Homocysteine Concentrations in Heterozygote MTHFR (677C-T) and Factor V (1691 G-A) Mutation-carrying Individuals with the History of Thromboembolic Disease

ORIGINAL INVESTIGATION

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ABSTRACT

Objective: Factor V (FV) (1691 G-A) and methylenetetrahydrofolate reductase (MTHFR) (677 C-T) mutations have been identified as potential risk factors for cardiovascular disease. In this study, we determined vitamin B_{12}, folate, and total homocysteine t(Hcy) concentrations in heterozygote MTHFR (677 C-T) and FV (1691 G-A) mutation-carrying individuals.

Materials and Methods: The study included a total of 74 individuals with MTHFR (677 C-T) or FV (1691 G-A) mutations and 70 controls. All subjects had the history of thromboembolic disease. t(Hcy), folate, and vitamin B_{12} concentrations were compared between the groups.

Results: A significant difference was found in vitamin B_{12} and folate concentrations between patients and controls in the MTHFR (677 C-T) group (p=0.041, p=0.049, respectively). Further, t(Hcy) concentrations were found to be higher in patients than in controls in the FV (1691 G-A) mutation-carrying group (p=0.002). No significant difference was found between the groups in relation with gender in both mutations.

Conclusion: t(Hcy) concentrations should be assessed to decrease the risk of future venous thromboembolism in the presence of heterozygote FV (1691 G-A) mutation.

Keywords: Homocysteine, Vitamin B_{12}, Folate, MTHFR, Factor V

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INTRODUCTION

Hyperhomocysteinemia is an independent risk factor for atherosclerotic and atherothrombotic vascular disease. One of the causes of hyperhomocysteinemia is the genetic mutation of enzymes in homocysteine metabolism (1). Methylenetetrahydrofolate reductase (MTHFR) catalyzes the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, and it plays a key role in the homocysteine-to-methionine methylation (2). It is indicated that the MTHFR (677 C-T) mutation causes increased plasma homocysteine concentrations (3). Hyperhomocystenemia is involved in endothelial dysfunction, and this condition contributes toward progressive thrombosis and atherosclerosis (1).

In addition, a single nuclear polymorphism in factor V (FV) gene was found to be associated with activated protein C (APC) resistance. The G→A missense mutation at position 1691 results in the replacement of Arg506 by Gln (FV_{Leiden}). This mutation is known as the most common genetic risk factor for thrombosis. The activated mutant FV molecule is resistant to proteolytic inactivation by APC and predisposes individuals to thromboembolic disease (4).

It is suggested that there is a pathophysiological correlation between total homocysteine [t(Hcy)] concentrations and impaired function of the protein C pathway (5, 6). However, the effects of FV_{Leiden} and MTHFR mutations on arterial vascular disease and plasma homocysteine concentrations are still contradictory (7, 8). Another cause of hyperhomocysteinemia is vitamin B_{12} and folate deficiencies. Plasma t(Hcy) can be moderately elevated in individuals with an inadequate intake of vitamin B_{12} and folate, which serve as cofactors in the enzymatic pathways of homocysteine metabolism (1, 9-11).

In the present study, we assessed vitamin B_{12}, folate, and t(Hcy) concentrations in heterozygote MTHFR (677 C-T) and FV (1691 G-A) mutation-carrying individuals with a history of thromboembolic disease. Our study might contribute to prevent future vascular events due to homocysteine in these mutation-carrying patients with a history of thromboembolic disease.

MATERIALS and METHODS

Human subjects

The study subjects comprised 74 mutation-carrying individuals [30 males and 44 females, aged between 18 and 84 years (mean age: 39±13.18)], who had heterozygote FV (1691 G-A) or MTHFR (677 C-T) mutations, without

MTHFR (1298 A-C) mutation and 70 controls [28 males and 42 females, aged between 26 and 65 years (mean age: 39±13.77)]. Control subjects were divided into two groups: one group comprised 37 subjects (18 males and 19 females) without the FV (1691 G-A) mutation and the other group comprised 33 subjects (10 males and 23 females) without the MTHFR (677 C-T) mutation. All members of the control and mutation groups had a history of brain embolism, stroke, and deep vein thromboembolism, which are classified as thromboembolic diseases. We evaluated t(Hcy) concentrations of all subjects at their first admission to our hospital. No significant differences were observed between the groups in terms of population number. The distributions of mutation-carrying individuals are shown in Table 1 as per gender and mutation types. We obtained data of the study population from the laboratory information systems of Ankara Numune Training and Research Hospital. The exclusion criteria were impaired renal and thyroid function and diabetes mellitus. Samples were sent by physicians from various medical inpatient and outpatient clinics. The protocol was approved by the ethical committee of Ankara Numune Training and Research Hospital.

