A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase


Hyperhomocysteinaemia has been identified as a risk factor for cerebrovascular, peripheral vascular and coronary heart disease1-4. Elevated levels of plasma homocysteine can result from genetic or nutrient-related disturbances in the trans-sulphuration or re-methylation pathways for homocysteine metabolism1-5. 5, 10-Methylenetetrahydrofolate reductase (MTHFR) catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulatory form of folate and carbon donor for the re-methylation of homocysteine to methionine. Reduced MTHFR activity with a thermolabile enzyme has been reported in patients with coronary and peripheral artery disease5-6. We have identified a common mutation in MTHFR which alters a highly-conserved amino acid; the substitution occurs at a frequency of approximately 38% of unselected chromosomes. The mutation in the heterozygous or homozygous state correlates with reduced enzyme activity and increased thermolability in lymphocyte extracts; in vitro expression of a mutagenized cDNA containing the mutation confirms its effect on thermolability of MTHFR. Finally, individuals homozygous for the mutation have significantly elevated plasma homocysteine levels. This mutation in MTHFR may represent an important genetic risk factor in vascular disease.

Severe MTHFR deficiency, the most common inborn error of folate metabolism, results in hyperhomocysteinaemia, homocystinuria, and hypomethioninaemia. Patients with severe MTHFR deficiency (0-20% residual activity in cultured fibroblasts) present in infancy or adolescence with developmental delay, motor and gait dysfunction, seizures, psychiatric disturbances and other neurological abnormalities; they are also at risk for vascular complications6. Individuals with 50% residual activity, due to a thermolabile form of the reductase, were first reported in approximately 17% of 212 North American patients with coronary artery disease. A recent study of the Netherlands population identified the thermolabile variant in different forms of premature vascular disease, and estimated its incidence to be 7% of vascular patients. The presence of a thermolabile MTHFR is predictive of coronary artery stenosis, independent of other risk factors, such as age, smoking, hypercholesterolemia and hypertension6.

Our recent isolation of a cDNA for human MTHFR10 has enabled us to identify nine mutations in this gene, in the severely-deficient group of patients, by SSCP analysis and direct sequencing of PCR fragments10,11. Using the same procedures, we identified a C to T substitution at nucleotide (nt) 677, which converts an alanine to a valine residue (Fig. 1a). This alteration creates a HinfI site (Fig. 1b), which was used to screen 114 unselected French Canadian chromosomes; the allele frequency of the substitution was 0.38. The frequency of the three genotypes were as follows: -/-, 37%; +/-, 51%; and +/+12% (+ indicates the presence of the HinfI site and a valine residue). As these individuals were not examined clinically or biochemically, they cannot be considered as a control group.

We next performed genotypic analysis and measured enzyme activity and thermolability in a total of 40 lymphocyte pellets from patients with premature vascular disease and controls (Table 1). We selected 13 vascular patients from our previous study, among whom five were considered to have thermolabile MTHFR. From a large reference group of 89 controls, we studied all seven individuals who had thermolabile MTHFR, and selected at random an additional 20 controls with normal MTHFR from the same reference group. The mean MTHFR activity for individuals homozygous for the Ala to Val substitution (+/+1) was approximately 30% of the mean activity for (-/-) individuals. Heterozygotes had a mean MTHFR activity of 65% compared to (-/-) individuals, intermediate between values for (-/-) and (+/+1) individuals. The ranges of activities showed some overlap for the heterozygous and (-/-) genotypes, but homozygous (+/+1) individuals showed virtually no overlap with the other two groups. A one-way

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Fig. 1 Sequence change and restriction enzyme analysis for the alanine to valine substitution. a, Sequence of two individuals, a homozygote for the alanine residue and a homozygote for the valine residue. The antennase strands are depicted. The primers for analysis of the A→V change are: 5′-TGAAGGAGAA GTGTCCTGCG GGA-3′ (exonic) and 5′-AGGACTGGTG CGTGGAAGTG-3′ (intronic); these primers generate a fragment of 198 bp. b, The substitution creates a HinfI recognition sequence which digests the 198 bp fragment into 175 and 23 bp fragments; the latter fragment has been run off the gel. All three possible genotypes are shown.
The values shown represent mean ± standard error for each experiment, as expressed as (nmol formaldehyde/hr/mg protein) were as follows: 3.8 and 5.3 (Exp. 1) and separate experiments (with 3-4 replicates construct experiment) were performed to measure thermostable activity of the wild-type and mutagenized MTHFR cDNAs.

