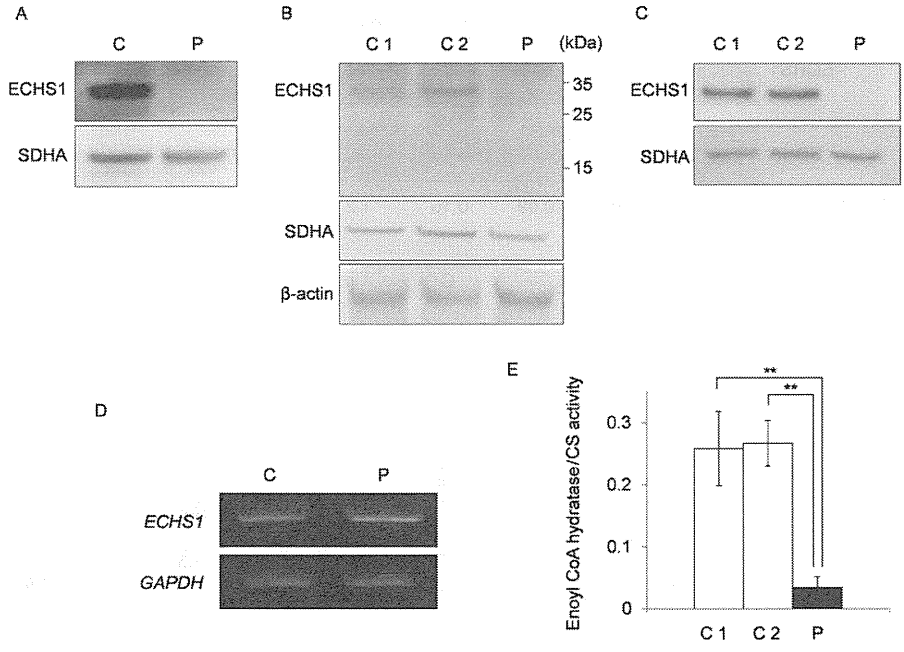


**Figure 3.** ECHS1 expression and enzyme activity. ECHS1 expression was analyzed by immunoblotting. C1/2, control; P, patient. Mitochondrial fraction prepared from patient's skeletal muscle (**A**) or whole-cell lysate (**B**) and mitochondrial fraction (**C**) prepared from the patient-derived myoblasts were analyzed via immunoblotting. All findings indicated that ECHS1 levels in patient samples were too low to detect by immunoblotting. **D**: RT-PCR was used to assess *ECHS1* mRNA levels in the patient. Notably, patient-derived myoblasts and control myoblasts did not differ with regard to *ECHS1* mRNA level. **E**: Mitochondrial fractions prepared from patient-derived myoblasts were used to estimate ECHS1 enzyme activity in the patient. All ECHS1 activity measurements were normalized to CS activity; ECHS1 activity in patient-derived samples was 13% of that in control samples. The experiments were performed in triplicate. Error bars represent standard deviations. (** $P < 0.005$ Student's *t*-test).

