Hyperhomocysteinemia and C677T Polymorphism of Methylenetetrahydrofolate Reductase Gene in Patients with Cardiovascular Disease

Zahira Houcher1, Bakhouche Houcher1, Abderezak Touabti2, Samia Begag1, Ayşenur Öztürk3, Yonca Egin3, Nejat Akar3, Farida Djabi2

1Department of Biology, Faculty of Sciences, University of Sétif, Sétif; 2Department of Medicine, Faculty of Medical Sciences; University of Sétif, Sétif, Algeria; 3Pediatric Molecular Genetics Department of Ankara University Medical School, Ankara, Turkey

Abstract

The aim of the present study was to explore the influence of age and gender, on the association between the methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism and plasma total homocysteine (tHcy) concentrations in patients with cardiovascular disease (CVD). Fasting tHcy and the MTHFR C677T mutation were evaluated in 98 patients with CVD, 46 were men and 52 women (aged 20-96 years). There was a significant elevation of plasma tHcy with age (<45 yr: 33.9 µmol/L vs. ≥75 yr: 43.6 µmol/L; p <0.01). The mean tHcy concentration increased significantly with age in men (<55 yr: 33.4 µmol/L vs. ≥55yr: 42.45 µmol/L; p 0.01). However, the plasma tHcy was not increased with older age in women. The frequency of the TT genotype was 19.6% in the younger patients group (>175 yr) compared with 4.7% in the older patients group (>55 yr; p <0.01). In conclusion, the data presented here are consistent with genetic factors that influence tHcy levels being more prominent in old patients (>55 yr). Then, the MTHFR mutation does not seem to be associated with either high tHcy or the occurrence of CVD.

Key words: hyperhomocysteinemia, methylenetetrahydrofolate reductase, C677T polymorphism, cardiovascular disease, age, sex, Algeria

Introduction

The homocysteine (Hcy) metabolism consist of two pathways, Hcy can be remethylated to methionine or it can undergo the irreversible transsulfuration to cystathionine (1). These lead to the formation of methionine and cystathionine, respectively (2). Methylenetetrahydrofolate reductase (MTHFR, EC 1.7.99.5) and cystathionine β-synthase (CBS) deficiency are two well-defined inborn errors of homocysteine metabolism (3).

A relationship between moderate hyperhomocysteinemia (HHcy) and cardiovascular disease is well established (4). Moderate increases of total plasma Hcy (tHcy) have been associated with a higher risk of cardiovascular disease (CVD) in observational studies, in particular case-control studies (5). Furthermore, a meta-analysis study pointed at a moderate increase in plasma THCY and the risk of CVD (6).

HHCY can result from genetic or environmental and dietary factors that disrupt HCY metabolism (7). Many factors are related to circulating tHcy concentrations (2). Fasting plasma tHcy concentration is consistently higher in men than in women, and increases with age (8). The male-female difference has been attributed mainly to sex differences in muscle mass (8, 9) and circulating sex hormones (9). The nutritional status of vitamin B12, B6 and folate is a major determinant of tHcy concentrations (10), and other identified nutritional and lifestyle factors may also influence circulating tHcy levels (11).

Genetic background also affects tHcy concentrations. A common gene variant of MTHFR is the enzyme that catalyses the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a cosubstrate for HCY remethylation to methionine (7), is the most frequent genetic cause of mild HHCY. The MTHFR 677C-T polymorphism was identified in 1995...
(12). The T allele causes an alanine to valine amino acid substitution (Ala222Val) within the catalytical domain of the enzyme, which results in the production of a thermostable enzyme (12) with decreased activity, with TT homozygotes having ~35-50% reduction in enzyme activity compared to control values (7). Then, the association of the C677T polymorphism genotypes and increased plasma tHCY concentrations has been controversial (13). The lack of homogeneity in the data and the high number of factors influencing plasma tHCY concentrations remain conflicting (14).

No data are available about the frequency of the MTHFR mutation and its relation to tHCY in the Algerian subjects with cardiovascular disease. Thus, the aim of the present study was to determine tHCY level and its relationship MTHFR polymorphism in patients with established cardiovascular disease, age and sex in the population of Sétif (Algeria).

