Identification of novel mutations of the \textit{HADHA} and \textit{HADHB} genes in patients with mitochondrial trifunctional protein deficiency

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Abstract. Patients with long-chain 3-hydroxyacyl coenzyme A dehydrogenase (LCHAD) deficiency manifest hypoketotic hypoglycemia, hepatomegaly, hypotonia, lactic acidemia, acute renal failure, cardiomyopathy, and sudden death. We describe four novel mutations of the \(\alpha\) - and \(\beta\)-subunits of the mitochondrial trifunctional protein in four patients from three unrelated families. Their plasma acylcarnitine profiles suggested the presence of LCHAD deficiency by demonstrating highly elevated 3-hydroxyacyl carnitines by tandem mass spectrometry (MS/MS). Patients 1 and 2 had siblings who had died of lactic acidemia during the neonatal period. These patients also manifested lactic acidemia and died in the neonatal period. Patient 3 had a family history of Reye-like syndrome. She exhibited acute renal failure, rhombomylolysis, pericardial effusion, and myopathy at the age of 12 years. DNA analysis of patients 1 and 2 revealed homozygosity for a c.1689+2T>G mutation of the \textit{HADHA} gene, resulting in the skipping of exon 16 with an in-frame 69-bp deletion. Patient 3 was a compound heterozygosity of the \textit{HADHB} gene, N307D/N389D. Patient 4, a 25-month-old baby, manifested recurrent episodes of lethargy, metabolic acidosis, elevated liver enzymes, and dark urine from the age of 10 months. Mutation analysis of the \textit{HADHB} gene of patient 4 identified compound heterozygosity of N114D/N307D.

Introduction

Long-chain 3-hydroxyacyl coenzyme A dehydrogenase (LCHAD; long-chain-(S)-3-hydroxyacyl-CoA:NAD\textsuperscript{+} oxidoreductase, EC 1.1.1.211) deficiency is one of a class of mitochondrial fatty acid \(\beta\)-oxidation disorders characterized by acute life-threatening episodes of hypoketotic hypoglycemia, seizures, hypertrophic cardiomyopathy, myoglobinuria, rhabdomyolysis, peripheral neuropathy, retinitis pigmentosa, and sudden and unexpected death (1). LCHAD is a component of the mitochondrial trifunctional protein (M-TFP), which is bound to the inner mitochondrial membrane. LCHAD catalyzes the third step in the long-chain mitochondrial \(\beta\)-oxidation pathway and carries out the dehydrogenation of 3-hydroxyacyl-CoA compounds with chain lengths of 12 to 18 carbons (2). The four enzymes involved in the mitochondrial \(\beta\)-oxidation of long-chain fatty acids are very long-chain acyl-CoA dehydrogenase (VLCAD), long-chain 2,3-enoyl-CoA hydratase (LCEH), LCHAD, and long-chain 3-ketoacyl-CoA thiolase (LCKT). Among them, LCEH, LCHAD, and LCKT comprise the mitochondrial trifunctional protein which catalyzes three steps in the mitochondrial \(\beta\)-oxidation of fatty acids (3). This protein is a heterocomplex composed of four \(\alpha\)-subunits that contain LCEH and LCHAD and four \(\beta\)-subunits that harbor LCKT activity (4). The active site of LCHAD is located in the C-terminal domain of each of the four \(\alpha\)-subunits of the trifunctional protein, and the site of LCEH activity is located in the N-terminal domain of each of the \(\alpha\)-subunits (5). Therefore, M-TFP deficiency is classified into two categories: the more common isolated LCHAD deficiency (McKusick 600890) with defects of the \(\alpha\)-subunits encoded by the \textit{HADHA} (hydroxyacyl-CoA dehydrogenase \(\alpha\)-subunit) gene and the less common pattern of complete M-TFP deficiency (McKusick 143450).
involving all three enzyme activities with defects of the α- or β-subunits encoded by the \textit{HADHB} (hydroxyacyl-CoA dehydrogenase β-subunit) gene (5,6).

This report describes clinical and biochemical findings of patients with M-TFP deficiency and four novel mutations of the \textit{HADHA} and \textit{HADHB} genes.

**Materials and methods**

**Patients 1 and 2.** Two premature female monozygotic twin babies were born by Cesarean section with body weights of 1,640 g (50-75th percentile) and 1,595 g (25-50th percentile) at the 31st gestational week to healthy, non-consanguineous parents. As a family history, their first baby succumbed to respiratory distress at the age of 11 days, the third baby developed severe lactic acidemia within 24 hours after birth and died at the age of 5 days without a specific diagnosis, and the fourth baby also manifested severe lactic acidemia and died of cardiac failure 7 days after birth. Urine organic acid analysis of the fourth baby showed severe lactic acidosis, massive excretion of pyruvate, 2-hydroxybutyrate, and prominent C6-C10 dicarboxylic acids. He was presumed to have a respiratory chain defect. The second one is the only healthy child, a boy now 8 years of age.

