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Mutation (677 C to T) in the methylenetetrahydrofolate reductase gene aggravates hyperhomocysteinemia in hemodialysis patients

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Mutation 677 C to T in the methylenetetrahydrofolate reductase gene aggravates hyperhomocysteinemia in hemodialysis patients. Hyperhomocysteinemia is frequent in hemodialysis patients and represents an independent risk factor for vascular disease in these patients. Elevated total homocysteine (tHcy) plasma levels can result from defective remethylation of Hcy to methionine due to decreased activity of the enzyme methylenetetrahydrofolate reductase (MTHFR). A genetic aberration in the MTHFR gene (677 C to T substitution) has been shown to result in reduced MTHFR activity. We tested the hypothesis that elevation of tHcy plasma levels in hemodialysis patients is influenced by the 677 C to T mutation of the MTHFR gene and examined the relation of the genotype with tHcy, folate and vitamin B_{12} plasma levels in these patients. The allelic frequency of the MTHFR mutation was evaluated in 203 patients maintained on chronic hemodialysis treatment. Total Hcy, folate, vitamin B_{12} levels and the MTHFR mutation were analyzed in 69 of the 203 patients and in 69 age- and sex-matched healthy control subjects. The allelic frequency of the 677 C to T transition in the MTHFR gene in hemodialysis patients was 34.7% versus 35.5% in healthy controls. Of 203 patients 26 (12.8%) were homozygous for the mutation (+/+) versus 10.2% in healthy subjects. The heterozygous (+/−) genotype was identified in 43.8% of patients versus 50.7% in controls. The mean tHcy level in hemodialysis patients was 28.7 ± 11.0 μmol/liter versus 10.0 ± 3.0 μmol/liter in control subjects. The mean tHcy levels were 36.4 ± 13.4 μmol/liter in (+/+) patients and 12.2 ± 4.5 μmol/liter in (+/+) controls, 28.7 ± 10.8 μmol/liter in (+/−) patients and 9.9 ± 2.7 μmol/liter in (+/−) controls and 25.4 ± 8.5 μmol/liter in (−/−) hemodialysis patients versus 9.7 ± 2.8 μmol/liter in (−/−) controls. There was no significant difference of folate and vitamin B_{12} concentrations in patients and controls with different MTHFR genotypes. Analysis of covariance including age, gender, folate concentrations, vitamin B_{12} levels, albumin and creatinine as covariables revealed a significant influence of the (+/+) genotype, albumin and folate status on tHcy levels in hemodialysis patients. Together, our data demonstrate that the extent of hyperhomocysteinemia in hemodialysis patients is not only the result of uremia or folate status, but is also genetically determined by the (+/+) MTHFR genotype. The presence of the 677 C to T mutation in the MTHFR gene does not appear to represent a risk factor for development of end-stage renal disease.

Homocysteine (Hcy), a sulfur containing amino acid, is the demethylated derivate of the essential amino acid methionine and is formed during protein metabolism by the adenosylmethionine-dependent conversion of methionine to cysteine [1]. Low intracellular concentrations of this potentially cytotoxic sulfur amino acid are maintained by remethylation to methionine or transsulfuration to cysteine. During transsulfuration the enzyme cystathionine-β-synthase (CBS) catalyzes the irreversibel condensation of Hcy and serine to cystathionine, using vitamin B_6 as a cofactor, and contributes to the maintenance of normal postprandial Hcy concentrations [2]. Remethylation to methionine can be accomplished by two pathways. In one pathway, the methyl donor for the conversion of Hcy to methionine is provided by the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR). 5-methyltetrahydrofolate, the active form of folate, then acts as the methyl donor for the vitamin B_{12} dependent remethylation of Hcy to methionine by the enzyme 5-methyltetrahydrofolate-homocysteine methyltransferase (methionine synthetase). Alternatively, betaine can serve as methyl donor to allow formation of methionine by the enzyme betaine-homocysteine methyltransferase. The remethylation enzymes serve to maintain normal fasting Hcy concentrations [2].

Hyperhomocysteinemia can be classified into moderate, intermediate and severe forms that are defined as basal plasma Hcy concentrations between 16 and 30, between 31 and 100, and above 100 nmol/ml, respectively [3]. Elevated Hcy levels have been reported to be associated with an increased risk for cerebrovascular, peripheral vascular, coronary artery disease and for deep vein thrombosis in patients without renal failure [4–6]. In end-stage renal disease patients who are on maintenance hemodialysis, increased plasma Hcy concentrations are common and have been shown to represent an independent risk factor for vascular disease [7–16].

