0.31) was significantly associated with venous thrombosis even after adjustment for age and sex. We conclude that, there was no evidence found for an association between MTHFR C677T and hyperhomocysteinemia and the risk of venous thrombosis. It is possible that thrombosis risk is influenced by the interaction with further prothrombotic factors. We note also that there is no rationale for genotyping the MTHFR C677T variant for clinical purposes.

Keywords: Hyperhomocysteinemia, Methylenetetrahydrofolate reductase, C677T mutation, Venousthromboembolism, Risk factor, Homocysteine.

INTRODUCTION

Venous thromboembolic disease (VTE) is a serious disorder accounting for high morbidity and mortality rates with an annual incidence of 1/1000[1,2]. Many genetic and acquired risk factors were identified to cause VTE including factor V Leiden mutation, lupus anticoagulants, pregnancy, use of oral contraceptives, major surgeries, cancer, inflammations, prothrombin G20210A mutation and genetic deficiencies of proteins C, protein S and antithrombin III[3]. A correlation between hyperhomocystinemia and arterial vascular disease is well established[4,5].

Several studies have investigated the role of hyperhomocysteinemia in recurrent vein thromboembolism(VTE). Some studies attribute it to a causal relation[6-8], whereas others demonstrate that hyperhomocysteinemia is not a frequent cause of VTE[9,10]. It is the result of genetic and nutritional disturbances in homocysteine metabolism[7,11]. A common point mutation in 5,10-methylenetetrahydrofolate reductase (MTHFR) gene was identified by Frosst[12]. A C/T substitution at nucleotide 677 changes a highly conserved alanine into a valine residue and results in an elevated plasma levels of homocysteine[13].

Similarly, there have been conflicting opinions about the importance of the MTHFR C677T polymorphism as a risk factor for VTE[14,15]. Moreover, the frequency of MTHFR 677T allele varies substantially in different regions of the world and among ethnic groups. For example, the allele frequency is 0.07 in sub-Saharan Africans and 0.06 in Canadian Inuit, whereas in whites, Japanese, and Chinese, the allele frequencies are 0.24–0.54[16,17]. Interestingly, an increase in allele frequency has also been observed in the north to south of Europe[18]. In the Maghreb countries, the results showed an allelic frequency of 17.8% in Tunisia[19] and 34.3% in Algeria[20]. Therefore, the variable distribution of the MTHFR C677T mutation show the importance to investigate this factor separately based on each country.

The aim of the present study was to determine the total homocysteine (tHcy) levels, and the genotypic distribution of MTHFR C677T polymorphism in patients with confirmed venousthromboembolism, and in age-sex matched healthy controls. Then to investigate if hyperhomocysteinemia (HHC) and C677T polymorphism of methylenetetrahydrofolate reductase gene were associated with a risk of venous thromboembolism.

MATERIALS AND METHODS

Study Population

Our clinical series consisted of 121 cases with venous thromboembolism and 146 sex-age matching healthy controls. Samples were collected from September 2012

to January 2014 within internal medicine and cardiology departments of Constantine University Hospital Center.

The mean ages for cases and controls were 38.7 and 37.8 years respectively. The age ranged from 12 to 85 years for cases and 16 to 85 years for controls. Deep venous thrombosis was diagnosed by Doppler ultrasound. Cases were excluded if thromboembolism was due to surgery or cancer.

Normal controls were selected among healthy laboratory workers, students and other employees healthy subjects without personal or familial history of VTE. All cases and controls participated in the study after they had given their full informed consent.

Methods

The quantitative determination of the total homocysteine was carried out using the Immulite 2000. Blood samples were collected from fasting participants on heparinized tubes, transported on ice and centrifuged during 15 minutes, after that plasma was frozen at -80°C until assayed.

Hyperhomocysteinemia was defined as the level of plasma homocysteine above the 95th percentile of the control group.