112 microcentrifuge at 14,000g and 100 °C (lanes 4-6) were prepared by homogenization in 0.25 M sucrose with aprotinin and leupeptin, followed by sonication on ice. The extracts were spun for 15 min in a table 1 correlation between MTHFR genotype and enzyme activity, thermostability and plasma homocysteine level.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Specific activity (mmol CH₃OH/mg protein/hr)</th>
<th>Residual activity after heating (%</th>
<th>Plasma homocysteine (μM) after fasting</th>
<th>Plasma homocysteine (μM) (post-methionine load)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/-</td>
<td>22.9 ± 1.7</td>
<td>66.8 ± 1.5</td>
<td>12.6 ± 1.1</td>
<td>41.3 ± 5.0</td>
</tr>
<tr>
<td>+/-</td>
<td>15.0 ± 0.6</td>
<td>58.2 ± 1.5</td>
<td>13.8 ± 1.0</td>
<td>41.2 ± 5.0</td>
</tr>
<tr>
<td>+/-</td>
<td>6.9 ± 0.6</td>
<td>21.8 ± 2.8</td>
<td>22.4 ± 2.9</td>
<td>72.6 ± 11.7</td>
</tr>
</tbody>
</table>

Enzyme activity and plasma homocysteine were determined as previously reported. Each value represents mean ± standard error. The range is given in parentheses below the mean.

a One-way anova P<.01; *paired t test; 1 for all combinations P<.01; 2 for +/- group versus +/- group or +/- group; P<.05 for +/- versus +/- group; n=18, n=11.

The alanine residue is conserved in porcine MTHFR and in the corresponding bacterial metF genes. We have also observed a region of homology in the human dihydrofolate reductase (DHFR) gene, although the alanine residue itself is not conserved; this region of homology has been implicated in folate binding of human DHFR. This region in MTHFR might also be involved in folate binding and the enzyme maybe stabilized in the presence of folate. This hypothesis is compatible with the well-documented influence of folate on homocysteine levels. We have used the original MTHFR cDNA (1.3 kb) to isolate a 2.2 kb cDNA, which contained an additional 900 bp at the 3' end; the latter contained a termination codon, 100 bp of 3' UTR and a poly A tail (GenBank #U09806). The open reading frame of 1980 bp predicts a protein of 74.6 kDa. The purified porcine liver enzyme has been shown to have subunits of 77 kDa. Western analysis (Fig. 2a) of several human tissues and of porcine liver reveals a polypeptide of 77 kDa, as well as an additional polypeptide of approximately 70 kDa in human fetal liver and in porcine liver, suggesting the presence of isozymes.

The wild-type cDNA and a mutagenized cDNA, containing the Ala to Val substitution, were expressed in E. coli and yielded a protein of approximately 70 kDa (Fig. 2a), which co-migrates with the smaller polypeptide mentioned above. Treatment of extracts at 46 °C for five minutes revealed that the enzyme containing the substitution was significantly more thermostable than the wild-type enzyme (P<.001; Fig. 2b). The expression experiments were not designed to measure differences in specific activity before heating, since variation in efficiencies of expression could contribute to difficulties in interpretation. Curiously, however, the specific activity for the mutant construct was higher in both experiments. It is possible that the mutant protein has increased stability in E. coli, or that inclusion bodies in our extracts contributed to differences in recovery of properly-assembled enzyme.

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and with the reported correction of mild hyperhomocysteinaemia by folic acid in individuals with premature vascular disease and thermolabile MTHFR.

Our data have identified a common genetic change in MTHFR which results in thermolability; our experiments do not directly address the relationship between this change and vascular disease. Nonetheless, this mutation represents a diagnostic test for evaluation of MTHFR thermolability in hyperhomocysteinaemia. Large case-control studies are required to evaluate the frequency of this genetic change in various forms of occlusive arterial disease and to examine the interaction between this genetic marker and dietary factors, such as folate intake. Well-defined populations need to be examined, as the limited data set thus far suggests that population-specific allele frequencies may exist. More importantly, however, the identification of a candidate genetic risk factor for vascular disease, which may be influenced by nutrient intake, represents a critical step in the design of appropriate therapies for the homocysteine-forming activity of atherosclerosis.

**Methodology**

**Mutation identification.** Primers were designed from the cDNA sequence to generate 250–300 bp fragments which overlapped 50–75 bp at each end. When PCR amplification of human genomic DNA yielded larger fragments than expected for the coding region alone, these fragments were presumed to contain introns and were sequenced directly (Cycle Sequencing kit, Gibco). Intronic primer sequences were obtained with this strategy. PCR products were analysed by a non-radioactive SSCP protocol as described. Fragments showing a shift on SSCP gels were subcloned into Bluescript and sequenced (Sequenase kit, USB). To confirm the sequence changes, a new PCR was performed with genomic DNA; the PCR product was digested with HindIII and analysed by polyacrylamide gel electrophoresis.

**Clinical material.** To determine the frequency of the A→V mutation, DNA from 57 individuals from Quebec was analysed by PCR and restriction digestion. The individuals, all French Canadian, were not examined clinically or biochemically. The 60 individuals analysed in Table 1 have been described. Of the 13 cardiovascular patients, eight had cerebrovascular arteriosclerosis and five had peripheral arteriosclerosis. Five had thermolabile MTHFR while eight had normal MTHFR activity. More importantly, however, the identification of a candidate genetic risk factor for vascular disease, which may be influenced by nutrient intake, represents a critical step in the design of appropriate therapies for the homocysteine-forming activity of atherosclerosis.

**Expression analysis.** Overnight cultures of M195 containing vector, wild-type or mutagenized MTHFR cDNA were grown at 37 °C. In 2 × YT media with 105 mg ml−1 ampicillin. Fresh 10 ml cultures of each vector alone (to subtract background formaldehyde/h/mg protein) were calculated for the 2 cDNA-containing constructs after subtraction of the values obtained with vector alone (to subtract background E.coli MTHFR activity).

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