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ASSOCIATION OF HOMOCYSTEINE AND METHYLENE TETRAHYDROFOLATE REDUCTASE (MTHFR C677T) GENE POLYMORPHISM WITH DEEP VEIN THROMBOSIS IN THE POPULATION OF NORTH MACEDONIA

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Abstract

The implications of the methylene tetrahydrofolate reductase (MTHFR) gene and the level of total homocysteine (tHcy) in the pathogenesis of deep vein thrombosis (DVT) have been extensively studied in various ethnic groups. Our aim was to discover the association of MTHFR (C677T) polymorphism and homocysteine level in healthy subjects and in patients with DVT in the Republic of North Macedonia.

The study group consisted of 123 healthy subjects, control group, and 93 consecutive ultrasonography and/or venography confirmed DVT patients. The concentration of plasma tHcy was determined by cyclical enzymatic method and the MTHFR gene polymorphism was analyzed by the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP).

The results obtained for mean plasma tHcy in the control group were 9.7±3.65 μmol/L, while tHcy level was significantly higher in patients with deep vein thrombosis - 15.19±3.63 μmol/L ($p<0.001$). The highest frequency of mutation of MTFHR gene *C677T* was found for genotype CT, followed by wild genotype CC and the lowest frequency of genotype TT was found in control and DVT groups.

The results have shown that plasma tHcy level is a contributing factor for development of this disease, DVT. Our findings have shown that MTHFR C677T polymorphisms were not associated with deep vein thrombosis in the selected population.

Key words: total homocysteine, deep vein thrombosis, methylentetrahydrofolate reductase

Introduction

Hyperhomocysteinemia is an error in the metabolic pathways of sulfur-containing amino acids and is characterized by an increase in the level of toxic homocysteine (Hcy) in the serum. Mutations in methylene tetrahydrofolate reductase (MTHFR), an enzyme present at the branch point between the methylation and remethylation, as well as trans-sulfuration pathways, are the basic cause of homocysteinemia. The term "hyperhomocysteinemia" is also used to describe the elevated Hcy serum level due to other genetic independent and environmental factors [1]. In a normal, healthy individual, the serum Hcy level is between 5–15 μM, but it can increase to 50 μM in mild cases and to 500 μM in severe cases of homocysteinemia [2]. Further studies have also revealed that elevated plasma Hcy level is one of the key factors associated with primary cases like neurodegeneration, Down syndrome, and megaloblastic anemia. Hyperhomocysteinemia has also been connected to various other clinical complications. Recent advances have proven that there is a close link between hyperhomocysteinemia and atherosclerosis, cancer, coagulation, in which case venous thromboembolism is the most common cause of death in patients [3]. The mechanism for the vascular lesions induced by hyperhomocysteinemia remains unclear. Experimental evidence suggests that Hcy facilitates the vascular oxidative process, thereby altering the

coagulation system, and reducing the vasomotor regulation of the endothelium [4]. There are data implicating coagulation effects of Hcy. It induces a reduced action of thrombomodulin on the endothelial surface, which results in inhibition of protein C activity. Decreased protein C activity reduces the inhibition of Va and VIIIa factors. Therefore, transformation of prothrombin into thrombin is intensified, which results in an increased creation of fibrin that is responsible for the coagulation cascade process. Moreover, increased platelets aggregation and endothelial adhesion are also involved in this cascade process [5,6].

Several polymorphisms in the enzymes involved in the Hcy detoxification pathways (the transsulfuration and remethylation) have close clinical ties and folates. Thay are pivotal for cell proliferation and hypomethylation, but they have an inverse relation with Hcy. In majority of cases methylenetetrahydrofolate reductase (*MTHFR*) genetic polymorphism is responsible for mild to moderate hyperhomocysteinemia and it is one of the rare genetic risk factors that has been proven [7,8]. In this sense, it has been shown that folic acid stabilizes and maintains the function of the mutated enzyme, a fact that can help in eventual treatment of hyperhomocysteinemia. The common C→T change in the sequence of the genetic code in MTHFR enzyme is at nucleotide 677 and the mutation results in substitution of alanine by valine at position 222 of the polypeptide. Mutation of *C677T* gene might influence composition of intracellular folate pool [8,9].

