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Molecular analysis of holocarboxylase synthetase deficiency: a missense mutation and a single base deletion are predominant in Japanese patients

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Abstract

Holocarboxylase synthetase (HCS) deficiency is an inherited disease of biotin metabolism characterized by a unique pattern of organic aciduria, metabolic acidosis, and skin lesions. By analysis of five patients in four unrelated families, two mutations were identified: a transition from T to C which causes an amino-acid substitution of proline for leucine at position 237 (L237P) and a single deletion of guanine (delG1067) followed by premature termination. One patient was homozygous for the L237P mutation, three patients in two families were compound heterozygotes of the missense and deletion alleles, and the other patient was heterozygous for the L237P mutation. Inheritance was successfully demonstrated in all of the patients' families by a modified PCR followed by restriction enzyme digestion. The two mutations accounted for seven of eight mutant alleles, while neither mutation was detected in 108 normal healthy Japanese children (216 alleles). Transient expression in cultured fibroblasts from a patient showed that the L237P mutation was responsible for decreased HCS activity. These results suggest that the L237P and delG1067 mutations are frequent disease-causing mutations in Japanese patients with HCS deficiency. This PCR-based technique may therefore be useful for detecting mutations among Japanese patients.

Keywords: Holocarboxylase synthetase deficiency; Molecular analysis; Single base deletion; Missense mutation; Biotin metabolism; (Human)

1. Introduction

Holocarboxylase synthetase (HCS) deficiency is an autosomal recessive disorder of organic acid metabolism in man [1]. HCS is an enzyme that catalyzes the binding of biotin to various carboxylases, using ATP for biotin activation [2]. Most patients with this disease manifest such symptoms as tachypnea, feeding difficulties, seizures, and dermatitis in the early infantile period. The elevation of various organic acids in the urine indicates a defect in multiple carboxylases. The activities of various mitochondrial carboxylases (pyruvate carboxylase, propionyl-CoA carboxylase and methylcrotonyl-CoA carboxylase) and cytosolic acetyl-CoA carboxylase have been reported to be decreased in fibroblasts obtained from HCS-deficient patients [3–5]. The clinical symptoms are usually improved dramatically with the oral administration of 10 to 20 mg of biotin per day. HCS deficiency is a clinically heterogeneous disorder. The age of onset varies from a few hours after birth to 15 months of age and the biotin dose required for controlling the clinical symptoms varies several fold [1,6]. The degree of decreased affinity of HCS for biotin has been demonstrated to correlate with the age of onset [6,7].

We have recently purified HCS from bovine liver [8]. Using sequence data deduced by analysis of peptides from purified enzyme, we isolated human HCS cDNA clones [9]. The clones encode a polypeptide of 726 amino acids with a calculated molecular mass of 80 759 Da. Isolation of HCS cDNA enabled us to identify a one base deletion (delG1067) that resulted in premature termination at amino acid 280 and a missense mutation (L237P) in cells from

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siblings with HCS deficiency. However, it has not been determined whether the missense mutation accounts for the disease phenotype.

The purpose of this study was to expand the analysis of HCS deficiency to new patients at the molecular level. Another goal was to develop a transient expression system of HCS and to determine whether the mutant protein was responsible for decreased HCS activity.

2. Materials and methods

2.1. Patients and cell lines

Table 1 shows the clinical and biochemical data and outcome of five Japanese patients. Parents were not consanguineous in any family. Patient 1 developed metabolic ketoacidosis associated with severe neonatal distress within one day following birth. This patient responded to 10 mg per day of oral biotin which led to remarkable improvement of the clinical and biochemical abnormalities. Physical and psychomotor development was normal on oral biotin (40 mg/day) at 9 months. However, her most recent intelligence quotient (IQ) score was only 66 at 5 years, although she has remained free of other symptoms. Patient 2 manifested symptoms on the first day of life. High doses of biotin (80 mg/day) were required to control associated dermatitis. Patient 3 was discovered by detection of elevated leucine in a blood sample submitted for newborn mass screening. The patient was treated with 10 mg of oral biotin per day and his IQ was 74 at 5 years of age. Patients 4a and 4b have been described elsewhere [4,9].

