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Identification and functional analysis of cystathionine beta-synthase gene mutations in patients with homocystinuria

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Abstract Homocystinuria is an autosomal recessive inborn error of metabolism that is most often caused by mutation in the cystathionine beta-synthase (CBS) gene. Patients may develop serious clinical manifestations such as lens dislocation, mental retardation, osteoporosis, and atherothrombotic vascular disease. Over 100 mutations have been reported, but so far, none have been reported in Korea. Mutation analysis of the CBS gene in six Korean patients with homocystinuria was performed by direct sequencing. Eight mutations were identified, including four known mutations (T257M, R336C, T353M, and G347S) and four novel mutations (L154Q, A155V, del234D, and A288T). All patients were compound heterozygotes. To characterize these mutations, normal or mutated forms of CBS were cloned into pcDNA3.1 expression vector followed by transfection into mammalian cells for transient expres-

sion. Whereas the expression levels of mutant proteins were comparable to that of normal control, enzyme activities of all the mutant forms were significantly decreased. In addition, a novel single nucleotide polymorphism, R18C, was identified, which showed one-third to two-thirds the enzyme activity of wild type and 1% of the allele frequency in normal control. The spectrum of mutations observed in Korean patients bears less resemblance to those observed in Western countries.

Keywords Homocystinuria · CBS · Cystathionine beta-synthase · Mutation · Expression · Functional analysis · Korean

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Introduction

Homocystinuria is the most common inborn error of sulfur amino acid metabolism. It is most commonly caused by cystathionine beta-synthase (CBS) deficiency, which is an autosomal recessively inherited genetic defect (MIM 236200). Cystathionine beta-synthase (L-serine hydrolyase, EC 4.2.1.22), the enzyme that catalyzes the first step of the transsulfuration pathway, condenses homocysteine and serine to produce cystathionine and ultimately cysteine (Miles and Kraus 2004).

The clinical manifestations of untreated homocystinuria include venous thromboembolic disease, arterial vascular disease, ectopia lentis, mental retardation, osteoporosis, and marfanoid skeletal features. Among these features, early thromboembolic and accelerated atherosclerotic vascular complications are fatal and the most common cause of death. About 50% of homocystinuria patients respond to pyridoxine, and these patients are usually associated with the less severe phenotype (Mudd et al. 1985).

Homocystinuria due to CBS deficiency has been known to be a very rare disease except in Ireland, where the prevalence is 1:65,000 births, compared with 1:300,000

worldwide (Naughten et al. 1998). It is far less prevalent in Japan—1:1,051,959 (Tada et al. 1984). However, there have recently been several reports that the prevalence of CBS deficiency has been underestimated and that the actual homocystinuria population is larger than previously thought (Gaustadnes et al. 1999). But the incidence of homocystinuria in Korea is not yet known.

The human CBS gene is located at chromosome 21q22.3 (Munke et al. 1988), and it was not too long ago that the entire CBS gene was cloned (Kraus et al. 1998). The coding sequence comprises from exon 1 to exon 14 and exon 16. Exon 15 is alternatively spliced and not yet detected in human tissues. The 5' UTR of the messenger RNA CBS gene is composed of one of the alternatively spliced exons of -1a to -1e in addition to the invariably present exon 0. Exons 16 and 17 comprise 3' UTR (Chasse et al. 1995; Kraus et al. 1998).

The human CBS protein is a homotetramer of 63 kDa subunits, each consisting of 551 amino acids (Kraus et al. 1998). The enzyme's structure consists of a catalytic domain located in the N-terminal with 409 amino acids and a regulatory domain of 142 amino acids located in the C-terminal (Shan and Kruger 1998). Fifty-three amino acid residues (416–469) in the 140 carboxy-terminal regulatory domain are called the "CBS domain," a protein folding motif that contains an autoinhibitory region removed by the allosteric activator AdoMet (Miles and Kraus 2004).