For DNA analysis, blood was sampled in K(3)-ethylenediaminetetraacetic acid (EDTA) vacuum tubes thoroughly mixed with the anticoagulant by inverting the tube several times. DNA was isolated from peripheral blood leukocytes by the NaCl method.

For the detection of the thermolabile variant of methylentetrahydrofolate reductase (MTHFR), a region of 198bp in the exon four was amplified with two primers; forward: 5'TGAAGGAGAAGGTGTCTGCGGGA3' and reverse: 5'AGGACGGTGCGGTGAGAGTG3'.PCR reaction was performed in a total volume of 50µl containing: genomic DNA, 0.032 pmole/µl of each primer, 0.2 mM of dNTP, 0.04U of Taq polymerase, 2.5 mM $MgCl_2$ and 1X buffer. Amplification was performed for 30 cycles with an annealing temperature of 65°C.

The fragment obtained by PCR was digested with HinfI enzyme over night at 37° C, then 1hour for 65°C. The products were visualized by ethidium bromide staining of a 3% agarose gel electrophoresis.

The heterozygous amplicons gave two bands of 198bp and 175 bp. In contrast, wild type and homozygous MTHFR mutant amplicons exhibited only one band: 198bp and 175bp respectively.

Statistical Analysis

The chi-square test was used to assess differences in frequencies, and ANOVA for differences in means of homocysteine levels. The Hardy-Weinberg equilibrium was analyzed by the Chi-square test for the MTHFR C677T genotype. Multiple logistic regression was used to assess the association of MTHFR C677T mutation and hyperhomocysteinemia with venous thrombosis. Odds ratio was derived from the logistic regression model and 95% confidence interval (CI) were calculated after adjustment for age and sex. To correlate potential factors to plasma Hcy levels stepwise linear regression was applied. All statistical analyses were performed with the statistical package R 3.1.2 (www.r-project.org). A p-value less than 0.05 was considered significant throughout the analyses work.

RESULTS

The MTHFR genotype distribution in patients and controls is shown in Table 1. Among the cases, the prevalence of MTHFR genotypes were 19.8% for T/T homozygotes, 50.4% for C/T heterozygotes and 29.7% for C/C homozygotes. Among the controls, they were 15% for T/T homozygotes, 46.6% for C/T heterozygotes and 38.3% for C/C homozygotes. The distribution of the MTHFR C677T mutation was not significantly different between cases and controls (p=0.29). Based on the sum of all the genotypes, there was a good fit to the Hardy Weinberg equilibrium in the cases (X^2 = 0.006 / P= 0.93) and the controls (X^2 = 0.0042 / P= 0.95). The present study failed to demonstrate a significant association between the C677T MTHFR mutation and VTE status (OR = 1.7, 95% CI =0.8-3.4, p=0.15). The odds ratio remains not significant after adjustment for sex and age (OR = 1.6, 95% CI =0.8-3.4, p=0.17).

The overall mean concentration of total fasting homocysteine was not significantly different (p=0.17) between the cases (17,2±13,9 µmol/l) and the controls (15,2±12,9 µmol/l). Hyperhomocysteinemia was observed in eight cases and six controls (11.1% versus 5.8%); yielding an odds ratio for venous thrombosis of 1.7 (95% IC, 0.6-4.9, p=0.31) or 1.8 (95% IC, 0.6-5.3, p=0.26) by adjusting for sex and age. The linear regression test does not show a correlation between tHcy plasma levels increase and age in cases (r = 0.19, p=0.1) and controls (r = -0.04, p=0.65). Table 2 shows the levels of tHcy according to the different MTHFR 677T genotypes. There was a significant association between the MTHFR C677T genotype and plasma levels of homocysteine observed among cases (p=0.034).

