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Case Report

A fetus with mitochondrial trifunctional protein deficiency: Elevation of 3-OH-acylcarnitines in amniotic fluid functionally assured the genetic diagnosis

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ABSTRACT

Mitochondrial trifunctional protein (TFP) is a multienzyme complex that catalyzes the last three steps of the β-oxidation cycle of long-chain fatty acids. In the prenatal diagnosis of TFP deficiency, acylcarnitine (AC) analysis has been considered difficult because of limited excretion of long-chain ACs into the fetal urine and hence into the amniotic fluid. Here, we report our experience with prenatally diagnosing TFP deficiency using AC analysis of amniotic fluid. The index case was a boy born at 38 weeks gestation and weighing 2588 g. He suddenly became unconscious and hypoglycemic and died on day 6 of life. Postmortem blood AC analysis and gene sequencing revealed TFP deficiency. Therefore, the parents underwent prenatal diagnoses for their subsequent 2 pregnancies. Mutation analysis suggested that one (Case 1) was affected and the other (Case 2) was not. AC analysis also demonstrated identical results, with significantly elevated 3-hydroxy-AC levels in the amniotic fluid of the affected pregnancy compared with those of heterozygotes and normal controls ($n = 2$ for heterozygotes and $n = 8$ for normal controls). Our findings suggest that AC analysis can functionally confirm results even in families with unidentified mutations, without raising issues related to maternal cell contamination. During prenatal diagnosis, misdiagnosis has to be avoided, and combining AC analysis with gene sequencing may result in more accurate prenatal diagnosis of TFP deficiency.

1. Introduction

Mitochondrial trifunctional protein (TFP) is a multienzyme complex consisting of trans-2,3-long-chain enoyl-CoA hydratase (LCEH, EC 4.2.1.74), long-chain 3-OH-acyl-CoA dehydrogenase (LCHAD, EC 1.1.1.211) located in the TFP α-subunit (HADHA, OMIM: 600890), and long-chain 3-ketoacyl-CoA thiolase (LCKT, EC 2.3.1.16) located in the TFP β-subunit (HADHB, OMIM: 143450). These enzymes catalyze the last three steps of the β-oxidation cycle of long-chain fatty acids [1,2]. TFP deficiency is clinically classified into three types: 1) lethal type (neonatal-onset form), which includes the development of profound hypoglycemia, lactic acidosis and cardiomyopathy during the neonatal period; 2) intermediate type (infant-onset form), which is accompanied by hypoketotic hypoglycemia that is generally observed following infection or long periods of fasting during the infantile period; and 3) myopathic type (adult-onset form), which includes muscular symptoms, such as intermittent myalgia or rhabdomyolysis, that are associated with prolonged exercise after adolescence. The neonatal form is normally lethal during the neonatal period, irrespective of any intensive treatments [3]. Therefore, families who have had such an affected child often undergo genetic counseling for prenatal diagnosis during subsequent pregnancies.

TFP deficiency is usually diagnosed based on increased levels of long-chain 3-OH-acylcarnitines (3-OH-ACs), such as C16-OH or C18:1-OH, which can be measured by blood acylcarnitine (AC) analysis using tandem mass spectrometry (MS/MS). However, instead of AC analysis, gene analysis is usually performed for the prenatal diagnosis of TFP deficiency [4]. Herein, we report our experience with prenatally diagnosing TFP deficiency using AC analysis and gene analysis. Our data indicate that AC analysis of amniotic fluid is useful for the prenatal diagnosis of TFP deficiency.

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2. Materials and methods

The protocol for this study was approved by the Ethical Committee of Shimane University Faculty of Medicine.

2.1. Case

The index case was a boy born at 38 weeks gestation via vaginal delivery and weighing 2588 g. He was the second child of non-consanguineous parents, and his elder brother was healthy (Fig. 1).