Since a respiratory chain defect was suspected in one child in this family, the twin babies were extensively screened for metabolic disease by analysis of serum lactate/pyruvate, ammonia, serum amino acid, and urine organic acid levels. Acylcarnitine analysis was also performed in the neonatal period. The patients were fed a special formula [140 kcal/kg/day; 59% carbohydrate, 10% protein, long-chain fatty acid <5%, 26% triglyceride with medium-chain triglyceride (MCT) oil] and an l-carnitine supplement (100 mg/kg/day). Serum amino acid and urine organic acid analysis revealed no specific abnormalities in either patient.

Laboratory findings of patient 1 showed a pH of 7.33, a bicarbonate level of 17 mEq/l, an ammonia level of 102 μmol/l (normal range 10-35 μmol/l), a lactate level of 3.4 mmol/l (normal range 0.03-0.08 mmol/l), a pyruvate level of 0.080 mmol/l (normal range 0.034-0.080 mmol/l). Patient 1 had been doing well and was discharged 37 days after birth with instructions to be fed MCT-fortified milk and vitamin B2 (riboflavin 100 mg/ day). After discharge, she remained in a healthy state. However, patient 1 was suddenly found in respiratory arrest at home at 48 days of age, and brought to the emergency room. She died 2 hours after arrival despite cardiopulmonary resuscitation.

Laboratory findings of patient 2 showed a pH of 7.39, a bicarbonate level of 20 mEq/l, an ammonia level of 85 μmol/l, a lactate level of 4.4 mmol/l, and a pyruvate level of 0.136 mmol/l. Patient 2 was admitted again at the age of 22 days because recurrent apneic episodes developed suddenly with severe lactic acidemia (serum lactate 16.7 mmol/l). Uncontrollable acidosis, hypoglycemia and profound cardiac failure had progressed relentlessly. At that time, hepatic dysfunction was found with elevated liver enzymes including aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatine kinase. Urine organic acid analysis revealed massive excretions of lactate, C6-C10 saturated and unsaturated dicarboxylic acids with 3-hydroxy C10-C14 dicarboxylic acids. She eventually died of multi-organ failure at the age of 26 days.

**Patient 3.** Patient 3, a 12-year-old female, was admitted due to myoglobinuria, acute renal failure, rhabdomyolysis and pericardial effusion. Her younger sister had died of a Reye-like illness at the age of 4 years. Patient 3 had experienced recurrent episodes of myoglobinuria and rhabdomyolysis associated with upper respiratory tract infections since she was 4 years old. There was no evidence of retinal dystrophy. Laboratory findings showed a blood urea nitrogen level of 110 mg/dl, a creatinine level of 4.3 mg/dl, an AST level of 1,019 IU/l, an ALT level of 1,331 IU/l, a creatine kinase level of 37,935 IU/l, and a lactate level of 2.5 mmol/l. Urine output increased after 5 days of hemodialysis and all laboratory findings were normalized. A chest X-ray and an electrocardiogram showed improved cardiomegaly and pericardial effusion. She was treated with a dietary restriction of long-chain fatty acid intake and an l-carnitine supplement.

**Patient 4.** A twenty-five-month-old male patient was brought to the hospital for the evaluation of recurrent attacks of lethargy, metabolic acidosis, elevated liver enzymes, and dark urine, which were aggravated by exercise, upper respiratory tract infection, and fasting. His symptoms had started at the age of 10 months. His height and weight were both between the 10th and 25th percentile. He started to walk alone at 18 months of age. The AST and ALT levels were elevated to 2,787 and 1,157 IU/l. Blood ammonia, lactate, and serum creatine kinase levels were not increased. Serum amino acid and urine organic acid analysis showed no prominent abnormalities. He was treated with a dietary restriction of long-chain fatty acid and an l-carnitine supplement.

**Tandem mass spectrometry (MS/MS).** A blood spot was collected from the original newborn screening card of the newborns with parental informed consent. Acylcarnitines were extracted with methanol and then derivatized with butanolic HCl. Butylated acylcarnitines were analyzed by tandem mass spectrometry (Perkin Elmer Wallac MS2) in all patients (7,8). The samples for laboratory analysis were collected during acute metabolic decompensation in patients 1, 2 and 3, and after overnight fasting before diet therapy in patient 4.

**DNA mutation analysis.** Genomic DNA was extracted from peripheral blood leukocytes. Polymerase chain reaction (PCR) amplification and direct sequencing of all 20 exons of the gene encoding the α-subunit of the M-TFP was performed in all patients. RT-PCR and direct sequencing were performed using cDNA extracted from cultured skin fibroblasts in 20 exons of the gene encoding the α-subunit in patients 1 and 2. Direct sequencing of 16 exons of the β-subunit of the M-TFP was performed with DNA isolated from peripheral blood leukocytes of patients 3 and 4.

