MTHFR Gene polymorphisms, B-vitamins and hyperhomocystinemia in young and middle-aged acute myocardial infarction patients

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Abstract

We have examined the prevalence of the C677T and A1298C single nucleotide polymorphisms (SNPs) in the methylenetetrahydrofolate reductase (MTHFR) gene in healthy Tamilians and in patients with acute myocardial infarction and related this polymorphism to plasma homocysteine concentrations, serum folate, serum cobalamin and riboflavin status. The SNPs in the MTHFR gene were determined by polymerase chain reaction–restriction fragment length polymorphism analysis. Plasma homocysteine, serum folate and serum cobalamin concentrations were analyzed using an automated chemiluminescence method and riboflavin status was assessed by measuring the erythrocyte glutathione reductase activity using spectrophotometric method. Out of the 200 young and middle-aged (<48 years) individuals included in the study, 100 were acute myocardial infarction (AMI) patients and 100 were healthy individuals with no documented history of heart diseases. There was a significant increase in homocysteine levels among the AMI patients as compared to the healthy controls (p<0.001). The results of this study indicate that hyperhomocystinemia is more prevalent in Tamilian AMI patients and that the MTHFR C677T and A1298C SNPs are not associated with hyperhomocystinemia. Folate status was found to be within normal range in all the study subjects. There was no correlation between homocysteine and different biochemical variables including cobalamin, folate and riboflavin. However, serum cobalamin was found to be significantly decreased in AMI patients when compared to controls (p<0.001). The simultaneous presence of decreased serum cobalamin status, hyperhomocystinemia and mutant genotype for both the SNPs might lead to an increased risk for the occurrence of AMI. Further intervention trials including the supplementation of cobalamin may prove whether homocysteine level decrease in response to the supplementation of cobalamin in individuals with hyperhomocystinemia and mutant genotype for both the above mentioned SNPs.

Keywords: MTHFR; Myocardial infarction; Hyperhomocystinemia

Introduction

There is increasing evidence that hyperhomocystinemia is a risk factor for cardiovascular disease (Verhoef et al., 1997). The plasma concentrations of homocysteine varies among different populations, ages, sexes, diet habits and individual health conditions (Nygard et al., 1995). Molecular defects in the genes encoding the enzymes involved in homocysteine metabolism may lead to hyperhomocystinemia. As such, individuals with reduced MTHFR activity may have elevated amounts of homocysteine in their blood and/or urine (Wiemels et al., 2001). The metabolism and degradation of homocysteine in the body requires the presence of the vitamin B12 (cobalamin), B2 (riboflavin) and folate, especially during the remethylation of homocysteine (Pietrzik et al., 1996; Verhoef et al., 2005). Thus, altered homocysteine metabolism has become the focus of increasing attention because of its potential role in the pathogenesis of coronary artery diseases.

Two common single nucleotide polymorphisms (SNPs) have been reported in the MTHFR gene, C677T (MTHFR NM_0059-57.3:c.665C>T (p.Ala222Val)) and A1298C (MTHFR NM_005957.3:c.1286C>T (p.Glu428Ala)), that lead to altered amino acids. The C677T SNP results in a common C to T transition in the MTHFR gene coding sequence, leading to the substitution of alanine to valine residue within the N-terminal catalytic domain (Frosst et al., 1995). The A1298C SNP leads to a glutamate to alanine substitution within the S-adenosylmethionine-regulatory
domain of the enzyme due to an A to C transversion that occurs in exon 7 (Robien et al., 2003; Kumagai et al., 2003).

A certain degree of heterogeneity in the prevalence and penetrance of the C677T and A1298C MTHFR genotypes among different ethnic groups has been reported (Esfahani et al., 2003; Gašparović et al., 2004). Hence, it will be of great importance if we establish the genetic frequency of these two MTHFR SNPs according to geographic regions as suggested by Morita et al. (1997) and Esfahani et al. (2003). Whether these MTHFR SNPs affect homocysteine levels and are a risk factor for AMI in the Tamilian population has not yet been examined. Published data are very limited with respect to the effect of these MTHFR SNPs on homocysteine levels among Tamilians.

