The Molecular Basis of Cystathionine β-Synthase Deficiency in Dutch Patients with Homocystinuria: Effect of CBS Genotype on Biochemical and Clinical Phenotype and on Response to Treatment

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Summary

Homocystinuria due to cystathionine β-synthase (CBS) deficiency, inherited as an autosomal recessive trait, is the most prevalent inborn error of methionine metabolism. Its diverse clinical expression may include ectopia lentis, skeletal abnormalities, mental retardation, and premature arteriosclerosis and thrombosis. This variability is likely caused by considerable genetic heterogeneity. We investigated the molecular basis of CBS deficiency in 29 Dutch patients from 21 unrelated pedigrees and studied the possibility of a genotype-phenotype relationship with regard to biochemical and clinical expression and response to homocysteine-lowering treatment. Clinical symptoms and biochemical parameters were recorded at diagnosis and during long-term follow-up. Of 10 different mutations detected in the CBS gene, 833T→C (I278T) was predominant, present in 23 (55%) of 42 independent alleles. At diagnosis, homozygotes for this mutation ($n = 12$) tended to have higher homocysteine levels than those seen in patients with other genotypes ($n = 17$), but similar clinical manifestations. During follow-up, I278T homozygotes responded more efficiently to homocysteine-lowering treatment. After 378 patient-years of treatment, only 2 vascular events were recorded; without treatment, at least 30 would have been expected ($P < .01$). This intervention in Dutch patients significantly reduces the risk of cardiovascular disease and other sequelae of classical homocystinuria syndrome.

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Introduction

Homocystinuria due to cystathionine β-synthase (CBS; L-serine hydrolyase [adding homocysteine]) deficiency (MIM 236200) is the most common inborn error in methionine metabolism. CBS is a pyridoxal 5′-phosphate (PLP)–dependent enzyme and condenses homocysteine and serine to cystathionine, an irreversible step in transsulfuration (Mudd et al. 1995).

Carson and Neill (1962) first described homocystinuria in mentally retarded individuals in Northern Ireland. Soon thereafter, it was shown that the primary defect in homocystinuria was an enzymatic defect of CBS (Mudd et al. 1964), with a recessive mode of inheritance (Finkelstein et al. 1964). The clinical manifestation of CBS deficiency is diverse, and four major organ systems are predominantly involved: the eye (high myopia and ectopia lentis), the skeleton (osteoporosis, scoliosis, and Marfanoid features), the vascular system (premature arteriosclerosis and thromboembolism), and the CNS (mental retardation, convulsions, and psychiatric disturbances).

Biochemically, patients with CBS deficiency are characterized by severe hyperhomocysteinemia and homocystinuria, hypermethioninemia, and decreased plasma cysteine levels. Furthermore, the CBS activities measured in either liver biopsy specimens (Mudd et al. 1964), cultured fibroblasts (Uhlendorf and Mudd 1968), or phytohemagglutinin-stimulated lymphocytes (Goldstein et al. 1972) are mostly well below the range of CBS activities observed in controls and heterozygotes for CBS deficiency. The first choice of therapy in CBS-deficient patients consists of administration of supraphysiological doses of pyridoxine (vitamin B_6), the precursor of PLP, the cofactor of CBS. A large international survey of >600 patients with homocystinuria showed that ~50% of the patients responded to high doses of pyridoxine with a substantial reduction in blood homocysteine concentrations (Mudd et al. 1985). Pyridoxine-nonresponsive patients usually are more severely affected than pyridoxine-

Table 1

CBS Mutations in Dutch Homocystinurics

DNA Mutation	Amino Acid Substitution	RFLP	Frequency[a]
373C→T	R125W	−*Aci*I	1/42
456C→G	I152M	−*Sau*3A	3/42
494G→A	C165Y	−*Bso*FI	4/42
539T→C	V180A	+*Hha*I	1/42
833T→C	I278T	+*Bsr*I	23/42
1105C→T[b]	R369C	−*Hha*I	2/42
1111G→A	V371M	+*Nla*III	2/42
1301C→A	T434N	None	1/42
1330G→A	D444N	−*Taq*I	2/42
1471C→T[b]	R491C	+*Bgl*II	2/42

[a] In *independent* alleles.
[b] Mutations were observed in *cis* in one patient.

responsive patients and are concomitantly treated with combinations of folic acid, hydroxycobalamin, and betaine, to stimulate remethylation of homocysteine to methionine.

