Received: 01 June 2013; Accepted: 28 July 2013
Conflict of interest: none declared.

DOI: 10.5455/msm.2013.25.170-174

ORIGINAL PAPER Mater Sociomed. 2013 Sep; 25(3): 170-174

Hyperhomocysteinemia and of Methylenetetrahydrofolate Reductase (C677T) Genetic Polymorphism in Patients with Deep Vein Thrombosis

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ABSTRACT

Aim: To determine the concentration of total plasma homocysteine (tHcy) as well as different genotypes of methylenetetrahydrofolate reductase MTHFR (C677T) in healthy subjects and patients with deep vein thrombosis (DVT). **Material and methods:** The investigation comprised a total of 160 subjects divided in two main groups: 80 healthy subjects (control group) and 80 patients with deep vein thrombosis. Concentration of tHcy was determined by spectrophotometric cyclic enzymatic method and mutation of MTHFR (C677T) gene was examined by polymerase chain reaction according to Schneider. **Results:** The results obtained for plasma tHcy in the control group were 11.62±3.43 μmol/L, while tHcy level was significantly higher in patients with deep vein thrombosis as compared to the control group, 15.19±3.63 μmol/L ($p<0.001$). The analysis of the results has shown that MTHFR (C677T) genetic polymorphism was responsible for mild to moderate hyperhomocysteinemia in the majority of subjects. **Conclusion:** The level of tHcy in the examined patients was significantly higher in comparison with the control group. Multiple regression analysis has shown that tHcy level in CT and TT genotypes of MTHFR (C677T) was statistically higher in comparison with CC genotype of MTHFR (C677T) in both, the control group and the DVT patients.

Key words: total plasma homocysteine, MTHFR (C677T) gene, deep vein thrombosis.

1. INTRODUCTION

In patients with homocysteinuria, 50% of vascular complications account for vein thromboembolism. A large number of studies that have analyzed the association between hyperhomocysteinemia and venous thrombosis have shown an increased total homocysteine (tHcy) concentration in patients with deep vein thrombosis. In the study of Ayala et al. conducted in patients with diagnosed first episode of deep vein thrombosis hyperhomocysteinemia has been presented as a risk factor for this disease (1). Dionisio et al. analyzed patients with DVT and they also confirmed that an increased tHcy level was a risk factor for DVT (2).

In spite of the large number of epidemiological data about the association of hyperhomocysteinemia and venous thrombosis, little is known about the pathophysiology. A spectrum of mechanisms might be involved, but it seems that some of them that are assumed to participate in clarification of vascular diseases might also help in explaining venous thrombosis. 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 (3, 4).

Recently the increased interest in examination of Hcy has been focused on genetic factors, which cause moderate hyperhomocysteinemia. The cloning of the enzymes cystathione β-synthase, methionine synthase and methylenetetrahydrofolate reductase (key enzymes in Hcy metabolism) has identified

many genetic mutations (5, 6). Lack/absence of these enzymes causes elevation of Hcy concentration in the circulation, its excretion in the urine (homocysteinuria) and generalized atherosclerosis. Vitamin B_6 and B_{12} and folic acid are co-factors of these enzymes. If there is an impaired Hcy metabolism as a result of a defect in some of the genes in the genetic code of the mentioned enzymes or if there is an intracellular lack of some of the mentioned cofactors of these enzymes, then Hcy is being accumulated in the cells and it enters the circulation, and then its concentration is increased. The latest investigations have demonstrated that the gene responsible for *MTHFR* polymorphism is localized at chromosome 1p 36.3 and it is composed of 11 exons and 10 introns. Codon 677 of *MTHFR* gene on exon 4 is responsible for hyperhomocysteinemia (7).

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 (8, 9). 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 (8, 10).

One of the less strong enzyme defects, which has impact on the Hcy metabolism and is very common, is the thermolabile variant of the enzyme methylenetetrahydrofolate reductase. 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 (11). Mutation of C677T gene might influence on the composition of intracellular folate pool, and hence, the homozygous form of this mutation and to a lesser degree the heterozygous one, is associated with an increased Hcy concentration and a low folate status (12, 13).

The aim of this study was to determine the tHcy concentration by spectrophotometric cyclic enzymatic method, which nowadays is still not regularly used in routine biochemical laboratories in R. Macedonia; to verify whether determination of the tHcy concentration as well as determination of polymorphism, especially of methylenetetrahydrofolate reductase *(MTHFR)* C677T genetic mutation might help in diagnosing DVT and at the same time whether the results obtained might help in clarifying these issues.