Moreover, most studies to date have focused mainly on elderly individuals or randomly chosen individuals. In this population-based study, we exclusively evaluated young and middle-aged acute myocardial infarction patients with no traditional cardiovascular risk factors to find out whether hyperhomocysteinemia is an independent risk factor for AMI among Tamilians. The purpose of the study was also to investigate the genetic contribution to hyperhomocysteinemia in a relatively native population of Tamil Nadu. Independent and interactive effects of the two functional polymorphisms of the MTHFR gene (C677T and A1298C) on tHcy levels were analyzed and the potential interactions with the most relevant nutritional variables of the remethylation pathway of homocysteine metabolism including serum folate, cobalamin and riboflavin status were also assessed. Thus, this study has been designed to compare the influence of genetic and nutritional factors on homocysteine levels in order to see whether MTHFR gene mutation could be a risk factor for acute myocardial infarction among young and middle-aged Tamilians.

Methods

Selection of subjects

In this study, fasting venous blood samples were drawn within 24 h after the onset of AMI from 100 hospitalized patients with the diagnosis of AMI episode. One hundred similarly age-matched healthy male control subjects without any cardiovascular risk factors were also included. The objective of the study was described and informed consent was obtained. The Tamils represented in this study are the people who not only live in Tamil Nadu, but also belong to ethnic group from Tamil Nadu. The identity has historically been primarily linguistic; with Tamils belonging to those whose first language is Tamil. They are a member of the Dravidian people of South India. The other Dravidian people including the Brahui people, Kannadigas, Malayalis, Telugus and Tulus were excluded from the study subjects. The vegetarians mentioned in this study are lactovegetarians, those who include dairy products but avoid animals. People with a history of vitamin B complex tablet intake were not included in this study.

Information was sought regarding smoking habits, alcohol intake, consumption of drugs and vitamins and major conventional risk factors of coronary heart disease, i.e. hypertension, diabetes, hypercholesterolemia, obesity and family history of coronary artery disease. Only individuals without any of the major conventional risk factors were included for the study.

Biochemical measurement

Plasma for tHcy determination was separated immediately from the collected blood by centrifugation at 4 °C within half an hour and stored at −20 °C until analysis. Serum was separated from the blood within half an hour of collection. Plasma homocysteine, serum cobalamin and serum folate concentrations were determined using an automated chemiluminescence immunoassay manufactured by Bayer on an ACS Centaur (Bayer Diagnostics, Tarrytown, NY, USA). Riboflavin status was assessed by evaluating the erythrocyte glutathione reductase activity using a spectrophotometric method (Tietz et al., 1999).

Cut off values

Hyperhomocysteinemia was considered to be present if the level of tHcy was >15 μM/L. Decreased serum cobalamin level was considered to be present if the level of serum cobalamin was less than ≤156 pmol/L. Decreased folate was considered to be present if the serum folate was ≤7 nmol/L. Decreased riboflavin status was considered to be present if the oEGRA ratio was >1.2.

DNA extraction

Genomic DNA was extracted from 300 μL of whole blood using a standard protocol (Sambrook et al., 1989). The isolated DNA was stored at −20 °C. The DNA quality was confirmed by electrophoresis using a 0.7% agarose gel and quantity determined using absorbance spectrophotometry.

PCR/RFLP analysis

Approximately 120 ng of genomic DNA was incubated in a total reaction volume of 50 μL containing both the forward and reverse primers for the MTHFR C677T (MTHFR NM_005957.3:c.665C>T (p.Ala222Val)) or A1298C (MTHFR NM_005957.3:c.1286C>T (p.Glu428Ala)) SNPs, respectively, using 2.5 U Taq DNA polymerase (Bangalore Genie, India). Amplification for the MTHFR C677T SNP was performed with an initial denaturation step at 93 °C for 2 min in a thermal cycler (Eppendorf Mastercycler personnel, Germany). The PCR amplification conditions were as follows: 34 cycles consisting of 1 min denaturation at 92 °C, 1 min annealing at 64 °C and 1 min extension at 72 °C. The final cycle included a 10-min extension step at 72 °C. For the MTHFR A1298C SNP, DNA was initially denatured at 93 °C for 2 min prior to amplification and PCR amplification was accomplished using 30 cycles consisting of 2 min denaturation at 92 °C, 1 min

<table>
<thead>
<tr>
<th>Table 1</th>
<th>MTHFR C677T polymorphism—genotype and allele frequency in AMI patients and controls</th>
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<tbody>
<tr>
<td>Genotype/allele</td>
<td>Total, n=200</td>
</tr>
<tr>
<td>Genotypes (C677T)</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>165</td>
</tr>
<tr>
<td>CT</td>
<td>34</td>
</tr>
<tr>
<td>TT</td>
<td>1</td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.91</td>
</tr>
<tr>
<td>T</td>
<td>0.09</td>
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</table>

<table>
<thead>
<tr>
<th>Table 2</th>
<th>MTHFR A1298C polymorphism—genotype and allele frequency in AMI patients and controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype/allele</td>
<td>Total, n=200</td>
</tr>
<tr>
<td>Genotypes (A1298C)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>86</td>
</tr>
<tr>
<td>AC</td>
<td>84</td>
</tr>
<tr>
<td>CC</td>
<td>30</td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.64</td>
</tr>
<tr>
<td>C</td>
<td>0.36</td>
</tr>
</tbody>
</table>
AMI patients was 23.5.

between patients (r = 0.1237) and controls (r = 0.0590; p ≥ 0.05). When correlation analysis of tHcy and serum folate was done for all the 200 study subjects, no correlation was observed (p > 0.05).

