Molecular genetic analysis of pyridoxine-nonresponsive homocystinuric siblings with different blood methionine levels during the neonatal period

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Abstract : Two mutations in the cystathionine β-synthase (CBS) gene were found in two Japanese siblings with pyridoxine non-responsive homocystinuria who had different methionine levels in their blood during the neonatal period. Both patients were compound heterozygotes of two mutant alleles : one had an A-to-G transition at nucleotide 194 (A194 G) that caused a histidine-to-arginine substitution at position 65 of the protein (H65R), while the other had a G-to-A transition at nucleotide 346 (G346A) which resulted in a glycine-to-arginine substitution at position 116 of the protein (G116R). The two mutant proteins were separately expressed in Escherichia coli, and they completely lacked catalytic activity.

Despite their identical genotypes and almost equal protein intake, these siblings showed different levels of blood methionine during the neonatal period, suggesting that the level of methionine in blood is determined not only by the defect in the CBS gene and protein intake, but also by the activity of other enzymes involved in methionine and homocysteine metabolism, especially during the neonatal period. Therefore, high-risk newborns who have siblings with homocystinuria, even if the level of methionine in their blood is normal in a neonatal mass screening, should be followed up and diagnosed by an assay of enzyme activity or a gene analysis so that treatment can be begun as soon as possible to prevent the development of clinical symptoms. In addition, a new, more sensitive method for the mass screening of CBS deficiency in neonates should be developed.
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Key words : *cystathionine β-Synthase Deficiency, homocystinuria, mutation analysis, neonatal mass screening*

INTRODUCTION

Homocystinuria (McKusick 236200) due to cystathionine β-synthase (CBS, EC 4.2.1.22) deficiency is an inborn error in the trans-sulfuration pathway by which methionine is converted to cysteine. CBS deficiency results in the accumulation of homocysteine and methionine, and a decreased formation of cystathionine and cysteine. Untreated patients are characterized by multisystemic clinical symptoms, such as dislocated optic lenses, mental retardation, skeletal abnormalities, and vascular disease with severe thromboembolic complications (1).

Patients with CBS deficiency usually show elevated concentrations of both homocystine and methionine in the blood and urine, even in the neonatal period, consequently, the blood level of methionine is routinely measured in neonatal mass screenings for CBS deficiency worldwide (2). We previously reported two homocystinuric siblings who showed different levels of methionine in their blood during the neonatal period ; one of them was missed by a mass screening due to a low level of methionine in the blood (3). Naughten *et al*. (1998) pointed out four possible reasons why a patient with homocystinuria could be missed in a neonatal mass screening ; i.e., early hospital discharge, low protein intake (especially if breast fed), a high blood methionine cut-off

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concentration and pyridoxine responsiveness. Our patient who was missed by mass screening did not show any of these conditions.

Therefore, in this study, we analyzed the CBS gene in these siblings at the molecular level to confirm the cause of their different levels of methionine in the neonatal period.

MATERIAL AND METHODS

Patients :

Clinical and biochemical findings for the patients have been reported previously (3, 4). In summary, case 1, a 14-year-old boy, was born without any complications to Japanese parents who were not consanguineous. Neonatal mass screening performed at 5 days after birth when the protein intake was about 2.5 g/kg with normal milk formula showed hypermethioninemia, but he was not diagnosed as having homocystinuria until 3 months of age because urinary homocystine was not detected. When he reached 3 months of age, urinary homocystine was detectable and markedly reduced CBS activity in cultured skin fibroblasts was identified. Since the boy did not respond to the oral administration of pyridoxine, he was treated with a low-methionine diet and betaine. His physical and psychomotor development was normal. Case 2 (the younger sister of case 1), a 12-year-old girl, was delivered after 41 weeks of gestation without any complications and weighed 3,352 g at birth. She was fed a normal milk formula and her protein intake was about 2.5 g/kg at a neonatal mass screening performed also at 5 days after birth. Her blood concentration of methionine was below the cut-off value (67 μmol/L) in a neonatal mass screening. Her physical development was normal, but her mental development, especially her speech development, was slightly retarded. At 21/2 years, she was found to have hypermethioninemia, hyperhomocystinemia and homocystinuria. Cystathionine synthase activity in cultured skin fibroblasts was undetectable. Oral administration of pyridoxine did not decrease the level of homocystine in the urine and blood. She was then treated with a low-methionine diet, which kept the level of blood methionine below 67 μmol/L and total homocyst(e)ine below 30 μmol/L. Subsequently, each value gradually rose and betaine was supplemented at 3 years of age. Her retardation has since improved, and her IQ score was 91 at 12 years of age.