Data on the clinical efficacy of homocysteine-lowering treatment are scarce. In pyridoxine-responsive patients and early-treated pyridoxine-nonresponsive patients, such treatment has reduced the number of initial thromboembolic events (Mudd et al. 1985). A recent report by Wilcken and Wilcken (1997) showed that treatment that effectively lowered plasma homocysteine concentrations markedly reduced the cardiovascular risk in a group of 32 patients with pyridoxine-responsive and pyridoxine-nonresponsive homocystinuria.

The human CBS gene, which has been mapped to 21q22.3 (Müncke et al. 1988), encodes a CBS subunit of 63 kD, which assembles into a homotetrameric protein (Kraus et al. 1978). So far, >60 mutations have been detected in the CBS gene (Kraus 1998), and functional relevance has been tested for some of them in either a bacterial (de Franchis et al. 1994; Marble et al. 1994; Kluijtmans et al. 1996*a*) or a yeast (Kruger and Cox 1995) expression system. Although most mutations seem to be private or restricted to only a few pedigrees, three mutations are relatively common among patients with homocystinuria. An 833T→C transition (I278T) (Kozich and Kraus 1992) has been found in alleles from homocystinuric patients of different ethnic backgrounds and has been reported to be associated with pyridoxine responsiveness and a relatively mild clinical phenotype when present in homozygous state (Shih et al. 1995). On the other hand, a 919G→A transition (G307S) is related to a more severe clinical phenotype and has been detected mainly in alleles from homocystinuric patients of Celtic origin (Gallagher et al. 1995). A third relatively common CBS mutation, an IVS11-2A→C splice mutation, which results in skipping exon 12, has been detected in patients from Central and Eastern Europe (Kraus 1998).

In the present study, we investigated the molecular basis of homocystinuria due to CBS deficiency in 29 Dutch homocystinuria patients from 21 unrelated pedigrees, and we studied a possible relationship between CBS genotype and biochemical and clinical phenotype. We therefore measured homocyst(e)ine concentrations at diagnosis, upon pyridoxine treatment, and upon maximal treatment, with pyridoxine and folic acid, with or without betaine, and then recorded whether homocysteine-lowering treatment had been able to prevent further clinical events or symptoms.

Patients, Material, and Methods

Patients

We studied 29 patients with homocystinuria due to CBS deficiency, from 21 unrelated pedigrees. Patients were initially diagnosed on the basis of clinical manifestations of homozygous CBS deficiency, in combination with a quantitative determination of severe hyperhomocysteinemia and hypermethioninemia. In two patients (19 and 29), the diagnosis was made only on the basis of severe homocystinuria, which was demonstrated by qualitative urine analyses. Homozygous CBS deficiency in these two patients has been confirmed by detection of a homozygous mutation (patient 19) and by the clinical manifestation of ectopia lentis (19 and 29), never observed in heterozygotes for CBS deficiency. Furthermore, the parents of patient 29 showed an abnormal response to a methionine-loading test, comparable to that observed in obligate heterozygotes. Homocysteine-lowering treatment was initiated in all patients immediately after the diagnosis had been made. Each patient was seen on a regular basis (once or twice each year) by two of us (Boers and Cruysberg), and the biochemical efficacy of homocysteine-lowering therapy was determined by measurement of homocyst(e)ine and methionine in blood. At diagnosis and during long-term follow-up, clinical manifestations were recorded by means of routine clinical, radiographic, or scintigraphic examination procedures.

Pyridoxine Responsiveness

Pyridoxine responsiveness was examined after 6 weeks of treatment with vitamin B_6, 750 mg/day in adults or 200–500 mg/day in children. Patients in whom non–protein-bound serum homocysteine had decreased to <20 μmol/L, or total plasma homocysteine (protein-*and* non–protein-bound) to <50 μmol/L, were classified as pyridoxine responsive. All other patients were categorized as pyridoxine-nonresponsive homocystinurics.

Biochemical Analysis

Homocystine, homocysteine-cysteine–mixed disulfide, and methionine levels in serum were determined as described by Boers et al. (1983). The amount of non–protein-bound homocysteine was calculated as the sum of twice the concentration of homocystine plus the concentration of the homocysteine-cysteine–mixed disulfide moiety. Since 1990, total plasma homocysteine concentrations (i.e., the total amount of protein- *and* non–protein-bound homocysteine moieties) were determined as described by Te Poele-Pothoff et al. (1995).

CBS activities in extracts of cultured fibroblasts were measured as initially described by Fowler et al. (1978), with some modifications (Boers et al. 1985). These CBS activities were measured with and without addition of 1 mM PLP to the incubation mixture.

