Methylenetetrahydrofolate reductase polymorphism (C-677T) and coronary artery disease

N. M. MALIK*, P. SYRRIS*, R. SCHWARTZMAN†, J. C. KASKI†, D. C. CROSSMAN‡, S. E. FRANCIS‡, N. D. CARTER* and S. JEFFERY*
*Medical Genetics Unit, St George’s Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K., †Department of Cardiological Sciences, St George’s Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K., and ‡Section of Cardiology, Department of Medicine, University of Sheffield, Sheffield S5 7AU, U.K.

ABSTRACT

1. Many studies have shown that hyperhomocysteinaemia is a risk factor for atherosclerotic vascular disease. A mutation (C-677T) in the gene coding for the methylenetetrahydrofolate reductase (MTHFR) enzyme has been shown to produce a thermolabile form of the enzyme. Homozygosity for this mutation has been correlated with an elevated plasma homocysteine concentration. The present study aimed to determine whether this mutation was a risk factor for coronary artery disease (CAD). This was achieved by comparing the frequency of the C-677T mutation in patients with angiographically proven CAD against angiographically normal patients in two separate U.K. samples. The analysis was repeated with CAD patients split into those with ≥ 99% stenosis of arteries and those without, to establish whether the C-677T mutation could be correlated with severity of CAD.

2. Two patient groups were selected from London and Sheffield. The London group comprised 174 cases and 148 controls. The Sheffield group comprised 93 cases and 85 controls. The DNA samples of the patients were genotyped by polymerase chain reaction and restriction enzyme digestion.

3. For London the homozygous C-677T frequencies were: 0.07 (controls), 0.09 (CAD without ≥ 99% stenosis) and 0.10 (CAD with ≥ 99% stenosis). For Sheffield the homozygous C-677T frequencies were: 0.08 (controls), 0.10 (CAD without ≥ 99% stenosis) and 0.11 (CAD with ≥ 99% stenosis). No association was found between the C-677T mutation and CAD in our sample geographical groups. Statistical comparison by genotype distribution for 0 VD (no vessel disease, i.e. 0% diameter reduction in all epicardial arteries) versus CAD without ≥ 99% stenosis: London, P = 0.19; Sheffield, P = 0.53; 0 VD versus CAD with ≥ 99% stenosis: London, P = 0.23; Sheffield, P = 0.55.

INTRODUCTION

An important study using meta-analysis of publications regarding plasma homocysteine and vascular disease has confirmed that elevated plasma homocysteine is an independent risk factor for atherosclerotic vascular disease. This study calculated that around 10% of the population risk for arterial vascular disease may be attributable to increased homocysteine levels [1]. A number of mechanisms have been proposed to explain

Key words: coronary artery disease, genetic polymorphism, methylenetetrahydrofolate reductase gene.
Abbreviations: CAD, coronary artery disease; MTHFR, methylenetetrahydrofolate reductase; PCR, polymerase chain reaction; VD, vessel disease.
Correspondence: Dr S. Jeffery.
how homocysteine damages endothelial cells. These include the generation of hydrogen peroxide and the formation of oxidized lipids and proteins [2]. Hyperhomocysteinaemia can result from a defect in the gene that encodes cystathionine β-synthase, which catalyses the first step in the trans-sulphuration of homocysteine [3]. It can also result from a defect in the gene that encodes 5,10-methylenetetrahydrofolate reductase (MTHFR), which catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The latter is a major form of folate in the plasma, and carbon donor for the remethylation of homocysteine to methionine [4].

Hyperhomocysteinaemia can be broadly classified into either severe or mild forms. Severe hyperhomocysteinaemia has an autosomal recessive mode of transmission and is caused by deficiencies in the cystathionine β-synthase or MTHFR enzymes, leading to premature atherosclerosis and thromboembolic events [5,6]. In heterozygotes, cystathionine β-synthase and MTHFR lymphocyte activities are 34% [5] and approximately 50% [7] of their respective normal means. Mild hyperhomocysteinaemia can be due to a variant of MTHFR that is thermolabile at 46 °C [4]. Thermolabile MTHFR has approx. 50% of the normal mean specific activity in homozygotes and approx. 75% in heterozygotes, and has been positively associated with coronary artery stenosis independently of confounding risk factors [8]. Frosst et al. [9] have shown that thermolabile MTHFR is caused by a substitution (C-677T) in the MTHFR gene that changes a highly conserved alanine residue to valine. They demonstrated that individuals heterozygous or homozygous for the mutation had reduced specific MTHFR activity and increased thermolability. Individuals homozygous for the mutation had significantly elevated plasma homocysteine concentrations. The mutation was therefore proposed as a candidate genetic risk factor for vascular disease [9].

