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Short Report

Holocarboxylase synthetase deficiency: novel clinical and molecular findings

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Multiple carboxylase deficiency (MCD) is an autosomal recessive metabolic disorder caused by defective activity of biotinidase or holocarboxylase synthetase (HLCS) in the biotin cycle. Clinical symptoms include skin lesions and severe metabolic acidosis. Here, we reported four unrelated Thai patients with MCD, diagnosed by urine organic acid analysis. Unlike Caucasians, which biotinidase deficiency has been found to be more common, all of our four Thai patients were affected by HLCS deficiency. Instead of the generally recommended high dose of biotin, our patients were given biotin at 1.2 mg/day. This low-dose biotin significantly improved their clinical symptoms and stabilized the metabolic state on long-term follow-up. Mutation analysis by polymerase chain reaction-sequencing of the entire coding region of the *HLCS* gene revealed the c.1522C>T (p.R508W) mutation in six of the eight mutant alleles. This suggests it as the most common mutation in the Thai population, which paves the way for a rapid and unsophisticated diagnostic method for the ethnic Thai. Haplotype analysis revealed that the c.1522C>T was on three different haplotypes suggesting that it was recurrent, not caused by a founder effect. In addition, a novel mutation, c.1513G>C (p.G505R), was identified, expanding the mutational spectrum of this gene.

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Multiple carboxylase deficiency (MCD) is a rare autosomal recessive metabolic disease caused by defects of enzymes, either biotinidase (biotinidase deficiency, MIM 253260) or holocarboxylase synthetase (HLCS deficiency, MIM 253270), in the biotin cycle. The clinical presentations include severe metabolic acidosis, feeding and breathing difficulties, hypotonia and lethargy. Some patients have dermatologic signs which are erythematous rashes and hair loss (1). Ten milligrams per day biotin usually improve clinical symptoms (1, 2). Patients with HLCS deficiency usually have the neonatal or early-onset form, while patients with biotinidase deficiency have the juvenile or late-onset form. However, the age of onset and phenotypes are highly variable. Reliable diagnosis of either MCD types therefore requires enzyme activity or genetic analysis.

HLCS (EC 6.3.4.10) catalyzes the biotinylation of biotin-dependent mitochondrial carboxylases. In mammals, there are four carboxylases that require biotinylation: acetyl-CoA carboxylase, pyruvate carboxylase, propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase. These carboxylases are essential for cellular biosynthesis. Therefore, defects in HLCS, which reduce the biotin-dependent enzyme activity, affect several important metabolic processes. HLCS is encoded by an 11-exon gene, *HLCS*, located on chromosome 21q22.1. To date, there are at least 35 mutations reported in *HLCS* (http://www.hgmd.cf.ac.uk, accessed August 2009).

Here, we report four Thai patients with MCD. All of them were diagnosed to have HLCS deficiency. Treatment with low dose of biotin was able to significantly improve their clinical

symptoms. In addition, a common mutation was identified in Thai patients and a novel mutation was described.

Materials and methods

Patients

Four patients were referred to King Chulalongkorn Memorial Hospital with skin rashes or coma and severe metabolic acidosis. The age of onset varied from 1 to 9 months. They were diagnosed with MCD by urine organic acid analysis (3) (Fig. 1a and Table 1).

Biotinidase activity assay

The assay was performed on plasma using a colorimetric method. The biotinyl-*p*-aminobenzoate (B-*p*-ABA) was used as a substrate, as described by Pettit et al. (4). Patients' biotinidase activities were compared to those of 245 normal Thai controls.

Mutation analysis

Reverse transcription polymerase chain reaction (PCR) was performed on white blood cells. Total RNA was isolated using QIAamp® RNA Blood Mini Kit (Qiagen, Valencia, CA). Reverse transcription was performed using ImProm-II™ Reverse Transcriptase (Promega, Madison, WI). The entire coding region of *HLCS* was PCR amplified by two primer pairs (Table 2). PCR products were directly sequenced. The mutations found were confirmed on genomic DNA of the patients obtained from whole blood by PCR sequencing.