Mutation Analysis

The procedure for mutation analysis of the CBS gene in patients with homocystinuria has been described elsewhere (Kluijtmans et al. 1996*a*). In brief, genomic DNA was isolated from peripheral blood leukocytes (Miller et al. 1988) and stored at 4°C. Total RNA was extracted from cultured fibroblasts by the method of Chomczynski and Sacchi (1987) and was stored as an ethanol precipitate at −80°C. We used 1–5 μg total RNA for first-strand cDNA synthesis with Superscript II Reverse Transcriptase (Life Technologies). First-strand cDNA was used as a template in PCR amplification reactions to amplify the CBS-encoding region in multiple overlapping cDNA fragments. These fragments were subsequently sequenced on an ABI 377 automated DNA sequencer (Applied Biosystems) with the Taq Dye Deoxy Terminator Cycle Sequencing Kit. All cDNA fragments were sequenced on both strands, and mutations were confirmed at the genomic DNA level by restriction enzyme analysis or DNA sequencing. Restriction enzymes were purchased from Life Technologies or from New England Biolabs and were used according to the manufacturers' recommendations. Screening for 833T→C was performed as described elsewhere (Kluijtmans et al. 1996*b*). Using this procedure, we were able to discriminate between the real 833T→C carriers and those with an 844ins68 duplication variant (Sebastio et al. 1995).

Cloning and Expression of Mutations

cDNA fragments containing the presumed functional mutations were amplified by PCR and subcloned into an expression cartridge as described elsewhere (de Franchis et al. 1994). Recombinant clones were selected and sequenced to verify the integrity of the cloned fragment. CBS expression was induced upon addition of isopropyl-β-thiogalactopyranoside (Kozich and Kraus 1992). The CBS assay was performed without addition of bovine serum albumin to the incubation mixture, to which cystathionine was added at a final concentration of 2 mM.

Statistics

Differences in clinical manifestation of CBS deficiency and efficacy of homocysteine-lowering treatment between separate groups were assessed by Yates' corrected χ^2 test. Differences in age at diagnosis, homocysteine, and methionine concentrations were assessed by nonparametric Wilcoxon–Mann-Whitney U tests. All P values reported are two-tailed, and $P < .05$ was considered statistically significant.

Results

Study Group

Twenty-nine homocystinuria patients from 21 pedigrees were included in this study. Six families had two affected siblings; one family had three affected siblings. The male–female ratio was 16:13. The diagnosis of CBS deficiency was established at a mean age of 26 years (median, 23 years [range 4–60]).

Genetic Basis of Homocystinuria

The CBS gene of the patients with homocystinuria was analyzed for mutations, either by direct sequencing of reverse transcription–PCR–amplified fragments (in 14 patients) or by RFLP analysis of genomic DNA fragments, to screen for previously recognized mutations (in 15 patients). The molecular basis of homocystinuria was resolved in 25 (86%) of the 29 patients. Overall, 10 different mutations were found (table 1), of which the 833T→C mutation was the most prevalent. This mutation was observed in 23 (55%) of 42 independent alleles. In four patients, including one in whom the entire cDNA was sequenced, only one mutation in heterozygous state was found; the mutation in the second allele has yet to be found (table 2).

CBS Activities in Cultured Fibroblasts

CBS activities were measured in extracts of cultured fibroblasts in 12 healthy controls, in 9 CBS-deficient patients homozygous for I278T, and in 12 patients with another CBS genotype (table 2). Without PLP addition to the incubation mixture, the mean (± SD) CBS activity in homozygotes was .17 (± .37) nmol cystathionine/mg protein/h, <2.5% of the control mean (7.4 [± 5.1] nmol cystathionine/mg protein/h). One homocystinuric patient (18) clearly exhibited CBS activities, in the range of obligate heterozygotes, and has been described elsewhere (Kluijtmans et al. 1996*a*).