DISCUSSION

In this study, the mean plasma of tHcy in controls was 15.2±7.9mmol /l, a rate similar found in an Algerian study (14.69 ± 7.3 µmol/l)[21]. Several studies have evaluated plasma levels of tHcy in healthy populations; Amouzou EK et al[22] found a frequency 62.3% of moderate HHC in healthy subjects of the Western Africa. The main determinant was deficiency in folate due to the high prevalence of poverty in these regions. Our results show that the plasma mean tHcy in controls, was closely compared to Europe[23] and neighboring countries such as Tunisia[24] and Morocco[25]. This can be explained by the Mediterranean diet. In addition, we also note that the polymorphism C677T MTHFR could become the main

determinant of these variations. Thus, it has been reported in coastal West Africa a high prevalence of hyperhomocysteinemia, explained by folate concentrations and the MTHFR 677T allele[22].

Our study shows that plasma tHcy levels did not increase with age which indicates that age was not considered as a major determinant of the HHC among cases and controls. This finding is consistent with other results in a meta-analysis of Den Heijer; where most studies found that the prevalence of HHC is higher in patients with relatively young age[26]. Conversely, in two other studies, age was the major determinant of HHC among cases with VTE[27,28].

Our data failed to demonstrate that hyperhomocysteinemia increased the risk of venous thrombosis; with an estimated odds ratio of 1.7 (95% IC, 0.6-4.9, p=0.31) or 1.8 (95% IC, 0.6-5.3, p=0.26) by adjusting for sex and age, although several epidemiological studies have found a direct association between elevated plasma homocysteine levels and venous thromboembolism[13]. In addition hyperhomocysteinemia was found to be prevalent in patients with an initial episode of leg DVT[29] or recurrent DVT[6]. In 1998, a meta-analysis of 10 case-control studies depicted that eight studies found hyperhomocysteinemia to be a risk factor for venous thrombosis however, the two others which were in accordance with our finding; reported that there is no relationship between hyperhomocysteinemia and venous thrombosis[30]. In a further meta-analysis of 9 studies, Ray[31] found that pooled odds ratio for DVT in the presence of HHC was 2.95 (95% CI, 2.08–4.17). Prospective cohort studies, which generally provide more rigorous evidence, yielded conflicting results, Petri[32] and Cattaneo[33] did not find significant association between elevated tHcy levels and subsequent venous thrombosis. In a nested case–control study of a subset of 14916 men, participating in Physician's Health Study, followed prospectively over 10 years, patients with HHC had no increase in risk of all venous thromboembolism but were subjected to increased risk of idiopathic thrombosis (OR, 3.4; p=0.002)[34]. In another prospective cohort study of 264 patients[35], the relative risk of recurrent DVT in patients with HHC was 2.7 (95% CI 1.3–5.8). In another meta-analysis on 24 retrospective studies including 3289 cases and 3 prospective studies including 476 cases[26], Den Heijer demonstrated a modest association between homocysteine and venous thrombosis. They reported that 5µmol/l increase of tHcy was associated with a 60% and 27% increased risk of venous thrombosis in retrospective and prospective studies, respectively.

The inconstancy of literature data about the relationship between moderate HHC and venous thrombosis is mainly related to methodological issues and suggests the weakness of such relationship. Moreover, the absence of a significant relationship in the present report; does not completely exclude the existence of a potential high risk of VTE associated with the HHC. Indeed, in some studies, lack of statistical significance may be masked by the acquired or genetic predisposition of the healthy population to have high plasma levels of tHcy. In a study conducted in Tehran, the serum homocysteine in the control group was unexpectedly high[36]. The association between homocysteine and deep vein thrombosis was not confirmed in the study, especially with men who had higher serum homocysteine, more than women. The authors suggested that this association fades away in populations with high prevalence of hyperhomocysteinema.