Restriction fragment length polymorphism analysis

Restriction fragment length polymorphism (RFLP) was used to confirm mutations on genomic DNA of the patients and to screen for the presence of each mutation in their parents. *HLCS* exons 7 and 8 were amplified and treated with *Fox*I or *Hha*I (New England Biolabs, Berverly, MA),

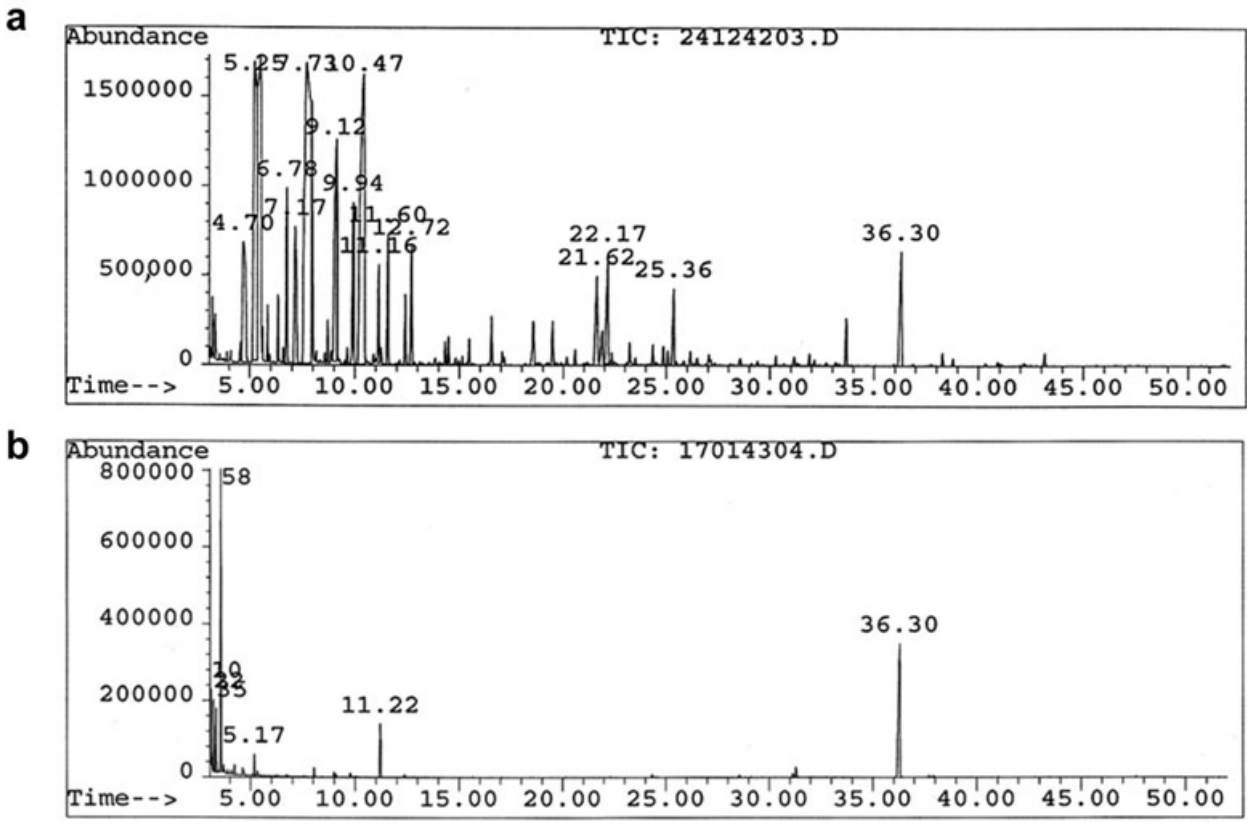

Fig. 1. Urine organic acid analysis of patient 1. **(a)** The tracing before the initiation of biotin revealing large peaks of lactic acid at 5.25 min, 3-hydroxypropionic acid at 7.17 min, 3-hydroxy-n-valeric acid at 9.94 min, and 3-methylcrotonylglycine at 22.17 min, consistent with the diagnosis of multiple carboxylase deficiency. **(b)** The tracing 24 days after the initiation of 1.2 mg/day of biotin showing no detectable levels of the abnormal acids. The peak at 36.30 of both panels represented the internal standard.

Table 1. Clinical description of four Thai patients with holocarboxylase synthetase deficiency

Patient	1	2	3	4
Gender	Female	Female	Female	Male
Consanguinity	−	+	−	−
Age of onset	1 month	8 months	9 months	9 months
Age at diagnosis	6 months	9 months	2 years 5 months	6 years
Age at last follow-up	8 years	3 years	6 years	12 years
Biotinidase activity (nmol/min/ml; normal: 5.63 ± 1.25 nmol/min/ml)	8.48	4.88	6.60	7.64
Development at last follow-up	Normal	Normal	Mildly delayed	Moderate mental retardation (IQ = 50)[a]

[a]Intelligence testing on Stanford-Binet Intelligence Scales at the age of 9 years and 3 months.

respectively. All of the reactions were performed according to the company's recommendations.