Table 2

The Molecular Basis of Homocystinuria Due to CBS Deficiency

Sibship	Patient	Code	Pyridoxine Responsiveness	CBS activity[a] (nmol Cystathionine/mg Protein/h)		Mutation	
				− PLP[b]	+ PLP	Allele 1	Allele 2
1	1	HvE51	+	ND	ND	C165Y	I278T
	2	HvE53	+	.06	0	C165Y	I278T
	3	vU58	+	ND	ND	C165Y	I278T
2	4	AB58	−	.32	.31	C165Y	Unidentified
	5	RB61	−	ND	ND	C165Y	Unidentified
3	6	Gr32	+	0	.12	I278T	I278T
	7	BGr28	+	0	.24	I278T	I278T
4	8	JJ68	−	ND	ND	I278T	I278T
	9	WSJ63	−	ND	ND	I278T	I278T
5	10	BH50	+	.05	.38	I278T	I278T
	11	HH49	+	ND	ND	I278T	I278T
6	12	MC62	ND	.08	1.03	I152M	I278T
	13	JC70	ND	.06	1.08	I152M	I278T
7	14	RvD66	−	.25	.31	R125W	T434N
	15	HvD67	−	0	0	R125W	T434N
8	16	YvH75	ND	0	0	I278T	I278T
9	17	AP67	−	0	.24	C165Y	C165Y
10	18	JL75	−	1.74	2.60	D444N	D444N
11	19	MK66	+	.62	.77	I152M	I152M
12	20	LK61	−	.12	.20	I278T	I278T
13	21	LH80	ND	.18	.26	I278T	I278T
14	22	GMV29	+	.09	.67	R369C + R491C	R369C + R491C
15	23	CE38	+	0	0	I278T	I278T
16	24	RB58	+	0	0	I278T	I278T
17	25	AdZ46	+	.06	.42	V180A	Unidentified
18	26	JM61	+	0	.12	I278T	V371M
19	27	JU78	+	.07	0	I278T	I278T
20	28	JR42	ND	.03	.08	I278T	V371M
21	29	RP72	ND	ND	ND	I278T	Unidentified

Note.—A plus sign (+) indicates pyridoxine responsive, and a minus sign (−) indicates pyridoxine nonresponsive; ND = not determined.

[a] Cystathionine β-synthase activity expressed in nmol cystathionine formed/mg protein/h.

[b] Mean (± SD) CBS activity in fibroblasts of healthy controls: 7.4 (± 5.1).

The mean CBS activity in fibroblasts of homozygotes for I278T ($n = 9$) versus patients with other genotypes ($n = 12$) was 0.05 ± 0.07 versus 0.13 ± 0.18 nmol cystathionine/mg protein/h ($P = .28$), and 0.13 ± 0.14 versus 0.42 ± 0.38 nmol cystathionine/mg protein/h ($P = .07$) in the assay without and with, respectively, 1 mM PLP. Patient 18 was excluded in these calculations.

In Vitro Expression of Mutations

The functional relevance of seven mutations was investigated in an *Escherichia coli* expression system: six constructs contained a single mutation, and one construct contained the 1105C→T (R369C) and 1471C→T (R491C) mutations in *cis*. All mutated constructs, except the one containing 1330G→A (D444N) (Kluijtmans et al. 1996*a*), showed a reduction in CBS activity of >90%, demonstrating the detrimental effects of each construct on CBS activity (fig. 1). Two mutations (373C→T [R125W] and 1301C→A [T434N]) have not yet been functionally tested.

Pyridoxine Responsiveness

Fourteen (48%) of 29 patients were classified as pyridoxine responsive and 9 (31%) patients as pyridoxine nonresponsive, on the basis of the criteria described in the Patients, Material, and Methods section. In six patients (12, 13, 16, 21, 28, and 29), responsiveness to pyridoxine alone could not be assessed. In view of their extremely high homocysteine levels at diagnosis, these patients were treated directly with a combination of therapeutic regimens (pyridoxine and folic acid, with or without betaine). Pyridoxine responders were diagnosed at a mean age of 29 years (median, 26 years [range, 7–54 years]), and nonresponders at a mean age of 19 years (median, 16 years [range, 4–30 years]; $P = .08$).