The results of our study indicate that other candidate inheritable thrombophilic defects, namely MTHFR 677C/T polymorphism was similarly distributed among cases and controls. Homozygosity and heteozygozity for the MTHFR C677T mutation were observed in respectively 47% and 15% of the controls; these frequencies were similar with those in an Algerian study[37]. In addition, the incidence of the T/T genotype in our healthy subjects was higher than the rates of Bahrain and Saudi Arabia[38], and was closely comparable with southern European communities including Spain, France, and Italy[39], together with Tunisia[38], hereby indicating that MTHFR C677T may have occurred on a founder haplotype[40]. Similarly to hyperhomocysteinemia, the relationship between C677T mutation and venous thromboembolism is still somewhat controversial.When some find an increased risk of DVT in homozygous for C677TMTHFR mutation without[41,42] or with[43,44] the coexistance of biological risk factors, others did not[45,46]. In a meta-analysis[47], only four studies among 31 independently demonstrated a significant association between the MTHFR 677 homozygous state and VTE[14,41,48,49]. Our finding showed that in cases of VTE heterozygozity and homozygosity for the C677T MTHFR genotype are not significantly associated with thrombotic risk, which is in agreement with previous studies[50-52]. However this result contrasts with data from other studies that reported an independent risk of venous thrombosis with the homozygous polymorphism TT of MTHFR[53,54] but, these associations remained relatively low (OR<2).

One possible explanation is that the risk due to this mutation is low or may be masked by other known thrombotic factors. The variation in selection criteria from a study to another, might explain, too, the discrepancy of results, particularly the difference in nutritional status populations studied, which may influence the frequency of folate deficiency, interfering with the phenotypic expression of the mutation[53]. In a meta-analysis of Den Heijer among 53 studies: 30 were carried out in Europe, 11 in North America and 12 were carried out elsewhere[28]. The risk estimates for 677TT genotype and venous thrombosis obtained from studies carried out in North America differed from those carried out in Europe and elsewhere, which may be explained by the higher dietary intake of folate and riboflavin in North America compared with Europe[43,55,56]. The variety of populations among different studies is another likely

source of heterogeneity, since ethnogeographic differences in the prevalence of the MTHFR C677T polymorphism have been demonstrated[47,57].

In our study, we also deduced that there was a significant association between plasma levels of Hcy and homozygous (TT) genotype in MTHFR gene mutation among cases. Based on our observations of the controls, the mean plasma Hcy levels of TT genotype carriers were not significantly higher than those of CC genotype carriers. This means that the mutation MTHFR C677T alone does not explain the HHC among controls. As in several studies[43, 50, 54] homozygous TT mutation of MTHFR investigated herein was not associated with DVT, but it had higher levels of plasma Hcys than CC or CT genotypes. This complies with the notion that both congenital and acquired factors affect plasma Hcys levels[12,58].

In conclusion, this study showed that there was no evidence found for an association between MTHFR C677T and hyperhomocysteinemia and the risk of venous thrombosis.

It is possible that thrombosis risk is influenced by the interaction with further prothrombotic factors. We also note that there is no rationale for genotyping the MTHFR C677T variant for clinical purposes.

Table 1: Distribution of genotypes of C677T mutation in the methylenetetrahydrofolate reductase gene and its thrombotic risk.

Genotypes of MTHFR C677T	Cases (n=121) n (%)	Controls (n=146) n (%)	Univariate OR (95 % CI) - P value	Adjusted OR (95 % CI) - P value
CC	36 (29.8)	56 (38.3)		
CT	61 (50.4)	68 (46.6)	1.4 (0.8-2.4) - 0.23	1.4 (0.8-2.4) - 0.23
TT	24 (19.8)	22 (15.1)	1.7 (0.8-3.4) - 0.15	1.6 (0.8-3.4) - 0.17

Table 2: Plasma levels of homocysteine according to the different MTHFR 677T genotypes.

Genotypes of MTHFR C677T	Plasma homocysteine (µmmol/l)		P-value
	Controls (n=104)	Cases (n=72)	
CC	13.7± 4.8	14.3± 8.6	0.77
CT	15.3± 8.2	16.8± 9.3	0.46
TT	19.0±12.3	23.0±13.8	0.41
P-value	0.078	0.034	

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