The adverse vascular effects of Hcy leading to atherothrombosis have been intensively studied. In animals, administration of Hcy thiolactone caused patchy desquamation of the vascular endothelium, followed by increased platelet consumption and thrombosis [17, 18]. The endothelial toxicity of Hcy is attributed to the sulfhydryl group that generates hydrogen peroxides, raising the possibility of an involvement of free radicals [19]. It has been shown that short-term exposition of endothelial cells (EC) to Hcy stimulates the production of nitric oxide, which is able to protect EC from Hcy-induced injury by formation of S-nitrosothiol [20,

Key words: gene mutation, hyperhomocysteinemia, MTHFR gene defect, uremia, amino acid, folate, vitamin B_{12}, hemodialysis.

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21]. With prolonged Hcy exposure, however, the protective mechanism is lost because of toxic effects of Hcy generated hydrogen peroxides. Consequently, accumulation of platelets occurs at the sites of vascular injury. Further mechanisms that are supposed to underly the accelerated atherogenesis in homocysteinemia are the interference with natural anticoagulants and the fibrinolytic system [22, 23], the initiation of vascular smooth muscle cell proliferation [24], and oxidation of low-density lipoproteins [25].

Elevated plasma Hcy levels can result from genetic defects of enzymes, necessary for either remethylation or transsulfuration of Hcy, such as severe MTHFR deficiency [26], inherited thermolability of MTHFR [27] and CBS deficiency [1] or nongenetic, acquired factors, such as vitamin B_{12} [28], vitamin B_6 [29] and folate deficiency [30], and can be lowered by vitamin intervention therapy [31].

Recently, a C to T mutation at nucleotide position 677 of the MTHFR coding sequence leading to an exchange of a highly conserved alanine to a valine residue was identified [32]. The allelic frequency of this substitution was 38% in 57 French Canadian individuals without renal disease. The presence of the mutation was shown to correlate with a reduced specific MTHFR activity and increased enzyme thermolability in lymphocyte extracts. Furthermore, homozygous individuals had significantly elevated plasma total Hcy (tHcy) levels, almost twice the values obtained in heterozygotes and non-carriers. The tHcy concentrations in heterozygous subjects and individuals without mutation were indistinguishable.

The allelic frequency of the 677 C to T MTHFR mutation and the impact of the mutation on hyperhomocysteinemia in hemodialysis patients is unknown. To investigate the hypothesis that tHcy plasma levels in these patients are influenced by the presence of the MTHFR mutation, we evaluated the frequency of the MTHFR gene defect in a cohort of 203 patients on maintenance hemodialysis, and examined whether or not the genotype is related to tHcy, folate and vitamin B_{12} levels in a large subset of these patients.

METHODS

Study population

To determine the allelic frequency of the 677 C to T transition in the MTHFR gene, 203 patients maintained on chronic hemodialysis treatment at the three dialysis units of the University Hospital of Vienna from December 1994 to July 1996 were investigated. The mean age of the patients (88 females, 115 males) was 56.9 ± 15.5 years. Dialysis treatment was initiated because of end-stage renal disease due to secondary kidney graft failure (N = 41), shrunken kidneys of unknown etiology (N = 40), glomerulonephritis (N = 31), diabetic nephropathy (N = 26), chronic interstitial nephritis (N = 15), urogenital malformations or malignancies (N = 14), polycystic kidney disease (N = 13), analgesic nephropathy (N = 10), nephrosclerosis (N = 8), and miscellaneous nephropathies (N = 5). The mean duration of hemodialysis treatment was 2.2 ± 2.4 years. All patients received a vitamin supplementation including 0.16 mg of folate and 10 mg of vitamin B_6 daily.

To find out whether tHcy plasma levels are influenced by the C677T mutation in the MTHFR gene, the MTHFR genotype, plasma tHcy, folate and vitamin B_{12} concentrations, creatinine and albumin levels were analyzed in 69 of the 203 hemodialysis patients (29 females, 40 men, mean age 54.4 ± 15.5 years), who were on dialysis treatment at one of the three dialysis units. Sixty-nine age- and sex-matched healthy subjects without renal disease or clinical signs of coronary artery disease, cerebrovascular or peripheral vascular disorders served as controls.