RNA, DNA and cDNA Preparation :

Total RNA was isolated from cultured skin fibroblasts from patients and normal control subjects by the guanidinium thiocyanate method (5). To confirm the detected mutations in family members, genomic DNA was isolated from peripheral mononuclear cells by the proteinase K method, as described elsewhere (6). Single-strand cDNA was synthesized from total RNA using a cDNA synthesis kit (GIBCO-BRL Inc).

Amplification of cDNA, subcloning and sequencing :

The full-length cDNA for CBS was amplified by the polymerase chain reaction (PCR) using the sense primer (nt- 31 to-12 ; 5 '-GAACTGTCAGCACCATCT GT-3 ') and the antisense primer (nt 1674 to 1655 ; 5 '-CGCCCAGCGCTCCGGACTTC-3 '). Thirty five cycles of PCR were performed according to the following program : denaturation at 94°C for 1 min, annealing at 57°C for 2 min and extension at 72°C for 3 min. Amplified full-length cDNA of CBS was subcloned into the vector pCR2.1 using a TA cloning kit (Invitrogen). Eight independent clones from case 1 and six clones from case 2 were sequenced in both directions by the dideoxy chain termination method using the Sequenase kit (United States Biochemical) and [^{35}S] dATP as the labeled deoxyribonucleotide.

Detection of mutations in genomic DNA :

To confirm the mutations in genomic DNA, part of exon 3, around the site of the A194G mutation, and part of exon 5, around the site of G346A, were amplified from genomic DNA using primer set A (tccttgctttgccaggTCCCAGC and atcaaaagcaggactta CGGGGC) and mismatch primer set B (TGGCCA AGTGTGAGTTCTTCAACGCCTGC and GATAAT CGTGTCCCCGGGCTTCAGCGTC), which creates a Pst I site in PCR products, respectively. After PCR amplification, PCR products were digested with Mae III or Pst I, respectively, and analyzed with 4% NuSieve agarose gel electrophoresis.

Expression of CBS protein in E. coli :

The cDNA encompassing the entire coding region of normal CBS cDNA was amplified by PCR and inserted into a pCR2.1 vector. After confirming the cDNA sequencing, a pCR2.1 vector containing normal CBS cDNA was digested with Eco RI and a CBS cDNA fragment was subcloned into the prokaryotic expression vector pKK223-3 (Pharmacia), and the direction of the cDNA was confirmed by digestion

with Pst I (pKK-CBS). To construct the expression vector of mutant CBS, segments of the patients ' CBS cDNA which contained the individual mutations were amplified by PCR and replaced by the corresponding segment of normal CBS cDNA in the pKK-CBS plasmid (pKK-CBS-A 194G and pKK-CBS-G346A). All of the mutant CBS constructs were confirmed by sequencing. The E. coli strain JM105 was transformed with pKK-CBS, pKK-CBS-A194G or pKK-CBS-G346A and cultured in 100 ml of the culture medium at 37°C overnight. The expression of CBS protein was induced by adding 1 mM isopropyl β-D-thiogalactoside (IPTG), and after incubation at 37°C for 5 hours, the cells were harvested by centrifugation. Cells were disrupted by sonication and centrifuged at 10,000 g for 30 min at 4°C. The CBS enzyme activity in the supernatant was measured by the method of Fleisher *et al*. (7) modified according to Kozich and Kraus (8).

RESULTS

Detection of mutation :

In case 1, four of eight sequenced clones had the A-to-G transition at nucleotide 194 (A194G), which changed an encoded histidine to arginine at codon 65 (H65R) (Fig1A). In case 2, four of six sequenced clones had the same A194G mutation. These clones had no other muation. The remaining clones from cases 1 and 2 had only the G-to-A transition at nucleotide 346 (G346A), which changed an encoded glycine to arginine at codon 116 (G116R) (Fig 1 B).