**Results**

**Tandem mass spectrometry (MS/MS).** The blood and plasma acylcarnitine profiles in patients 1 and 2 revealed highly...
Figure 1. Acylcarnitine profiles in patient 2 with LCHAD deficiency performed during acute metabolic decompensation, showing highly elevated levels of 3-hydroxydicarboxylic derivatives of the C16:0, C18:1 and C18:2 species.

Figure 2. (A) Partial genomic sequence of the \textit{HADHA} gene identified a c.1689+2 T>G homozygote mutation in patients 1 and 2, which is a novel mis-splicing mutation. (B) PCR products of \textit{HADHA} cDNA showed using primer set, 5'-GAAAACTTCAAAGACACCA-3' and ACACCCTCCTGATAGATGT-3' compared to normal control. Partial cDNA sequences of \textit{HADHA} from cultured skin fibroblasts showed deletion of exon 16, leading to an in-frame 69-bp deletion in the lower panel. Normal sequences including exon 16 are showed in the upper panel.
elevated levels of 3-hydroxydicarboxylic derivatives of the C16:0, C18:1 and C18:2 species (Fig. 1). The concentration of 3-hydroxypalmitoylcarnitine (OH-C16, m/z=472), the typically elevated metabolite in LCHAD deficiency, was significantly increased. The urine organic acid profile showed enormous amounts of 3-hydroxydicarboxylic acid species. The acylcarnitine analysis of patient 3 using the blood spot in a Guthrie filter paper identified massive elevations of C14(m/z=444)-, C16(m/z=472)-, and C18:1(m/z=498)-hydroxyacylcarnitine levels, which are consistent with a diagnosis of LCHAD or M-TFP deficiency. Acylcarnitine analysis of patient 4 revealed mild elevations of C14-, C16:1-, C16-, and C18:1-hydroxyacylcarnitine levels. Although the patient was not on an MCT-rich diet, large amounts of C8 and C10 were observed along with long-chain and hydroxyl long-chain acylcarnitine species. The reason for this finding remains to be elucidated at this time.

**DNA mutation analysis.** DNA analysis using genomic DNA revealed a novel homozygous mis-splicing mutation, c.1689+2T>G in patients 1 and 2. Further DNA analysis of the gene for the ß-subunit of the M-TFP using cDNA isolated from patient skin fibroblasts showed a deletion of exon 16, leading to an in-frame 69-bp deletion (Fig. 2). Molecular analysis of patient 3 showed that the coding region and the sequences of the exon-intron boundary of the CPT2 and HADHA genes were normal. Direct sequencing of the ß-subunit of the M-TFP (HADHB) revealed compound heterozygosity of two novel mutations, leading to the substitution of aspartic acid for asparagine at two different positions.

**Figure 3.** Partial genomic sequences of the HADHB gene in patient 3. Direct sequencing of genomic DNA extracted from peripheral blood leukocytes revealed compound heterozygotes of the ß-subunit of M-TFP (HADHB); asparagines were substituted by aspartic acid at codon 307 (N307D) in one allele and at codon 389 (N389D) in the other allele.

**Figure 4.** Partial genomic sequences of the HADHB gene in patient 4. Direct sequencing of genomic DNA extracted from peripheral blood leukocytes revealed compound heterozygotes of the ß-subunits of M-TFP (HADHB); asparagines were substituted by aspartic acid at codon 114 (N114D) in one allele and at codon 307 (N307D) in the other allele.
codons 307 and 389 (N307D and N389D) (Fig. 3). In 100 alleles of the \textit{HADHB} gene in 50 healthy DNA, all of the alleles revealed a normal sequence; adenine at c.916 for the N307D mutation, and c.1162 for the N389D mutation. The allele frequencies of N307D and N389D were <1% in the population. Patient 4 turned out to be a compound heterozygosity of the \textit{HADHB} carrying two different missense mutations, N114D/N307D (Fig. 4).

Discussion

Features of LCHAD deficiency vary from acute life-threatening episodes of hypoketotic hypoglycemia, lethargy, cardiomyopathy, hepatopathy, coma or sudden death to later onset myopathy or retinopathy. Clinical features are not clearly distinct between isolated LCHAD deficiency and complete M-TFP deficiency (4). Furthermore, complete M-TFP deficiency often presents chronic, nonspecific symptoms of patients (9,10). Three affected siblings of patients 1 and 2 manifested severe lactic acidosis in their neonatal periods. Initially, a disorder of the respiratory chain complex was suspected in these patients. Patients 1 and 2 had eventually developed severe lactic acidosis during metabolic decompensation. They were diagnosed as LCHAD deficient based on acylcarnitine profiles. It is also suggested that lactic acidosis in these patients can be explained by a secondary impairment of several enzymes of the respiratory chain complexes and an increased size and number of mitochondria with a swollen appearance caused by the accumulation of toxic metabolites of fatty acid \(\beta\)-oxidation (11,12). Acute fatty liver of pregnancy (AFLP) and HELLP (hemolysis, elevated liver enzymes, and low platelet counts) syndrome are the most common manifestations of maternal complications of pregnancy (13). However, there was no such history of maternal complications in our patients.