Biochemical assays

Citrated blood was collected after an overnight fast from 69 hemodialysis patients of one dialysis unit prior to dialysis and from the 69 age- and sex-adjusted controls. Samples were immediately placed on ice and centrifuged within 30 minutes at 2000 g at 4°C (20 min). Plasma aliquots were stored at −70°C.

Total (that is, free plus protein-bound) plasma Hcy concentrations were determined by automated high-performance liquid chromatography (HPLC) with reverse-phase separation and fluorescent detection using a commercially available kit (Immundiagnostik, Bensheim, Germany) according to the method originally described by Araki and Sako [33]. Hyperhomocysteinemia was defined as tHcy levels above 15 μmol/liter. This cut off was determined from the mean tHcy level (9.7 ± 2.8 μmol/liter) plus 2 SDs in control subjects who were negative for the 677 C to T mutation in the MTHFR gene.

Plasma folate (5-methyltetrahydrofolate) and vitamin B_{12} levels were measured with a radioassay, which allowed simultanous determination of both vitamin concentrations in a single reaction tube (SimulTRAC-SNB; Becton Dickinson, Ontario, Canada). Folate deficiency was defined as plasma concentration of less than 3.4 nmol/liter. Vitamin B_{12} deficiency was defined as a concentration of less than 118 pmol/liter, respectively.

PCR analysis of MTHFR gene

Identification of the 677 C to T transition in the MTHFR gene was performed as previously described [32]. In brief, 500 μl of citrated blood was frozen twice at −70°C. Cellular DNA was obtained by thawing and boiling for 10 minutes, followed by centrifugation at 12,000 g (10 min). The supernatants were collected and 5 μl of a 1:10 dilution were used in a 50 μl PCR reaction containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 1.25 mM $MgCl_2$, 0.2 mM of each nucleoside triphosphate, 30 pmol of each primer and 1.25 units of AmpliTaq DNA Polymerase (Perkin Elmer Cetus, Norwalk, CT, USA). PCR was performed using the primers described by Frosst et al: 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′ and 5′-AGGACG-GTGCGGTGAGAGTG-3′ [32]. Thermocycling conditions consisted of 40 cycles of denaturation at 95°C for one minute, annealing at 60°C for one minute and extension at 72°C for one minute, preceded by an initial denaturation step at 95°C for three minutes and followed by a terminal extension of five minutes at 72°C. Two microliters of the 198-bp PCR product were subjected to *Hinf* 1 digestion (0.5 units enzyme in a 20 μl digest). The presence of a mutation creates a *Hinf* 1 recognition sequence that leads to a digestion of the 198 bp PCR product into fragments of 175 bp and 23 bp, respectively. Heterozygous subjects show three fragments (198 bp, 175 bp and 23 bp), and a homozygous C to T substitution results in the production of two fragments of 175 bp and 23 bp. *Hinf* 1 digests of PCR amplification products were analyzed by electrophoresis through 6% polyacrylamide gels (Novex, San Diego, CA, USA) followed by ethidium bromide staining.

Table 1. Genotype and allelic frequency of the (C677T) MTHFR mutation in hemodialysis patients and control subjects

MTHFR (C677T) genotype	Hemodialysis patients $N = 203$	Hemodialysis patients $N = 69$	Control subjects $N = 69$
(+/+)	26 (12.8%)	12 (17.4%)	7 (10.2%)
(+/−)	89 (43.8%)	29 (42.0%)	35 (50.7%)
(−/−)	88 (43.4%)	28 (40.6%)	27 (39.1%)
Allelic frequency	34.7%	38.4%	35.5%

There was no significant difference of the MTHFR (C677T) genotype or allelic frequency between hemodialysis patients ($N = 203$), control subjects ($N = 69$) and the subgroup of hemodialysis patients ($N = 69$) in whom tHcy plasma levels were determined.

Statistical analyses

Comparison of the frequency of the different MTHFR genotypes in hemodialysis patients and the control group was performed by chi square test. For analysis of covariance, tHcy plasma levels were transformed (natural logarithm, ln). Descriptive statistics included means ± SD, geometric means, medians, full ranges and 10th to 90th percentile ranges.

Separate comparisons of the tHcy plasma levels between the three groups of (−/−) subjects with (+/−) and (+/+) subjects were performed by analysis of covariance in both the patient and the control group. The covariables were folate, vitamin B_{12}, age, gender, albumin and creatinine for hemodialysis patients, and folate, vitamin B_{12}, age and gender for healthy subjects. For assessment of the interaction of suboptimal folate status and MTHFR genotype on tHcy plasma levels, a less than median plasma folate (yes/no) term was included as covariable. Individual comparisons between the groups were conducted by post hoc analysis (Scheffe's test). All calculations were performed by the statistical software package SAS (SAS Institute Inc., Cary, NC, USA).