One hundred and thirty-two mutations in CBS have been identified to date, with the majority being missense mutations. The most frequent among them is I278T (c.833T > C), commonly found in Caucasians, of which clinical manifestation is relatively mild and vitamin B₆-responsive (Kruger et al. 2003). The second most common mutation is G307S (c.919G > A), which is common in patients of Celtic origin and is more severe and vitamin B₆-nonresponsive (Kraus et al. 1999). Among Asians, H65R and G116R mutations have been found in Japanese (Chen et al. 1999) and the c.844ins68 mutation in Chinese (Zhang and Dai 2001). Besides CBS deficiency, N(5,10)-methylene tetrahydrofolate reductase deficiency and various defects in vitamin B₁₂ metabolism can cause homocystinuria (Engbersen et al. 1995; Ubagai et al. 1995).

Materials and methods

Patient recruitment and sampling

Six patients with homocystinuria and their families were recruited from Soonchunhyang University Hospital (Seoul, South Korea) and Asan Medical Center (Seoul, South Korea). They were diagnosed by biochemical profiles of increased serum homocysteine and methionine. Diagnosis was possible because of complications of CBS deficiency, mostly dislocated lens, and by neonatal screening and family screening. The patients who were diagnosed several years after birth had critical

complications such as mental retardation, and two of them had thrombotic vascular disease. After diagnosis, the patients were treated with a low-methionine diet, vitamin B₆, folate, vitamin B₁₂, and betaine. All of the patients provided written informed consent prior to participating in this study.

Mutation analysis of the CBS gene

Samples of 5 ml of peripheral venous blood were collected in acid-citrate dextrose tubes, and genomic DNA was extracted with the QIAamp DNA Blood Maxi kit (Qiagen, Valencia, CA, USA). All 16 coding exons were polymerase chain reaction (PCR)-amplified with primer pairs as described previously (Kruger et al. 2003), with minor modification. PCR amplification was performed under standard conditions of 94°C for 1 min, each annealing temperature for 30 s, and 72°C for 1 min for 35 cycles using rTaq DNA polymerase (Takara, Kyoto, Japan). After PCR, the products were purified with the QIAquick gel extraction kit (Qiagen) and directly sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 3.1, and an automated sequencer (ABI PRISM 3730, Applied Biosystems, Foster City, CA, USA) with sense and antisense primers. Deletion mutant was sequenced after cloning into pGEM T easy vector (Promega, Madison, WI, USA). The reference sequence for sequence analysis was NM_000071 of GenBank, NCBI.

Construction of human CBS complementary DNA and cloning into pBS

Human CBS RNA was extracted from human hepatoma cell line HepG2 using TRI Reagent (Ambion, Austin, TX, USA), and RT-PCR was performed. The primers were derived from 5' UTR (5' AC-GTCTCCTTACAGAGTTTGAGCG 3') and 3' UTR (5' TGTGCGCA CTAACCATTGAC 3'), and the product size was 1,813 bp. After cloning into pGEM T easy vector, it was cut with *EcoRI* to produce 1,831 bp insert and subcloned into pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA). Due to the technical difficulties in dealing with the relatively large size of pcDNA3.1 for mutagenesis, it was cut again with *KpnI* and *NotI* to produce 1,889 bp insert and cloned into smaller pBS II SK- (Stratagene, La Jolla, CA, USA) to produce 4,808 bp DNA.

Site-directed mutagenesis

Sense and antisense primers for each detected known and novel mutation were made according to the manufacturer's guidelines (QuikChange XL site-directed mutagenesis kit, Stratagene). Using the PCR amplification method, primers containing the desired mutation

expand along template DNA by *pfu* Turbo DNA polymerase. After parental DNA templates were digested with *DpnI* treatment, the aimed mutagenized plasmid DNA was synthesized. Each mutagenized cDNA was confirmed by PCR, restriction enzyme cut, and bidirectional sequencing done. The plasmids including wild type CBS cDNA, nine mutagenized CBS cDNA, and mock cDNA were purified with QIAprep (Qiagen) for transfection.