The early identification and treatment of affected individuals before catastrophic events are likely the most effective ways to improve the outcome in LCHAD deficiency. This goal could possibly be achieved through acylcarnitine analysis in newborns using tandem mass spectrometry (14-16). The presumptive diagnosis of isolated LCHAD deficiency or M-TFP deficiency is based on the demonstration of an accumulation of 3-hydroxyacylcarnitines in the plasma by acylcarnitine analysis and the presence of 3-hydroxydicarboxylic acids with a chain length of 10 to 14 carbon atoms in urine by gas chromatography/mass spectrometry (GC/MS) (14-16). However, differentiation between isolated LCHAD
deficiency and complete M-TFP deficiency by MS/MS, and/or GC/MS remains unclear. The diagnosis is usually confirmed by a demonstration of significantly decreased enzyme activities of the three involved enzymes in lymphocytes, fibroblasts, muscle or liver biopsy specimens. Although mutations in either subunit gene can result in M-TFP deficiencies with reduced activities of all three enzymes, molecular genetic analysis is becoming an increasingly important approach to the differential diagnosis between isolated LCHAD and complete M-TFP deficiency. Up to date, 28 different mutations have been described in the HADHA gene of M-TFP deficient patients (reviewed in the HADHA gene mutation database at the URL http://www.hgmd.org/). The first reported molecular defect and the most common mutation of LCHAD deficiency is a c.1528G>C mutation resulting in glutamic acid to glutamine substitution at codon 510 (E510Q) in exon 15 of the HADHA gene, which has been found only in patients with isolated LCHAD deficiency (6,9,12,17). The mutation is directly responsible for the loss of LCHAD activity without structural alteration of the enzyme complex (6). The E510Q mutation was initially screened for its presence in our cases and in 100 normal unrelated Korean individuals, but was identified in none of the tested subjects, indicating that this mutation is rare in the Korean population (data not shown). The monozygotic twin patients 1 and 2 turned out to be homozygous for a novel c.1689+2T>G mutation of the HADHA gene, leading to an exon 16 skipping by an aberrant splicing. This resulted in the in-frame deletion of an entire exon 16 composed of 21 amino acids, accounting for the C-terminal region of the α-subunit of M-TFP which mainly carries LCHAD activity. Although the enzymatic activities of each component of M-TFP were not assayed in the patients, the fact that the sequence of exon 16 is highly conserved across various species and encodes for the C-terminal region suggests that it may abolish the activity of other components of M-TFP as well as LCHAD by destabilizing the structure of the protein (Fig. 5). It is also hypothesized that the genotype may explain the patients severe clinical phenotype including neonatal onset with relentless progression to early death, probably caused by the complete loss of the M-TFP enzymatic function.

On the other hand, patient 3 manifested a milder presentation, later onset recurrent rhabdomyolysis. Two novel missense mutations (N307D/N389D) were identified in the HADHB gene of the patient. DNA analysis of patient 4 also identified a compound heterozygosity of two missense mutations (N114D/N307D) of the HADHB gene. These mutations were not found in more than 100 normal tested alleles. To note, both mutations replace the same basic amino acid by the same acidic amino acid residue. So far, 20 different mutations have been reported in the HADHB gene of M-TFP deficient patients and the vast majority of them are point mutations in the coding regions (http://www.hgmd.org/). The majority of patients with the β-subunit mutations are compound heterozygosity (18). Three clinical phenotypes with defects of the β-subunit have been described: a severe neonatal form with hypoketotic hypoglycemia, Reye-like syndrome, and early death; a hepatic form with recurrent hypoketotic hypoglycemia; and a milder late-onset neuro-myopathic form with episodic myoglobinuria. Missense mutations are predominantly associated with milder myopathic phenotypes (18). Patients 3 and 4 with two missense mutations of the HADHB gene can be categorized clinically as having a neuromyopathic form with a defect of the LCAT domain.

In conclusion, four different novel mutations of the HADHA or HADHB genes have been described in patients with M-TFP deficiency. This suggests that their genotypes are correlated with phenotypes, even though the number of subjects is small in this report. Therefore, the identification of a genetic mutation will make prenatal molecular diagnosis feasible in family members at risk.

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References