RESULTS

MTHFR genotype

The allelic frequency of the 677 C to T transition in the MTHFR gene in hemodialysis patients was 34.7% versus 35.5% in healthy controls. Twenty-six of 203 patients (12.8%) showed a homozygous (+/+) mutation versus 7 of 69 healthy subjects (10.2%). The heterozygous (+/−) transition was identified in 89 of 203 patients (43.8%) versus 35 of 69 controls (50.7%). The mutation was not present in 88 of 203 (−/−) patients (43.4%) and in 27 of 69 (−/−) control subjects (39.1%). In the 69 hemodialysis patients of one hemodialysis unit in whom tHcy plasma levels had been analyzed, the (+/+) genotype was identified in 12 of 69 patients (17.4%), which was not significantly different compared with the prevalence of 12.8% of the whole study population of 203 patients. In 29 of these 69 hemodialysis patients the (+/−) genotype was found (42.0%), and 28 of 69 patients (40.6%) were negative for the MTHFR mutation (Table 1).

Total plasma homocysteine levels

The MTHFR genotype, fasting tHcy levels, folate and vitamin B_{12} concentrations were determined in 69 hemodialysis patients and in 69 age- and sex-matched control subjects. The mean tHcy level in hemodialysis patients was 28.7 ± 11.0 μmol/liter versus 10.0 ± 3.0 μmol/liter in control subjects. In (+/+) patients the mean tHcy level was 36.4 ± 13.4 μmol/liter and 12.2 ± 4.5 μmol/liter in (+/+) controls. In (+/−) patients the mean tHcy concentration was 28.7 ± 10.8 μmol/liter versus 9.9 ± 2.7 μmol/liter in (+/−) controls, and in (−/−) hemodialysis patients the mean tHcy level was 25.4 ± 8.5 μmol/liter versus 9.7 ± 2.8 μmol/liter in (−/−) controls (Fig. 1). Only 5 of 69 patients (7.3%) had tHcy levels below the cut off point of 15 μmol/liter, in contrast to 63 of 69 healthy subjects (91.3%). None of these 5 patients was homozygous for the MTHFR gene variation [2 patients were (+/−) and 3 patients (−/−), respectively], whereas in the control group 5 of 63 (7.9%) subjects with tHcy levels < 15 μmol/liter were identified as homozygous carriers of the MTHFR mutation.

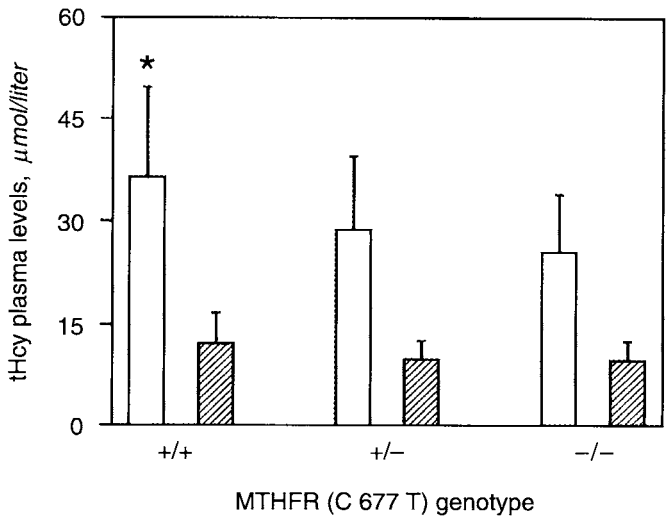

Fig. 1. Plasma tHcy levels (mean ± SD) in 69 hemodialysis patients (□) and 69 age- and sex-matched healthy subjects (▨) with different MTHFR genotypes. *$P < 0.05$, for comparison of (+/+) versus (−/−) hemodialysis patients.

There was a significant influence of folate and serum albumin levels on tHcy levels in hemodialysis patients (F test, $P = 0.039$ and $P = 0.041$). Post hoc analysis revealed a significant influence of the (+/+) C677T MTHFR-gene mutation on tHcy plasma levels compared with non-carriers (Scheffe's test, $P < 0.05$). The geometric mean tHcy level in (+/+) patients was 27.4% higher than tHcy level of the (+/−) patients and 41.3% higher than in patients without mutation (Table 2). An increase of serum albumin levels of 1 g/liter resulted in an estimated increase of ln tHcy plasma levels of 0.027 μmol/liter. The other covariables (vitamin B_{12}, creatinine, age and sex) had no significant influence on tHcy levels.