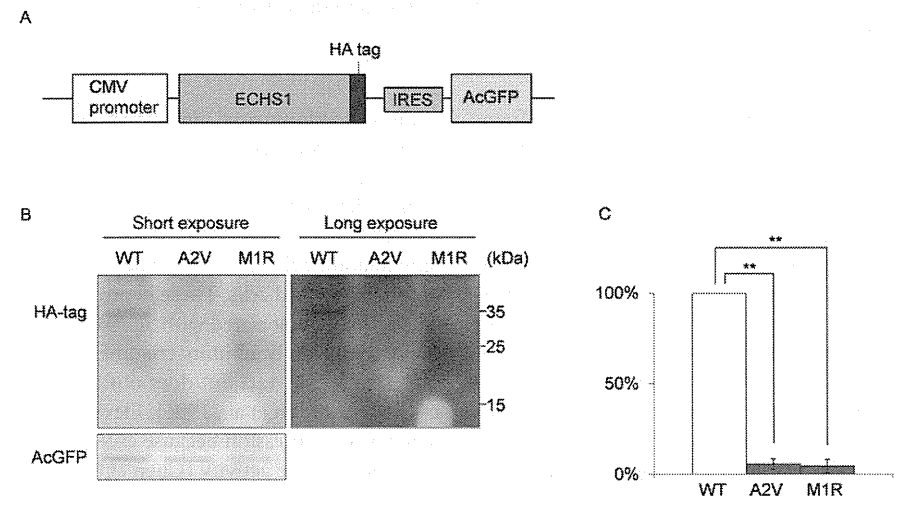


**Figure 4.** Exogenous expression of mutant ECHS1 protein in cancer cells. **A**: Schematic diagram of the pIRES mammalian expression vector. **B**: Representative image of an immunoblotting containing AcGFP, an internal control, and each HA-tagged ECHS1 protein; all proteins were isolated from DLD-1 cells that transiently overexpressed wild-type, A2V, or M1R HA-tagged ECHS1 from pIRES. The images obtained by short exposure (left) and long exposure (right). **C**: Overexpressed HA-tagged ECHS1 protein levels. Both mutant ECHS1 proteins showed dramatically decreased expression compared to wild-type ECHS1 protein, when ECHS1 was normalized relative to the internal control. Each experiment was performed in triplicate. Error bars represent standard deviations (** $P < 0.005$ Student's *t*-test).

## Discussion

Here, we described a patient harboring compound heterozygous mutations in *ECHS1*. Immunoblotting analysis revealed that ECHS1 protein was undetectable in patient-derived myoblasts; moreover, these cells showed significantly lower ECHS1 enzyme activity than controls. Exogenous expression of two recombinant mutant proteins in DLD-1 cells showed c.2T>G; p.M1R and c.5C>T; p.A2V mutations affected ECHS1 protein expression. Cellular complementation experiment verified the patient had ECHS1 deficiency.

The c.2T>G; p.M1R mutation affected the start codon and therefore was predicted to impair the protein synthesis from canonical