The mutation A194G created a new Mae III site, and so a 250-bp genomic DNA fragment around this mutation was amplified by PCR and digested with Mae III to confirm this mutation in genomic DNA from patients and their parents. After digestion with Mae III, a normal control and the father had only a 250-bp DNA fragment. On the other hand, these siblings and their mother had three DNA fragments : 250 bp, 217 bp and 33 bp (Fig 2 A). The mutation G346A created no new restriction enzyme sites, and so a 112-bp DNA fragment around this mutation was amplified using a mismatch primer, which created a Pst I site in the mutant gene. After Pst I digestion, a normal control and the mother had only 112-bp fragments, while the patients and their father had three fragments : 112 bp, 83 bp and 29 bp (Fig 2 B). These results indicated that these siblings were compound heterozygotes for these mutations and their mother and father were heterozygous for A194G and G346A, respectively.

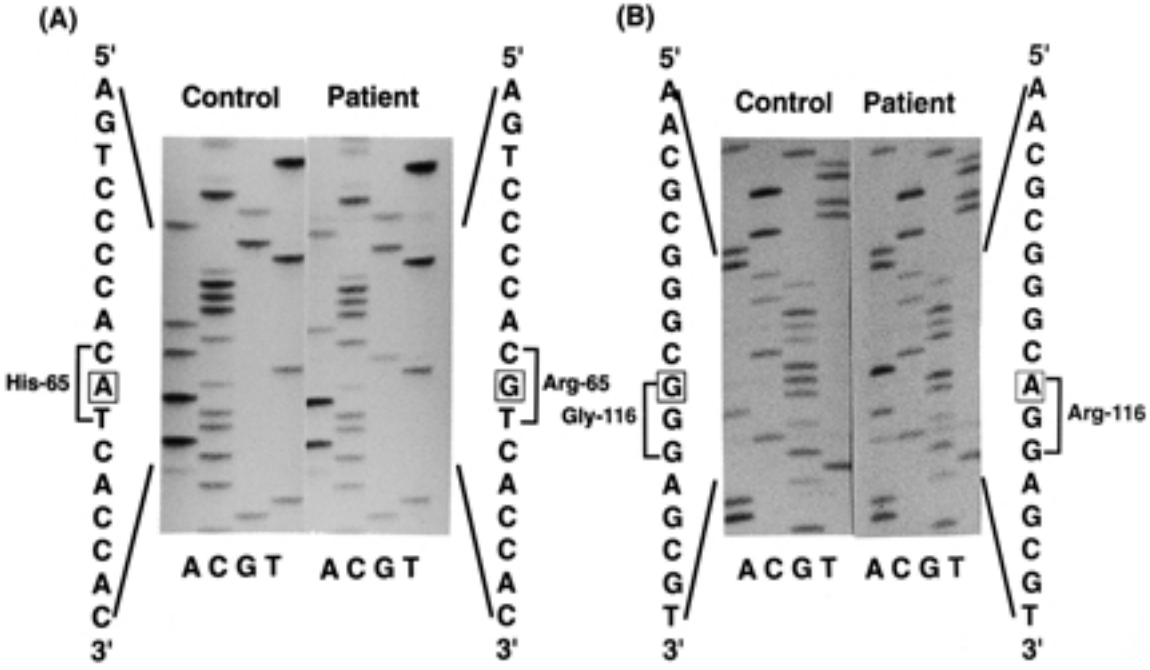

Fig.1. Sequence analysis of CBS cDNA obtained from a normal control and two siblings with CBS deficiency. (A) Four of eight sequenced clones from case 1 and four of six sequenced clones from case 2 showed an A-to-G transition at nucleotide 194 (A194G), resulting in a predicted histidine-to-arginine substitution at amino acid 65 (H65R). (B) Four of eight sequenced clones from case 1 and two of six sequenced clones from case 2 showed a G-to-A transition at nucleotide 346 (G346A), resulting in a predicted glycine-to-arginine substitution at amino acid 116 (G116R)