The mean ±SD of αEGRA in AMI patients and controls were 1.00±0.05 and 1.01±0.01, respectively, which indicates that there is adequate supply of riboflavin, due to the occurrence of decreased αEGRA ratio (<1.2). All the individuals including both AMI patients and controls in the study group had an αEGRA < 1.2.

Serum cobalamin levels were significantly decreased among AMI patients compared to the controls (p < 0.01) (Table 3). Decreased cobalamin status was observed in 69% of the AMI patients whereas it was observed in only 21% of the controls. The correlation analysis indicated that there is no significant correlation between homocysteine and cobalamin levels between patients (r = -0.1237) and controls (r = -0.0590; p ≥ 0.05). When correlation analysis of tHcy and serum folate was performed, a significant correlation was found between tHcy and serum folate (r = 0.1237).

The genotype distributions in both groups of individuals were found to be normal in both the control and AMI groups. AMI patients showed homocysteine levels similar to those of controls (Table 3). There was also no correlation between the level of serum folate and plasma tHcy in both patients and controls (r = -0.1146 and -0.0661, respectively). When correlation analysis of tHcy and serum folate was done for all the 200 study subjects, no correlation was observed (p > 0.05).

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Table 3
Levels of different biochemical parameters in AMI patients and controls

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AMI patients, n=100</th>
<th>Healthy individuals, n=100</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine (μmol/L)</td>
<td>32.35±10.3 (30.48–34.52)</td>
<td>13.62±3.56 (12.82–14.42)</td>
<td>S [&lt; 0.001]</td>
</tr>
<tr>
<td>Cobalamin (pmol/L)</td>
<td>141.4±54.23 (129.28–153.59)</td>
<td>239.5±112.11 (214.42–264.66)</td>
<td>S [&lt; 0.01]</td>
</tr>
<tr>
<td>Folate (nmol/L)</td>
<td>26.5±6.4 (25.07–27.93)</td>
<td>27.75±6.86 (26.21–29.29)</td>
<td>NS [&gt;0.05]</td>
</tr>
<tr>
<td>αEGRac ratio</td>
<td>1.00±0.05 (0.9888–1.011)</td>
<td>1.01±0.01 (1.01–1.078)</td>
<td>NS [&gt;0.05]</td>
</tr>
</tbody>
</table>

Table 4
Effect of compound genotypes (MTHFR-A1298C and C677T) on homocysteine levels in AMI patients and controls

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>AMI patients, mean±SD (%) (n=100)</th>
<th>Controls, mean±SD (%) (n=100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1298C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>31.81±10.77 (35)</td>
<td>13.18±3.41 (44)</td>
</tr>
<tr>
<td>AC</td>
<td>32.1±10.83 (37)</td>
<td>14.91±3.78 (30)</td>
</tr>
<tr>
<td>CC</td>
<td>32.25±7.83 (9)</td>
<td>12.97±3.66 (10)</td>
</tr>
</tbody>
</table>

| C677T     |                                   |                                |
| CC        | 32.05±0.4 (2)                     | 13.03±2.78 (4)                 |
| CT        | 35.71±9.89 (9)                    | 13.15±3.28 (8)                 |
| TT        | 28.82±11.36 (7)                   | 12.04±3.79 (4)                 |

The genotype distributions in both groups of individuals were in Hardy–Weinberg equilibrium. The MTHFR-C677T mutation itself or in conjunction with the A1298C genotype had no effect on the homocysteine value of an individual. The results of the present study showed that combined heterozygosity for both MTHFR mutations (677 CT/1298 AC genotype) does not result in significantly elevated tHcy levels in AMI patients and controls when compared to individuals with normal genotype (677CC/1298AA) in AMI patients and controls (p > 0.05), demonstrating that the mutation in the MTHFR gene does not affect the tHcy concentration (Table 4). However, irrespective of the genotypes, the total homocysteine levels were found to be elevated in AMI patients when compared to the controls.
cobalamin was done for all the 200 study subjects, no correlation was observed ($p > 0.05$). Among the AMI patients, 65% of them had decreased cobalamin status and hyperhomocystinemia whereas among controls, the prevalence of such incidence was only 8% (Table 5). The calculated odds ratio showed that decreased concentration of serum cobalamin was 8-fold as frequent in the patient group than in the control group. Relative risk analysis indicated that individuals with decreased cobalamin concentrations and hyperhomocystinemia have a 2.4-fold risk for the occurrence of AMI.