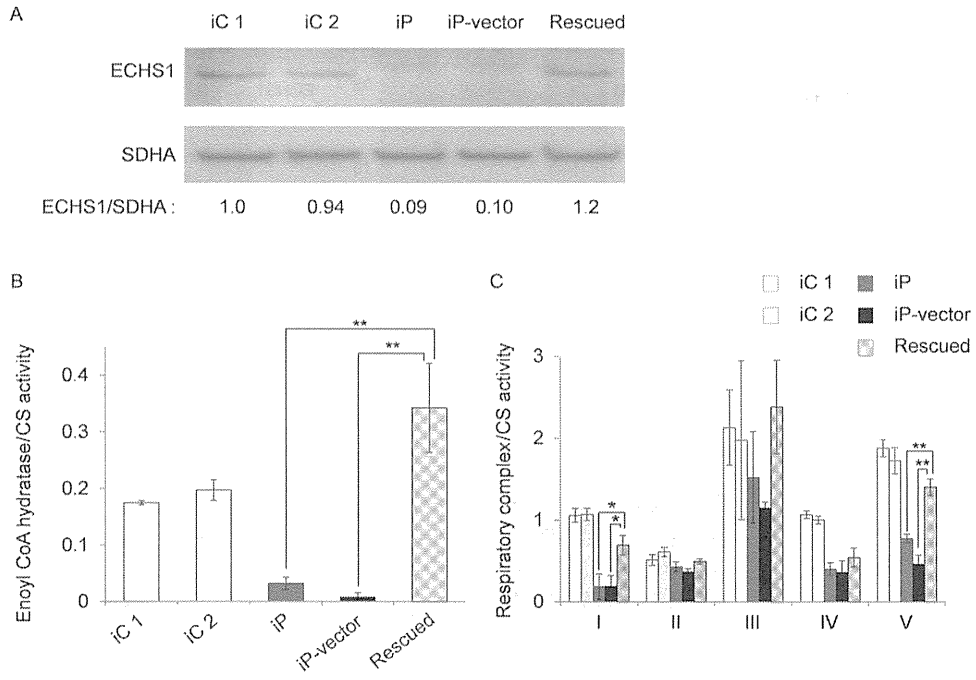


**Figure 5.** ECHS1 protein expression and enzyme activity in rescued myoblasts. An empty vector or a construct encoding wild-type ECHS1 was introduced into immortalized patient-derived myoblasts. iC1/2, immortalized control myoblasts; iP, immortalized patient-derived myoblasts; iP-vector, immortalized patient-derived myoblasts transfected with empty vector; Rescued, immortalized patient-derived myoblasts stably expressing wild-type ECHS1. **A**: ECHS1 levels were assessed on immunoblotting using mitochondrial fractions prepared from rescued myoblasts. ECHS1 level in "rescued" is 11 times higher than that in "iP-vector". **B**: Mitochondrial fractions prepared from rescued myoblasts were also used to measure ECHS1 enzyme activity. ECHS1 activity normalized to CS activity in "rescued" was 49 times higher than that in "iP-vector." Each experiment was performed in triplicate. Error bars represent standard deviations (** $P < 0.005$ Student's *t*-test). **C**: Mitochondrial fractions prepared from rescued myoblasts were used to measure enzyme activities of mitochondrial respiratory complexes. Activity values were normalized to CS activity. Activities of complexes I, IV, and V were mostly restored from "iP" and "iP-vector." In "rescued," the enzyme activities of complexes I, IV, and V were 3.5, 1.3, and 2.2 times higher, respectively, than the "iP-vector." Each experiment was performed in triplicate. Error bars represent standard deviations (** $P < 0.005$, * $P < 0.05$ Student's *t*-test).

initiation site. In the reference *ECHS1* sequence, the next in-frame start codon is located in amino acids 97 (Fig. 2C). Even if translation could occur from this second start codon, the resulting product would lack the whole transit peptide and part of the enoyl-CoA hydratase/isomerase family domain (Fig. 2C). The c.5C>T; p.A2V mutation was located in the mitochondrial transit peptide and the mutation may affect the mitochondrial translocation of ECHS1. Surprisingly, the MitoProt-predicted mitochondrial targeting scores for the wild-type and A2V-mutant proteins were 0.988 and 0.991, respectively [MitoProt II; http://ihg.gsf.de/ihg/mitoprot.html; Claros and Vincens, 1996] and not markedly different from each other. Nevertheless, mislocalized mutant protein may have been degraded outside of the mitochondria. Consistent with this speculation was the finding that immunoblotting of lysate from patient-derived myoblasts (Fig. 3B) or from transfected cells that overexpressed the recombinant p.A2V-mutant ECHS1 (Fig. 4B, Supp. Fig. S2) did not show upper shifted ECHS1 bands that indicated ECHS1 with the transit peptide. Another possible explanation is that the mutation affected the translation efficiency because it was very close to the canonical start codon. It can change secondary structure of ECHS1 mRNA or alter the recognition by the translation initiation factors. As stated above, even if there was a translation product from the second in-frame start codon, that product would probably not function.

This patient presented with symptoms that are indicative of fatty acid oxidation disorders (e.g., hypotonia and metabolic acidosis), but he also presented with neurologic manifestations, including developmental delay and Leigh syndrome, that are not normally associated with fatty acid $\beta$-oxidation disorders. Interestingly, developmental delay is also found in cases of SCAD deficiency [Jethva et al., 2008]. In the absence of SCAD, the byproducts of butyryl-CoA—including butyrylcarnitine, butyrylglycine, ethylmalonic acid (EMA), and methylsuccinic acid—accumulate in blood, urine, and cells. These byproducts may cause the neurological pathology associated with SCAD deficiency [Jethva et al., 2008]. EMA significantly inhibits creatine kinase activity in the cerebral cortex of Wistar rats but does not affect levels in skeletal or heart muscle [Corydon et al., 1996]. Elevated levels of butyric acid modulated gene expression because excess butyric acid can enhance histone deacetylase activity [Chen et al., 2003]. Moreover, the highly volatile nature of butyric acid as a free acid may also add to its neurotoxic effects [Jethva et al., 2008].