Blood sample collection
Overnight fasting blood samples were collected from all subjects. Two types of blood collection tubes [red top tube (Becton Dickinson, UK) and ethylenediaminetetraacetic acid (EDTA)-containing tube (Becton Dickinson, UK)] were used. Red top tube was used for the analysis of homocysteine, folate, and vitamin B_{12}. EDTA-containing tube was used for the molecular analysis of MTHFR (677 C-T) polymorphism and FV (1691 G-A) mutation.

Biochemical measurement
1. Folate and vitamin B_{12}
Folate and vitamin B_{12} concentrations were determined in serum using a chemiluminescence immunoassay with Unicel DXI 800 (Beckman Coulter, USA). The folate assay had a precision of 5.2% at 3.78 ng/mL, 4.05% at 6.46 ng/mL, and 3.33% at 15.7 ng/mL. The reportable range was 0.5–20 ng/mL with a normal range of 3.56–20 ng/mL. The vitamin B_{12} assay had a precision of 8.5% at 88 pg/mL, 6.6% at 374 pg/mL, 7.5% at 775 pg/mL, and 11.4% at 975 pg/mL. The reportable range was 50–1500 pg/mL, with a deficiency range of ≤141 pg/mL.

2. Homocysteine
t(Hcy) concentration was determined using a competitive immunoassay with Immulite 2000 (Siemens Healthcare Diagnostics Prod-

ucts Ltd., Lianberries, Gwynedd, UK). The assay precision was 15.1% at 4.2 µmol/L, 8.5% at 11 µmol/L, 7.8% at 16.5 µmol/L, and 8.1% at 24.5µmol/L. The reportable range was 2–50 µmol/L, with a reference range of 5–15 µmol/L.

Genotyping of MTHFR (C677T) polymorphism and FV (1691G-A) mutation
Deoxyribonucleic acid (DNA) was extracted from whole blood samples using a High Pure PCR Template Preparation Kit (Roche Applied Science, Penzberg, Germany). Mutation analysis was performed by LightCycler 2.0 RT-PCR thermal cycler (Roche Applied Science, Germany) based on real-time polymerase chain reaction (RT-PCR). Mutation and polymorphism detection processes were performed using commercial kits: FV$_{Leiden}$ kit (Roche Molecular Systems, Branchburg, New Jersey, USA) and LightMix C677T kit (Tib Molbiol GmbH, Berlin, Germany). The increase in the fluorescence of the product obtained through DNA amplification using RT-PCR was monitored in real time. Based on a detailed melting curve analysis of PCR products, gene polymorphism was detected. The heterozygote genotype FV (1691G-A) melting temperatures were 65±2.5°C and 57±2.5°C. The MTHFR (677 C-T) melting temperatures were 63±2.5°C and 54.5±2.5°C

Statistical analysis
Data normality was assessed by Shapiro–Wilk's test and histogram; q-q plots were plotted. Chi-square analysis was used to compare the differences of categorical variables, and a two-sided independent samples t test was used to compare the differences of continuous variables. Analyses were performed using IBM SPSS software (release 20.0, IBM, SPSS Inc.; Chicago, IL, USA), considering p<0.05 as statistically significant.

RESULTS

A total of 144 subjects were included in this study. MTHFR (677 C-T) polymorphism and FV (1691 G-A) mutation frequencies were 25.70% (n=37) and 25.70% (n=37), respectively.

Table 2 shows the overall laboratory findings of the mutation-carrying individuals and controls according to gender. In MTHFR (677 C-T) and FV (1691 G-A) mutation-carrying individuals, the median serum t(Hcy) concentrations were 12.4 (8.78–16.65) µmol/L and 12.5 (9.5–17.95) µmol/L, respectively. 14 (37.84%) out of 37 heterozygote FV (1691 G-A) mutation-carrying subjects and 12 (32.43%) out of 37 heterozygote MTHFR (677 C-T) polymorphism carrying subjects had concentrations greater than 15 µmol/L t(Hcy).

Table 3 shows the comparison of t(Hcy), folate, and vitamin B_{12} concentrations between patients and controls in the MTHFR (677 C-T) and FV (1691 G-A) mutation groups. A significant difference was found in vitamin B_{12} and folate concentrations between patients and controls in the MTHFR (677 C-T) group (p=0.041, p=0.049, respectively). Furthermore, t(Hcy) concentrations were found to be higher in patients compared to controls in the FV (1691 G-A) group (p=0.002).