Haplotype analysis

Tetra-nucleotide repeats, CAAA and ATTC in introns 8 and 9, respectively, were amplified as described by Yang et al. (5). Primer pairs and PCR conditions were shown in Table 2. Haplotypes were determined by a combination of the CAAA and ATTC repeats. EH program was employed to estimate the haplotype frequencies of 100 control alleles (6).

Results

Clinical presentations

MCD was suggested in four patients by urine organic analysis (Table 1). All patients had large amounts of 3-hydroxypropionic acid, 3-hydroxyisovaleric acid, 3-methylcrotonylglycine and lactic acid. The urine organic acid profile from patient 1 was shown in Fig. 1a. They had skin lesions around their mouth, eyes, neck and perineum, but none around the nose area (Fig. 2). After the diagnosis of MCD, they were treated with biotin. For all of the patients, treatment with biotin 1.2 mg/day, with the exception of periods of acute infection when the biotin dose was increased to threefold, was sufficient to eliminate skin rashes (Fig. 2) and restore normal acid–base balance. The urine organic acids in all patients were restored to normal. The urine organic acid profile of patient 1 at 24 days after the initiation of treatment with 1.2 mg/day of biotin showed undetectable 3-hydroxypropionic acid, 3-hydroxyisovaleric acid, 3-methylcrotonylglycine and lactic acid (Fig. 1b). For patients 1 and 2, who were early diagnosed, their development during their last follow-ups remained appropriate (Table 1).

Biotinidase activity analysis

To differentiate the two forms of MCD, biotinidase activities were measured and compared to those of unaffected controls ($n = 245$) (Table 1). All four patients were found to have biotinidase activity in the normal range (4.88–8.64 nmol/min/ml in patients compared to 5.63 ± 1.25 nmol/min/ml in

Table 2. Oligonucleotides and polymerase chain reaction (PCR) conditions for *HLCS* mutation analysis

Name	Primer sequences from 5′ to 3′	Annealing Temperature (°C)	Note
*HLCS*1-F	CTGGGGATCCTTATCGGCTA	64	PCR cDNA
*HLCS*1-R	CATGTCACAGCTGAGGCCAA		
*HLCS*2-F	TCCCAGCTCCAACATAGTG	62	PCR cDNA
*HLCS*2-R	CAGATGCATGGGCACGGACA		
*HLCS*3	TTTCTCAGGGAGGGAAGGTG	—	Sequencing
*HLCS*4	TTCAGACACCGCAGGAAATG	—	Sequencing
HLCS exon 7-F	CTCATGGCTCCACATTCCTG	58	PCR gDNA
HLCS exon 7-R	CTCCATTCCAGGCGGTTATG		
HLCS exon 8-F	GAGTGTGTGGCCCTGGCATA	60	PCR gDNA
HLCS exon 8-R	GCTGAGGTTCTACAGCCACC		
CAAA-F	CTGTAGTCCCAGCTAGTTGA	58	microsatellite
CAAA-R	CATTTTCCACCACAGCTGAG		
ATTC-F	CTCTGGTGAATGGAAGAACC	60	microsatellite
ATTC-R	CAGCAGGAGACCAGTATAGG		