On the other hand, it is very rare for fatty acid $\beta$-oxidation disorders causing Leigh syndrome. Therefore, the most noteworthy manifestation in this patient was Leigh syndrome. Leigh syndrome is a neuropathological entity characterized by symmetrical necrotic lesions along the brainstem, diencephalon, and basal ganglion [Leigh, 1951]. It is caused by abnormalities of mitochondrial energy generation and exhibits considerable clinical and genetic heterogeneity [Chol et al., 2003]. Commonly, defects in the mitochondrial respiratory chain or the pyruvate dehydrogenase complex are responsible for this disease. This patient's skeletal muscle samples exhibited a combined respiratory chain deficiency, and this deficiency may be the reason that he presented with Leigh syndrome. Although it

remained unclear what caused the respiratory chain defect, cellular complementation experiments showed almost complete restoration, indicating there was an unidentified link between ECHS1 and respiratory chain. One of the possible causes of respiratory chain defect is the secondary effect of accumulation of toxic metabolites. For example, an elevated urine glyoxylate was observed in this patient. Although the mechanism of this abnormal accumulation is not clear at the moment, it was shown that glyoxylate inhibited oxidative phosphorylation or pyruvate dehydrogenase complex by in vitro systems [Whitehouse et al., 1974; Lucas and Pons, 1975]. Therefore, we speculate that in our patient, ECHS1 deficiency induced metabolism abnormality including glyoxylate accumulation, and glyoxylate played a role in decreased enzyme activities of respiratory chain complexes. Interestingly, a recent paper describing patients with Leigh syndrome and ECHS1 deficiency showed decreased activity of pyruvate dehydrogenase complex in fibroblasts [Peters et al., 2014], (Supp. Table S5). BN-PAGE showed the assembly of respiratory complex components in the patient was not clearly different from the control (Supp. Fig. S1). This result suggests that the respiratory chain defect in the patient is more likely because of the secondary effect of accumulation of toxic metabolites. On the other hand, many findings indicate interplays between mitochondrial fatty acid $\beta$-oxidation and the respiratory chain. For example, Enns et al. [2000] mentioned the possibility of the physical association between these two energy-generating pathways from overlapping clinical phenotypes in genetic deficiency states. More recently, Wang and his colleagues actually showed physical association between mitochondrial fatty acid $\beta$-oxidation enzymes and respiratory chain complexes (Wang et al., 2010). Similarly, Narayan et al. demonstrated interactions between short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD) and several components of the respiratory chain complexes including the catalytic subunits of complexes I, II, III, and IV via pull-down assays involving several mouse tissues. Considering the role of SCHAD as a NADH-generating enzyme, this interaction was suggested to demonstrate the logical physical association with the regeneration of NAD through the respiratory chain [Narayan et al., 2012]. Still more recently, mitochondrial protein acetylation was found to be driven by acetyl-CoA produced from mitochondrial fatty acid $\beta$-oxidation [Pougovkina et al., 2014]. Because the activities of respiratory chain enzymes are regulated by protein acetylation [Zhang et al., 2012], this finding indicated that $\beta$-oxidation regulates the mitochondrial respiratory chain. Remarkably, acyl-CoA dehydrogenase 9 (ACAD9), which participates in the oxidation of unsaturated fatty acid, was recently identified as a factor involved in complex I biogenesis [Haack et al., 2010; Heide et al., 2012]. Cellular complementation experiments that involve overexpression of wild-type ACAD9 in patient-derived fibroblast cell lines showed restoration of complex I assembly and activity [Haack et al., 2010]. Accumulating evidence indicates that there are complex regulatory interactions between mitochondrial fatty acid $\beta$-oxidation and the respiratory chain.