His mother had no abnormalities during the pregnancy, including no HELLP (hemolysis, elevated liver enzymes, and low platelet counts) syndrome or AFLP (acute fatty liver of pregnancy). On the 2nd day after birth, the boy suddenly became unconscious and hypotonic, accompanied by severe hypoglycemia and lactic acidosis. Despite various treatments, including continuous hemodiafiltration that was performed to address the potential of septic shock, his clinical condition deteriorated, and he died of heart failure on the 6th day. Postmortem blood AC analysis revealed the accumulation of 3-OH-ACs, suggesting that the boy had the lethal type of TFP deficiency. Gene analysis and Western blotting confirmed the diagnosis. Therefore, the parents underwent prenatal diagnoses for their subsequent 2 pregnancies (Cases 1 and 2).

After obtaining informed consent from the parents, AC analysis of the amniotic fluid was performed as well as gene analysis and Western blotting.

2.2. Amniotic fluid

Amniotic fluid was collected at 16 and 18 weeks of gestation for Cases 1 and 2, respectively. For comparison purposes, amniotic fluid samples were obtained after 15 weeks from 8 normal controls and from 2 heterozygotes for TFP deficiency who had both undergone prenatal genetic testing (heterozygote-A: c.442+614 A>G and heterozygote-B: c.1364T>G in the *HADHB* gene).

2.3. Gene analysis

Genomic DNA was extracted from the pellets of centrifuged amniotic fluid using the QIAamp DNA Micro Kit (Qiagen GmbH, Hilden, Germany). Both the *HADHA* and the *HADHB* genes, which encode TFP, were sequenced as previously reported [5].

2.4. Western blot analysis

Western blot analysis of cultured fibroblasts or amniocytes was performed using a rabbit polyclonal antibody raised against both the α- and β-subunits of TFP; this antibody was kindly provided by Dr. T. Hashimoto, Professor Emeritus, Shinshu University, Matsumoto, Japan. The signals were visualized using the ImmunoPure NBT/BCIP Substrate Kit™ (Promega, Madison, WI, USA), as previously described [6].

2.5. Acylcarnitine analysis

AC analysis was performed according to the modification of the method as reported [7]. Briefly, 10 μL of amniotic fluid supernatant was obtained after centrifugation at 3000 rpm for 5 min. The sample was then subjected to butyl derivatization using API 3000 triple–quadrupole tandem mass spectrometer (MS/MS) in combination with an SIL-HTc autosampler (Shimadzu, Kyoto, Japan).

3. Results

3.1. Gene analysis

Direct DNA sequencing of the index case revealed the compound heterozygote mutations c.1392+1G>A and c.1689+2T>G in the *HADHA* gene, both of which induce mRNA splicing errors. The same mutations were identified in Case 1, whereas no mutations were identified in Case 2.

3.2. Western blot analysis

Western blot analysis of TFP in the cultured amniotic cells showed that both the α- and β-subunits were detected in Case 2, but not in Case 1, whereas the very-long-chain acyl-CoA dehydrogenase (VLCAD, EC 1.3.8.9) protein was expressed normally in both cases (Fig. 2).

3.3. Acylcarnitine analysis

AC analysis of the amniotic fluid of Case 1 demonstrated significant elevations of 3-hydroxy-ACs: C14-OH measured at 55 nmol/L (control, 3.9 ± 5.0; +10.3 SD), C16-OH measured at 120 nmol/L (control, 0.8 ± 1.5; +77.6 SD), C18-OH measured at 31 nmol/L (control, 3.2 ±