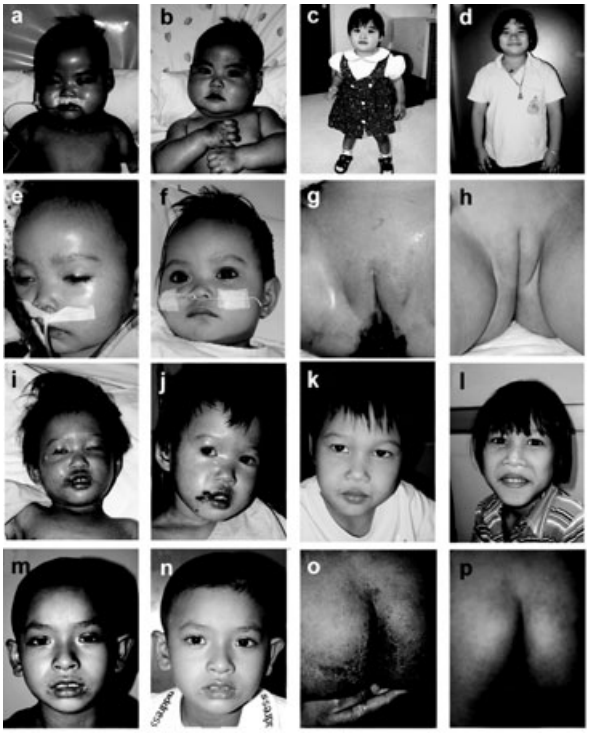

Fig. 2. Clinical features. **(a–d)** Patient 1, (**e–h**) patient 2, (**i–l**) patient 3, (**m–p**) patient 4. (**a**, **e**, **i**, **m**) Patients' faces before the biotin treatment. (**b**, **f**, **j**, **n**) Patients' faces approximately 1 week after the initiation of biotin. (**c**, **d**) Patient 1 at the ages of 18 months and 8 years, respectively. (**g** and **h**) Patient 2's perineum before and 1 week after the treatment, respectively. (**k** and **l**) Patient 3 at the ages of 4 and 6 years, respectively. (**o** and **p**) Patient 4's buttock before and 1 week after treatment, respectively.

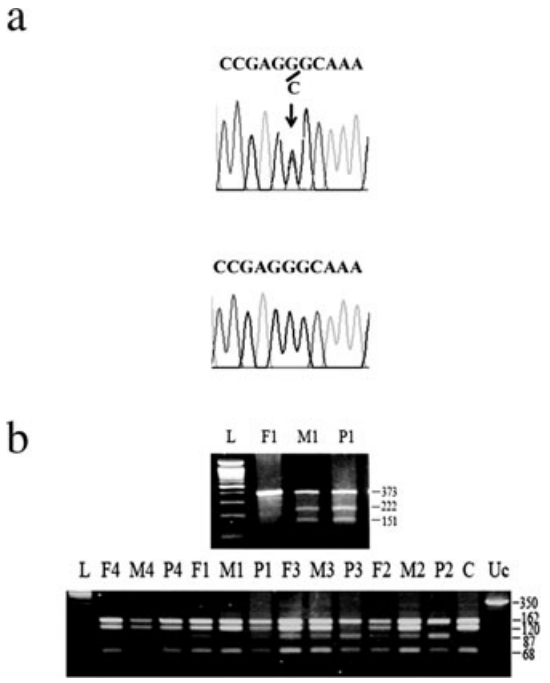

Fig. 3. Mutation analysis. **(a)** Chromatograms demonstrate a nucleotide change detected in the patient 1 (top) compared to an unaffected control (bottom). A black arrow shows the c.1513G>C mutation. (**b**) Restriction fragment length polymorphism (RFLP) analysis for the nucleotide changes. Top, RFLP analysis for the c.1513G>C change in patient 1 (P1) and her parents (F1 = father, M1 = mother). The mutation creates a new restriction site for *Hha*I in exon 7 and enables it to cut the 373-bp polymerase chain reaction (PCR) product into 222- and 151-bp products. Bottom, genomic DNA of the patients (P1–P4) and their parents (F = father, M = mother) was amplified for *HLCS* exon 8, yielded 350-bp products, which were treated with *Fox*I restriction enzyme. *Fox*I digested the wild-type allele of a normal control (C) into 162-, 120-, and 68-bp products. The c.1522C> T mutation creates one of the *Fox*I restriction sites. The digestion of its PCR product therefore revealed 162-, 87-, 68-, and 33-bp products. Note that the 33-bp band is not visible in this figure. (L = DNA ladder, C = normal control, Uc = uncut amplified product).

controls). This finding excluded the diagnosis of biotinidase deficiency and suggested that all the patients had the HLCS deficiency form of MCD.

Mutation analysis

PCR sequencing revealed sequence variants in seven alleles; six were c.1522C>T (p.R508W) and the other one was c.1513G>C (p.G505R) (Table 3 and Fig. 3a). No other nucleotide changes were found in and around exonic regions. The parents' genotypes for the corresponding changes were identified by RFLP analysis. The results were consistent with the genotypes of the probands (Fig. 3b).