Fibroblasts were maintained in minimum essential medium (MEM) supplemented with antibiotics and 10% FCS at 37°C in 5% CO_2. Lymphoblastoid cell lines were established by infection with Epstein–Barr virus and maintained under the same conditions as fibroblasts except that RPMI 1640 was used instead of MEM. HCS activity in the cultured cells was determined by monitoring the rate of holoenzyme synthesis using apopropionyl-CoA carboxylase from HCS-deficient cells as the substrate [8]. HCS activity in all the patients was less than 6% of the normal control value.

2.2. Nucleotide sequence analysis of cDNA

Total RNA was extracted from cultured lymphoblasts using a guanidinium thiocyanate/phenol/chloroform RNA extraction method [10]. Poly(A^+) RNA fractions were purified with an mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden) and cDNA was synthesized as described previously [9]. The entire coding region of HCS cDNA was amplified by polymerase chain reaction (PCR) with four pairs of oligonucleotide primers in two overlapping fragments [9]. The DNA was subcloned into the *Xho*I-*Eco*RI site of pBluescript II KS + (Toyobo, Tokyo, Japan) and sequenced on an automated laser fluorescent (A.L.F.) sequencing apparatus (Pharmacia Biotech).

2.3. Direct sequencing

Genomic DNA was extracted from cultured lymphoblastoid cells using the Sepa Gene kit (Sanko Junyaku, Tokyo, Japan). One μg of genomic DNA was subjected to PCR amplification with primers S4 and AS5 (Table 2). Each amplification was performed in a volume of 30 μl containing 200 pmol of each primer, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 100 μM of each dNTP, and 2.5 units of *Taq* DNA polymerase (Wako, Osaka, Japan). Each of the 25 amplifi-

Table 1
Clinical data of patients with HCS deficiency

Patient	1	2	3	4a	4b
Sex	F	M	M	F	F
Age of onset, day	1	1	? [a]	2	PT [b]
Respiratory distress	+	+	−	+	−
Seizure	−	−	−	+	−
Hypotonia	−	+	−	−	−
Coma	−	−	−	−	−
Skin rash	−	+	−	+	−
Metabolic acidosis	+	+	−	+	−
Hyperammonemia	−	+	−	+	−
Organic aciduria	+	+	−	+	−
Age at evaluation, years	5	7	5	10	6
Intelligence quotient score	66	80	67	69	64
%HCS activity of normal value	6	UD [c]	3	UD [c]	2

Note: '+' = presence of symptom and '−' = absence of symptom.
[a] Found by detection of elevation of leucine in neonatal mass screening.
[b] Prenatal treatment.
[c] Undetectable.

cation cycles consisted of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min. One μl of the first amplification was used as the template for the second PCR amplification. The sense primer had an M13 reverse sequence at the 5′ end (5′-GGAAACAGCTATGACTTCTCTACCACCTGCTGGAG) and the antisense primer had a biotin group at the 5′ end (5′-biotin CTCAAGACGCTGAGCTTCACCTC). The thermoprofile of the second PCR was 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were separated on a 1.5% low melting point agarose gel (FMC , Rockland, ME) and DNA was recovered by using a Magic prep purification kit (Promega, Madison, WI). The purified DNA was immobilized onto Dynabeads M-280 streptavidin (Dynal, Oslo, Norway) and denatured in 0.1 M NaOH to single-stranded DNA. The single-stranded DNA was then sequenced on an A.L.F. sequencing apparatus using an M13 reverse primer as the sequencing primer to improve the quality of sequencing reactions.

2.4. Construction of expression vectors

The λgt10 DNA containing full-length HCS cDNA [9] was subjected to PCR in a 100-μl reaction mixture containing 20 pmol of each primer (S9 and AS9, Table 2), 20 mM Tris-HCl (pH 8.2), 2 mM $MgCl_2$, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 10 μg/ml BSA, 100 μM of each dNTP, and 2.5 units of *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Each of the 30 amplification cycles consisted of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 74°C for 1 min. The PCR product was digested with *Xho*I (Takara, Kyoto, Japan) and subcloned into the *Xho*I site of pBluescript II KS + . The nucleotide sequence was confirmed. The *Xho*I fragment containing the HCS coding sequence was isolated from the cloning vector and subcloned into the mammalian expression vector pCAGGS, described by Niwa et al. [11]. To construct the mutant expression vector, the fragment between the *Bbs*I (New England Biolabs, Beverly, MA) sites (positions 966 and 1025) was replaced with the cDNA with L237P. The upstream and downstream ligation sites and the orientation of the cDNA were determined by sequencing. The vectors containing the normal and mutant (L237P) HCS cDNAs were designated as 'pCAGGS-N' and 'pCAGGS-L237P', respectively.