2. MATERIAL AND METHODS

The investigation comprised 160 subjects divided into two main groups:

- 80 healthy subjects (control group)
- 80 patients with deep vein thrombosis

For comparative purposes the control group of subjects comprised blood donors from the Republic Institute of Transfusion Medicine, 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.

Group of patients with DVT consisted of 80 patients at the age of 35-65 years. Diagnosis of DVT was established by application ultrasonography and/or venography at the Republic Institute of Transfusion Medicine. Patients who were receiving anti-coagulation therapy, which according to literature data has no influence on the homocysteine concentration, were candidates to be included in this study.

Exclusion criteria in this group of patients were the following: positive family history for hereditary diseases associated with impaired homocysteine metabolism, diabetes mellitus, cerebrovascular insult, autoimmune diseases (systemic lupus erythematodes), severe form of psoriasis, 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 subject was given 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). One tube with whole blood was transported at +4°C to the Institute of Molecular Biology and Human Genetics at the Faculty of Natural and Mathematical Sciences for determination of genetic polymorphism of C677T in MTHFR enzyme. Plasma from the second vacutainer was immediately put on dry ice and was transported to the Institute of Medical and Experimental Biochemistry; it was centrifuged within 30 min. after blood specimens were collected and tHcy was determined.

Principle of cyclic and spectrophotometric enzymatic method for determination of plasma tHcy

Concentration of tHcy was determined by cyclic enzymatic method. In general, the principle of this method is based on previous reduction of the protein-bound or oxidized homocysteine into free homocysteine. Free homocysteine in a reaction catalyzed by S-methyltransferase reacts with S-adenozylmethionin (SAM), it is transformed into methionine and S-adenosylhomocysteine (SAH). SAH is immediately hydrolized into homocysteine and adenosine in presence of S-adenosylhydrolase. The created homocysteine re-enters into reaction in which it is transformed into methionine and SAH. In this cyclic transformation of homocysteine, adenosine is accumulated and it is transformed into inosine and ammonia in presence of adenosine hydrolase. Ammonia with 2-ketoglutarate in a reaction catalyzed by glutamate dehydrogenase that, being a coenzyme, contains NADH is transformed into glutamate, when NADH oxidizes to NAD^+, which is manifested by decline of the absorbency to 340 nm. Homocysteine concentration is proportional to absorbency reduction that is to transformation of NADH into NAD^+ (14).

Isolation of genomic DNA

Standard isolation of genomic DNA from nucleic cells (leukocytes) was done, with sodium chloride–extraction and subsequent precipitation with ethanol (15). 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

Amplification of regions of *MTHFR* gene was done by polymerase chain reaction (PCR). The following pairs of primers were used for amplification: 5'-TTT GAG GCT GAC CTG AAG CAC TTG AAG GAG-3' и 5'-GAG TGG TAG CCC

TGG ATG GGA AAG ATC CCG-3' (16), which resulted in an amplification product of 173 base pairs (bp) length. In each reaction tube (with thin walls) calculated volume was pipette for: prepared PCR buffer; mix of deoxynucleodites (dNTP); pair of oligonucleotide primers: thermostabile *Taq* polymerase and DNA sample collected from each individual. Amplification was done on PCR-machine (thermocycler) (*Perkin-Elmer GeneAmp System 2400*) by using an adequate program with thirty cycles in three phases each.

Homocysteine	Valid N	Mean	Confidence -95,00%	Confidence +95,00%	Minimum	Maximum	Std.Dev.
Examined group	80	15.19	14.38	16.00	8.30	34.30	3.63
Control group	80	11.62	10.86	12.39	6.10	28.30	3.43

Table 1. Values of total homocysteine in patients with DVT and control group

Homosysteine	Rank Sum Exam. gr.	Rank Sum Control gr.	U	Z	p-level	Z	p-level	Valid N	Valid N
	8380,50	4499,50	1259,50	6,62	0,000***	6,62	0,000***	80	80

Table 2. Difference of homocysteine between patients with DVT and control group p<0.001***

Detection of mutation C677T in MTHFR gene

Detection of C→T *missense* mutation in *MTHFR* gene was made by restriction analysis (*Polymerase Chain Reaction-Restriction Fragment Length Polymorphism*–PCR-RFLP). Amplification products were digested by restriction endonuclease *Hinf*I under optimal conditions (buffered solution adequate for each enzyme, at a temperature of 37°C). Presence or absence of either certain normal, or mutant sequence from PCR products was accompanied with subjectivity or resistance to restriction digestion with the enzyme, thus resulting with onset of digested fragments of 125 and 48 bp length or non-digested electrophoretic band of 173 bp during electrophoretic analysis.