Haplotype analysis

As the c.1522C>T mutation was present in six out of eight alleles in our four patients, it

Table 3. Mutations in the *HLCS* gene and haplotypes of Thai patients with holocarboxylase synthetase deficiency

Patient	Nucleotide change	Amino acid change	CAAA repeat	ATTC repeat	Haplotype
1	c.1522C>T/c.1513G>C	p.R508W/p.G505R	8–9	9–9	8–9/9–9
2	c.1522C>T/c.1522C>T	p.R508W/p.R508W	7–7	10–10	7–10/7–10
3	c.1522C>T/c.1522C>T	p.R508W/p.R508W	8–8	9–9	8–9/8–9
4	c.1522C>T/U[a]	p.R508W/U	8–8	7–10	8–7/8–10

[a]U, unidentified.

was interesting to investigate whether it was a founder mutation in our Thai population. Two microsatellite markers, CAAA and ATTC, in *HLCS* introns 8 and 9 were used, as described by Yang et al. (2000) (5). Fifty unaffected Thai individuals were examined for both markers by PCR and sequencing (Table 4). A haplotype with nine CAAA repeats and eight ATTC repeats, accounting for 25%, was found to be the most frequent haplotype in Thais. The c.1522C>T (p.R508W) change was associated with at least three haplotypes: 7–10, 8–9 and either 8–7 or 8–10. The c.1522C>T in patient 1 and patient 4 may associate with the haplotype 8–9 or 9–9, and 8–7 or 8–10, respectively. Our findings suggested that the c.1522C>T was a recurrent rather than a founder effect mutation in our population.

Discussion

In our 10-year experience at King Chulalongkorn Memorial Hospital, all Thai patients with MCD diagnosed by urine organic acid analysis had normal serum biotinidase activities, excluding biotinidase deficiency. Our findings were different from those found in western countries in which a larger group of children with MCD were deficient in biotinidase activity (7). Although our study included only a small number of patients, it suggested that majority of Thai patients with MCD could have defects in HLCS, instead of biotinidase. This finding was without ascertainment bias, as the phenotypes of the two forms of MCD are variable and overlap with each other and all MCD patients we identified were included in the study.

Another interesting finding from this cohort of patients is that dermatitis has been found in the periorbital and perioral areas, neck and perineal area, but never been detected around the nose area similar to those previously reported (1, 8, 9) (Fig. 2).

Table 4. Haplotype frequency of the *HLCS* gene in 100 control alleles

Haplotype repeat number (CAAA-ATTC)	Frequency (%)
7–7	0.8
7–8	1.8
7–9	1.4
8–7	7.6
8–8	16.7
8–9	13.7
9–7	11.6
9–8	25.5
9–9	20.9

At the time of their diagnosis, the availability of biotin in our institute was limited; a dose of 1.2 mg/day of biotin was therefore given. With close observation, we found that this low level of biotin significantly improved their symptoms and on the long-term follow-up, it was able to stabilize the metabolic state and maintain their developmental milestones. Although the dose of 1.2 mg per day is lower than the lowest dose ever reported in the literature, it is about a hundred times higher than daily requirement for normal children, which is 12 μg/day (Food and Nutrition Board, Institute of Medicine, National Academies). It is generally recommended to give 10 mg/day of biotin to patients with HLCS (1) and a massive dose of enteral biotin of 100 mg/day was also given in some cases (10). In our patients, it could be possible that the disease onset after neonatal period might indicate a reasonable remaining amount of enzymatic activity, which might explain the relatively low biotin dosage required in these cases. This relatively small dose could result in a normal growth and development without acidosis in patients 1 and 2 who were diagnosed and treated within 9 months of age. However, delayed diagnosis and delayed treatment in patients 3 and 4 resulted in developmental delay. Notably, developmental outcomes of our four patients correlated with their ages when biotin was started; the earlier the initiation of biotin, the better the developmental outcomes (Table 1).

To our knowledge, this is the first mutation study of HLCS deficiency in Southeast Asia. Of our four patients, seven mutant alleles were identified; six were c.1522C>T (p.R508W) and the other one was c.1513G>C (p.G505R). The finding of p.R508W in all four patients (100%) and in six out of eight alleles (75%) makes it possible the most common mutation in the Thai population. Although it has been reported in American, Chinese, Japanese, French, and Iranian (8, 11), it has not been demonstrated as the most common in these previously studied ethnic groups. The closest ethnic group studied for mutations in this gene was probably the Japanese in which their most common mutations were a truncating mutation (c.1067delG) and a missense mutation (p.L237G) (11). This information will certainly help facilitate the diagnosis of HLCS deficiency in Thai patients.