Cell line preparation and transfection

NIH3T3 and COS7 cell lines were used for the transient expression system. They were subcultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum. NIH3T3 and COS7 cells were transfected with normal or mutant type CBS gene at 70% and 90% of cell confluence, respectively. β -galactosidase (β -gal) gene was used as internal control for transfection efficiency. We used the liposomal method for transfection as per the manufacturer's guidelines (Lipofectamine Reagent, Invitrogen).

Cell harvest and protein extraction

After 48 h of transfection, the cells were harvested with trypsin, and protein was extracted by sonication. The protein concentration was measured spectrophotometrically at 420 nm. The β -gal activity was assayed by the method described previously (Kim and You 2004), and the final calibrated protein concentration was determined by dividing the raw values with each of the β -gal concentrations.

In vitro enzyme reaction and TLC

The CBS enzyme activity was determined by modification of previously reported methods (Jhee et al. 2000; Shan and Kruger 1998). A total of 50 μ l of reaction volume contained 5 μ l of 1-M Tris-HCl (pH 8.6), 2.5 μ l of 5-mM PLP, 2.5 μ l of 10 mg/dl BSA, 5 μ l of 10-mM cystathionine, 1 μ l of 14 C-serine, 1 μ l of 50-mM Ado-Met, 165 μ g of calibrated CBS protein for the NIH3T3 cell line, and 80 μ g of calibrated CBS protein for the COS7 cell line. After preincubation of the mixture at 37°C for 5 min, the reaction was initiated by the addition of 2.5 μ l of 200-mM homocysteine. After incubation for 2 h at 37°C, the reaction was terminated by immediately placing the reaction mixture into ice for at least 5 min. After centrifugation at 12,000 \times g for 5 min, 15 μ l of the supernatant was spotted on a silica gel (SiO₂) TLC plate. The product, L-[14 C] cystathionine, was separated from L-[14 C] serine by ascending thin-layer chromatography in the mobile phase of butanol:acetic acid:water = 60:15:25 (v:v:v) for 20 h. The

TLC plate was exposed to an IP plate for 4 h and developed in a BAS imaging analyzer (Fuji, Japan). The radioactivity was measured with TINA software (TINA 2.0, Raytest, Courbevoie, France).

Western blot

Fifty micrograms of total protein was loaded into a well of 10% SDS-PAGE. After electrophoresis, the proteins were transferred to a PVDF membrane (Invitrogen). The membrane was hybridized with a 1:7,500 dilution of rabbit antibody against human CBS (the anti-CBS antibody was kindly provided by Warren D. Kruger) and subsequently with a horseradish peroxidase-conjugated anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The signals were then visualized using the ECL-Plus enhanced chemiluminescence detection system (Santa Cruz Biotechnology).

Results

Phenotypic characteristics of patients with homocystinuria

Clinical and biochemical characteristics of each patient are described in Table 1. Patient 3, diagnosed during family screening, is a sibling of patient 2. Blood sampling from the parents of patient 6 was not available. Neonatal screening was effective in only one case (patient 1), and the others, who had not undergone neonatal screening, presented with clinical symptoms including lens dislocation. Unfortunately, most of the recently detected patients already had mental sequelae, and patient 4 had fully developed homocystinuric syndrome (Table 1).

