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Molecular Genetic Analysis in Mild Hyperhomocysteinemia: A Common Mutation in the Methylenetetrahydrofolate Reductase Gene Is a Genetic Risk Factor for Cardiovascular Disease

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Summary

Mild hyperhomocysteinemia is an established risk factor for cardiovascular disease. Genetic aberrations in the cystathionine β-synthase (CBS) and methylenetetrahydrofolate reductase (MTHFR) genes may account for reduced enzyme activities and elevated plasma homocysteine levels. In 15 unrelated Dutch patients with homozygous CBS deficiency, we observed the 833T→C (I278T) mutation in 50% of the alleles. Very recently, we identified a common mutation (677C→T; A→V) in the MTHFR gene, which, in homozygous state, is responsible for the thermolabile phenotype and which is associated with decreased specific MTHFR activity and elevated homocysteine levels. We screened 60 cardiovascular patients and 111 controls for these two mutations, to determine whether these mutations are risk factors for premature cardiovascular disease. Heterozygosity for the 833T→C mutation in the CBS gene was observed in one individual of the control group but was absent in patients with premature cardiovascular disease. Homozygosity for the 677C→T mutation in the MTHFR gene was found in 9 (15%) of 60 cardiovascular patients and in only 6 (~5%) of 111 control individuals (odds ratio 3.1 [95% confidence interval 1.0–9.2]). Because of both the high prevalence of the 833T→C mutation among homozygotes for CBS deficiency and its absence in 60 cardiovascular patients, we may conclude that heterozygosity for CBS deficiency does not appear to be involved in premature cardiovascular disease. However, a frequent homozygous mutation in the MTHFR gene is associated with a threefold increase in risk for premature cardiovascular disease.

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Introduction

Over the past decade, mild hyperhomocysteinemia has been recognized as a risk factor for occlusive arterial disease and thrombosis (Wilcken and Wilcken 1976; Boers et al. 1985*b*; Kang et al. 1992; Malinow 1994; Den Heijer et al. 1995). Homocysteine levels are influenced by environmental (folate, vitamin B6, and vitamin B12 intake) as well as genetic factors (Boers et al. 1985*b*; Kang et al. 1992; Miller et al. 1992; Daly et al. 1993; Selhub et al. 1993; Guttormsen et al. 1994).

Classic homocystinuria, which is inherited as an autosomal recessive trait, is characterized by severely elevated concentrations of homocysteine and methionine in blood and urine and is caused by a genetic deficiency of cystathionine β-synthase (CBS) (Finkelstein et al. 1964). In the transsulfuration pathway, CBS catalyzes the condensation of homocysteine and serine to cystathionine. Life-threatening complications of CBS deficiency are premature arteriosclerosis and thrombosis (Boers 1986). To date, 17 mutations have been found in the CBS gene in homozygous CBS-deficient patients (Kraus 1994; Kluijtmans et al. 1995). Reduced activities in the range of obligate heterozygotes for CBS deficiency have been reported in vascular patients with mild hyperhomocysteinemia (Boers et al. 1985*b*; Clarke et al. 1991), suggesting a causal role for heterozygosity for CBS deficiency in premature vascular disease. However, Mudd et al. (1981) reported a normal incidence of heart attacks or strokes in a large group of obligate heterozygotes for CBS deficiency.

A less frequent form of severe hyperhomocysteinemia is methylenetetrahydrofolate reductase (MTHFR) deficiency. MTHFR is a regulating enzyme in folate-dependent homocysteine remethylation; it catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. Compared with controls, patients with severe hyperhomocysteinemia due to MTHFR deficiency show virtually no residual MTHFR activity in isolated lymphocytes and also have an in-

creased risk of arteriosclerosis and thrombosis (Rosenblatt 1989).

In 1988, a thermolabile variant of the MTHFR enzyme was described with a specific MTHFR activity <50% of the control value in lymphocytes and with decreased thermostability after inactivation at 46°C (Kang et al. 1988*a*, 1988*b*). On the basis of the biochemical phenotype evaluation of subjects with thermolabile MTHFR and of their family members, Kang et al. (1991) concluded that thermolability of MTHFR is inherited as an autosomal recessive trait. They reported a frequency of this thermolabile MTHFR enzyme of 17% in coronary artery disease and of 5% in controls. In another study, the same group demonstrated a positive association between the severity of coronary artery stenosis and the presence of thermolabile MTHFR (Kang et al. 1993). This association was independent of other known risk factors for coronary artery disease. In a previous study from our group, on patients with cardiovascular disease, we observed an incidence of a thermolabile MTHFR enzyme in 11 (~28%) of 39 hyperhomocysteinemic cardiovascular patients and in 1 (~5%) of 23 controls (Engbersen et al. 1995). All these studies indicate that thermolabile MTHFR may be a significant risk factor for hyperhomocysteinemia-related cardiovascular disease.