ECHS1 has been shown to interact with several molecules outside the mitochondrial fatty acid $\beta$-oxidation pathway [Chang et al., 2013; Xiao et al., 2013] and the loss of this interaction can affect respiratory chain function in a patient. Further functional analysis of ECHS1 will advance our understanding of the complex regulation of mitochondrial metabolism.

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*Disclosure statement*: The authors have no conflict of interest to declare.

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# Acute fatty liver of pregnancy associated with fetal mitochondrial trifunctional protein deficiency

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## Abstract

Acute fatty liver of pregnancy (AFLP) is a devastating disorder of the maternal liver in the third trimester. Recent studies have demonstrated an association between AFLP and fetal fatty acid oxidation disorders. Here, we report a case of AFLP caused by fetal mitochondrial trifunctional protein (TFP) deficiency. A 21-year-old parous woman presented with nausea, genital bleeding and abdominal pain at 33 weeks of gestation. Laboratory data revealed hepatic failure and disseminated intravascular coagulopathy. The patient underwent emergency cesarean section and was diagnosed with AFLP from the clinical characteristics. She was successfully treated with frequent plasma exchange. The newborn presented severe heart failure and died on the 39th day after birth. Tandem mass spectrometry indicated long-chain fatty acid oxidation disorder. Gene analysis demonstrated homozygous mutation in exon 13 of *HADHB*, the gene responsible for mitochondrial TFP deficiency. The parents carried a heterozygous mutation at the same location in *HADHB*.

**Key words:** acute fatty liver of pregnancy, coagulopathy, fatty acid oxidation disorder, gene analysis, mitochondrial trifunctional protein deficiency.

## Introduction

Acute fatty liver of pregnancy (AFLP) is a serious condition occurring in the third trimester of pregnancy, with significant perinatal mortality.[1] Starting from nonspecific manifestations including headache, nausea and fatigue, hepatic dysfunction and coagulopathy progress rapidly, with renal failure and hepatic encephalopathy. Although the etiology of AFLP is still unclear, recent evidence has linked it to an inherited fetal disorder of mitochondrial fatty acid oxidation.[1–5] Fatty acid oxidation involves the activities of four enzymes: long-chain acyl-coenzyme A dehydrogenase (VLCAD), long-chain enoyl-coenzyme A hydratase (LCEH), long-chain 3-hydroxyl- coenzyme A dehydrogenase (LCHAD) and long-chain 3-ketoacyl-coenzyme A thiolase (LCKT). Three of them, LCEH, LCHAD and LCKT, are known as trifunctional protein (TFP). TFP deficiency can cause diseases of various degrees of severity which include a form with neonatal onset, a hepatic form with infant onset and a myopathic form with onset in late adolescence. Here, we report a case of AFLP, which was caused by fetal mitochondrial TFP deficiency, diagnosed by gene analysis.

## Case Report

A 21-year-old woman (gravida 1, para 1) was referred to Wakayama Medical University Hospital with genital bleeding and abdominal pain at 33 weeks and 4 days of gestation. She bore a healthy baby without any obstetric problems. Prior history and family history were unremarkable, including in terms of inherited disorders. She had pharyngeal pain, nausea and anorexia for

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1 week and laboratory tests revealed elevated liver enzymes with hyperbilirubinemia. On admission, the blood pressure was 136/85 mmHg and other vital signs were normal. On vaginal examination, there was a small amount of genital bleeding. Laboratory data revealed alarmingly disturbed liver function: aspartate aminotransferase (AST), 313 IU/L; alanine aminotransferase (ALT), 454 IU/L; lactate dehydrogenase (LDH), 649 IU/L; total bilirubin, 7 mg/mL; and direct bilirubin, 5.1 mg/dL. Prothrombin time was prolonged to 36.6%, fibrinogen was decreased to 22 mg/dL, antithrombin activity was only 9% and fibrin degradation product was increased to 186.7 μg/dL, demonstrating disseminated intravascular coagulopathy (DIC). Glucose was 89 mg/dL and ammonia was 51 μg/dL. Viral serology tests including hepatitis A, B, C and Epstein–Barr virus were all negative. Her data did not show hemolysis or anemia (hemoglobin, 11.5 g/dL), and the platelet count was relatively low ($11.1 \times 10^4/\mu L$).