Material and Methods

Subjects

This study group was collected by a group from the University Hospital of Sétif (Algeria). Total of 98 subjects were included in the study, the CVD group comprised 46 men and 52 women, aged 20-96 years (mean ±S.D.; 56 ± 17 yrs) who were recruited into a retrospective study assessing a possible relationship between HHHCY, MTHFR genotype and CVD. Blood samples for the study were obtained from the patients admitted to Sétif University Hospital (Algeria) and transferred to Ankara/Turkey. Written informed consent was obtained from all the blood patients, and the study was approved by the Local Ethics Committee of Sétif University Hospital. The current study was carried on four groups: The group 1 consisted of patients <45 years (Gp1), age in group 2 ranged between 45-54 years (Gp2), in group 3 age ranged between 55-74 years (Gp3) and the age of group 4 was ≥75 years (Gp4). All patients were living in the same geographic area of Northern Algeria (Sétif).

Homocysteine and laboratory measurements

For the determination of tHCY, peripheral venous blood was collected into EDTA vacutainer tubes following an overnight fast. Plasma was separated immediately to prevent ex vivo leakage of HCY by erythrocytes. Plasma was stored in plastic vials at -20°C until analysis. The concentration of tHCY was determined using a competitive immunoassay on the IMMULITE and IMMULITE 1000 Analyzers (Siemens Diagnostics) using reagents and calibrators. Moderate, intermediate and severe HHHCY are defined as plasma tHCY concentrations in the range 16-30, 31-100, and >100 μmol/L, respectively.

Analysis of polymorphisms

DNA extraction was performed using the conventional phenol-chloroform method. After haemolysis of the blood in hypotonic solution, the DNA was isolated by using a simple proteinase K treatment at 65°C in the presence of SDS, followed by ammonium acetate precipitation of debris and ethanol precipitation of the DNA. Genetic analysis MTHFR 677C-T polymorphism was determined by real-time polymerase chain reaction (PCR) method by melting curve analysis performed on Light Cycler (Roche Molecular Biochemicals, Mannheim, Germany) in borosilicate capillaries with MTHFR 677CT polymorphism detection kit (Roche Molecular Biochemicals, Mannheim, Germany). The identification of MTHFR genotype has been performed by the analysis of the melting peaks of the run of real-time PCR. The presence of just one melting peak at 63.0 C indicates a wild-type genotype, two melting peaks at 54.5°C and 63.0°C indicate a heterozygous mutant, and one melting peak at 54.5°C indicates a homozygous mutant.

Statistical analysis

Results are expressed as the mean ± SD. Statistical analysis of the data was carried out using the ANOVA test. Prevalence of alleles and genotypes among patient groups were done by a Chi-square test ($\chi^2$, Fisher's exact test). For correlation studies, Pearson correlation test was used. Statistical significance was accepted at $p<0.05$. Statistical analysis was performed using SPSS 10 statistical Package.

Results

HHHCY concentrations ranged from 16.1 to 51 μmol/L in CVD subjects, 35.7% of patients had concentrations >15 and ≤30 mol/L comprising 16.3% of men and 19.4% of women, whereas 64.3% of patients had concentrations ≥30 μmol/L in group of CVD, comprising 30.6% of men and 33.7% of women.

Previously reported MTHFR genotype frequencies in the “healthy” Algerian population were shown as 14.3% of subjects to be homozygous for the mutated allele (TT), 45.6% homozygous for the wild-type allele (CC), and 40.1% heterozygous (CT), the overall frequency of the T and C allele was 34.4% and 65.6%, respectively (15). Comparing our patients to this reported distribution, we found that T allele was not different (36.7%). The mean tHCY concentration was not higher in men compared to women.

The frequencies of C677T/MTHFR in women were 46.1% for CC, 40.4% for CT, and 13.5% for TT genotypes; whereas those in men were 40.8% for CC, 44.9% for CT, and 14.3% for TT genotypes. There was no significant difference in T allele frequencies
between sex groups (Table 1).

**Table 1.** Serum concentration of total homocysteine (tHCY) and polymorphism of methylenetetrahydrofolate reductase (MTHFR) by sex and age in patients with cardiovascular disease

<table>
<thead>
<tr>
<th>Age</th>
<th>tHCY</th>
<th>CC n (%)</th>
<th>CT n (%)</th>
<th>TT n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;55 yrs (n = 46)</td>
<td>34.0 ± 10.2</td>
<td>16 (34.8)</td>
<td>21 (45.6)</td>
<td>9 (19.6)</td>
</tr>
<tr>
<td>≥55 yrs (n = 43)</td>
<td>38.9 ± 10.1*</td>
<td>20 (46.5)</td>
<td>21 (48.8)</td>
<td>2 (4.7) *</td>
</tr>
</tbody>
</table>