Previous studies have failed to find any association of the C-677T mutation with coronary artery disease (CAD) [10–12]. In contrast, a recent report from a Tokyo clinic [13] has demonstrated a clear association of the C-677T mutation with CAD. It also found that the frequency of the mutation was increased in patients with ≥ 99% stenotic lesions and also in patients with triple-vessel disease as compared with single- or double-vessel disease. This has prompted us to re-evaluate the pathophysiological role of the mutation. In order to vigorously test this we divided our clinical data using the same criterion as the Tokyo study, by splitting our CAD patients into those with ≥ 99% stenotic lesions and those without. We then compared the frequency of the C-677T mutation in patients with angiographically proven CAD against angiographically normal patients in two groups recruited from London and Sheffield, to determine whether the C-677T mutation is a genetic risk factor for CAD.

METHODS

Subjects

The London patient group

Three-hundred and twenty-two unrelated Caucasian subjects were recruited from the Cardiological Sciences Department at St George’s Hospital. Patients with CAD (≥ 50% narrowing of at least one major coronary artery) were divided into two groups based on the degree of stenosis in their arteries as shown by coronary angiography. These were 94 subjects with stenosis (without ≥ 99% diameter reduction) of one or more major epicardial artery and mean age (± S.D.) 60.4 ± 11.5 years (± 2.03 S.E.M.), and 80 subjects with stenosis (with ≥ 99% diameter reduction) of one or more major epicardial artery and mean age 62.7 ± 7.9 years (± 1.00 S.E.M.). The controls comprised 148 subjects, mean age 56.8 ± 10.3 years (± 1.16 S.E.M.), who were angiographically proven to have 0% diameter reduction in all epicardial arteries (0 VD). From a clinical point of view these controls were angiographically normal patients with chest pain but no other cardiac problem (referred to as Syndrome X patients), and the remaining controls had valvular heart disease.

The Sheffield patient group

One-hundred and seventy-eight unrelated Caucasian subjects were recruited from the Northern General Hospital in Sheffield. Patients were investigated for either ischaemic chest pain or for valvular, myocardial or congenital heart disease. Patients with CAD (≥ 30% narrowing of at least one major coronary artery) were divided into two groups based on the degree of stenosis in their arteries as shown by coronary angiography. These were 67 subjects with stenosis (without ≥ 99% diameter reduction) of one or more major epicardial artery and mean age 57 ± 8.4 years (± 1.14 S.E.M.), and 26 subjects with stenosis (with ≥ 99% diameter reduction) of one or more major epicardial arteries and mean age 63.2 ± 7.8 years (± 1.00 S.E.M.). The controls comprised 85 subjects, mean age 58 ± 4.4 years (± 0.5 S.E.M.), who were angiographically proven to have 0% diameter reduction in all epicardial arteries (0 VD). The selection criteria for these controls were similar to those for the London group controls (either Syndrome X patients or patients with valvular heart disease).

At both centres patients attended for diagnostic angiography. Coronary angiograms were interpreted by two independent cardiologists.

Genotype determination

Blood from all individuals was collected into sterile EDTA tubes and stored at −20 °C. Genomic DNA was extracted using a Nucleon II DNA extraction kit.
(Bioscience, Scotland). The following primers [8] were used to carry out the polymerase chain reactions (PCRs) with a Hybaid Omnigene thermal cycler: forward primer, 5′-TGAAGGAGAAAGTTGTCTGCGGGA-3′; reverse primer, 5′-AGAACGGGTGCGGTAAGAGTG-3′.

For every sample, 1.0 µg of DNA was amplified with the following reagents: 1.5 units of Red Hot Taq DNA polymerase (Advanced Biotechnologies Ltd), 10 µl of PCR buffer [20 mM (NH₄)₂SO₄, 75 mM Tris/HCl, pH 9.0, 1.5 mM MgCl₂], and 0.2 mM dNTPs in a final reaction volume of 25 µl. The following programme was used for the PCR amplification: 1 cycle of 95 °C for 4 min; 35 cycles of 95 °C for 1 min, 64 °C for 1 min, 72 °C for 1 min, and 1 cycle at 72 °C for 10 min. For genotyping, 8 µl of PCR product was digested by the Hinfl restriction enzyme (1.5 units/reaction mixture). The C-677T mutation creates a recognition site for the enzyme that cleaves the 198-bp PCR fragment into 175-bp and 23-bp fragments. The bands were resolved by electrophoresis on a 2.5% agarose gel.

Statistical analysis
A χ² test was used to determine whether the genotype distributions for subgroups from both geographical groups were in Hardy–Weinberg equilibrium so as to rule out selection for a specific MTHFR genotype. The χ² test was also used to determine whether there was a significant difference in the genotype and allele distributions for the mutation between the cases and controls. In all tests a significant difference was accepted if \( P < 0.05 \).

RESULTS

No subgroup had a genotype distribution that deviated significantly from that expected for a population in Hardy–Weinberg equilibrium. The results are presented in Table 1.

First we examined the allele distribution of the C-677T mutation for cases and controls in London and Sheffield.

