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CASE REPORT

Normal Levels of Plasma Free Carnitine and Acylcarnitines in Follow-Up Samples from a Presymptomatic Case of Carnitine Palmitoyl Transferase 1 (CPT1) Deficiency Detected Through Newborn Screening in Denmark

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Abstract Carnitine palmitoyl transferase (CPT) 1 A deficiency is a rare disorder of hepatic long-chain fatty acid oxidation. CPT1 deficiency is included in newborn screening programs in a number of countries to allow presymptomatic detection and early treatment of affected patients.

We present a case of presymptomatic CPT1A deficiency detected through newborn screening in Denmark with diagnostic levels of carnitine and acylcarnitines in the initial dried blood spot. Levels of plasma-free carnitine and acylcarnitines in follow-up samples were normal, but reverted to diagnostic levels when the patient developed clinical symptoms at the age of 8 months. At that time, a diagnosis of CPT1A deficiency was confirmed by sequence analysis of the *CPT1A* gene revealing homozygosity for a novel c.167C>T variation in exon 3. Enzyme activity measurements showed a relatively mild enzyme defect with a decreased residual enzyme activity of 17–25%. We conclude that *CPT1A* gene testing and/or enzyme assay is mandatory to confirm an abnormal newborn screen suggesting CPT1A deficiency to avoid delayed diagnoses.

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Introduction

Carnitine palmitoyl transferase (CPT) 1A deficiency (MIM ID #255120) is a disorder of long-chain fatty acid beta-oxidation. It is a rare disorder with only approximately 30 cases in the published literature (Bennett 2010). CPT1A deficiency is inherited in an autosomal recessive manner.

The CPT1 enzyme exists in three tissue specific isoforms: the liver isoform (CPT1A), the muscle isoform (CPT1B), and the brain isoform (CPT1C). The three isoforms are encoded by three different genes. CPT1A deficiency is caused by mutations in the *CPT1A* gene, which is localized on the long arm of chromosome 11(11q13.1–q13.2), and codes for the liver enzyme carnitine palmitoyl transferase 1A (CPT1A) (EC 2.3.1.21). CPT1A is localized in the outer mitochondrial membrane and is mainly expressed in liver, kidney, leukocytes, and fibroblasts. The enzyme converts the long-chain fatty acyl-CoA molecules to their respective acylcarnitine molecules, which are then transported across the

inner mitochondrial membrane via the carnitine acylcarnitine translocase. In the mitochondria, they undergo fatty acid beta-oxidation (Bonnefont et al. 2004).

Mitochondrial fatty acid oxidation by the liver provides an alternative source of energy when glycogen reserves are depleted by the increased energy demand during fasting or during a concurrent illness. The clinical symptoms are caused by the reduced ability to use long-chain fatty acids for ketogenesis.

The patients present with hepatomegaly and hypoketotic hypoglycaemia, which can cause seizures and coma (Bennett 2010).

To prevent symptoms, the long-term treatment is to prevent hypoglycaemia. This is accomplished by providing a constant supply of a low-fat, high-carbohydrate diet, and a supply of medium-chain fatty acids. Fasting periods should be kept at a minimum. During acute episodes when the child is unwilling to eat or during surgery, intravenous 10% glucose should be given (Bonnefont et al. 2004).

Because prevention of hypoglycaemia reduces the risk of neurologic damage, it is important to diagnose these patients early. CPT1A deficiency has been included in newborn screening programs world-wide using tandem mass spectrometry to detect abnormal and diagnostic carnitine to long-chain acylcarnitine ratios in dried whole blood spots (Fingerhut et al. 2001; Sim et al. 2001).

Here, we report a newborn diagnosed with CPT1A deficiency through newborn screening in Denmark. The initial newborn screen and a repeat blood spot was consistent with CPT1A deficiency but a plasma sample taken when the patient was stable came out normal reflecting that genetic testing and/or enzyme assay is mandatory to avoid delayed or false-negative diagnoses.

Clinical Case History

Patient M.G is a girl of Turkish descent and she is the second child of consanguineous parents (3rd–4th degree cousins). After a normal pregnancy, she was delivered by caesarean section because of prolonged labour. Her gestational age was 38 + 0, Apgar score 10/1, 10/5, 10/10, birth weight: 3,880 g. She was admitted to the neonatal unit age 3 days under suspicion of a bacterial infection because of respiratory problems, and increased C-reactive protein 50 mg/L (0.0–5.0 mg/L). Further laboratory findings in her blood were normal.