2.5. Transient expression study

HCS deficient fibroblasts from patient 4b were maintained as described above. The transfection of fibroblasts was carried out by using Lipofectamine Reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's recommendations. Twenty μg of plasmid DNA was transfected with 80 μl of Lipofectamine Reagent per 150-mm dish. Five μg of pCAGGS-βgal, the vector containing *Escherichia coli* β-galactosidase cDNA, was cotransfected as an internal standard. The transfected cells were harvested by scraping 48 h after transfection, washed twice with PBS, and stored at −80°C until use.

Propionyl-CoA carboxylase (PCC) activity in the transfected fibroblasts was measured by a radioactive assay entailing the fixation of [^{14}C]bicarbonate [12]. Lysate buffer (50 mM Tris-HCl (pH 8.0), 3 mM EDTA, and 2.5 mM reduced glutathione) was used instead of cell extracts as a blank assay. Protein concentration was determined by the method of Bradford [13]. β-galactosidase activity was measured by β-galactosidase enzyme assay kit (Promega).

2.6. Modified PCR for the detection of L237P and delG1067 mutations

Genomic DNA was extracted from either cultured fibroblasts or lymphoblastoid cells as described above. Ex-

Table 2
Oligonucleotides used as PCR primers and ASO hybridization probes

PCR Primer [a]	Location [b]	Sequence
S4	656	5′-GTCTGCCGAGCATATGCCAGAC-3′
S8	955	5′-AGTCCATTCTCGAGGACCTGTAC-3′
S9	253	5′-GTGTGGACAACTCGAGCAAGCTG-3′
AS5	1122	5′-CTCAAGACGCTGAGCTTCACCTC-3′
AS6	1042	5′-GCTTGTCACCTGAATTCCACCAA-3′
AS9	2503	5′-ATTAGATCTCGAGATGCATGGGCA-3′
Oligonucleotide probe		**Sequence**
L237P		
Wild		5′-GGCCTATCTTTCTCAGG-3′
Mutant		5′-GGCCTATCCTTCTCAGG-3′
delG1067		
Wild		5′-ACAAGCAAGGGTGCACT-3′
Mutant		5′-ACAAGCAAGGTGCACTG-3′

[a] S = sense primer, AS = antisense primer.
[b] Refers to the most 5′-nucleotide with respect to the (+) orientation.

tracting DNA from blood-blotted paper was carried out according to the method of Ogasawara et al. [14].

To facilitate the DNA analysis of the two mutations, we developed a method using restriction-site-generating PCR [15]. A forward primer (S10; 5'-TGTACCAGAAGTTCATGGCGGATC) mismatches the HCS gene at the 20th and 21st positions to create a *Bam*HI site in PCR products amplified from the allele carrying the L237P mutation. To detect the delG1067 mutant alleles, the underlined nucleotides in the forward primer (S11; 5'-GTGGCTTTCAGGTGACAAGCCCGG) were changed from A to C to make a recognition site for *Sma*I in the PCR products amplified from the normal allele (Fig. 2). The first PCR was conducted with primers S4 and AS5 in the same condition as described before. One μl of the first amplification reaction was used as a template for the second PCR amplification using a pair of primers, S10 and AS5 or S11 and AS5 (Table 2). The thermoprofile of the second PCR was 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The restriction enzyme digestion was performed in a total volume of 40 μl which contained 34 μl of PCR products, 20 U of *Sma*I or *Bam*HI (New England Biolabs), and 4 μl of 10 × digestion buffers as supplied by the manufacturer. The electrophoresis was performed on a 6% agarose gel (2% SeaPlaque (FMC) plus 4% Agarose S (Nippongene, Toyama, Japan)). The DNA fragments were stained with 0.5 μg/ml of ethidium bromide.