If a 'folate below/above median' term was included, a significant influence of low folate levels (≤ sample median of 23.1 nmol/liter) on tHcy levels was obtained in all 69 hemodialysis patients (F test, $P = 0.049$). Post hoc analysis in this model again showed a significant influence of the (+/+) MTHFR genotype on tHcy plasma levels compared with (−/−) patients (Scheffe's test, $P < 0.05$).

Separate analysis of patients with folate levels below the sample median ($N = 34$) revealed a significant trend for an influence of the MTHFR genotype on tHcy plasma levels (F* test, $P = 0.07$; Scheffe's test for comparison of (+/+) versus (−/−) genotype, $P < 0.05$). There was no influence of the MTHFR genotype on

Table 2. MTHFR (C677T) genotype, tHcy, folate and vitamin B_{12} plasma levels in 69 hemodialysis patients and 69 age- and sex-matched controls

MTHFR genotype	Number of subjects	mean ± SD		Geometric mean	Median	Range	10th Percentile	90th Percentile
tHcy plasma levels *μmol/liter*								
(+/+)	12 (7)	36.4 ± 13.4	(12.2 ± 4.5)	33.9 (11.4)	35.0 (14.4)	45.2 (11.1)	16.3 (6.8)	52.4 (17.9)
(+/−)	29 (35)	28.7 ± 10.8	(9.9 ± 2.7)	26.6 (9.5)	27.2 (9.7)	49.6 (10.1)	11.6 (6.3)	43.4 (13.6)
(−/−)	28 (27)	25.4 ± 8.5	(9.7 ± 2.8)	24.0 (9.3)	24.4 (8.9)	32.9 (9.5)	15.3 (6.8)	37.7 (14.9)
Total	69 (69)	28.7 ± 11.0	(10.0 ± 3.0)	26.6 (9.6)	27.6 (9.4)	50.0 (12.3)	15.3 (6.8)	44.3 (14.9)
Folate plasma levels *nmol/liter*								
(+/+)	12 (7)	25.1 ± 16.7	(14.2 ± 7.0	22.1 (12.8)	22.6 (12.2)	63.7 (19.4)	12.4 (6.2)	27.9 (25.6)
(+/−)	29 (35)	26.1 ± 15.8	(15.5 ± 6.2)	22.8 (14.5)	23.0 (13.3)	73.4 (25.7)	13.0 (10.5)	46.9 (26.2)
(−/−)	28 (27)	27.7 ± 12.5	(18.8 ± 7.9)	25.2 (17.5)	25.6 (15.3)	48.2 (29.7)	15.1 (11.5)	47.8 (33.2)
Total	69 (69)	26.6 ± 14.6	(16.7 ± 7.1)	23.6 (15.4)	23.1 (14.2)	73.4 (33.1)	13.6 (10.0)	46.9 (27.4)
Vitamin B_{12} plasma levels *pmol/liter*								
(+/+)	12 (7)	230.5 ± 103.5	(174.5 ± 72.1)	213.3 (162.5)	208.2 (150.6)	366.2 (220.6)	140.1 (86.4)	344.8 (307.0)
(+/−)	29 (35)	293.0 ± 157.5	(235.2 ± 104.0)	255.1 (216.3)	253.3 (222.0)	509.5 (508.3)	114.3 (121.3)	571.2 (350.0)
(−/−)	28 (27)	280.1 ± 134.6	(223.0 ± 86.6)	252.4 (208.2)	250.3 (209.8)	518.8 (404.5)	135.0 (135.3)	491.7 (354.0)
Total	69 (69)	276.9 ± 140.2	(224.2 ± 95.1)	246.2 (207.0)	242.8 (209.8)	524.4 (528.7)	126.6 (122.0)	491.7 (350.0)

Data are presented as hemodialysis patients' (control) levels.

tHcy levels in 35 patients with folate levels above the sample median (F-test, $P = 0.43$; Scheffe's test, NS).