Irrespective of the genotypes, the cobalamin levels were found to be decreased in AMI patients when compared to the controls. The MTHFR-C677T mutation itself or in conjunction with the A1298C genotype had no effect on cobalamin, riboflavin and folate values of an individual (Table 6).

Relative risk analysis indicated that individuals with mutant genotypes for C677T SNP decreased serum cobalamin concentrations and hyperhomocystinemia have a 4.3-fold risk for the occurrence of AMI. Similarly, relative risks for individuals with mutant genotypes for the A1298C SNP, decreased cobalamin concentrations and hyperhomocystinemia have a 4.5-fold risk for the occurrence of AMI (Table 7).

**Discussion**

In recent years, MTHFR gene polymorphisms have gained enormous attention due to its association with cardiovascular diseases. In the present study, the allelic frequencies of the two most common MTHFR gene polymorphisms (C677T and A1298C) were calculated for both AMI patients and controls. The ‘T’ allele frequency established by this study (0.09) is slightly lower compared to that of North Indian subjects (0.184) (Vasisht et al., 2002) and higher compared to Sri Lanka (0.049). Mexican population has the highest ‘T’ allele frequency of 0.59. The Japanese population showed a relatively high ‘T’ allele frequency of 0.37 (Morita et al., 1997).

Frequencies of MTHFR 677TT in AMI cases and controls in this study were 1% and 0%, respectively. These values are comparable with those reported by Mukherjee et al. (2002) who have found these frequencies to be 2% in coronary artery disease patients and zero in controls among Asian Indians. Another study conducted among North Indian men by Vasisht et al. (2002) showed the presence of mutant 677TT genotype in 7.8% of coronary artery disease patients and in 3.6% controls which is higher when compared to that of South Indian Tamils. The ‘C’ allele frequencies for A1298C polymorphism established by this study (0.36) is similar to Canadians (0.36), Western Europe (0.36) and Israelis (0.34) and higher compared to Chinese (0.17) and Africans (0.21) (Song et al., 2001; Gebhardt et al., 2001; Isotalo et al., 2000).

The TT genotype is completely absent in controls and it was present in only one AMI patient. This may be explained by the fact that the occurrence of the 677TT genotype may be deleterious and it might have its impact on fetal viability as suggested by Devi et al. (2004). The present study indicates that the individual with the 677TT genotype also had the 1298AA genotype. This is in accordance to the suggestion by Van der Put et al. (1998), that these two common mutations are always in a trans configuration as they arose on different alleles, and...
because of their small distance on the chromosome the chance for crossing over to occur may be less. The exceptional genotype (677CT/1298CC) was present only in 7% of AMI patients and 4% of controls which may be due to cross over that rarely form recombinant chromosomes (Van der Put et al., 1998; Hanson et al., 2001).

While several studies suggest an association between the MTHFR 677TT genotype and hyperhomocystinemia, the present study did not find any such association. This may also be due to the absence of such genotype (except in one individual) in the study population.

Some authors have suggested that MTHFR C677T and A1298C SNPs with homozygous/heterozygous mutant genotype or combined heterozygous for both the SNPs contribute to cardiovascular diseases (Verhoef et al., 1997; Froos et al., 1995; Christensen et al., 1997; Gemmati et al., 1999; Kluijtmans et al., 1997). However, the results of the present study indicate that there was no significant difference in the occurrence of double heterozygosity in AMI patients and controls. Thus, the present study also reports no significant association of MTHFR SNPs and hyperhomocystinemia.

The MTHFR-C677T mutation itself or with the A1298C genotype had no effect on the Hcy value of an individual. Irrespective of the genotypes, the total homocysteine levels were found to be elevated in AMI patients when compared to the controls. This shows that the MTHFR-C677T or A1298C genotype itself has no effect on the concentration of homocysteine.