We found there was no significant difference when comparing the allele frequency of controls against patients without ≥ 99% stenosis in the London group (\( P = 0.18 \)) or in the Sheffield group (\( P = 0.19 \)). There was also no significant difference when comparing the allele frequency of controls against patients with ≥ 99% stenosis in the London group (\( P = 0.20 \)) or in the Sheffield group (\( P = 0.55 \)).

Next we examined the genotype distribution of the C-677T mutation for cases and controls in the two groups. We found that there was no significant difference when comparing the genotype distribution of controls against patients without ≥ 99% stenosis in the London group (\( P = 0.19 \)) or in the Sheffield group (\( P = 0.53 \)). There was also no significant difference when comparing the genotype distribution of controls against patients with ≥ 99% stenosis in the London group (\( P = 0.23 \)) or in the Sheffield group (\( P = 0.55 \)).

Heterozygous C-677T mutation causes an approximate 25% reduction in MTHFR activity but only homozygous C-677T mutation causes a reduction of 50% in MTHFR activity and an elevation of homocysteine in the circulation [8,9]. Therefore, to take this into account, the analysis was repeated using only CC and TT results to examine the association of the TT genotype with CAD (1 degree of freedom). Again it was shown that there was no association between the TT genotype and CAD: 0 VD versus CAD without ≥ 99% stenosis (London) \( P = 0.36 \), (Sheffield) \( P = 0.88 \); 0 VD versus CAD with ≥ 99% stenosis (London) \( P = 0.25 \), (Sheffield) \( P = 0.40 \).

DISCUSSION

In our study we found an allele frequency for the C-677T mutation of 0.30 in the London control group and 0.33 in the Sheffield control group. This compares well with a previously published allele frequency of 0.35 in a Caucasian myocardial infarction control group from the U.K. [11]. The paper by Frostell et al. [9] reported a slightly higher allele frequency of 0.38 for unselected French Canadians. We found homozygote frequencies of

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<tr>
<th>C-677T genotype</th>
<th>London group</th>
<th>Sheffield group</th>
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<tr>
<td></td>
<td>0 VD</td>
<td>CAD without St.</td>
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<tr>
<td>CC n (%)</td>
<td>70 (47)</td>
<td>35 (37)</td>
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<tr>
<td>CT n (%)</td>
<td>68 (46)</td>
<td>51 (54)</td>
</tr>
<tr>
<td>TT n (%)</td>
<td>10 (7)</td>
<td>8 (9)</td>
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<tr>
<td>Allele frequency (C/T)</td>
<td>0.70/0.30</td>
<td>0.64/0.36</td>
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0.07 in the London controls and 0.08 in the Sheffield controls. These are slightly lower than previously reported frequencies of 0.11, 0.10 and 0.13 in Australian [10], Japanese [13] and U.K [11] control groups respectively. Our results confirm those of previous studies that report a lack of association of the C-677T mutation with CAD [10–12], and are in clear contrast to the positive association found by Morita et al. [13]. In their Japanese Mongolid population Morita et al. [13] reported an allele frequency of 0.33 for the mutation which is similar to that in the French Canadians reported by Frostell et al. [9] and suggested that there were no population differences in the MTHFR gene. Even though the distributions of the C-677T mutation in Japanese and French Canadian populations are similar, evidence shows that the MTHFR gene does vary in certain ethnic populations. For example, Ubbink et al. [14] have demonstrated that black South Africans had significantly lower plasma homocysteine concentrations after a methionine load that was adjusted for body mass, compared with white subjects of a similar age and on the same diet. This suggests a genetic difference in homocysteine metabolism between black and white people and is supported by a homozygote frequency for the C-677T mutation of 0.01 in African Americans [15]. Future work should be undertaken to evaluate genotype–phenotype correlation in different ethnic groups to determine the risk spectrum of specific MTHFR polymorphisms.

We found a lack of association of the C-677T mutation with CAD in our U.K. groups, both CAD subgroups failing to show any association with the C-677T mutation as suggested by Morita et al. [13]. Nutrition is an important factor when considering MTHFR polymorphisms and may be a reason why we found no association with the C-677T mutation. A low dietary intake of vitamins B or folate plasma levels, and increasing age have been correlated with an increased risk of homocysteinuria due to cystathionine beta-synthase deficiency. Morita et al. [13] reported an allele frequency of 0.33 for the mutation which is similar to that in the French Canadians reported by Frostell et al. [9] and suggested that there were no population differences in the MTHFR gene. Even though the distributions of the C-677T mutation in Japanese and French Canadian populations are similar, evidence shows that the MTHFR gene does vary in certain ethnic populations. For example, Ubbink et al. [14] have demonstrated that black South Africans had significantly lower plasma homocysteine concentrations after a methionine load that was adjusted for body mass, compared with white subjects of a similar age and on the same diet. This suggests a genetic difference in homocysteine metabolism between black and white people and is supported by a homozygote frequency for the C-677T mutation of 0.01 in African Americans [15]. Future work should be undertaken to evaluate genotype–phenotype correlation in different ethnic groups to determine the risk spectrum of specific MTHFR polymorphisms.

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REFERENCES


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