In healthy subjects there was a slight, but not significant difference in tHcy plasma levels between the three groups with different MTHFR genotypes (F test, $P = 0.29$; Scheffe's test, NS). Folate concentration, age and sex had a significant influence on tHcy level (F test, $P = 0.029$, $P = 0.039$ and $P = 0.003$, respectively). An increase of age of one year resulted in an estimated increase of the ln tHcy plasma level of 0.004 μmol/liter. In male subjects the estimated elevation of the natural logarithm (ln) tHcy plasma level was 0.204 μmol/liter compared with female subjects. No influence of the vitamin B_{12} concentration on tHcy plasma levels was detectable. Inclusion of a 'folate below/above median' term revealed a significant influence of folate levels below the sample median (< 14.2 nmol/liter) on tHcy plasma levels (F test, $P = 0.040$).

Separate analysis of healthy subjects with folate levels below the sample median ($N = 34$) revealed no influence of the MTHFR genotype on tHcy plasma levels [F* test, $P = 0.12$; Scheffe's test for comparison of (+/+) versus (−/−) genotypes, NS]. No influence of the MTHFR genotype on tHcy levels in 35 healthy subjects with folate levels above the sample median was observed (F-test, $P = 0.29$; Scheffe's test, NS). In this model, age was the only covariable with significant influence on tHcy levels (F-test, $P = 0.0061$) if high folate levels were present. Geometric and arithmetic means of tHcy levels in healthy subjects are depicted in Table 2.

Vitamin concentrations

The mean folate concentration was 26.6 ± 14.6 nmol/liter (normal range 3.4 to 38 nmol/liter) in hemodialysis patients versus 16.7 ± 7.1 nmol/liter in controls. In (+/+) patients the mean folate level was 25.1 ± 16.7 nmol/liter and 14.2 ± 7.0 nmol/liter in (+/+) controls. In (+/−) patients the mean folate concentration was 26.1 ± 15.8 nmol/liter and 15.5 ± 6.2 nmol/liter in (+/−) controls, and in (−/−) dialysis patients the mean folate level was 27.7 ± 12.5 nmol/liter and 18.8 ± 7.9 nmol/liter in (−/−) controls. The mean vitamin B_{12} concentration was 276.9 ± 140.2 pmol/liter in the patient group (normal range 118 to 720 pmol/liter) and 224.2 ± 95.1 pmol/liter in healthy subjects. The mean B_{12} level in (+/+) patients was 230.5 ± 103.5 pmol/liter and 174.5 ± 72.1 pmol/liter in (+/+) controls. In (+/−) patients the mean B_{12} concentration was 293.0 ± 157.5 pmol/liter and 235.2 ± 104.0 pmol/liter in (+/−) controls, and in (−/−) patients the mean B_{12} level was 280.1 ± 134.6 pmol/liter and 223.0 ± 86.6 pmol/liter in (−/−) controls. The folate and vitamin B_{12} concentrations did not differ significantly between the different MTHFR genotypes in the patient and the control groups. The mean vitamin and tHcy levels of the patients and the controls with different MTHFR genotypes are summarized in Table 2.

Not one of the patients or the healthy controls had a folate deficiency. Deficiency of vitamin B_{12} was identified in 4 of 69 patients (5.8%) and in 4 of 69 control subjects (5.8%). The mean vitamin B_{12} level in B_{12} deficient patients was 111.1 ± 3.2 pmol/liter and 96.0 ± 14.8 pmol/liter in B_{12} deficient control subjects. The mean tHcy level in this patient group was 37.3 ± 15.7 μmol/liter and 11.6 ± 2.5 μmol/liter in the control individuals. One patient and one control showed the wild-type MTHFR genotype. Three patients and two controls were identified as heterozygous carriers and one control had a homozygous C677T mutation in the MTHFR gene.

DISCUSSION

The present study provides evidence that the extent of hyperhomocysteinemia in hemodialysis patients is not only due to the impairment of renal function or folate status, but is also genetically determined. We demonstrate that tHcy plasma levels in these patients are influenced by a genetic defect localized in the MTHFR gene. In patients with a homozygous 677 C to T MTHFR mutation the mean tHcy plasma level was 36.4 ± 13.4 μmol/liter, which was significantly higher compared to tHcy concentrations of patients without mutation (25.4 ± 8.5 μmol/liter), and was about thrice the mean normal value of 9.7 ± 2.8 μmol/liter of the age- and sex-matched healthy (−/−) subjects.