The results of this study, in addition to validating the earlier view on the association of homocysteine levels with coronary artery disease, establishes the occurrence of hyperhomocystinemia in the absence of major coronary risk factors in young and middle-aged AMI patients. Another striking factor noted was that the controls had high basal levels of homocysteine when compared to western populations. This may be attributed to the dietary behavior of the study subjects. The prevalence of inadequate cobalamin status was greater (69%) for AMI patients compared to controls (21%). Hence, the presence of decreased concentrations of cobalamin may account for the higher plasma tHcy concentrations among Tamils. However, the correlation analysis showed no significant correlation between serum cobalamin and plasma homocysteine levels of patients and controls. The basal level of cobalamin in the present study was found to be very low in controls indicating that even the healthy individuals have baseline cobalamin levels among the South Asian Tamils when compared to western populations. Refsum et al. (2001) conducted a study among Indians living in the west and identified an unusually high rate of cobalamin deficiency in vegetarians than in non-vegetarians.

In the present study, the observed normal folate level in the presence of decreased cobalamin concentrations may therefore be due to impaired intracellular folate retention. While serum cobalamin deficiency was more prevalent among AMI patients, folate concentrations were found to be normal in both AMI patients and controls. It may be explained by the fact that decreased concentrations of serum cobalamin reduces the methionine synthase activity thereby leading to an accumula- tion of circulating methyl THF. Thus, the folate may become trapped extracellularly as methyl THF, which can again enter the methylation cycle as dihydrofolate, bypassing the cobalamin-dependent remethylation of homocysteine (Van Guelpen et al., 2006). Therefore, even with adequate folate intake, reduced cobalamin concentrations may lead to elevated plasma homocysteine levels (Blander-Gouaille and Bottiglieri, 2003).

In situations when there is abundant intracellular folate, the folate molecule may be able to hold the variant MTHFR protein in the appropriate and functional three dimensional structure (Robien et al., 2003). Thus, it was hypothesized that mutation in the MTHFR gene may lead to a higher folate requirement in tHcy regulation (Siri et al., 1998). However, the results indicated that the AMI patients with mutant genotypes had hyperhomocystinemia in the presence of normal serum folate levels. This finding suggests that serum folate might not be responsible for hyperhomocystinemia among Tamils.

Riboflavin status was reported as a modulator of plasma homocysteine concentrations in healthy adults, especially among subjects homozygous for the MTHFR-C677T mutation. The findings of Strain et al. (2004) pointed out that riboflavin supplementation appear to be effective at lowering plasma homocysteine only in those individuals homozygous for the T allele of the MTHFR C677T polymorphism. McNulty et al. (2002) suggested that a higher riboflavin status may prevent the FAD cofactor from leaving the active site or may allow its quick replacement, thus stabilizing the variant form of the enzyme.

The results of this study showed the adequate riboflavin status in AMI patients and that the riboflavin status may not influence tHcy among Tamils. Moreover, there was no correlation between riboflavin status and plasma homocysteine concentration in both patients and controls. Thus, this study provide evidence that riboflavin status as measured by red blood cell glutathione reductase activity is not a determinant of fasting plasma tHcy concentration in AMI patients among Tamils. It was also noted that riboflavin status with respect to mutant MTHFR genotype does not influence homocysteine levels.

When the two MTHFR gene single nucleotide polymorphisms were individually and together related to other biochemical variables (serum folate, serum cobalamin and riboflavin), there was no statistical difference in the occurrence of genotypes and genotype combinations and the concentration of different biochemical variables included in the study. As no interactions could be detected between the MTHFR gene variants and biochemical variables on the risk of AMI, this study does not provide any direct evidence for MTHFR gene disease associations. However, the calculated relative risk indicated that simultaneous presence of decreased cobalamin status, hyperhomocystinemia and mutant genotype for both the SNPs individually have at least 4-fold risk for the occurrence of AMI whereas, when individuals with decreased cobalamin concentrations and hyperhomocystinemia were taken into account without considering the genotypes, it was identified that there was only a 2.4-fold risk for the occurrence of AMI.
The combination of low cobalamin, and high tHcy, caused by the Indian vegetarian diet or by malabsorption, may also be a factor for the increased risk of myocardial infarction. As dietary cobalamin is typically present in foods of animal origin, a strict vegetarian diet may be associated with an increased risk of cobalamin deficiency (Refsum et al., 2001; Herrmann et al., 2001). National food balance data indicated that adequate intake of fruits and vegetables which are good source of folate but decreased consumption of cobalamin-rich animal products causes good folate but inadequate cobalamin intake (Kark et al., 2002). Further intervention trials including the supplementation of cobalamin may prove whether homocysteine level decrease in response to the supplementation of cobalamin in individuals with hyperhomocystinemia and mutant genotype for both the above mentioned SNPs.

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