**Men (n = 40)**

<table>
<thead>
<tr>
<th>Age</th>
<th>tHCY</th>
<th>CC n (%)</th>
<th>CT n (%)</th>
<th>TT n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;55 yrs (n = 28)</td>
<td>33.4 ±10.4</td>
<td>9 (32.1)</td>
<td>14 (50.0)</td>
<td>5 (17.9)</td>
</tr>
<tr>
<td>≥55 yrs (n = 12)</td>
<td>42.5 ±8.17**</td>
<td>5 (41.7)</td>
<td>7 (58.3)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

**Women (n = 49)**

<table>
<thead>
<tr>
<th>Age</th>
<th>tHCY</th>
<th>CC n (%)</th>
<th>CT n (%)</th>
<th>TT n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;55 yrs (n = 18)</td>
<td>34.9 ±9.86</td>
<td>7 (38.9)</td>
<td>7 (38.9)</td>
<td>4 (22.2)</td>
</tr>
<tr>
<td>≥55 yrs (n = 31)</td>
<td>37.5 ±10.5</td>
<td>15 (48.4)</td>
<td>14 (45.2)</td>
<td>2 (6.4)</td>
</tr>
</tbody>
</table>

**Discussion**

An elevated concentration of plasma tHCY has been recognized as a risk factor for vascular diseases (16). The genetic factors affecting plasma tHCY include mutations in the genes of the key enzymes participating in HCY metabolism.

The most important genetic determinant of tHCY in the general population is the common C677T variant in MTHFR that results in higher tHCY. Individuals with the homozygous mutant (TT) genotype have a significantly higher (14-21%) risk of heart disease (17). The homozygous mutant TT genotype for the MTHFR C677T polymorphism typically affects about 10% of individuals worldwide but can be as high as 14% (Algeria) (15), 26% (south Italy) and 32% (Mexico) in some areas (18). The association between the C677T variant in the MTHFR gene and CVD is controversial in several populations worldwide (19). Our research is the first in Algeria which studied CVD patients in order to determine the association of the T allele and tHCY with CVD in the region of Sétif (Algeria).

The principle findings of this study include the fol-
Houcher Z. et al.: Hyperhomocysteinemia and MTHFR C677T Polymorphism in CVD

lowings: first plasma tHCY was significantly higher in the patients with CVD and second, MTHFR gene mutation does not seem to be associated with the occurrence of CVD or the elevation of plasma tHCY in the studied patients.

In the present study, the data are consistent with genetic factors that influence tHCY levels being more prominent in old male patients (≥ 54 yr). The MTHFR 677TT genotype strongly interacted with low folate levels to produce a high tHCY phenotype, an effect that was more pronounced in males than in females (17). Nevertheless, the proportion of the variance in

Table 2. Serum concentration of total homocysteine (tHCY) and polymorphism of methylenetetrahydrofolate reductase (MTHFR) by the four age groups in patients with cardiovascular disease.

<table>
<thead>
<tr>
<th>Gp1 (&lt;45 yrs)</th>
<th>Group2 (45-54 yrs)</th>
<th>Gp3 (55-74 yrs)</th>
<th>Gp4 (≥75 yrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 21)</td>
<td>(n = 25)</td>
<td>(n = 27)</td>
<td>(n = 16)</td>
</tr>
</tbody>
</table>

Serum tHCY

<table>
<thead>
<tr>
<th></th>
<th>Gp1</th>
<th>Group2</th>
<th>Gp3</th>
<th>Gp4</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmol/L</td>
<td>33.9 ± 8.14</td>
<td>3.15 ± 11.7</td>
<td>36.1 ± 10.3</td>
<td>43.6 ± 7.99*</td>
</tr>
</tbody>
</table>

MTHFR genotype

<table>
<thead>
<tr>
<th></th>
<th>Gp1</th>
<th>Group2</th>
<th>Gp3</th>
<th>Gp4</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>9 (42.8)</td>
<td>7 (28.0)</td>
<td>12 (44.4)</td>
<td>8 (50.0)</td>
</tr>
<tr>
<td>CT</td>
<td>10 (47.6)</td>
<td>11 (44.0)</td>
<td>14 (51.8)</td>
<td>7 (43.7)</td>
</tr>
<tr>
<td>TT</td>
<td>2 (9.6)</td>
<td>7 (28.0)</td>
<td>1 (3.8)†</td>
<td>1 (6.3)</td>
</tr>
</tbody>
</table>

* p < 0.01 (Gp4 vs. Gp3, Gp2 and Gp1); † p < 0.02 (TT; Gp3 vs. Gp2)