Mutation analysis

A total of nine variations were detected, and four of them, T257M, R336C, G347S, and T353M, had been previously reported (de Franchis et al. 1999; Gaustadnes et al. 2002; Dawson et al. 1997; Kraus 1994). Five variations, R18C, L154Q, A155V, A288T, and del234D, were novel (Table 2). All patients were compound heterozygotes. The origins of the alleles were documented through parental analysis. In patient 1, three variations were detected, and two of them were found to have originated from her father. One had a possibility of a benign polymorphism; therefore, we carried out direct sequencing of exon 1 in 400 chromosomes and exon 4 in 100 chromosomes of normal controls to detect the mutation rate in the normal population. R18C variation was found in four out of 400 chromosomes (1%), and no L154Q mutation was found among 100 chromosomes. Thus, R18C variation had a high probability of benign polymorphism rather than true pathogenic mutation, although functional

Table 1 Clinical and biochemical characteristics of patients (*pre Met* pretreatment methionine, *pre Hcy* pretreatment homocysteine, *pre tHcy* pretreatment total homocysteine, *post Met* posttreatment methionine, *post Hcy* posttreatment homocysteine, *post tHcy* posttreatment total homocysteine, *ND* not determined, *NA* not available)

Patient ID	Sex	Age at diagnosis	Basis for diagnosis	Lens dislocation	Mental retardation	Marfan syndrome	Vascular occlusion	Pre Met (μM)	Pret Hcy (μM)	Pre tHcy (μM)	Post Met (μM)	Post Hcy (μM)	Post tHcy (μM)
1	F	1 month	Neonatal screening	–	–	–	–	1,239	28	ND	123	0	144
2	M	8 years	Lens dislocation	++	+	–	–	609	78	ND	352	5.9	136
3	M	1 month	Family screening	–	–	–	–	141	16	ND	152	4	119
4	F	7 years	Lens dislocation	++	++	++	++	24	34	ND	575	0	194
5	F	6 years	Lens dislocation	++	–	–	–	697	3	ND	648	ND	121
6	F	19 years	Lens dislocation	++	+	–	–	90	3	181	ND	ND	115

Normal reference ranges: plasma methionine, 7–47 μM ; plasma homocysteine, 0–5 μM ; plasma total homocysteine, 5–15 μM

activity needs to be determined. All of the mutations were missense mutations, except one deletion mutation, which was found in two out of six patients. The deletion did not result in frameshift or premature termination of translation; however, one amino acid, aspartate, was missing. Neither of the worldwide most common mutations, I278T and G307S, was found in our study.

In vitro expression of CBS mutations

The expression studies to evaluate the functional activity of the nine variations showed that all eight mutations were nearly completely devoid of CBS activity in vitro, relative to wild type. As predicted, CBS activities of R18C were about 30% of wild type in NIH3T3 and over 70% in COS7, which is compatible with benign polymorphism. In the rest of the mutations, there was no significant difference in activity between NIH3T3 and COS7 in each mutation and also no significant difference among mutations in the cell lines (Fig. 1, Tables 3, 4). The Western blot analysis showed that, compared with

wild type, the abundance of CBS mutant proteins was not significantly decreased, except A155V mutation, suggesting that the decreased enzyme activity detected on TLC was not due to decreased amount of protein expression and/or stability, but rather due to the functional defect itself (Fig. 2).

Discussion

In the present study, four novel mutations and one single nucleotide polymorphism (SNP) in addition to four known mutations were identified in Korean homocystinuria patients. These mutations were assessed for their enzyme activities and protein expressions in mammalian systems.

Until now, 132 CBS mutations in 553 alleles have been reported (<http://www.uchsc.edu/cbs/>), most of them originating from Caucasians, especially those of European descent, and very few from African-Americans and Asians (Kruger et al. 2003; Watanabe et al. 1997). Therefore, although the incidence seems to be much lower

Table 2 Genotypes of the CBS gene in the patients (*NA* not available)