In Table 4 and 5, we compared serum t(Hcy), folate, and vitamin B_{12} concentrations as per gender for individuals with heterozygote MTHFR (677 C-T) and FV (1691 G-A) mutations and controls,

Table 1. Distribution of mutation-carrying individuals and controls according to gender and mutation type

| Mutation type | Mutation-carrying individuals | | |
	Male n (%)	Female n (%)	p
MTHFR (677 C-T)	13 (35.1)	24 (64.9)	0.861
FV (1691G-A)	17 (45.9)	20 (51.4)	0.999

FV: Factor V; MTHFR: methylenetetrahydrofolate reductase

Values are expressed as frequencies and the percentages indicate the significance between females and males.

p: indicates the significance between male and females in both mutations.

Table 2. Serum t(Hcy), folate, vitamin B_{12} concentrations, and mean age in the controls and mutation-carrying individuals according to gender

	Age (years)	t(Hcy) (µmol/L)	Folate (ng/mL)	Vitamin B_{12} (pg/mL)
Controls (n=70)				
Males (n=28)	45.46±11.61	13.2 (9.50–17.72)	5.1 (3.87–7.14)	205 (149–288)
Females (n=42)	35.64±10.21	8.3 (7.23–10.17)	7.03 (4.93–9.31)	208 (185–289)
MCI (n=74)				
Males (n=30)	44.58±12.53	13.3 (8.91–17.71)	5.21 (3.56–7.67)	183 (147–252)
Females (n=44)	35.93±13.91	11.7 (8.76–15.85)	5.75 (4.28–8.35)	177 (146–250)

MCI: mutation-carrying individuals; t(Hcy): total homocysteine
Results are expressed as median (25th and 75th percentiles) and mean±SD (standard deviation) with 95% confidence intervals.

Table 3. Comparison of t(Hcy), folate, and vitamin B_{12} concentrations between patients and controls in the MTHFR (677 C-T) and FV (1691 G-A) groups

	MTHFR (677 C-T)			FV (1691 G-A)		
	MCI	Controls	p*	MCI	Controls	p**
t(Hcy) (µmol/L)	12.4 (8.8–16.7)	10.1 (8.6–3.8)	0.180	12.5 (9.6–18.0)	7.9 (6.3–13.1)	0.002
Folate (ng/mL)	5.4 (4.0–7.7)	7.5 (4.9–9.4)	0.049	5.8 (3.5–8.5)	5.6 (4.1–7.6)	0.711
Vitamin B_{12} (pg/mL)	177 (146–250)	204 (180–288)	0.041	146 (188–260)	210 (181–290)	0.106

MCI: mutation-carrying individuals; t(Hcy): total homocysteine
p*: indicates the significance between controls and the heterozygote MTHFR (677 C-T) mutation-carrying individuals.
p**: indicates the significance between controls and the heterozygote FV (1691 G-A) mutation-carrying individuals.
Results are expressed as median (25th and 75th percentiles) with 95% confidence intervals.

respectively. We did not find any significant difference between the subjects and control groups.

DISCUSSION

To the best of our knowledge, this is the first study to investigate the effect of vitamin B_{12} and folate on total homocysteine concentrations in heterozygote MTHFR (677C-T) and FV (1691 G-A) mutation-carrying individuals with a history of thromboembolic disease.

Median serum t(Hcy) concentrations were 12.4 (8.8–16.7) µmol/L in individuals with the MTHFR (677 C-T) mutation. Our result was similar with the studies made by Yilmaz et al. (12) and Ilhan et al. (13), in which the reported median homocysteine concentrations in individuals carrying the heterozygote MTHFR (677 C-T) mutation were 14±7.8 and 14.8±4.7 µmol/L, respectively. In the study made by Mahfouz et al. (14), it was reported that t(Hcy) concentrations in heterozygote MTHFR (677 C-T) mutation-carrying individuals were 13.0±4.6 µmol/L. The small difference between our study and the aforementioned studies in terms of t(Hcy) might be due to the difference in concentrations of vitamin B6, B_{12}, and folate and the smoking status of the population.

Although serum vitamin B_{12} and folate concentrations were found to be significantly lower in patients with the MTHFR mutation, we did not find any significant difference in t(Hcy) concentrations. Our results might indicate that other factors such as vitamin B6 concentrations, smoking status, and renal functions, as well as folate and vitamin B_{12} on t(Hcy) concentrations in the heterozygote MTHFR (677 C-T)

mutation-carrying individuals, were important. We thought that this result supports the result in the previous paragraph.