Seven (58%) of 12 homozygotes for the I278T mu-

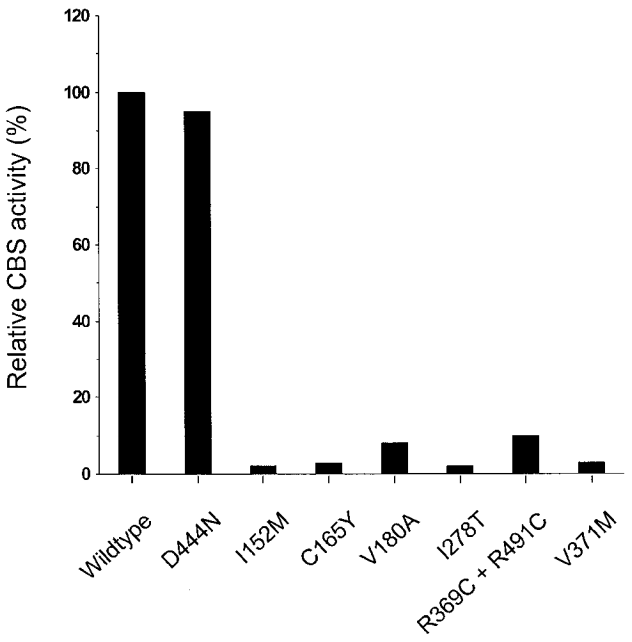

Figure 1 CBS activities measured in an *E. coli* expression system. Mutations were introduced as described in the Patients, Material, and Methods section. Bacterial lysates were assayed for CBS activity, and the mean CBS activity of a control construct (40.3 nmol cystathionine formed/mg protein/h) has been set to 100%. Each bar represents another mutated construct and is the mean of two independently performed CBS assays.

tation showed in vivo pyridoxine responsiveness, 3 (25%) were nonresponders, and, in 2 (17%) patients, this specific responsiveness could not be assessed. In 17 individuals with other genotype combinations, including compound heterozygotes for I278T, these numbers were 7 (41%), 6 (35%), and 4 (24%), respectively (χ^2 = 0.13; P = .7). Conversely, in 14 pyridoxine-responsive patients, 18 (64%) of 28 alleles carried the I278T mutation (7 homozygotes, 4 heterozygotes), versus 6 (33%) I278T alleles in 9 nonresponsive patients (3 homozygotes) (χ^2 = 1.39; P = .5). There was an absolute concordance of pyridoxine responsiveness between siblings.

Clinical Characteristics at Diagnosis

The clinical manifestation of homocystinuria due to CBS deficiency in this study group is very diverse and is depicted in detail in table 3. At diagnosis, some patients (2, 13, and 15) showed virtually no clinical symptoms and were investigated because of a homocystinuric sibling. In other patients (6, 19, 22, 23, and 25), all four major organ systems were involved. Ocular abnormalities and skeletal abnormalities were the most consistent findings among these 29 patients with homocystinuria: 25 (86%) suffered from either high myopia or ectopia lentis. Twenty-six (90%) patients exhibited osteoporosis, scoliosis, or Marfanoid features, and 13 (45%) patients had complications in the vascular system. The CNS was involved in 16 (55%) patients, and psychiatric illness was noticed in only 4 (14%) patients. No significant differences in clinical presentation of CBS deficiency were observed between I278T homozygotes and patients with other genotypes (data not shown).

Biochemical Characteristics at Diagnosis

At diagnosis, non–protein-bound homocysteine concentrations had been measured in 21 patients and total homocysteine concentrations in 6 patients. In two patients, no baseline homocysteine blood levels were available; in these two cases the initial diagnosis had been made in 1975, by means of qualitative examination of urine only. The mean non–protein-bound homocysteine concentration was 135 μmol/L (range, 42–266 μmol/L; n = 21) and mean total plasma homocysteine concentration was 240 μmol/L (range, 134–299 μmol/L; n = 6). Mean serum methionine concentration was 130 μmol/L (range, 52–549 μmol/L; n = 23). Homozygotes for the I278T mutation tended to have a higher mean homocysteine concentration than homocystinuria patients with other genotypes (160 ± 73 μmol/L versus 116 ± 57 μmol/L non–protein bound; P = .16), whereas methionine concentrations were not significantly different between both genotype groups (89 ± 35 μmol/L versus 152 ± 166 μmol/L; P = .49).

Response to Homocysteine-Lowering Treatment

Long-term homocysteine-lowering therapy (mean term, 13 years [range, 1–29 years]) consisted of maximally 750 mg pyridoxine. Fifteen (56%) patients were concomitantly treated with 5 mg folic acid per day, and eight (30%) patients also with 6 g betaine per day. Only one patient (17) had a methionine-restricted diet, with a methionine content of 600 mg/d. Intramuscular injections with 1 mg hydroxycobalamin every 1–2 mo were given to four patients, because of the development of a vitamin B_{12} deficiency. Two patients could not be followed after diagnosis had been made: patient 6 refused treatment and patient 13 moved to another country. Hence, follow-up was recorded in 27 (93%) of the patients with homocystinuria. The mean length of follow-up was 11 years (range, 1–20 years; n = 11) in homozygotes for the I278T mutation and 16 years (range, 3–27 years; n = 16) in patients with other genotypes (P = .09).

Biochemically, pyridoxine treatment resulted in a marked decrease in homocysteine concentrations of 90% in homozygotes for the I278T mutation and of 67% in patients with other genotypes (P < .02). Extended intervention with folic acid, with or without betaine, further decreased homocysteine concentrations in

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