Patient no.	Exons	Nucleotide changes	Predicted AA changes	Novel or known	Origin of allele	References
1	1	c.52 CGC > TGC	R18C ^b	Novel	Paternal	
	4	c.461CTG > CAG	L154Q	Novel	Paternal	
	7	c.770 ACG > ATG	T257M	Known	Maternal	^c
2, 3 ^a	6	c. del 700–702 GAC	Del 234D	Novel	Paternal	
	7	c.770 ACG > ATG	T257M	Known	Maternal	^c
4	9	c.1006 CGC > TGC	R336C	Known	Paternal	^d
	10	c.1058 ACG > ATG	T353M	Known	Maternal	^e
5	4	c.464 GCC > GTC	A155V	Novel	Paternal	
	9	c.1039 GGT > AGT	G347S	Known	Maternal	^f
6	6	c. del 700–702 GAC	Del 234D	Novel	NA	
	8	c.862 GCA > ACA	A288T	Novel	NA	

^aSiblings

^bSNP

^cMutation reported by Sebastio et al. (1995)

^dMutations reported by de Franchis et al. (1999)

^eMutation reported by Dawson et al. (1997)

^fMutation reported by Gaustadnes et al. (2002)

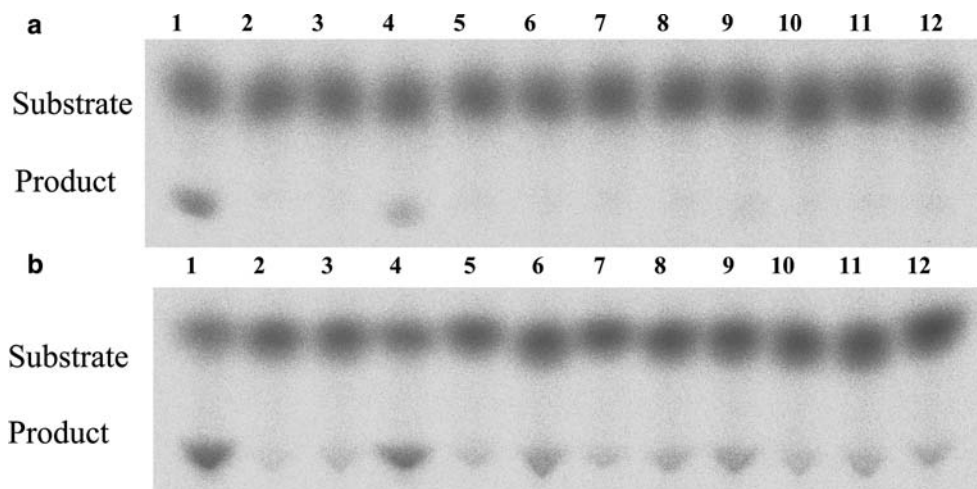


Fig. 1 TLC analysis of CBS activities expressed in NIH3T3 (a) and in COS7 cell lines (b). Products were separated from substrates after running for 20 h on a TLC plate. Lane 1, wild type CBS cDNA transfectant in each cell line. Lane 2, nontransfected, mock cell lines. Lane 3, non-CBS containing vector-transfected mock cell lines. Lanes 2 and 3 showed absent radioactivity of products in the NIH3T3 cell line and nearly absent radioactivity of products in the COS7 cell line, which had weak endogenous CBS activity

(confirmed in RT-PCR, data not shown). Lane 4, R18C variation showed a comparable amount of radioactivity of products to wild type, thus favoring SNP rather than pathogenic mutation. Lanes 5–12, L154Q, A155V, T257M, A288T, R336C, G347S, T353M, and del234D, respectively. Lanes 5–12 showed nearly absent radioactivity of products in NIH3T3, and weak radioactivity in COS7 cell lines, which were compatible to pathogenic mutations

in Asians than in Caucasians, the prevalence of homocystinuria in Asia might have been underestimated.

Nearly one-quarter of the missense mutations were found in exon 3, which is the most evolutionary conserved part of the CBS enzyme. The c.833T > C (I278T) is known as the most common mutation in Caucasians

of European descent, manifesting pyridoxine responsiveness in homozygote (Kozich and Kraus 1992) but various pyridoxine responsiveness in heterozygote (Kruger et al. 2003). The c.919G > A (G307S) is the second most common mutation, especially prevalent in Celts in Ireland, with pyridoxine unresponsiveness and relatively severe clinical manifestations (Hu et al. 1993). None of these mutations were found among Korean patients with homocystinuria, although the number of patients in the study is small.