Digested products as well as some of the non-digested PCR products in presence of an electrophoretic marker (PCR Marker, Sigma-Aldrich) were separated by horizontal agarose electrophoresis (Bio-Rad) and were visualized by fluorescence staining with ethidium bromide under UV light (312 nm). Gels were photographed with a digital camera (Canon A70) and digital images were processed with Adobe Photoshop. Digital analysis (identification of electrophoretic bands and determination of their length in base pairs) was realized with the Image J software from NIH.

Statistical methods

Statistical analysis of the results was made by means of the Statistica, version 7.1 program.

In series with numeric features descriptive statistics (mean ± std.dev., ±95.00 CI., minimal value, maximal value) was applied;

In series with numeric features and when there were deviations from the normal distribution, the difference between two independent parameters was tested with Mann-Whitney U test (U/Z);

Correlation between total plasma homocysteine as an independent variable and MTHFR (C677) genetic polymorphism as an independent variable (CC; CT; TT) was analyzed with multiple regression (R).

3. RESULTS

Concentration of tHcy in the control group was 11.62±3.43 μmol/L and it varied in the interval 10.86-12.39 μmol/L ±95.00%CI. Minimal concentration was 6.10 μmol/L, and maximal 28.30 μmol/L (Table 1 and Figure 1).

Concentration of tHcy in the group of patients with DVT was 15.19±3.63 μmol/L and it varied in the interval 14.38-16.00 μmol/L ±95.00%CI. Minimal concentration was 3.30 μmol/L, and maximal 34.30 μmol/L (Table 1 and Figure 1).

Table 2 presents the results of the difference of homocysteine between patients with DVT and control group. Patients with DVP had significantly higher levels of homocysteine in comparison with control group, by Z=6.62 and p<0.001(p=0.000). Correlation between plasma tHcy as a dependent variable and MTHFR (677) genetic polymorphism as an independent variable (CC; CT; TT) in the control group was also examined (Table 3).

R= 0,82 / F(2,77)=78,312 p<0.000***						
	Beta	Std.Err.	B	Std.Err.	t(77)	p-level
Intercept			9.16	0.34	26.77	0.000***
CT	0.43	0.07	2.94	0.48	6.13	0.000***
TT	0.86	0.07	8.56	0.69	12.37	0.000***

Table 3. Multiple regression of total plasma homocysteine versus MTHFR (C677T) genotypes in control group p<0.001***

R= 0.78 / F(2.77)=59.367 p<0.000***						
	Beta	Std.Err.	B	Std.Err.	t(77)	p-level
Intercept			11.96	0.46	25.96	0.000***
TT	0.90	0.08	6.79	0.63	10.72	0.000***
CT	0.33	0.08	2.52	0.64	3.94	0.000***

Table 4. Multiple regression of total plasma homocysteine versus MTHFR (C677T) genotypes in patients with deep vein thrombosis p<0.001***

There was a very strong significant correlation for the examined ratio, by R=0.82 (p<0.0). Wild CC genotype was taken as a reference category. Homozygous mutated gene TT (Beta=0.86) had greater influence while heterozygous mutated gene CT (Beta=0.43) had milder influence. Subjects with homozygous mutated TT gene had on average 8.56 μmol/L higher plasma tHcy levels, which was statistically significant in comparison with patients with wild CC genotype, p<0.001 (p=0.000). Subjects with heterozygous mutated CT gene had on average 8.56 μmol/L higher plasma tHcy levels, which was statistically significant in comparison with patients with wild CC genotype, p<0.001 (p=0.000). Correlation between plasma tHcy as a dependent variable and MTHFR (677) genetic polymorphism as an independent variable (CC; CT; TT) in patients with DVT was examined (Table 4). There was a very strong significant correlation for the examined ratio, by R=0.78 (p<0.0). Wild CC genotype was taken as a reference category. Homozygous TT mutated gene (Beta=0.90) had greater influence while heterozygous CT mutated gene (Beta=0.33) had milder influence.