In an earlier work, we reported the isolation of the human cDNA for MTHFR and the assignment of the gene to chromosome 1p36.3 (Goyette et al. 1994). Genetic analysis in severe MTHFR deficiency revealed nine mutations (Goyette et al. 1994, 1995). Very recently, we observed a common 677C→T transition in the MTHFR-coding sequence, which changed a highly conserved alanine into a valine residue; this mutation introduced a *Hin*FI restriction site. Individuals who are homozygous for the mutation showed reduced specific MTHFR activity, increased thermolability, and elevated homocysteine concentrations (Frosst et al. 1995). *Escherichia coli* expression studies with a mutagenized MTHFR cDNA demonstrated that this mutation is responsible for the thermolabile phenotype (Frosst et al. 1995).

In the present study, we assessed whether the 833T→C transition in the CBS gene, observed in 50% of the alleles in Dutch homozygous CBS-deficient patients, was implicated in premature cardiovascular disease. Furthermore, we examined the prevalence of the homozygous 677C→T mutation in the MTHFR gene in patients with premature cardiovascular disease and in controls. Finally, we related genotype to biochemical phenotype at the level of homocysteine concentration and MTHFR activity.

Subjects and Methods

We studied 60 patients (age 13–68 years) with documented premature cardiovascular disease. Ten patients had suffered from myocardial infarction, 32 from cerebral arterial occlusive disease, and 18 from peripheral arterial occlusive disease. Cardiovascular disease had been diagnosed in these patients by use of standard methods and techniques as reported by us elsewhere (Boers et al. 1985*b*). Excluded from this study were patients with the following known risk factors for vascular disease: hyperlipoproteinemia (fasting serum levels of cholesterol >6.5 mmol/liter and triglycerides >2.0 mmol/liter), hypertension (systolic and diastolic blood pressure >150 and >90 mmHg, respectively), and diabetes mellitus (fasting plasma glucose levels >5.6 mmol/liter). No other exclusion criteria were employed. The study group did not consist of patients reported by us elsewhere (Engbersen et al. 1995). Five patients had reduced vitamin B12 concentrations (<150 pmol/liter), and two patients were folate deficient (<5.0 nmol/liter).

Control subjects (n = 111; age 23–75 years) were recruited from a general practice in The Hague (Den Heijer et al. 1995). All subjects agreed to participate in this study.

Fifteen unrelated patients with homocystinuria due to CBS deficiency were studied for the 833T→C transition. The diagnosis was established by severe hyperhomocysteinemia, hypermethioninemia, and decreased levels of cysteine in plasma. In addition, CBS activities in cultured fibroblasts were <1% of the control mean. Most patients showed responsiveness to pyridoxine in vivo. The study protocol had been approved by the hospital ethics committee.

Biochemical Analysis

Cardiovascular patients (n = 60) and controls (n = 111) were subjected to an oral methionine-loading test (0.1 g L-methionine/kg body wt) as described by Boers et al. (1985*b*). Total homocysteine concentrations (fasting and post methionine loading) were measured in EDTA plasma by high-performance liquid chromatography (HPLC) and fluorescence detection (Te Poele-Pothoff et al. 1995).

CBS activity was measured as described elsewhere (Fowler et al. 1978; Boers et al. 1985*a*) and is expressed as nanomoles of cystathionine formed per milligram protein per hour. Assays were performed without the addition of pyridoxal 5′-phosphate to the incubation mixture.

Specific and residual MTHFR activities in isolated lymphocytes were determined radiochemically as described extensively by Engbersen et al. (1995). Activities are expressed as nanomoles of formaldehyde formed per milligram protein per hour. Protein concentrations in fibroblast and lymphocyte extracts were determined as described by Lowry et al. (1951). Folic acid and vitamin B12 concentrations were determined in heparinized plasma, and vitamin B6 concentrations were determined

in whole blood, all by routine hospital assays (Steegers-Theunissen et al. 1994).