Table 3 TLC analysis of mutations expressed in NIH3T3 cells

Mutations	Substrate (S)/ product (P)	PSL value ^a	Reaction rate (%) ^b	Enzyme activity (% wild type) ^c
Wild type	S	22.13	43.89	100
	P	17.31		
R18C	S	34.31	13.14	30
	P	5.19		
L154Q	S	37.01	0.48	1.1
	P	0.18		
A155V	S	37.81	0.74	1.7
	P	0.28		
T257M	S	43.21	1.32	3
	P	0.58		
A288T	S	46.76	1.79	4.1
	P	0.85		
R336C	S	51.03	2.54	5.8
	P	1.33		
G347S	S	48.81	2.14	4.9
	P	1.07		
T353M	S	41.14	1.67	3.8
	P	0.7		
del234D	S	47.12	1.48	3.4
	P	0.71		

^aPhoto-stimulated luminescence (PSL-background radioactivity)/mm³

^bThe reaction rate was calculated by dividing the PSL value of the product by the sum of the substrate and product of each mutation after adjustment by β -gal activity (data not shown)

^cThe enzyme activity of each mutation was nearly absent, whereas R18C, the SNP, retained some amount of enzyme activity

The c.770C > T (T257M) mutation was first found as homozygote in an Italian patient who was unresponsive to pyridoxine treatment. The enzyme activity expressed in *Escherichia coli* was sharply decreased (Sebastio et al. 1995). The c.1006C > T (R336C) was detected in an English ancestor, unresponsive to pyridoxine, although the genotype was compound heterozygote with G307S. It had no measurable enzyme activity in *E. coli* (de Franchis et al. 1999). The c.1039G > A (G347S) was first found in a Caucasian of Anglo-Celtic descent. This mutation was completely devoid of enzyme activity in *E. coli*, and the expression was as abundant as wild type in Western blot. Its pyridoxine responsiveness is not known (Gaustadnes et al. 2002). The c.1058C > T (T353M) was known to be prevalent in African-Americans and some individuals of European origin without pyridoxine responsiveness. Its catalytic activity was negligible, but the expression in *E. coli* was similar to wild type (Dawson et al. 1997).

The four novel mutations [c.461T > A (L154Q), c.464C > T (A155V), c.862G > A (A288T), and c.del700-702 GAC (del234D)] were also devoid of enzyme activity in TLC analysis, but protein expression was comparable to wild type in Western blot analysis. Another novel mutation, c.52C > T (R18C), revealed in patient 1, had

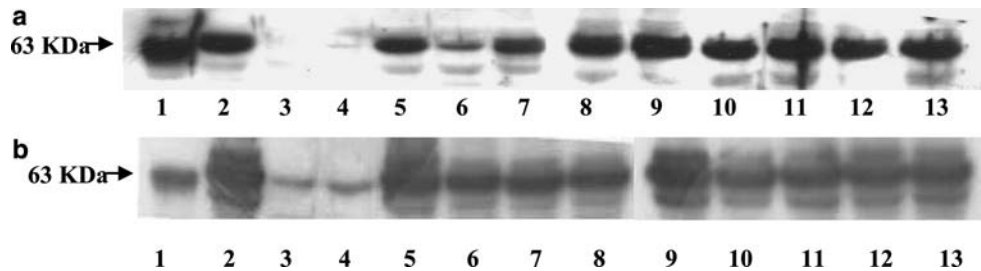


Fig. 2 Western blot analysis of CBS mutants expressed in NIH3T3 (a) and COS7 cell lines (b). CBS proteins of 63 kDa were expressed in NIH3T3 and COS7. Lane 1, endogenous expression of CBS in HepG2 cell line. Lane 2, wild type CBS cDNA transfectant in each cell line. Lane 3, nontransfected, mock cell line. Lane 4 non-CBS containing vector-transfected mock cell line. Lanes 3 and 4 showed

absent CBS expression in NIH3T3 and markedly decreased CBS expression in COS7 cell lines. Lanes 5–13, R18C, A155V, T257M, A288T, R336C, G347S, T353M, and del234D, respectively. Lanes 5–13 showed nearly equal amounts of CBS expression in each mutant compared with wild type