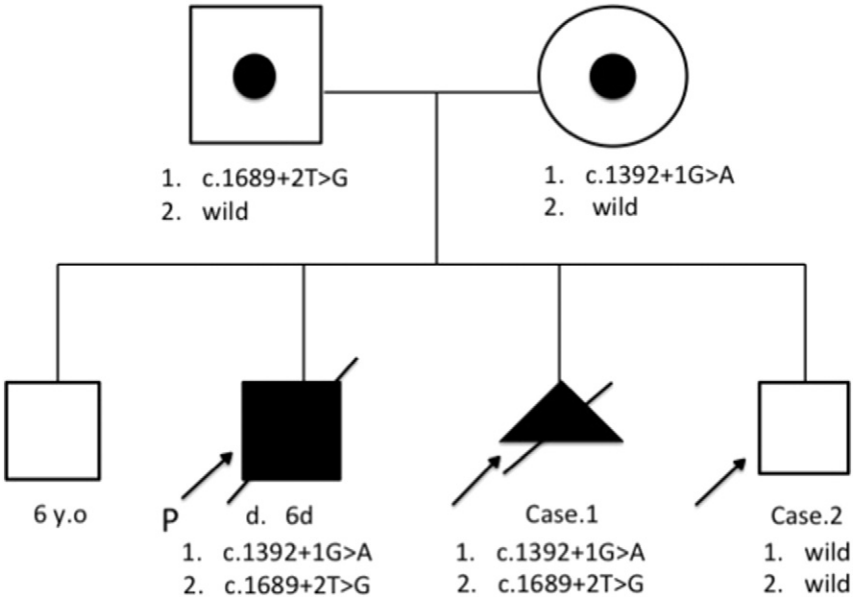

Fig. 1. Family tree The age of each patient and sibling is described for the time at which the amniotic fluid of Case 2 was obtained.

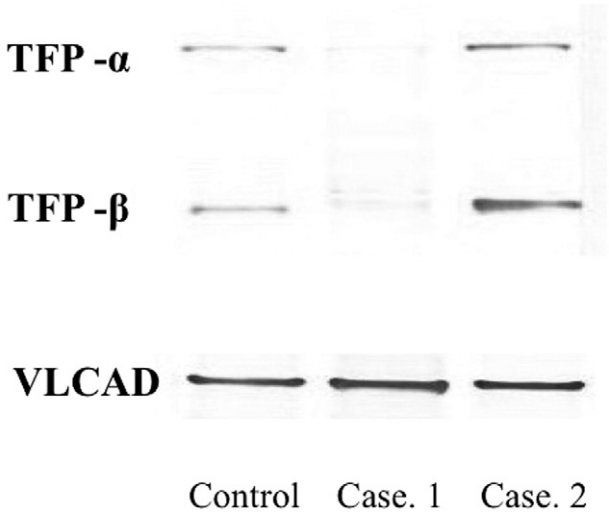

Fig. 2. Western blot analysis of mitochondrial trifunctional protein in the cells in the amniotic fluid. TFP-α and TFP-β were not detected in Case 1. Abbreviations: TFP-α and TFP-β, α- and β-subunits of TFP, respectively; VLCAD, very-long-chain acyl-CoA dehydrogenase.

7.4; +3.5 SD), and C18:1-OH measured at 44 nmol/L (control, 4.6 ± 4.9; +7.9 SD), as shown in Table 1. Conversely, 3-hydroxy-AC levels were not elevated in Case 2 compared with the normal control. There was a marginal increase in the amounts of C14-OH (24 nmol/L; control 3.9 ± 5.0; +1.6 SD) and C16-OH (13 nmol/L; control 0.9 ± 1.5; +4.6 SD) in heterozygote-A, although these values were lower than those in Case 1. Meanwhile, the AC values of the specimen from heterozygote-B overlapped with those of the normal controls.

3.4. Follow-up

It was concluded that Case 1 was affected but that Case 2 was normal. Eventually, Case 1 was artificially aborted at 21 weeks of gestation after obtaining consent from the parents. The diagnosis was validated by AC analysis of umbilical cord blood. In Case 2, the pregnancy was continued, and a boy was born healthy at 38 weeks and 5 days of gestation.