As for being a potent natural anticoagulant by inactivating FV and factor VIII, protein C is one of the major components required for providing hemostasis. In normal conditions, the activation of thrombomodulin by thrombin and activation of thrombomodulin later induce the complex formation of APC. APC cleaves and inactivates the activated forms of FV and factor VIII (factors Va and VIIIa). In the presence of FV_{Leiden}, FV cannot be inactivated by anticoagulant APC. Some studies have shown that Hcy inhibits thrombomodulin-dependent protein C activation and interferes with the expression of thrombomodulin (15-17). In our study, we found a higher homocysteine concentration in the mutation-carrying group than in the control group. The reason for the presence of a significant difference between the two groups in terms of homocysteine concentrations could be the positive feedback for the influence of increased concentrations of APC, protein C, and thrombomodulin. This finding supports the hypothesis that t(Hcy) analysis should be made for individuals with FV_{Leiden} and who are genetically predisposed to thrombosis to decrease the risk of future cardiovascular disease. However, in a meta-analysis conducted by Keijzer et al. (18), it was reported that published studies in a large case-only study did not show evidence for interaction between FV_{Leiden} and hyperhomocysteinemia in the risk for venous thrombosis. When we consider our results, further studies that exclude all events associated with homocysteinemia should be made to assess the relationship between FV_{Leiden} and hyperhomocysteinemia.

Table 4. Comparison of t(Hcy), folate, and vitamin B_{12} concentrations according to gender between the heterozygote MTHFR (677 C-T) mutation-carrying individuals and controls.

| | Control | | MCI | | p^ϵ | p^Φ |
	Male	Female	Male	Female		
t(Hcy) (µmol/L)	15.81 (10.88–20.86)	10.16 (9.05–11.20)	20.12 (15.10–26.78)	10.84 (9.04–12.66)	0.650	0.936
Folate (ng/mL)	5.71 (3.50–8.18)	8.22 (5.59 –9.64)	4.47 (3.75–5.39)	6.52 (4.44–8.11)	0.376	0.131
Vitamin B_{12} (pg/mL)	189 (147.75–267.75)	208 (186–289)	156 (146–226)	185 (146–254)	0.976	0.344

MCI: mutation-carrying individuals; t(Hcy): total homocysteine
p^Φ: indicates the significance between the controls and the mutation-carrying individuals in females.
p^ϵ: indicates the significance between the controls and the mutation-carrying individuals in males.
Results are expressed as median (25th and 75th percentiles) with 95% confidence intervals.

Table 5. Comparison of t(Hcy), folate, and vitamin B_{12} concentrations according to gender between the heterozygote FV (1691 G-A) mutation-carrying individuals and controls

| | Control | | MCI | | p^ϵ | p^Φ |
	Male	Female	Male	Female		
t(Hcy) (µmol/L)	15.81 (10.88–20.86)	10.16 (9.05–11.20)	20.12 (15.10–26.78)	10.84 (9.04–12.66)	0.650	0.936
Folate (ng/mL)	4.80 (3.93–6.48)	6.71 (4.61–8.22)	3.64 (3.06 –7.13)	6.78 (4.4–8.77)	0.691	0.492
Vitamin B_{12} (pg/mL)	227 (174–292)	208 (182–289)	204 (147–267)	184 (145–250)	0.192	0.956

MCI: mutation-carrying individuals; t(Hcy): total homocysteine
p^Φ: indicates the significance between the controls and the mutation-carrying individuals in females.
p^ϵ: indicates the significance between the controls and mutation in males.
Results are expressed as median (25th and 75th percentiles) with 95% confidence intervals.

One of the limitations of the present study was that all study populations included the same ethnic group, so the results could not be generalized to other ethnic groups. As a result of being designed as a retrospective study, populations included were highly heterogeneous. Further, it should be noted that there is a high intra-individual variability of serum folate and vitamin B_{12} concentrations, which may need another measurement to represent the average concentration of the individual. Other limitations were the less number of subjects to evaluate the gender-specific effects of MTHFR and FV_{Leiden} mutations on t(Hcy) concentrations and the lack of data about the patients' vitamin B6 concentrations and cigarette-smoking status.

CONCLUSION

As well as vitamin B_{12} and folate, other factors leading to hyperhomocysteinemia, such as vitamin B6 deficiency and smoking status, are important to prevent the progression of vascular events due to high t(Hcy) concentrations in heterozygote MTHFR (677 C-T).

Thromboembolic diseases of venous thromboembolic disease (VTE), thrombotic stroke, and peripheral vascular disease are associated with elevated levels of homocysteine. Additionally, it was reported in some studies (16, 18) that the coexistence of hyper-

homocysteinemia and Leiden mutations are increasing the risk of developing future VTEs. In this manner, we thought that the assessment of t(Hcy) concentrations reduced the risk of future thrombotic events for patients with an FV (1691 G-A) mutation. Further investigations should be made in different study populations and ethnic groups by considering other factors relevant to hyperhomocysteinemia and their effects on vascular events.

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