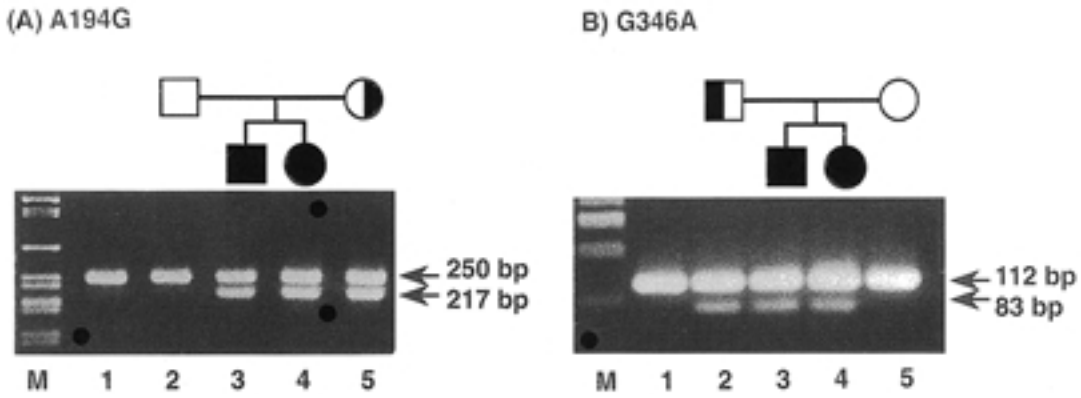

Fig. 2. Detection of mutations in genomic DNA. Lane M, size marker. Lane 1, normal control. Lane 2, father. Lane 3, case 1. Lane 4, case 2. Lane 5, mother. (A) Detection of A 194 G in the CBS gene. PCR products (250bp), amplified by primer set A, were digested by Mae III enzyme, which either gives two fragments of 217 bp and 33 bp in the case of the mutated sequence (194 G) or leaves the product undigested in the case of the wild-type sequence (194 A). (B) Detection of G 346 A in the CBS gene. PCR products (112 bp), amplified by mismatch primer set B, were digested by Pst I enzyme, which either gives two fragments of 83 bp and 29 bp in the case of the mutated sequence (346 A) or leaves the product undigested in the case of the wild-type sequence (346 G).

Table 1. CBS Activity in the Sonicated Supernatant of Transformed E. coli Cells

Plasmid	CBS Activity[a] (nmol/min/mg of total protein)
Non-transformed JM105	n.d.
pKK223-3	n.d.
pKK-CBS-WT	7.63
pKK-CBS-A194G	n.d.
pKK-CBS-G346A	n.d.

n.d. : not detectable

a) CBS activities were measured in two or three separate experiments and the mean value is shown.

Enzyme activities of the wild-type and mutant CBS in E. coli :

The wild-type and mutant CBS protein were expressed by transforming the E. coli strain JM105 with pKK223-3, pKK-CBS-WT, pKK-CBS-A194G or pKK-CBS-G346A. After the induction of CBS protein with IPTG, the enzyme activity in E. coli cells, containing the wild-type or one of the two mutant CBS cDNAs, was measured. CBS enzyme activities in E. coli without plasmid vector and with pKK-233-3 were not detected. The enzyme activity of wild-type CBS was 7.63 nmol/min/mg of protein, however, the activities of CBS from cDNAs with the mutations A194G and G346A could not be detected, similar to E. coli without CBS cDNA (Table 1).

DISCUSSION

Since the isolation and characterization of the human CBS cDNA sequence in 1993, studies on the CBS mutations in patients with homocystinuria in the USA and Europe have lead to the identification of about 64 mutations within the CBS gene (9). In this study, two mutations in the CBS gene, A194G and G346A, were found in pyridoxine-nonresponsive homocystinuric siblings who showed different blood methionine levels during the neonatal period. The A194G mutation is new while G346A has been reported previously in European patients (10). Since CBS proteins from these mutations had no detectable enzyme activity by an expression analysis in E. coli, these mutations were pathogenic in these patients. The results of a mutation analysis on genomic DNA from these patients and their parents indicated that these siblings were compound heterozygotes of maternal A194G and paternal G346A, and did not have any other mutations.