4. Discussion

Our study clearly demonstrates that AC analysis using amniotic fluid can distinguish a fetus affected with TFP deficiency from a healthy fetus. In certain organic acidemias, such as methylmalonic acidemia, propionic acidemia and glutaric acidemia type 1, prenatal diagnosis by gas chromatography/mass spectrometry (GC/MS) and MS/MS using amniotic fluid has been reported [8–11]. In contrast, the prenatal diagnosis of long-chain fatty acid oxidation disorders (FAODs) using MS/MS has not been considered as an optimal approach because ACs derived from long-chain fatty acids do not seem to accumulate in the fetus in response to the inhibition of β-oxidation via malonyl-CoA-mediated suppression of carnitine palmitoyltransferase (CPT)-1 activity and seem to have limited excretion into the fetal urine and hence into the amniotic fluid due to poor solubility [12,13].

Table 1
Acylcarnitines in amniotic fluid of Case 1, Case 2 and heterozygotes.

	Amniotic fluid					Serum
	Case 1	Case 2	Hetero zygote-A	Hetero zygote-B	Control	Index case
C14	27	0	7.0	0	4.6 ± 3.4	1160
C14:1	21	0	3.0	0	5.8 ± 6.5	430
C14-OH	55	0	24	0	3.9 ± 5.0	310
C16	52	0	17	4.0	6.9 ± 8.6	6300
C16-OH	120	0	13	3.0	0.9 ± 1.5	3100
C18	14	10	0	0	2.9 ± 4.6	780
C18-OH	31	0	0	0	3.6 ± 7.8	760
C18:1-OH	44	0	0	8.0	5.1 ± 5.0	1900

Normal control values are the mean ± SD (n = 9).
Unit of AC values: nmol/L.
Abbreviations: C14, myristoylcarnitine; C14:1, tetradecenoylcarnitine; C14-OH, hydroxy-tetradecanoylcarnitine; C16, palmitoylcarnitine; C16-OH, hydroxy-hexadecanoylcarnitine; C18, stearoylcarnitine; C18-OH, hydroxy-octadecanoylcarnitine; C18:1-OH, hydroxy-octadecenoylcarnitine.

However, a recent report demonstrated that fatty acid oxidation is activated and plays a significant role even during the fetal periods [14–16], suggesting that the potential use of AC analysis to detect fetal FAODs is feasible. Although long-chain ACs are considered to be hydrophobic, the presence of a polar hydroxyl group can make ACs (C14:1, C16-OH, C18:1-OH) more soluble in amniotic fluid, thus making their detection in amniotic fluid feasible. Moreover, as identified in Case 1, unsaturated ACs such as C14:1 was elevated. Although it has been suggested that AC analysis using MS/MS is not appropriate for the prenatal diagnosis of long-chain FAODs, the results presented here show that ACs specific for other long-chain FAODs, such as VLCAD deficiency, could be detected during the prenatal period.

AC analysis of amniotic fluid has several advantages over gene analysis and Western blotting. AC analysis allows for the rapid detection of accumulated ACs in a small volume of amniotic fluid. Moreover, this analysis makes prenatal diagnosis feasible, even when the mutation of the index case cannot be identified and there are no issues related to contamination by maternal cells, in contrast to gene analysis. In our study, the AC values overlapped between heterozygotes and normal controls, although those with affected specimens were undoubtedly distinguishable from the heterozygotes and the controls. These findings suggest that the accurate diagnosis of milder cases may be complicated, although prenatal diagnosis may not be necessary in these cases.

Because only one case was studied in this report, additional studies need to be performed. Nevertheless, our study demonstrates that AC analysis can be used for prenatal diagnosis of TFP deficiency.

5. Conclusion

During prenatal diagnosis, misdiagnosis has to be avoided in all cases. Therefore, multiple approaches from several angles are desirable, and their results should be evaluated comprehensively. In this regard, combining AC analysis with genetic testing (gene sequencing) may result in more effective prenatal diagnoses of TFP deficiency and may avoid misleading results.

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