Fetal ultrasonography showed cardiac dilation and the cardiothoracic area ratio was 40%. Estimated fetal bodyweight was 1802 g (−1.1 standard deviations [SD]). There was no evidence of retroplacental hematoma. A cardiotocogram showed a normal baseline with decreasing variability and some late decelerations.

In view of the severe liver dysfunction, maternal DIC and non-reassuring fetal status, emergency cesarean delivery was performed after fresh frozen plasma (FFP) and fibrinogen transfusion. The mother and the baby were admitted to the intensive care unit and the neonatal intensive care unit, respectively. On the 2nd day after delivery, the mother was somnolent because of severe hypoglycemia. Results of abdominal ultrasound and computed tomography scans demonstrated her fatty liver. Combined with laboratory findings such as liver failure and severe coagulopathy, as well as the clinical features, she was diagnosed with AFLP, which fulfilled the Swansea criteria.[6] Frequent plasma exchange and transfusion of platelets and FFP led to gradual recovery of her general condition (Fig. 1). Liver biopsy was performed on the 58th day. Pathologic examination showed hepatocyte dropout, cholestasis and portal fibrosis, lacking microvesicular steatosis, which is a typical finding of AFLP. She was discharged on the 60th day after cesarean delivery.

The newborn was a female with a birthweight of 1659 g (−1.2 SD). Apgar scores were 9 and 9 at 1 and 5 min, respectively. The baby showed severe metabolic acidosis, while the liver function was within the

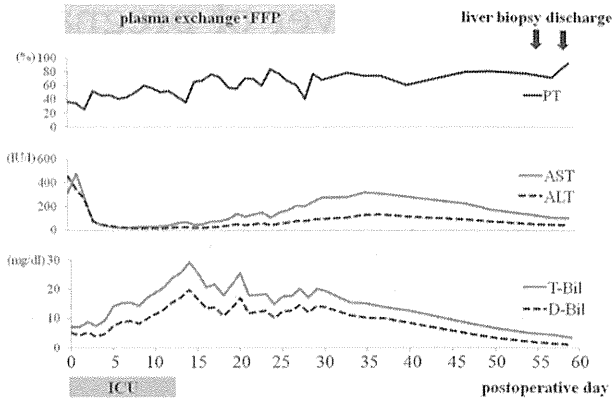


**Figure 1** Trend of the maternal laboratory data after cesarean delivery. ALT, alanine aminotransferase; AST, aspartate aminotransferase; D-Bil, direct bilirubin; FFP, fresh frozen plasma; ICU, intensive care unit; PT, prothrombin time; T-Bil, total bilirubin.

normal range: lactic acid, 140.8 mg/dL; AST, 26 IU/L; ALT, 9 IU/L; LDH, 355 IU/L; total bilirubin, 1.7 mg/dL; and direct bilirubin, 0.3 mg/dL. The baby had severe heart failure. Despite intensive therapy, the baby died of cardiomyopathy on the 39th day after birth. Tandem mass spectrometry revealed that C16-OH and C18:1-OH were increased to 1.42 and 2.17 nmol/mL, respectively, indicating long-chain fatty acid oxidation disorder. In order to clarify the inherited disorder associated with fatty acid oxidation, gene analysis was performed on skin fibroblasts obtained from the baby and on blood samples from the parents, after acquiring informed consent. Gene analysis of the baby demonstrated homozygosity of 1136A>G, PH 379R, in exon 13 of *HADHB*, the gene responsible for mitochondrial TFP deficiency. The parents carried a heterozygous mutation at the same location in *HADHB*. We did not perform gene analysis on the parents' first baby because this baby was 2 years old and could not consent to genetic examination. The parents' first baby had undergone acylcarnitine profile analysis, showing normal results. We provided genetic counseling for the parents about subsequent pregnancies including preimplantation genetic diagnosis.