Patients with homozygous TT mutated gene had on average 6.79 μmol/L higher plasma tHcy levels, which was statistically significant as compared to patients with wild CC genotype, p<0.001 (p=0.000). Patients with heterozygous CT mutated gene had on average 2.52 μmol/L higher plasma tHcy levels, which was statistically significant as compared to patients with CC gene, p<0.001 (p=0.000).

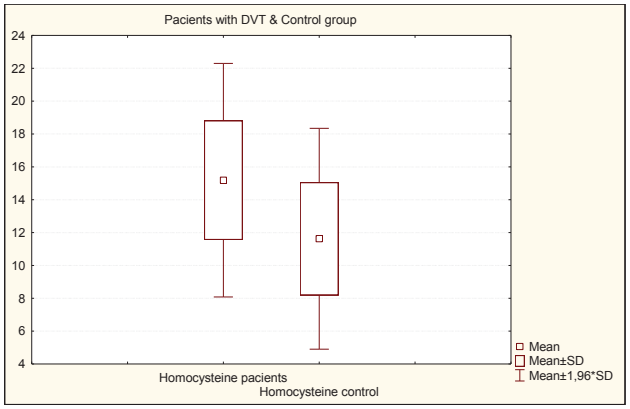

Figure 1. Distribution of homocysteine in patients with DVT and in healthy subjects (control group)

4. DISCUSSION

The mean plasma tHcy value of the control group obtained with the cyclic enzymatic method was 11.62±3.43 μmol/L, and to a great extent this result was in agreement with data in the literature (3, 17). However, distribution of tHcy values in the healthy population was disturbed, that is, shifted to higher levels, which coincided with distribution presented by Krstevska and Vaya (17, 18). This distribution is still under discussion and it is assumed to be a result of the unhealthy lifestyle (insufficient intake of cofactors with food, age, sex, life habits) (19). Some authors, Refsum, Clarke (20, 21) consider tHcy frequency distribution in control subjects to be in the range between 5 and 15 μmol/L.

The levels obtained for total plasma homocysteine were significantly higher in patients with deep vein thrombosis in comparison with the control group, 15.19±3.63 μmol/L ($p<0.001$). Our data are consistent with observations from a number of other studies (22, 23). These studies performed in patients with DVT have confirmed our finding that hyperhomocysteinemia is a risk factor for development of this disease.

MTHFR (C677T) genetic polymorphism is responsible for mild to moderate hyperhomocysteinemia in majority of cases and it is one of the rare genetic risk factors that has been confirmed. Statistical analysis showed that patients with CT genotype of MTHFR (C677T) had by 2.94 μmol/Lhigher tHcy level when compared to subjects with wild CC genotype of MTHFR (C677T), $p<0.001$. Subjects with TT genotype of MTHFR (C677T) had by 8.56 μmol/L higher plasma tHcy level when compared to subjects with wild CC genotype of MTHFR (C677T), $p<0.001$. This fact indicates that mutation of MTHFR (C677T) gene can influence on the composition of intracellular folate pool and the homozygous form of this mutation, and to a lesser extent the heterozygous form, is accompanied with an elevated tHcy concentration (24). In this sense, it has been shown that folic acid stabilizes and maintains the function of the mutated/mutant enzyme, and this fact represents a ground for eventual treatment of hyperhomocysteinemia (22).

There are a large number of studies on the association of homocysteine and mutations of MTHFR (C677T) gene and they all, more or less, show the association of T allele with the increased plasma homocysteine level (25). Our research has shown that patients with DVT with CT genotype of MTHFR (C677T) had by 2.52 μmol/L higher tHcy level when compared to patients with wild CC genotype of MTHFR (C677T), $p<0.001$. On the other hand, patients with TT genotype of MTHFR (C677T) had by 6.69 μmol/L higher plasma tHcy level when compared to patients with wild CC genotype of MTHFR (C677T), $p<0.001$. The risk of development deep vein thrombosis in patients with TT and CC genotypes is associated with mild to moderate hyperhomocysteinemia and reduced folic acid status.

5. CONCLUSION

Concentration of total homocysteine in patients with deep vein thrombosis was statistically significantly increased in comparison with healthy subjects.

MTHFR (C677T) genotypes are associated with plasma tHcy level both in the control group and in the patients with deep vein thrombosis.

Acknowledgements

This research is part of the project "Blood Homocysteine Level and Prevalence of C667T Mutation of Enzyme Methylentetrahydropholate Reductase (MTHFR) as Risk Factors for Blood Vessel Diseases" supported by Ministry of Education and Science of the Republic of Macedonia (No. 13-1672/4-02).

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