2.7. Allele-specific oligonucleotide (ASO) hybridization

The DNA fragment containing both the L237P and delG1067 sites was amplified with 1 μg of genomic DNA by semi-nested PCR with primers S4 and AS5 followed by primers S8 and AS5 (Table 2). The PCR products (189 bp length) were subjected to electrophoresis on an agarose gel (4 × 11 cm) and transferred onto a Gene screen plus nylon membrane (New England Nuclear, Boston, MA) by an alkaline transfer method [16]. The ASO detection probes are shown in Table 2. The probes were radiolabeled with T4 polynucleotide kinase (Toyobo) and [γ-^{32}P]ATP (Amersham, Buckinghamshire, UK). The filter was finally washed in 2 × SSC/0.1% SDS at 37°C for 10 min.

3. Results

3.1. Identification of the mutation in patient 1

During the course of sequencing cDNA from patient 1, a T to C substitution at nucleotide position 997 (L237P) was found in all five clones isolated. To determine if the patient was homozygous for alleles containing the L237P mutation, the genomic DNA fragment containing the mutation was amplified and directly sequenced using the solid phase method. Fig. 1 shows that patient 1 is a homozygote for the L237P mutation.

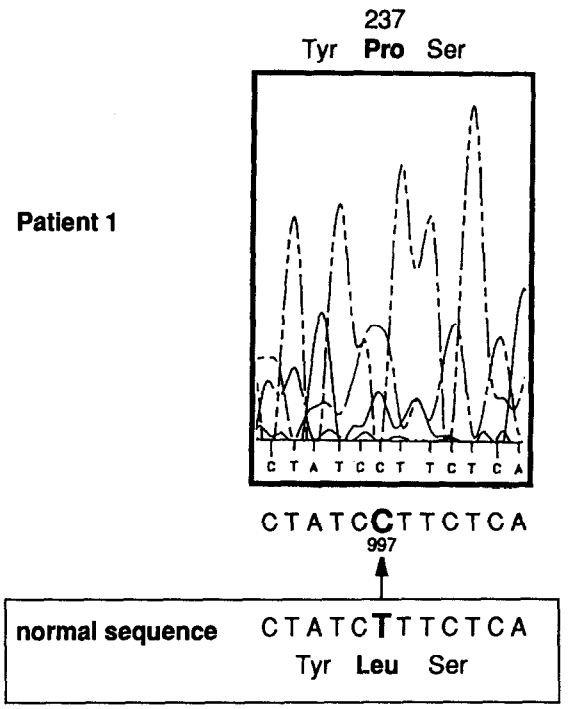

Fig. 1. Direct sequencing of genomic DNA from patient 1. The arrow indicates a T to C substitution at nucleotide position 997, suggesting homozygosity for this mutation.

3.2. Transient expression study

To determine whether this substitution was responsible for decreased HCS activity, we made wild and mutant (L237P) expression constructs. Vectors containing normal and mutant (L237P) cDNA were transfected into HCS deficient fibroblasts obtained from patient 4b. PCC activity in the transformants 48 h after transfection with the wild-type cDNA (pCAGGS-N) was 12.1 ± 1.0 (mean ± S.D., $n = 4$), which was about 3-fold higher than that of the intrinsic activity (4.2 ± 0.6; mean ± S.D., $n = 2$) (Table 3). In contrast, no significant increase in PCC activity was observed in fibroblasts transfected with the pCAGGS-

Table 3
Analysis of PCC and β-galactosidase activities in HCS-deficient fibroblasts transfected with either normal or mutant HCS cDNA

	Propionyl-CoA carboxylase [a] (pmol/min per mg)	β-Galactosidase (mU/mg)
HCS (−) fibroblasts ($n = 2$)	4.2 ± 0.6	
with pCAGGS-N ($n = 4$)	12.1 ± 1.0 [b]	2.4 ± 0.3
with pCAGGS-L237P ($n = 4$)	4.7 ± 1.4	2.2 ± 0.2

[a] Activity was assayed in duplicate for each dish.
[b] A significant difference between HCS-deficient cells and pCAGGS-N transfected cells. $P < 0.001$, as determined by t-test).