Mutation Analysis

DNA was extracted from peripheral lymphocytes as described elsewhere (Miller et al. 1988), and ~100 ng was used for PCR amplification. The PCR mixture for analysis of the 833T→C transition in the CBS gene consisted of 100 ng of the forward oligonucleotide (5′-GAAGCTGGACATGCTGGTGGC-3′; cDNA position 738–758; Kraus et al. 1993), 100 ng of the reverse oligonucleotide (5′-CGCACAGCAGCCCCTCTTG-3′; cDNA position 1039–1021), 200 μM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg gelatine/ml, 1 mg Triton X-100/ml, and 1 U *Taq* polymerase (Life Technologies), in a total volume of 100 μl. The cycle parameters were as follows: 5 min initial denaturation at 96°C, followed by 35 cycles of 1 min at 93°C, 1 min at 59°C and 2 min at 72°C. Final extension was performed at 72°C for 10 min. The 833T→C mutation introduces a *Bsr*I restriction site, so the PCR product was subjected to *Bsr*I restriction enzyme (New England Biolabs) analysis, after which the digestion fragments were resolved in a 6% polyacrylamide gel containing 5% glycerol.

For detection of the 677C→T transition in the MTHFR gene, PCR was performed by use of 100 ng forward and reverse primer (Frosst et al. 1995) in the same buffer as described above. An initial denaturation step was carried out for 5 min at 96°C, followed by 35 cycles of denaturation for 50 s at 93°C, primer annealing for 50 s at 55°C, and primer extension for 30 s at 72°C. A final extension step was performed for 7 min at 72°C. *Hin*FI restriction enzyme (Life Technologies) analysis and subsequent electrophoresis in a 3% agarose gel revealed the mutational status of the subject.

Statistics

Results are expressed as the mean ± SD. We calculated mean differences (MD) and 95% confidence intervals (95% CI) for homocysteine concentrations, specific MTHFR activity, and residual MTHFR activity, as estimates of statistical significance between different groups. Odds ratios and 95% CI were calculated (Morris and Gardner 1989) to estimate the relative risk of the homozygous mutation and of a homocysteine concentration (fasting and post methionine loading) that exceeded the 90th percentile of the control group. These odds ratios were adjusted for age and gender, by use of a logistic regression model. Correlation analyses were performed by Spearman's rank correlation test (r_s).

Results

Fasting and post-methionine-loading homocysteine concentrations were measured in 58 patients and in 111 controls. The mean homocysteine concentrations (fasting and post methionine loading) were higher in vascular patients than in controls (table 1). The 90th percentile of the fasting homocysteine concentration in the control group was 17.1 μmol/liter. In the patient group, 13 (22.4%) of 58 individuals exceeded this concentration, versus 11 individuals (10%, by definition) in the control group. The calculated crude odds ratio for fasting homocysteine concentrations >17.1 μmol/liter was 2.9 (95% CI 1.1–6.3). The 90th percentile of the post-methionine-loading homocysteine concentration in the control group was 56.5 μmol/liter. In the patient group, 12 (20.7%) of 58 exceeded this cutoff point, versus 10 individuals (10%) in the control group, which resulted in a crude odds ratio of 2.6 (95% CI 1.1–6.5). After adjustments for age and gender, we found odds ratios of 5.7 (95% CI 1.8–17.4) and 4.0 (95% CI 1.4–11.6] for the fasting and post-methionine-loading homocysteine concentrations, respectively.

To evaluate heterozygosity for CBS deficiency in cardiovascular disease, we first investigated the prevalence of the 833T→C transition in 15 Dutch homozygous CBS-deficient patients. This 833T→C transition accounted for 50% of the Dutch homocystinuric alleles. Next, we screened cardiovascular patients and controls for the same mutation. We failed to detect the 833T→C transition in the patient group (n = 60), whereas 1 heterozygote for this mutation was observed among the 111 control subjects.

In cardiovascular patients and in controls, we measured specific MTHFR activities in isolated lymphocytes, as well as the residual MTHFR activity after heat inactivation, as a percentage of the specific MTHFR activity. The observed mean specific MTHFR activity tended to be lower in cardiovascular patients versus controls (17.5 ± 7.1 vs. 19.1 ± 6.8 nmol CH_2O/mg protein/h; MD 1.6 [95% CI −0.9–4.1]). The residual MTHFR activity after heat inactivation showed a significant difference between patients and controls (53.3% ± 15.7% vs. 60.2% ± 12.1%; MD 6.9 [95% CI 2.1%–11.7%]). There was no correlation between age and specific MTHFR activity (data not shown).

The prevalence of the homozygous 677C→T mutation in the MTHFR gene was examined in patients with cardiovascular disease, as well as in control subjects. In the patient group, we observed an incidence of the homozygous transition (+/+ genotype) in 9 (15%) of 60 cases, which is significantly higher than 6 (5.4%) of 111 controls (table 2). The calculated odds ratio for the +/+ genotype was 3.1 (95% CI 1.0–9.2).