Table 3. Genotype and allele frequency of the MTHFR (677 C→T) polymorphism among moderate- and intermediate HHcy patients with cardiovascular disease

<table>
<thead>
<tr>
<th>Genotype/Allele</th>
<th>Moderate HHcy (n = 35)</th>
<th>Intermediate HHcy (n = 63)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotypes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>14 (40.0)</td>
<td>27 (42.8)</td>
<td>1</td>
</tr>
<tr>
<td>CT</td>
<td>16 (45.7)</td>
<td>27 (42.8)</td>
<td>0.88 (0.36-2.25)</td>
</tr>
<tr>
<td>TT</td>
<td>5 (14.3)</td>
<td>9 (14.4)</td>
<td>0.93 (0.26-3.31)</td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>44 (62.8)</td>
<td>70 (60.9)</td>
<td>1</td>
</tr>
<tr>
<td>T</td>
<td>26 (37.2)</td>
<td>45 (39.1)</td>
<td>1.09 (0.59-2.01)</td>
</tr>
</tbody>
</table>

Values in parentheses denote genotype frequencies (columns 2 and 3) or 95% CI (column 4).
tHcy levels that is attributable to genetic factors is relatively modest.

Several population-based studies have demonstrated that tHcy levels are influenced by age and gender (20). The current study did not show a positive correlation between tHcy and age which is not keeping with previous results in different ethnic populations (21). However, when patients were stratified according to age groups and reanalyzed for the plasma tHcy, there was significant increase in tHcy in the patients in the different groups. In our study, the risk of HHCY was increased in men over 55 yr of age than in women of the same age group. Previous work suggests that the sex difference may be explained by tHcy formation in connection with the testosterone/creatinine synthesis that is proportional to muscle mass (22). Nevertheless, our data is not in agreement with those who reported a higher Hcy in women than in men. It has been demonstrated that estrogen-induced an increased turnover of folate, thus determining the increase of tHcy (23).

Part of the relationship between tHcy and age in woman might be explained by menopause, since tHcy concentration was found to be higher in postmenopausal compared with premenopausal women (24). This suggests that higher postmenopausal tHcy levels may be related to the lower methionine transamination or estrogen deficiency (25).

Regarding the relationship between tHcy and MTHFR mutation, in this study, we did not find a significant correlation between high tHcy levels and MTHFR genotypes in our patients. This finding is in keeping with a report which found no association between the plasma tHcy and the MTHFR mutation (26). The implications of this finding are still unclear so far, especially that the association between the C677T polymorphism and the development of CVD has not been studied in Algeria and the Arab world particularly. However, some authors suggested that the homozygosity for the MTHFR C677T polymorphism was associated with an increased risk of CHD (27).

The moderate HHCY that is dependent on the thermolabile MTHFR mutation is observed for plasma folate concentrations <15.4 nmol/L, at higher concentrations, no increase in circulating tHcy is observed (13).

Furthermore, in the current study, when cases were stratified according to expression of the T allele and reanalyzed for the plasma tHcy, there was no significant increase in tHcy in the TT genotype individuals in our patients. This finding supports more the lack of association between the presence of the T allele and the high tHcy levels. This could be explained by the presence of other factors that control tHcy (13) especially plasma folate and vitamin B12 (28). The differences in tHcy concentrations can be attributed to variations in the blood concentrations of folate and vitamin B12 in the population of each country (29). Thus, homozgyosity for MTHFR may only be a risk factor for CVD in some ethnic groups and not in others (14). In other words, if folate intake is sufficient, subjects with the TT genotype would not have increased risk of CVD via HHcy (30). The divergence between populations raises the question whether dietary factors could play a significant interactive role in C677T. The measurement of vitamin B12 and folate was not done in this study. Further analysis combining MTHFR polymorphism, nutrition, and disease prevalence is needed.

In the present study, which the first be conducted in Algeria, we postulate that the C677T polymorphism might not be associated with a higher risk of developing CVD relatively compared to several populations worldwide. Our results will serve as a baseline to investigate on the possible causes of CVD in our population.

Acknowledgements

This study was supported by Ankara University, Turkey and the National Agency for the Development of Health Research, Algeria (project No. 03/03/00/07/093). We extend our special thanks to the personnel of cardiovascular sections in Sétif University Hospital (Algeria) and the patients who participated in this study.

References

5 Ford ES, Smith SJ, Stroup DF, Steinberg KK, Mueller PW, Thacker SB. Homocyst(e)ine and cardiovascular disease: a systematic review of the evidence with special emphasis on case-control studies and nested case-control studies. Int J Epidemiol