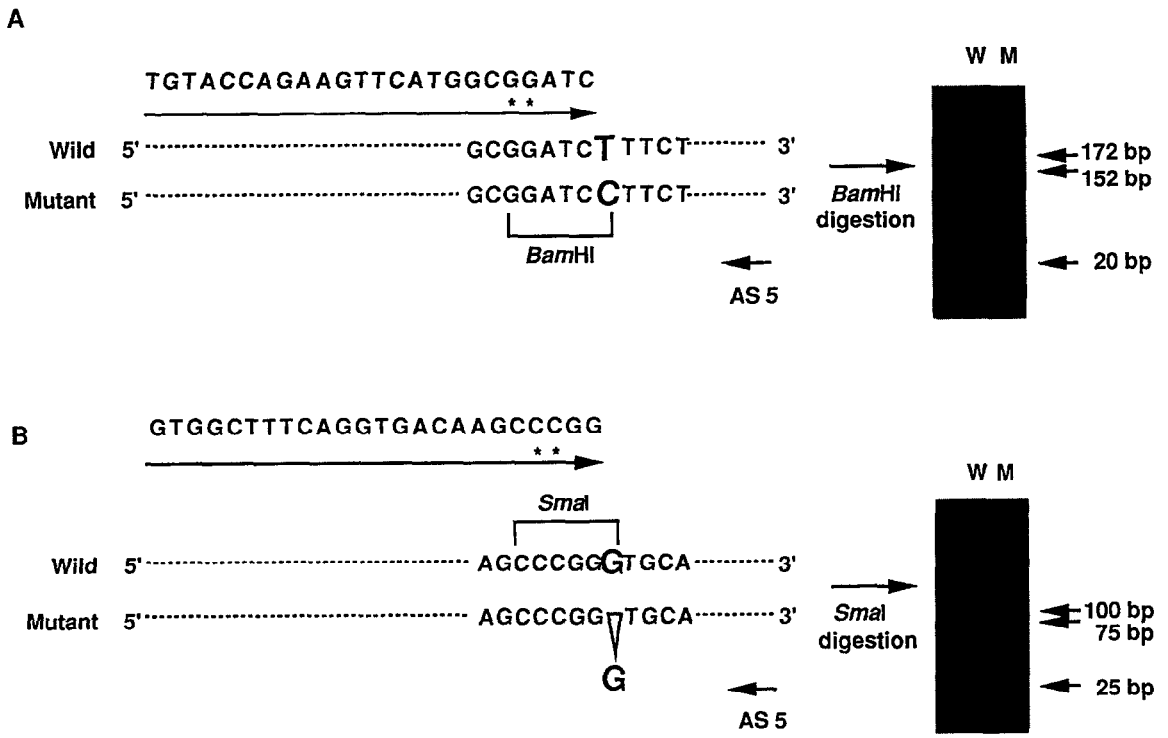

Fig. 2. Schematic illustration of a PCR-generating restriction enzyme method for detecting the (A) L237P and (B) delG1067 mutations. The asterisks indicate mismatched residues generating restriction sites. In regard to the L237P mutation, the PCR products amplified from the mutant allele can be digested by *Bam*HI (A). The products from the normal allele can be digested with *Sma*I and the products carrying the delG1067 allele retain the original size (B). Lane W, normal allele. Lane M, mutant allele.

L237P (4.7 ± 1.4; mean ± S.D., $n = 4$). Western blot analysis of transfected fibroblasts revealed that the size and amount of the mutant enzyme expressed in the cells were similar to those of the wild-type enzyme (data not shown). There was no significant difference in β-galactosidase activity between dishes. These results suggest that while the isolated cDNA clone boosted HCS activity in eukaryotic cells, the mutant HCS failed to increase PCC activity.