To study the correlation between genotype and biochemical phenotype, we divided all individuals into three distinct subgroups based on their MTHFR genotype (+/+, +/−, and −/−). Individuals with the +/+ genotype showed markedly elevated homocysteine, compared with

Table 1

Fasting and Post-Methionine-Loading Homocysteine Concentrations in Cardiovascular Disease Patients and Controls

	Cardiovascular Disease Patients	Controls
Fasting homocysteine[a] (μmol/liter)	14.1 ± 5.1 (n = 58)	12.5 ± 4.0 (n = 111)
Post-methione-loading homocysteine[b] (μmol/liter)	44.5 ± 18.6 (n = 58)	38.6 ± 13.0 (n = 110)

NOTE.—Results are expressed as mean ± SD.
[a] Mean difference 1.6 (95% CI 0.2–3.0).
[b] Mean difference 5.9 (95% CI 1.0–10.8).

individuals with the −/− genotype (table 3). A striking correlation was observed between genotype and specific MTHFR activity or residual MTHFR activity, with only a small overlap between the +/+ genotype and the two other genotypes (fig. 1). Patients and controls with the +/+ genotype showed a mean specific MTHFR activity of <50% of the mean specific MTHFR activity of the −/− group (7.8 ± 4.5 [n = 10] vs. 22.1 ± 6.2 [n = 81] nmol CH_2O/mg protein/h). The percentage of mean residual MTHFR activity in the +/+ group was significantly lower than that observed in the +/− and −/− groups (24.9% ± 10.6% [n = 10] vs. 53.6% ± 9.0% [n = 53] and 65.4% ± 8.1% [n = 81], respectively; fig. 1). The mean folic acid, vitamin B12, and vitamin B6 concentrations did not differ significantly between the three MTHFR genotypes (data not shown). In individuals with the +/+ genotype, a clear negative correlation was observed between fasting homocysteine and plasma folate ($r_s = -.94$; $P < .001$), contrary to the nonsignificant correlations in individuals with the +/− and −/− genotypes. Exclusion of a patient with folate deficiency and the +/+ genotype did not change the significance of this correlation. No correlations were found between fasting homocysteine and vitamin B12 or vitamin B6, for any of the three MTHFR genotypes (data not shown).

Discussion

In line with several previous studies (Boers et al. 1985*b*; Brattström et al. 1990; Clarke et al. 1991; Kang et al. 1992), we found elevated plasma homocysteine concentrations in cardiovascular disease patients compared with controls. In patients with cardiovascular disease and mildly elevated homocysteine concentrations, we (Boers et al. 1985*b*) and others (Clarke et al. 1991) have reported reduced CBS activities, within the range of obligate heterozygotes for CBS deficiency. In both studies, the enzymatic CBS analyses were performed in one and the same laboratory in Manchester (U.K.). Over the past 2 years, we have been unable to reproduce those previous findings and found normal CBS activities in cultured fibroblasts of 9 of 10 hyperhomocysteinemic vascular disease patients studied in our own laboratory (Engbersen et al. 1995). To date, we have studied 25 vascular disease patients with elevated homocysteine levels and have found normal CBS activities in 24 (96%) of these patients (H. J. Blom, G. H. J. Boers, and J. M. F. Trijbels, unpublished data). Because of the contradiction between previous studies and our present enzymatic findings, we examined this inconsistency by molecular genetic studies. Among 15 unrelated Dutch homozy-

Table 2

MTHFR Genotype Distribution among Cardiovascular Disease Patients and Control Groups

Genotype	Cardiovascular Disease Patients	Controls
+/+	15% (n = 9)	5.4% (n = 6)
+/−	35% (n = 21)	37.8% (n = 42)
−/−	50% (n = 30)	56.8% (n = 63)

Table 3

Relationship between Fasting and Post-Methionine-Loading Plasma Homocysteine Concentrations and MTHFR Genotype

	+/+ (n = 15)	+/− (n = 61)	−/− (n = 93)
Fasting homocysteine[a] (μmol/liter)	16.3 ± 8.3	13.4 ± 4.0	12.3 ± 3.6
Post-methionine-loading homocysteine[b] (μmol/liter)	49.8 ± 20.0	41.9 ± 18.0	38.4 ± 11.7[c]

NOTE.—Results are expressed as mean ± SD.
[a] Mean difference 2.9 (95% CI 0–5.8) for +/+ vs. +/− and 4.0 (95% CI 1.5–6.5) for +/+ vs. −/−.
[b] Mean difference 7.9 (95% CI −2.7–18.5) for +/+ vs. +/− and 11.4 (95% CI 4.2–18.6) for +/+ vs. −/−.
[c] n = 92 for this parameter.