## Discussion

Acute fatty liver of pregnancy is a rare liver disorder with an incidence of 1/13 000 deliveries, and has significant perinatal morbidity and mortality.[1] Clinically, non-specific manifestations including abdominal pain, nausea/vomiting and fatigue are presented for 1 or

2 weeks at an early stage. It is common that women with AFLP exhibit elevated liver enzymes and bilirubin, elevated ammonia, hypoglycemia, coagulopathy, acute renal failure and hepatic encephalopathy.[7] Pregnant women with AFLP and their fetuses are at risk of death even if they are delivered quickly.[8]

Histologically, AFLP is characterized by microvesicular hepatic steatosis, while widespread necrosis or inflammation is absent. Although liver biopsy is needed to make a definitive diagnosis of AFLP, it is seldom performed considering the complications in the presence of coagulopathy and hepatic dysfunction. In our case, there were no typical histological characteristics of AFLP in specimens of liver biopsy performed on the 58th day, possibly because the subject's liver tissues had recovered. Our case was diagnosed as AFLP from the clinical features, including imaging and laboratory findings based on the Swansea criteria.[6]

Despite manifestations of the clinical features of AFLP, its pathogenesis remains to be clarified, and until recently, it has been considered a mysterious disorder. Recent evidence has demonstrated that AFLP is associated with a fetal disorder of mitochondrial fatty acid oxidation. Mitochondrial β-oxidation of fatty acids is a complex process that consists of four enzymatic reactions resulting in the sequential removal of two-carbon, acetyl-coenzyme A units (Fig. 2). One of the mitochondrial enzymes, TFP, is a hetero-octamer of four α- and β-subunits and catalyzes the last three reactions of the mitochondrial fatty acid β-oxidation spiral with longer chain substrates. The α-subunit has LCEH and LCHAD activities, while the β-subunit has LCKT activity (Fig. 2). The genes that encode the α- and β-subunits are *HADHA* and *HADHB*, respectively.[1,9]

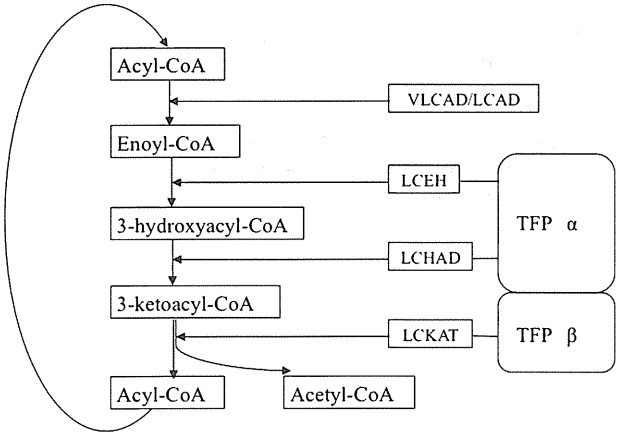


**Figure 2** Mitochondrial fatty acid β-oxidation spiral pathway. Trifunctional protein (TFP) catalyzes the last three reactions of mitochondrial fatty acid β-oxidation. The α-subunit has long-chain enoyl-coenzyme A hydratase (LCEH) and long-chain 3-hydroxyl-coenzyme A dehydrogenase (LCHAD) activities, while the β-subunit has long-chain 3-ketoacyl-coenzyme A thiolase (LCKT) activity.