In the 406 chromosomes of our 203 hemodialysis patients we found an allelic frequency of 34.7% for the 677 C to T transition in the MTHFR gene that was almost identical with the frequency of 35.5% in the control group. The frequency of the homozygous mutation in this Austrian cohort of hemodialysis patients was 12.8%, which corresponds with the frequency described in a mixed

population of patients who were either maintained on peritoneal or hemodialysis treatment [34]. The frequency of 10.2% in the healthy (+/+) control subjects is in line with the observation in Canadian individuals and in populations of European, Middle Eastern and Japanese origin [32, 35, 36]. In contrast, the frequency described in Finnish (5.4%), Dutch (5.2%) and African American (1.4%) population groups was significantly lower [35, 37], suggesting ethnic variations.

The enzyme MTHFR represents an important cofactor involved in the Hcy remethylation pathway. Decreased MTHFR activity results in inadequate production of 5-methyltetrahydrofolate, required for the remethylation of Hcy to methionine, with the consequence of elevated plasma Hcy concentrations [26, 27]. Frosst and collegues [32] recently demonstrated that the homozygous and the heterozygous 677 C to T mutation in the MTHFR gene represent the causative genetic defect associated with a thermolabile MTHFR phenotype originally described by Kang and co-workers [27]. The thermolabile mutant leads to elevated Hcy levels due to decreased MTHFR activity. In homozygous subjects the presence of mutant MTHFR was associated with elevated tHcy levels of 22.4 ± 2.9 μmol/liter that were almost twice the values for (−/−) individuals [32]. Homocysteine concentrations did not differ between heterozygotes and non-carriers. In our control group the mean tHcy plasma level in (+/+) subjects was 12.2 ± 4.5 μmol/liter, which did not differ significantly from the tHcy levels of (+/−) or (−/−) individuals. Folate status, age and sex significantly influenced tHcy levels, whereas the MTHFR genotype had no influence even in healthy subjects with folate levels below the sample median.

Hyperhomocysteinemia is a well established independent risk factor for development of vascular disease [4–6]. Elevated Hcy concentrations resulting from the homozygous thermolabile MTHFR mutant have been shown to be associated with coronary artery disease, myocardial infarction, cerebral artery disease, peripheral artery disease and venous or arterial thrombosis in individuals without renal failure [37–41]. This was initially diagnosed by biochemical methods [38, 39] and recently on the basis of molecular genetic analysis [37, 40, 41]. Atherothrombotic complications are the leading cause of death in maintenance hemodialysis patients [42, 43]. The age-specific cardiovascular disease risk of mortality is at least 3.5 to 10 times higher in the dialysis population than among individuals without dialysis treatment [44–46]. However, atherothrombotic cardiovascular disease in end-stage renal disease patients cannot be completely explained by conventional risk factors such as hypertension, hypercholesterolemia, obesitas, age and smoking. Hyperhomocysteinemia is commonly observed in end-stage renal disease. It was first reported in hemodialysis patients in 1980 by Wilcken, Gupta and Reddy [7] and later confirmed by other groups [8–15]. Elevated Hcy levels in hemodialysis patients have been shown to be associated with an increased frequency of atherosclerotic vascular disease [11, 13–16]. In our study the mean plasma tHcy concentration in predialysis probes of chronic hemodialysis patients was 28.7 ± 11.0 μmol/liter, which is similar to the tHcy levels reported previously [10, 12, 13, 15, 16, 47].

The pathogenesis leading to elevated Hcy concentrations in hemodialysis patients is currently unclear. Increased Hcy concentrations are already found at the incipient stage of chronic renal failure, and hyperhomocysteinemia progresses in parallel with the decline of renal function [7, 10, 11, 48–50]. There is evidence in the literature that the kidney may play a substantial role in Hcy metabolism. About 30% of plasma Hcy is not protein-bound (free fraction, consisting of a mixture of cysteine-Hcy, Hcy and homocystine) and can freely pass the glomerular basement membrane with a daily filtration rate of about 550 μmol [51, 52]. More than 99.5% of Hcy is reabsorbed from the ultrafiltrate by the renal tubule and is suggested to be converted into metabolites of the transsulfuration pathway (mainly cystathionine), as was demonstrated in isolated rat renal cortical tubules [53]. Alternatively, remethylation by the enzyme betaine-Hcy methyltransferase [54] or 5-methyltetrahydrofolate-Hcy methyltransferase [55], which have been identified in human kidneys, might be involved. Based on the assumption that the human kidney might be important for Hcy metabolism it was speculated that in the case of decreased glomerular filtration, Hcy might not reach the renal metabolic site, the tubule cell, and may not be degraded because of tubular cell dysfunction in end-stage renal disease. Uremic toxins may also contribute to hyperhomocysteinemia by inhibition of Hcy metabolism in various organs [56]. However, a recent study by Perna and collegues [57] suggests that the high Hcy concentrations found in uremia result in accumulation of highly toxic intracellular S-adenosyl-Hcy, an inhibitor of methyltransferases that is normally eliminated by hydrolysis to Hcy.