Folate is not only involved in nucleotide biosynthesis, but is is also required for the conversion of deoxyuridine monophosphate (dUMP) into thymidine monophosphate. Under normal conditions, thymidylate synthetase (TYMS) converts dUMP into thymidine monophosphate using 5,10-methylenetetrahydrofolate (derived from folate) as a methyl group donor. If folate is limiting, dUMP accumulates because its key methyl donor, 5,10-methylenetetrahydrofolate, is absent [3,7].

In this sense, it has been shown that folic acid stabilizes and maintains the function of the mutated enzyme, a fact that can help in eventual treatment of hyperhomocysteinemia.

The aim of this study was to determine the concentration of tHcy and prevalence of *C677T* mutation of *MTHFR* enzyme in healthy subjects, and in patients with deep vein thrombosis. It was also our aim to assess the relationship between MTHFR gene polymorphism and plasma homocysteine level as risk factors for deep vein thrombosis in the Macedonian population.

Material and methods

The investigation comprised 216 subjects divided into two groups:

1. Healthy subjects (control group), n = 123
2. Patients with deep vein thrombosis, n = 93

1. The control group of subjects was consisted of blood donors from the Institute of Transfusion Medicine in the Republic of North Macedonia, who were declared to be healthy by a medical doctor. Exclusion criteria in the control group included positive family history for hereditary disease associated with impaired homocysteine metabolism as well as diseases that were also known to be associated with impaired homocysteine metabolism.

2. Deep vein thrombosis in patients was diagnosed by ultrasonography and/or coronarography at the Institute of Transfusion Medicine in the Republic of North Macedonia. Exclusion criteria in this group of patients was positive family history for hereditary diseases associated with impaired homocysteine metabolism, diabetes mellitus, cerebrovascular insult, autoimmune diseases, all types of carcinoma, kidney failure and transplantation, thyroid gland diseases, usage of hypolipidemics and vitamin B_6, B_{12} and folic acid.

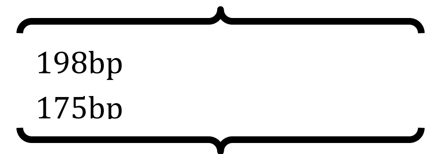
All patients and healthy individuals included in this study signed a written consent to participate in the study, which was approved by the Committee of the Ministry of Education and Science from the Republic of Macedonia (No. 13-1672/4-02).

Several days prior to blood analyses, each respondent got specific instructions: to avoid protein-rich food or fatty food 24 hours prior to examination. Blood samples were drawn from antecubital vein in

the morning, after 10-12 hours fast and collected in two vacutainer tubes of 5 ml preserved with the anticoagulant potassium ethylene diamine tetraacetic acid (K_3EDTA). Concentration of serum tHcy in first blood sample was determined by cyclic enzymatic method at the Institute of Medical and Experimental Biochemistry, Medical Faculty in Skopje. The assay method was based on enzymatic conversion of homocysteine to S-adenosyl homocysteine, followed by quantification of S-adenosyl-L-homocysteine by glutamate dehydrogenase. NADH like coenzyme of glutamate dehydrogenase, transform into NAD^+ and this is manifested as decline absorbency to 340 nm [10].

Genetic polymorphism of C677T in MTHFR enzyme was analyzed in second peripheral blood sample by polymerase chain reaction by Schneider [11], at the Institute of Molecular Biology and Human Genetics at the Faculty of Natural and Mathematical Sciences in Skopje. Isolation of genomic DNA from nucleic cells (leukocytes) was done with sodium chloride - extraction and subsequent precipitation with ethanol and DNA isolates were aliquoted in several test-tubes, of which one was kept at +4 to 8°C and was used for analyses, while the remaining were kept as a reserve in the sample bank at -18 to 20°C.

Amplification of regions of *MTHFR* gene was done by polymerase chain reaction and detection of C→T *missense* mutation in C677T of *MTHFR* gene was made by restriction analysis (*Polymerase Chain Reaction-Restriction Fragment Length Polymorphism* - PCR-RFLP) (Figure 1).