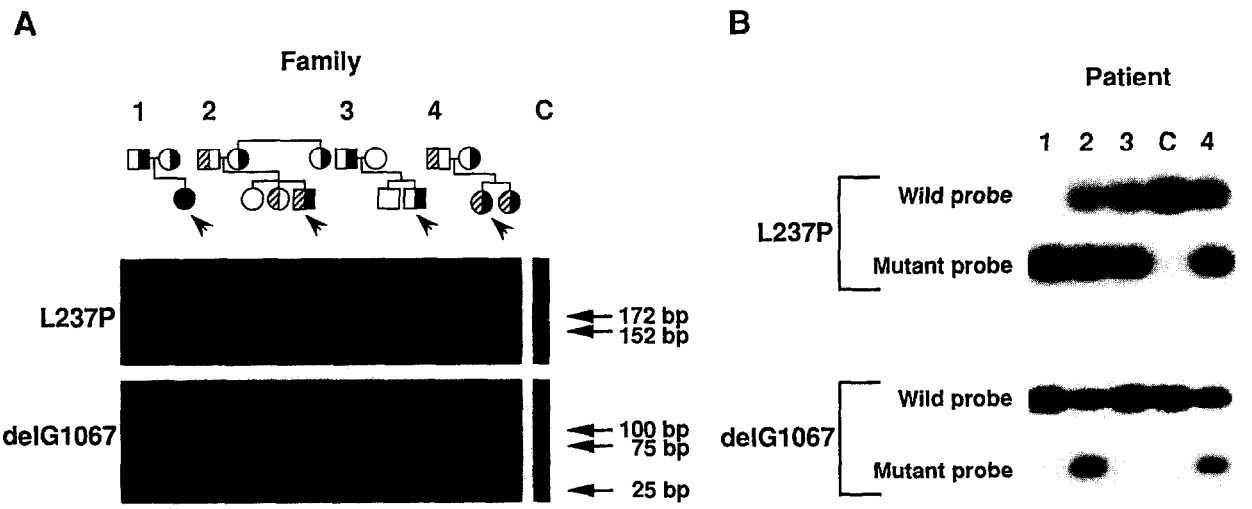

Fig. 3. (A) Detection of mutations in family members of patients with HCS deficiency by restriction-site generating PCR. Modified PCR was performed followed by either *Bam*HI or *Sma*I digestion. The digested DNA fragments were separated on a 6% agarose gel and stained by ethidium bromide. The patients are numbered as indicated in Table 1. Carriers are shown by half-blackened boxes (L237P) and half boxes with an oblique line (delG1067). The L237P mutation was found in patients 2, 3, 4a, and 4b in the heterozygous form and in patient 1 in the homozygous form. The single guanine base deletion was found in patients 2, 4a, and 4b in the heterozygous form. Lane C, normal control. (B) Allele-specific oligonucleotide (ASO) hybridization for detecting the L237P mutation and the delG1067 mutation in Japanese patients. The results were compatible with those of the PCR-restriction enzyme method.

3.3. Analysis of genotype in five patients and their family members

Genomic DNAs were extracted from dried blood spots from patient 3 and family members of the five patients or from cultured lymphoblasts of patients 1, 2, 4a, and 4b. When the DNA which contained the L237P mutation was amplified by PCR with primers S10 and AS5, digestion with *Bam*HI produced fragments of 152 bp and 20 bp (Fig. 2). The PCR product with the normal allele retained the original size after digestion. For detecting the delG1067 mutant allele, PCR with primers S11 and AS5 followed by *Sma*I digestion was carried out. The PCR products containing the normal sequence were digested into 75 bp and 25 bp products, while those containing the mutant were not.

The L237P mutation was identified in patients 2, 3, 4a, and 4b in the heterozygous form and in patient 1 in the homozygous form (Fig. 3A, the 20bp bands are not shown in this figure). The single guanine deletion was found in patients 2, 4a, and 4b in the heterozygous form. Of the eight alleles analyzed, the L237P mutation occurred in five alleles and the delG1067 in two. Another mutation was not identified in cDNA of patient 3. The same approach was applied to the detection of two mutations in family members of the patients. The two mutations showed a Mendelian inheritance pattern in all families without exception. The results obtained by this method were confirmed by ASO hybridization analysis (Fig. 3B). The L237P and delG1067 mutations were screened by ASO analysis in DNAs from blood spots from 108 normal healthy Japanese children (216 alleles, data not shown). No alleles contained the two mutations.