It is well established that plasma Hcy levels are also influenced by the nutritional status and renal function. In our study, albumin levels had a significant influence on tHcy plasma levels in hemodialysis patients. This observation is in line with the findings of Hultberg, Andersson and Sterner in chronic renal failure patients [10]. In contrast to Bostom and co-workers, who studied a dialysis patient group consisting of hemodialysis and peritoneal dialysis patients [58], we did not find a significant interaction of serum creatinine with tHcy levels in our patient population. It has been shown that plasma tHcy concentrations are amplified by low serum folate and vitamin B_{12} levels [28, 30], and even marginal deficiency of folate can result in moderate hyperhomocysteinemia [30, 58, 59]. In our study folate and vitamin B_{12} levels were generally higher in hemodialysis patients than in controls probably due to supplementation of 0.16 mg of daily folate and nutritional factors. In homozygous patients the mean folate concentration was 25.1 ± 16.7 nmol/liter, which is within the upper normal range. The mean vitamin B_{12} level of 230.5 ± 103.5 pmol/liter in homozygous hemodialysis patients was clearly above the lower normal value of 118 pmol/liter. Therefore, we can exclude vitamin B_{12} and folate deficiency as a cause of high tHcy levels in homozygous dialysis patients. In heterozygous individuals and in subjects with the wild-type MTHFR genotype, vitamin B_{12} deficiency was identified in 5.8% of patients showing a mean tHcy level of 37.3 ± 15.7 μmol/liter and in 5.8% of the control group exhibiting a mean tHcy level of 11.6 ± 2.5 μmol/liter. These data suggest a trend to higher tHcy concentrations in B_{12} deficient hemodialysis patients and in healthy individuals.

Recent studies suggest that the homozygous carrier status for the 677 C to T MTHFR mutation only affects tHcy plasma levels, if suboptimal folate concentrations are present [59, 60]. In homozygous subjects without renal failure fasting tHcy concentrations were 24% greater among individuals with plasma folate concentration below the sample median (< 15.4 nmol/liter) than in subjects without mutation [60]. No difference in Hcy levels was observed between genotypes in individuals with folate levels > 15.4 nmol/liter. Similar observations have been described in

patients with myocardial infarction [36] and in a mixed population of dialysis patients who were either on peritoneal or hemodialysis treatment [58]. In the present study we found no significant influence of the (+/+) MTHFR genotype on tHcy levels in the control subjects whether or not suboptimal folate concentrations (< sample median of 14.2 nmol/liter) were present. In contrast, in hemodialysis patients a significant influence of homozygosity for the MTHFR polymorphism on tHcy plasma levels was present if folate levels were below the sample median.

Numerous investigators have demonstrated that plasma Hcy levels can be lowered by vitamin intervention therapy in patients with and without renal failure [12, 31, 47, 61–63]. Bostom and collegues observed a trend to normalization of tHcy levels in 5 of 15 hemodialysis patients by administration of 15 mg of folate, 100 mg of vitamin B_6 and 1 mg of vitamin B_{12} in addition to a routine vitamin treatment schedule [47]. However, in 10 of 15 patients studied, Hcy concentrations did not return to normal values raising the possibility of an influence of genetic determinants on therapeutic efficacy in hyperhomocysteinemia.

In summary, our data provide evidence that severity of hyperhomocysteinemia in hemodialysis patients is not only the result of uremia, folate status and albumin levels, but is also genetically determined. We found significanly higher tHcy levels in hemodialysis patients with a homozygous 677 C to T MTHFR gene mutation than in non-carriers. The allelic frequency of the 677 C to T transition in a large cohort of Austrian hemodialysis patients was similar to the allelic frequency in healthy subjects. Therefore, the presence of the 677 C to T mutation in the MTHFR gene does not appear to be a risk factor for development of end-stage renal disease. Further studies are required to determine the prevalence of vascular disease in patients with different MTHFR genotypes as well as the impact of the MTHFR genotypes on treatment efficacy of hyperhomocysteinemia in hemodialysis patients.

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