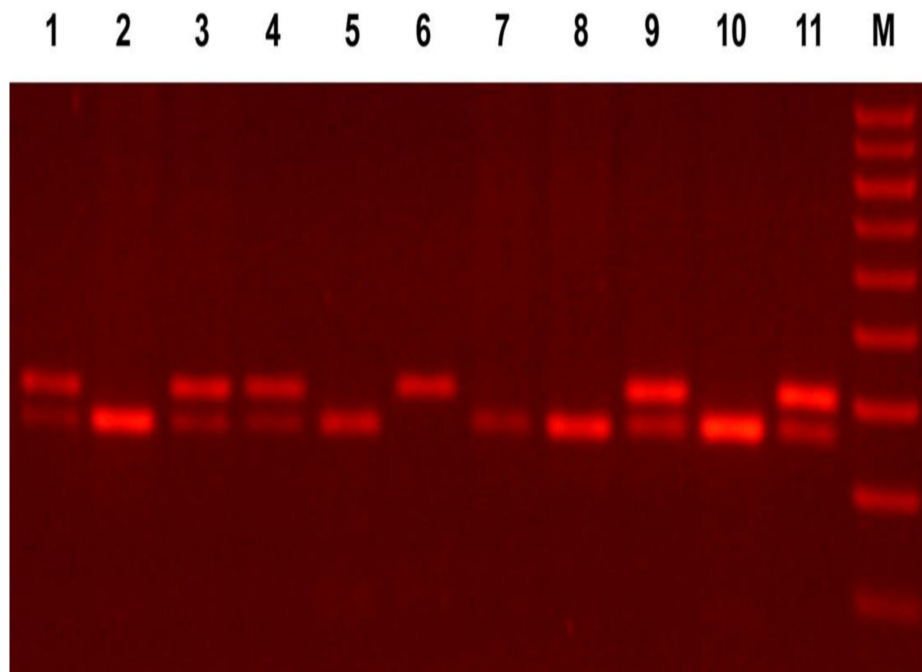


Figure 1. Detection of *MTHFR* polymorphism. On lane 6 there is wild type (CC) genotype, on lanes 1, 3, 4, 9 and 11 there are mutant heterozygous (CT) genotype and on lines 2,5,7,8 and 10 mutant homozygous (TT) genotype; M, molecular weight marker

Statistical methods

Genotype and allele frequencies in DVT and control groups were compared by Chi-square testing. The characteristics of patients and controls were evaluated by comparing biochemical findings using the Student t-test. Additionally, we performed multiple logistic regression model testing on the interaction of genotype and total Hcy levels in both group. All analyses were performed using Mendelian

randomization program by Rodriguez S, Gaunt TR, Day IN.*Am J Epidemiol*. 2009 doi: 10.1093/aje/kwn359. A two-tailed p value of $p < 0.05$ was considered statistically significant.

Results

The results of concentration of serum total homocysteine in both investigated groups are presented on Table 1. Patients with DVT had significantly higher levels of tHcy compared to control group, p<0.001.

Table 1. Concentrations of tHcy (μmol/L) in control group and patients with DVT

tHcy μmol/L $\overline{X} \pm SD$	**N**	**Control group**	**N**	**Patients with DVT**	**p**
	123	9.7±3.65	93	14.1±3.08	p<0.001

The results are expressed with mean ± standard deviation, p statistical significance

The highest frequency in healthy subjects and patients with mutations in the MTFHR gene had heterozyguos genotype CT in control 46% *vs*. 50 % in patients. Frequency in homozygous wild genotype CC was 44% in control *vs*. 32% in patients and the lowest frequency had the of genotype TT with 10% in control *vs*. 18% in patients (Table 2) .