4. Discussion

It is likely that the L237P and delG1067 mutations are predominant among Japanese patients, although the number of alleles studied is too small for a definitive conclusion. Among the five patients in the four unrelated families analyzed, one patient was homozygous for L237P, three patients in two families were compound heterozygotes of the missense and deletion alleles, and the other patient was heterozygous for the L237P mutation. Thus these two mutations accounted for seven of eight mutant alleles in these unrelated families, while neither mutation was detected in 216 alleles from normal healthy children. It is unknown why these mutations are prevalent in Japan. The L237P mutation does not occur at a CpG dinucleotide, which tends to be a hot spot for mutation. It is conceivable that these patients share a common ancestor although the four families reside in various regions of Japan and are not consanguineous as far as we know. The lack of polymorphic markers at or near the HCS locus precludes genetic analysis of relatedness. It would be of interest to determine the nature of mutations in patients from other ethnic origins for a more complete understanding of the range of HCS mutant genotypes.

The following observations suggest that the L237P mutation was causative for HCS deficiency. First, introduction of an expression vector containing mutant cDNA failed to increase PCC activity in transfected cells. Second, the mutation was not detected in 108 healthy Japanese children, being segregated exclusively among patients with HCS deficiency. The substitution of proline for leucine has been reported in various hemoglobinopathies [17,18], α-thalassemia [19], and Gaucher's disease [20]. This substitution has been shown to destabilize the protein secondary structure in these diseases [17,18].

High doses of biotin (20–60 mg/day) were required to control clinical symptoms in patients 2, 4a, and 4b. Intermittent respiratory infections have led to metabolic acidosis or organic aciduria in spite of biotin therapy in patients 4a and 4b. In contrast, patients 1 and 3 responded to 10 to 20 mg biotin per day and abnormal urinary metabolites virtually disappeared. Homology to BirA, which has DNA binding and HCS activity in *E. coli* [21,22], has suggested that the ATP and biotin binding sites are located in the 3′-region downstream from amino acid 500 of the HCS protein [9]. Since the single base deletion of G at nucleotide position 1067 results in premature termination at position 280, the truncated polypeptide would lack the biotin and ATP binding domains, which are essential for HCS activity. It is very likely that this mutant polypeptide shows no enzymatic activity. The current evidence suggests that the allele containing one base deletion is related to the more severe symptoms in patients 2, 4a, and 4b.

In the transient expression study, the level of PCC activity increased significantly in fibroblasts transfected with wild-type HCS cDNA. This observation strongly suggested that the expressed HCS catalyzed biotinylation of apo-PCC in HCS-deficient fibroblasts. HCS activity is found in both mitochondria and cytosol in chicken [23] and mammalian cells [8,24,25]. Little is known about the nature of the two enzymes and it is unknown where and by which type of HCS mitochondrial carboxylases are biotinylated. There are two possible hypotheses to explain where expressed HCS protein catalyzes the incorporation of biotin into apo-PCC. First, the protein expressed from HCS cDNA is translocated into mitochondria and catalyzes the incorporation of biotin into existing or newly translocated apo-PCC. Second, the HCS remains in the cytosol and incorporates biotin into the newly synthesized PCC precursor. The biotinylated PCC precursor is then transported into the mitochondria. Although we have no direct experimental evidence, we believe that the isolated cDNA encodes a cytosolic protein because no authentic mitochondrial leader sequence exists at the NH_3 terminus [9]. It has been reported that the α-subunit precursor of PCC accumulates in the cytoplasm and is biotinylated before translocation occurs [26]. We speculate that PCC is biotinylated by the expressed cytosolic HCS, translocated

into the mitochondria and then processed to the active protein. Analysis of the intracellular localization of expressed HCS is necessary to confirm this hypothesis.

Early diagnosis of HCS deficiency is crucial, because administration of biotin can prevent the occurrence of life-threatening ketoacidosis. Assays for either multiple carboxylases or HCS have been the only means available to establish the diagnosis of HCS deficiency. DNA analysis will provide another tool for diagnosing the disease. We developed a PCR-restriction enzyme method to detect two frequent mutations accurately and rapidly. Dried blood spots on filter paper, which may be stored and transported easily, were utilized as the source of DNA for this method. Although DNA analysis is not always informative, this method should facilitate, at least in Japan, definitive and rapid prenatal/postnatal diagnosis in families with HCS deficiency.

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