To begin treatment for homocystinuria as soon as possible and to prevent patients from developing clinical symptoms, the mass screening of newborns for homocystinuria due to CBS deficiency is performed in many countries by measuring the methionine concentration in blood, since blood methionine concentrations are usually elevated in patients with CBS deficiency even during the neonatal period (2). In Japan, a nationwide newborn mass screening program was started in 1972 by analyzing the methionine concentration in dried blood spots using a semi-quantitative bacterial inhibition assay. We found two Japanese siblings ; one

was identified by mass screening based on a high concentration of methionine in the blood and the other was missed by a mass screening due to a low level of methionine in blood, and was diagnosed after presenting clinical symptoms (3). Yap and Naughten reported four patients who were missed by newborn screening for CBS deficiency in Ireland (11) and Naughten *et al*. (2) suggested four possible reasons why a patient with homocystinuria could be missed by a neonatal mass screening : 1) early hospital discharge, 2) low protein intake, especially if breast fed, 3) high blood methionine cut-off concentration, and 4) pyridoxine responsiveness. However, there was no difference in these conditions between our siblings ; i.e., blood collection was performed at 5 days of age, their protein intakes were 2.5 g/kg/day with normal milk formula, they did not respond to the administration of a high dose of pyridoxine, and the cut-off methionine concentration in the screening program was 67 μmol/L (3), which is the lowest cut-off value in the world (2). We analyzed the CBS gene in these siblings at the molecular level because it was possible that their genotypes were different although they were siblings, and so that they showed different methionine concentrations in the neonatal period. However, they had identical genotypes for the CBS gene ; i.e., compound heterozygote with A194G and G346A.

CBS is a homotetramer of 63 kd subunits (1). In tissues of CBS-deficient patients with two mutant alleles, this enzyme may consist of various heterotetramers of two mutant subunits and/or the formation of heterotetramers of this enzyme may be disturbed according to the ratio of amounts of the two mutant subunits. Although the enzyme activities of a mutated homotetramer of CBS from our patients expressed in E. coli could not be detected, heterotetramers of CBS with two mutant subunits might have some residual enzyme activities. Furthermore, correct CBS folding requires the presence of heme (9), and the CBS activity depends on the contribution of several factors, including pyridoxal 5 -phosphate (12), heme biosynthesis (13), and adenosylmethionine formation (14). Therefore, a difference in the amount of each type of heterotetramer or in these other factors, which may result in the disturbance of the heterotetramer formation, likely affected the CBS activity during the fetal and neonatal periods in these siblings to cause different concentrations of methionine when blood was collected for screening of CBS deficiency, despite their identical genotypes.

Blood methionine concentrations are determined by the activities of enzymes involved in methionine and homocysteine metabolism, including methionine adenosyltransferase, CBS, 5-methyltetrahydrofolate-homocysteine methyltransferase, and betaine-homocysteine methyltransferase (1). The specific activities of methionine adenosyltransferase and betaine-homocysteine methyl-transferase are lower in fetal liver than in the adult liver, whereas the activity of 5-methyltetrahydrofolate-homocysteine methyltransferase is higher in fetal tissue than in adult tissue (15, 16). This fetal enzyme pattern should direct a large portion of the available homocysteine to the 5-methyltetrahydrofolate-dependent methylation cycle rather than to the synthesis of cystathionin. The failure to detect hypermethioninemia in some newborn infants with a deficiency of CBS might be explained by a quantitative difference in the activities of enzymes involved in the metabolism of methionine and homocysteine. This suggests that in our patients, the development of these enzymes, except CBS, differed during the fetal and neonatal periods, or the genotypes were different for these enzymes, which are not pathogenic and result in different enzyme activities or development patterns, so that their blood methionine concentrations during the neonatal period were not the same although they had identical genotypes for the CBS gene, which had two pathogenic mutations.

These results indicate that measurement of the methionine concentration in blood is not adequate for identifying all patients with pyridoxine-nonresponsive CBS-deficiency even if they intake enough protein and are not discharged early, and if the cut-off value of methionine in neonatal screening is low. Therefore, to begin treatment as soon as possible and to prevent patients from developing clinical symptoms, a CBS gene analysis and/or an assay of CBS activity should be performed in high-risk newborns, who have siblings with CBS deficiency, even if the results of a neonatal screening for CBS deficiency are normal. In addition, a new, more sensitive method for the mass screening of CBS deficiency in neonates, by which all patients with CBS deficiency can be detected, should be developed.

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