Table 2. Frequencies of polymorphism of *MTHFR* gene C677T in both groups

Genotype (%)	**Control** N=123	**DVT** N=93	**p**
CC	44	32	<0.05
CT	46	50	<0.05
TT	10	18	<0.05

p, statistical significance

Comparison of tHcy plasma concentrations with a specific *C677T* polymorphism genotype in the *MTHFR* gene in all of examined groups is shown in Table 3. The lowest concentrations of tHcy were obtained in both investigated groups with genotype CC, higher in the heterozygous CT genotype, and the highest in those with a variant genotype TT. The analysis of the differences have shown that statistical significance was found only among the genotypes CC and CT in the control group (p <0.05), as well as between CC and CT in the group with DVT (p <0.05). Differences in concentrations relative to the genotypes of both analyzed groups were not statistically significant (p> 0.05).

Table 3. Comparison of tHcy concentration (µmol/L) with the *MTHFR C677T* polymorphism genotype in control and DVT group

Genotype	Control group tHcy (µmol/L) N=123	Group of DVT tHcy (µmol/L) N=93
CC	9.32 ± 1.83	15.07 ± 4.65
CT	12.24 ± 2.82	19.01 ± 6.04
TT	14.70 ± 5.54	20.04 ± 3.12
CC and CT	p = 0.001	p = 0.054
CC and TT	p = 0.146	p = 0.013
CT and TT	p = 0.444	p = 0.582

The results are expressed with mean ± standard deviation, p statistical significance

The statistical comparison of the wild-type allele C with those of the variant T of the *MTHFR C677T* polymorphism is present on Table 4. Also, genotype the wild type CC with those of the heterozygous CT or homozygous variant TT of the *MTHFR C677T* polymorphism of the control group in relation to examined group of patients with DVT is shown in Table 4. The presence of either of the two alleles, C or T, did not have a significant correlation with the risk of DVT in relation to the control, healthy group. Namely, none of the p-values calculated according to the two types of analyses from the group of x^2 tests, nor did they show statistical significance ($p>0.05$) according to Fischer's test. Similarly, there was no connection either between the presence of the wild type genotype CC, nor the CT and TT genotypes containing the variant allele, in the examined group of patients with DVT. Although with greater variations, additional statistical analysis of the probability ratio OR, risk ratio RR, and confidence interval (95% CI) confirmed the absence of association of these genetic parameters in the group of patients with DVT.

Therefore, the risk of DVT does not differ significantly in persons with examined alleles and their combinations into the genotypes.

Table 4. Comparison of frequencies of allele and genotypes of *MTHFR C677*T polymorphism in control and DVT group

Parameters	Control (N=123)	DVT (N= 93)	x^2-test Yates *P*	Pearson *p*	Fisher p	OR	RR	95%CI
Alel								
C	174	100	0.166;	0.121	0.141	1.665	0.767	0.872-3.180
T	72	86	0.166;	0.121	0.141	0.601	1.304	0.314-1.147
Genotype								
CC	60	30	0.292;	0.200	0.254	1.812	0.720	0.727-4.513
CT/TT	63	63	0.292;	0.200	0.254	0.552	1.389	0.227-1.375

* The statistical significance was calculated according to the x^2 test with Yates correction and according to Pearson. **Fisher's Exact Test for Probability Two-way distribution; OR, Odds Ratio; RR, Risk Ratio; CI, confidence Interval at 95%.

Discussion

The levels obtained for total plasma homocysteine were significantly higher in patients with deep vein thrombosis in comparison to those in the control group, p<0.001. Our data are in compliance with observations from a number of other studies in which hyperhomocysteinemia, even moderate, was found to be an independent factor for development of deep vein thrombosis [12,13].

The association between higher risk for development of DVT in individuals with hyperhomocysteinemia is still unclear. This fact proves that the mutation of the *MTHFR* gene *C677T* may affect the composition of the intracellular folate capacity (pool), which is involved in the metabolism of homocysteine, so that the homozygous of this mutation, genotype TT, and to a lesser extent the heterozygous, is accompanied by an increased concentration of tHcy. In this sense, it has been proven that folic acid stabilizes and preserves the function of the mutated enzyme, a fact that will be the basis for the eventual treatment of hyperhomocysteinemia [14,15].