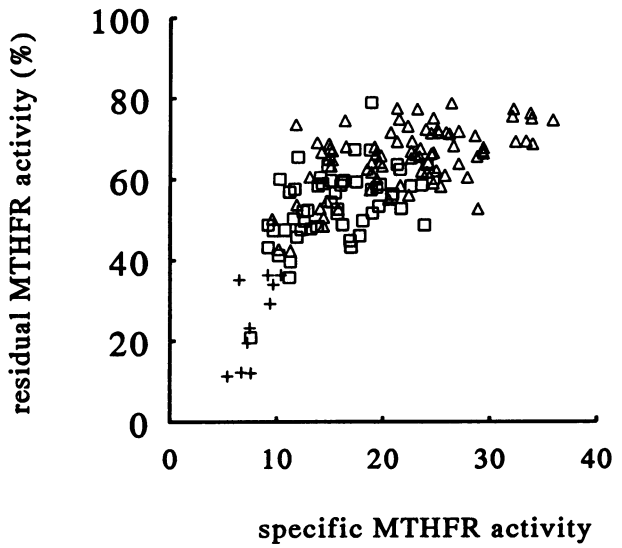

Figure 1 Residual MTHFR activity after heat inactivation, versus specific MTHFR activity in patients and controls with the three different genotypes. A plus sign (+) denotes individuals with the +/+ genotype; a square (□) denotes individuals with the +/− genotype; and a triangle (△) denotes individuals with −/− the genotype.

gotes for CBS deficiency, we observed the 833T→C transition in 50% of the alleles. We did not identify any 833T→C mutation in 60 cardiovascular disease patients, whereas in the controls (n = 111) the normal carrier frequency (~0.5%) (Boers et al. 1985*b*) was observed. This finding corroborates our enzymatic data and the recent results of Whitehead et al. (1994). They were unable to detect the 919G→A (G307S) mutation in the CBS gene, observed in 70% of Irish homocystinuric alleles, in a group of 100 Irish patients with premature vascular disease. Very recently, Kozich et al. (1995) were unable to detect mutations in the CBS cDNA in four hyperhomocysteinemic patients with peripheral occlusive arterial disease. Taken together, all enzymatic and molecular genetic studies do not provide any evidence that heterozygosity for CBS deficiency is a risk factor for cardiovascular disease.

We recently demonstrated that the 677C→T mutation in the MTHFR gene results in a thermolabile enzyme in vitro (Frosst et al. 1995). In that study and in the present report, a correlation was observed between MTHFR genotype and specific MTHFR activity in isolated lymphocytes. In the present study, low MTHFR activities were demonstrated in subjects with the +/+ genotype; intermediate activities occurred in the +/− genotype, while the highest MTHFR activities were observed in subjects with the −/− genotype. The genotype-phenotype correlation was even more striking when the residual MTHFR activity after heat inactivation was examined for the three genotypes. All subjects with the +/+ genotype showed a residual MTHFR activity, after heat inactivation, of <37%, with almost no overlap with the two other genotypes.

The mean homocysteine level was elevated in individuals homozygous for the mutation in the MTHFR gene, although fasting homocysteine concentrations were <17.1 μmol/liter (90th percentile of the control group) in 60% of the individuals with the +/+ genotype. Homocysteine concentrations are obviously determined not only by genetic factors but also by environmental parameters, especially nutritional factors. The homocysteine levels of subjects with decreased MTHFR activity might be more sensitive to low folate intake than those in other individuals. Plasma folate appeared to be strongly correlated to fasting homocysteine concentrations only in individuals with the +/+ genotype, in contrast with the situation in individuals with the +/− and −/− genotypes. This indicates that plasma folate is critical in homocysteine homeostasis in individuals with the +/+ genotype. Variations in dietary folate intake in these subjects could therefore lead to a diversity in homocysteine concentration.

Kang et al. (1988*a*, 1991) reported an incidence of thermolabile MTHFR of 17% among 212 patients with coronary artery disease. In the present study, we demonstrate that the homozygous 677C→T mutation, the cause of thermolabile MTHFR, is a risk factor for arteriosclerotic disease in general, including peripheral and cerebral arterial occlusive disease. From our results, an odds ratio of 3.1 could be calculated for the +/+ genotype, indicating that the mutation in the homozygous state may be an important risk factor for arteriosclerotic disease, comparable to hypercholesterolemia or cigarette smoking (Clarke et al. 1991).

In summary, we have no evidence that heterozygosity for CBS deficiency predisposes to premature cardiovascular disease. On the other hand, we show that a common mutation in the MTHFR gene, causing elevated homocysteine levels, is a genetic risk factor for premature cardiovascular disease.

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