For the polymorphic markers in the *HLCS* gene in the Thai population, the number of CAAA and ATTC repeats ranged from 7 to 9 and 7 to 10, respectively, compared to 6 to 8 and 10 to 15 in the Japanese population (5). While the Japanese study revealed that two most common mutations, p.L237P and c.1067delG, were founder mutations,

our data indicating that the most common mutation in the Thai population, p.R508W, was associated with at least three haplotypes, suggesting that it was rather a recurrent mutation.

The c.1513G>C (p.G505R) has never been previously reported (Fig. 3). This expands the mutational spectrum of *HLCS* to at least 36 (The Human Gene Mutation Database: http://www.hgmd.cf.ac.uk/ac/index.php; searched on August, 2009). Both mutations identified in this study are in the HLCS biotin-binding domain. It has been reported that mutations in this domain are responsible for the increased Km for biotin, which may explain the clinical biotin responsiveness and the disease onset after neonatal period (1, 12).

For an unidentified mutation in another allele of patient 4, several possibilities include it being in unexplored regions, for example, regulatory element, promoter, or intronic regions, or it being undetectable by our methods, such as an exonic deletion mutation.

In summary, we reported four unrelated Thai patients with MCD. An interesting clinical finding includes the lack of dermatitis of the perinasal area. In our Thai population, HLCS deficiency is probably more common than biotinidase deficiency. The low-dose biotin has been able to stabilize the metabolic state and maintain the patients' developmental milestones on long-term follow-up. The recurrent c.1522C>T (p.R508W) mutation is the most common mutation in the Thai population. In addition, a novel mutation, c.1513G>C (p.G505R), was identified.

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References

1. Wolf B. Disorders of biotin metabolism. In: Schriver CR, Beaudet AL, Sly WS et al., eds. The metabolic and molecular basis of inherited diseases. New York, NY: McGraw-Hill, 2000: 3935–3962.
2. Suormala T, Fowler B, Duran M et al. Five patients with a biotin-responsive defect in holocarboxylase formation: evaluation of responsiveness to biotin therapy in vivo and comparative biochemical studies in vitro. Pediatr Res 1997: 41: 666–673.
3. Shotelersuk V, Srivuthana S, Wacharasindhu S et al. Establishing gas chromatography-mass spectrometry to diagnose organic acidemias in Thailand. Southeast Asian J Trop Med Public Health 2000: 31: 566–570.
4. Pettit DA, Wolf B. Quantitative colorimetric assay of biotinidase activity. In: Hommes F, ed. Techniques in diagnostic human biochemical genetics: a laboratory manual. New York, NY: Wiley-Liss, 1991: 561–565.
5. Yang X, Aoki Y, Li X et al. Haplotype analysis suggests that the two predominant mutations in Japanese patients with holocarboxylase synthetase deficiency are founder mutations. J Hum Genet 2000: 45: 358–362.
6. Tervilliger J, Ott J. Handbook of human genetic linkage. Baltimore, MD: John Hopkins University Press, 1994.
7. Fenton WA, Gravel RA, Rosenblatt DS. Disorders of propionate and methylmalonate metabolism. In: Schriver CR, Beaudet AL, Sly WS et al., eds. The metabolic and molecular bases of inherited disease. New York, NY: McGraw-Hill, 2001: 2165–2194.
8. Morrone A, Malvagia S, Donati MA et al. Clinical findings and biochemical and molecular analysis of four patients with holocarboxylase synthetase deficiency. Am J Med Genet 2002: 111: 10–18.
9. Seymons K, De Moor A, De Raeve H, Lambert J. Dermatologic signs of biotin deficiency leading to the diagnosis of multiple carboxylase deficiency. Pediatr Dermatol 2004: 21: 231–235.
10. Van Hove JL, Josefsberg S, Freehauf C et al. Management of a patient with holocarboxylase synthetase deficiency. Mol Genet Metab 2008: 95: 201–205.
11. Suzuki Y, Yang X, Aoki Y, Kure S, Matsubara Y. Mutations in the holocarboxylase synthetase gene HLCS. Hum Mutat 2005: 26: 285–290.
12. Dupuis L, Leon-Del-Rio A, Leclerc D et al. Clustering of mutations in the biotin-binding region of holocarboxylase synthetase in biotin-responsive multiple carboxylase deficiency. Hum Mol Genet 1996: 5: 1011–1016.