Trifunctional protein deficiency is classified into two different biochemical phenotypes: one represents the existence of both subunits and the lack of only LCHAD activity, and the other represents the absence of both subunits and the lack of all three TFP activities, although their clinical features are similar.[10] LCHAD defect is usually caused by the common 1528G>C transversion in *HADHA* in Caucasians, and over 60 cases have been described to date.[11] More than 50 cases of TFP deficiency have been reported and 26 of them showed mutations of *HADHB*.[11] In Japan, nine cases of TFP deficiency with mutations of *HADHB* were reported until now, whereas LCHAD defect characterized by *HADHA* mutations were not reported. Inheritance of TFP defects occurs in an autosomal recessive manner, which means affected individuals must have two mutated alleles of the TFP gene for which the enzymatic activities of their products are impaired and both parents are heterozygous carriers.[9]

In 1991, Schoeman *et al.* first reported the association between recurrent AFLP cases and LCHAD defect, suggesting that affected women may have an inherited enzyme deficiency in β-oxidation, predisposing them to AFLP.[2] Chakrapani *et al.* reported the association of maternal liver disease and complete TFP deficiency in four cases.[4] In the present study, homozygous mutation of *HADHB*, which is the gene responsible for TFP deficiency, was found in a newborn baby. The parents showed heterozygosity at the same location in *HADHB*. Purevsuren *et al.* reported compound heterozygous mutations of *HADHB*,[11] one of which was the same as ours. Their transient expression analysis of mutant cDNA revealed that the enzyme activity of the mutated TFP was extremely reduced.

It is hypothesized that unmetabolized free fatty acids return to the mother's circulation via the placenta in cases in which the fetal enzyme activity of TFP is extremely impaired. Toxic metabolites strain maternal hepatic activity and overwhelm any diminished maternal hepatic enzyme activity, resulting in the symptoms of AFLP (Fig. 3). Environmental stress including a high-fat diet may lead to the further accumulation of toxic metabolites in the genetically susceptible mother.[1] In terms of fetal pathophysiology, intrauterine

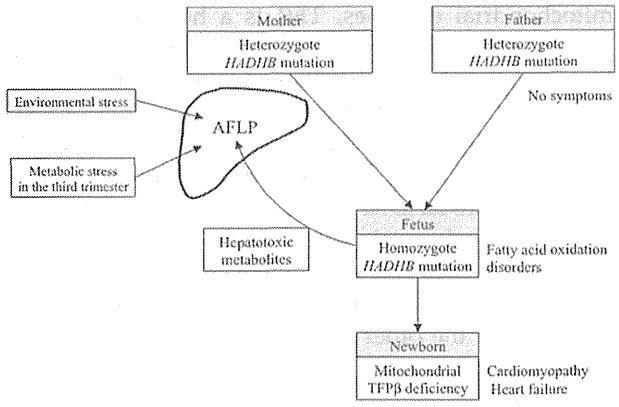


**Figure 3** Possible mechanisms of the pathogenesis of acute fatty liver of pregnancy (AFLP) in our case. Toxic fatty acid metabolites from the fetus lacking trifunctional protein (TFP) activity via the placenta return to the mother's circulation, resulting in the symptoms of AFLP. Environmental stress including a high-fat diet or metabolic stress in the third trimester of pregnancy may lead to the further accumulation of toxic metabolites in the genetically susceptible mother.

cardiomyopathy caused by severe cardiac mitochondrial proliferation in TFP deficiency may lead to lethality.[12]

In summary, AFLP is a serious maternal disorder occurring in the third trimester of pregnancy with significant perinatal mortality. Recent evidence demonstrates that fetal fatty acid oxidative disorders are one of the mechanisms underlying the pathophysiology of AFLP. Early detection and treatment are essential for better prognosis for both mother and newborn. Genetic counseling should be provided to the parents in subsequent pregnancies including pre-implantation genetic diagnosis.

## Disclosure

The authors have no conflict of interest.

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# Ⅵ. 研究構成員

新しい先天代謝異常症スクリーニング時代に適応した治療ガイドラインの作成および生涯にわたる診療体制の確立に向けた調査研究

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