30–70% wild type enzyme activity in vitro. Furthermore, the allele was detected in four out of 400 alleles in a normal control population. Therefore, it was concluded that the mutation was benign polymorphism rather than pathogenic mutation. Expression analysis of R18C mutant in the *E. coli* system could be more helpful to discriminate whether it is a mutation or a polymorphism. In addition, all the patients and their families showed c.1080C/T polymorphism, and c.699C/T polymorphism was found in patient 4 and her family. The c.1080C/T and c.699C/T are known as benign polymorphisms in CpG islands (Kraus 1994).

Therefore, it can be predicted that there are many heterogeneous mutations in the Korean homocystinuria population sharing a considerable number of mutations with European descents. However, as expected, new

mutations that have not been seen in Western populations were detected, implying racial differences or founder effect.

Although these patients were not assessed for pyridoxine responsiveness, the reported correlation between phenotype and residual enzyme activity is weak, indicating that other genetic or epigenetic factors or modifier loci might affect pyridoxine responsiveness and disease penetrance (Kruger et al. 2003).

The mutations found in this study were missense mutations or short in-frame deletions located in the active core of the CBS enzyme, except R18C, which is in the N-terminal domain. Consequently, the above consideration seems to explain the result of nearly no enzyme activity in TLC analysis but intact protein expression, as well as the fact that mutations in the active core cause defects of enzyme activity or changes of protein folding without affecting the mRNA expression. Further investigation is needed to determine exactly by which mechanism the mutations disrupt enzyme activity.

In this study, we used NIH3T3 and COS7 cells for expression of CBS cDNA. COS7 is a monkey-kidney cell line and is commonly used for transfection of various DNAs. Although it had weak endogenous CBS expression, its transfection efficacy was found to be good, and its power to discriminate wild type and mutations was sufficiently strong. NIH3T3 is an embryonic mouse-kidney cell line frequently used for transfection and lacks endogenous CBS expression; however, the transfection efficiency was low, and the power to discriminate wild type and mutations was relatively weak. Although the agreement between the two expression systems used was not very good, we used these two systems to complement each other.

Further evaluation of enzyme activity in vitro and/or in vivo may predict the response to pyridoxine and prognosis and can help manage patients in clinics, and identification of pathogenic mechanisms may accelerate the development of novel therapeutic strategies.

Table 4 TLC analysis of mutations expressed in COS7 cells

Mutations	Substrate (S)/ product (P)	PSL value ^a	Reaction rate (%) ^b	Enzyme activity (% wild type) ^c
Wild type	S	4	71	100
	P	9.8		
R18C	S	5.06	54.3	76.5
	P	6.01		
L154Q	S	10.25	0.29	0.4
	P	0.03		
A155V	S	9.71	12.68	17.9
	P	1.41		
T257M	S	13.01	1.36	1.9
	P	0.18		
A288T	S	13.98	4.5	6.3
	P	0.66		
R336C	S	13.6	6.4	9
	P	0.93		
G347S	S	14.88	4.62	6.5
	P	0.72		
T353M	S	12.71	7.5	10.6
	P	1.03		
Del234D	S	12.04	2.33	3.3
	P	0.29		

^aPhoto-stimulated luminescence (PSL-background radioactivity)/mm³

^{b, c}Each mutation showed nearly absent enzyme activities, whereas R18C showed a comparable amount of enzyme activity compared with wild type. The calculation methods were the same as in Table 3

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