The relationship between total homocysteine in the serum as a dependent phenomenon and the MTHFR gene (C677T) as an independent isolated polymorphism is observed through genotypes (CC; CT; TT) in the control group.CT genotype had a higher level of 2.94 μmol / L for tHcy, compared to subjects with the wild genotype CC of MTHFR (C677T) (p <0.001); while subjects with TT genotype of MTHFR (C677T) had a higher level of 8.56 μmol / L of plasma tHcy, compared to subjects with the wild type CC MTHFR (C677T), p <0.001. In this regard, the function of the mutated enzyme is fact that will be the basis for the possible treatment of hyperhomocysteinemia [16]. MTHFR (C677T) genotypes were associated with plasma tHcy levels in the control group.

Our patients with DVT had a relative RR risk of 1.30 for T allele, which resulted in a higher serum tHcy level than in patients with an allele C relative RR risk of 0.76 of MTHFR (C677T) gene mutations [17]. However, neither of the p-values calculated according to the two types of analysis of the χ^2-test group, nor the one of the Fisher test showed a statistical significance (p> 0.05). Our results showed that patients with DVT with TT and CT genotype had a relative RR risk of 1.38 higher tHcy, compared to patients with wild MTHFR CC (C677T) with RR of 0.72 . Similarly, there was no association between the presence of the wild type CC genotype, or the CT and TT genotypes containing the variant allele, in the tested group of patients with DVT. Although with greater variations, additional statistical confirmation of the absence of association of these genetic parameters with the tested group was also obtained with the values of probability (OR), risk (RR), and confidence interval (95% CI). .

It resulted that patients with deep vein thrombosis had mild to moderate hyperhomocysteinemia, but the relative risk did not show a statistically significant difference between the tested alleles and their combinations in genotypes (p> 0.05).

There have been numerous studies on homocysteine binding and MTHFR (C677T) gene mutations and mainly all show T cell alleles with elevated serum homocysteine levels in patients with deep vein thrombosis [18]. Our study demonstrated that patients with DVT with genotype CT had a 2.52 µmol / L higher level of tHcy, compared to patients with wild type genotype CC, p <0.001; while patients with TT genotype of MTHFR (C677T) had a 6.79 µmol / L higher level of tHcy in plasma compared to patients with wild type CC, p <0.001.

The presence of either of the C or T alleles had no significant association with the risk of DVT in the healthy control group. Namely, neither of the p-values calculated according to the two types of analysis in the χ^2-test group, nor the one according to the Fisher test showed statistical significance (p> 0.05). Similarly, there was no association either between the presence of the wild type CC genotype or CT and TT genotypes containing the variant allele in the studied group of patients with DVT. Although with greater variations, additional statistical confirmation of the absence of association of these genetic parameters with the tested group was also obtained with the values of probability (OR), risk (RR), and confidence interval (95% CI).

Epidemiological studies of the association between MTHFR (C677T) and deep vein thrombosis, performed as a meta-analysis in southern Indians, yield conflicting results [19], suggesting that mutation causes increased homocysteine values, but is not a risk factor for vascular disease. To some extent this coincides with our results. However, in the study of Cohen *et al*., who performed genetic testing for variations in MTHFR (C677T), their clinical manifestation had shown that this mutation had poor symptomatology as a risk factor for vascular disease [20].

Thus, the risk of DVT is not significantly different in individuals with the tested alleles and their combinations in genotypes. The risk of deep vein thrombosis in patients with TT genotype and CT genotype is associated with mild to moderate hyperhomocysteinemia and decreased folic acid status [21]. It has to be mentioned that there are also other risk factors besides hyperhomocysteinemia, such as fibrinogen, C-reactive protein, for development of DVT as a complex disorder [22].

Conclusions

Patients with DVT have significantly higher mean levels of tHcy compared to healthy subjects. We have not demonstrated a direct association of genetic mutations *MTHFR C677T* with DVT occurrence, although it is clear that they influence the increase in tHcy levels. Therefore, it can be concluded that DVT is a complex disorder, the occurrence of which is influenced by other genetic markers as well as environmental factors.

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Declaration of interest

Authors declare no conflict of interest.

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