On admission, she suffered from tachypnoea, had very loose stools and a fever of 38.7°C. A white blood cell count was normal and blood cultures were negative. Chest X-ray was normal. The patient was discharged after 24 h in good condition.

When the girl was 16 days old, a newborn screening sample obtained at 7 days of age showed increased blood free carnitine 133 (ref <66.9 μmol/L) and a slightly increased C0/(C16 + C18) ratio of 51 (ref <30). The abnormal results were confirmed in a new blood spot sample obtained when the girl was 20 days old, showing free carnitine of 75 (ref <66.9 μmol/L) and a C0/(C16 + C18) ratio of 68 (ref <30), which is consistent with CPT1A deficiency (Fingerhut et al. 2001; Sim et al. 2001). For further confirmation of the diagnosis, a blood sample for plasma-free carnitine of 35 (ref 24–64 μmol/L) and a C0/(C16 + C18) ratio of 700 (ref <750) were done. These tests showed no signs of CPT1A deficiency. At this point, no *CPT1A* gene sequencing or CPT1 enzyme activity measurements were done. The patient was considered healthy and was discharged from the hospital. The lack of confirmatory diagnostic tests may have led to what later showed to be a premature conclusion.

At age 8 months, the girl was admitted to the paediatric department again after 2 days with diarrhoea and fever. She was severely hypotonic and lethargic, and had a heart murmur at the physical examination. Echocardiography was normal. She was diagnosed with an enteroviral infection.

Laboratory findings in her blood were: pH 7.4, base excess – 6.1 mmol/L (ref – 4.0–2.0 mmol/L), HCO_3 19.4 (ref 21.8–26.2 mmol/L), ammonium 74 (ref <48 μmol/L), lactate 2.7 mmol/L (ref 0.5–2.2 mmol/L), thrombocytes 55×10^9/L (ref $160–360 \times 10^9$/L), Hgb 5.8 mmol/L (ref 6.2–9.0 mmol/L), alkaline phosphatase 145 IU/L (ref 55–425 IU/L), alanine aminotransferase 30 IU/L (ref 5–45 IU/L), bilirubin 19 μmol/L (ref 4–17 μmol/L), plasma glucose 8 mmol/L (3.3–5.5 mmol/L). These blood samples were repeated a week later and were unchanged except for lactate and ammonium that had normalized.

A new determination of amino acids and organic acids in urine was normal. Also plasma amino acids were within the control range, but plasma-free carnitine was slightly increased (65 μmol/L, ref. range 24–64 μmol/L) with an increased C0/C16 + C18 ratio of 2,167 (ref. <750). CPT1A deficiency was reconsidered and confirmed by *CPT1A* gene sequencing and CPT1 enzyme activity measurements (see below).

At 12 months of age, the girl was tested by a child physiotherapist to evaluate whether the girl had suffered any neurologic damage due to her possible episodes of hypoketotic hypoglycaemia associated with CPT1A deficiency. The test showed slight motor retardation and hypotonia. At 13 months of age, the girl started to crawl and at 17 months of age she walked.

Molecular Genetic Findings

Sequence analysis of the *CPT1A* gene (Ensemble gene: ENST00000110090) revealed homozygosity for a novel

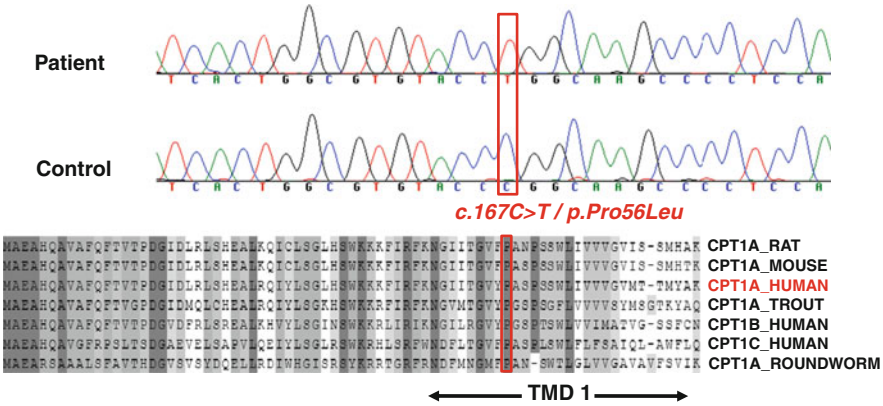

Fig. 1 The *CPT1A* c.167C>T variation. *Upper*: Sequence analysis of genomic DNA from the patient and a control. Shown is position c.152–181 of human *CPT1A*. *Lower*: Amino acid sequence (aa 1–76) alignment of human (*Homo sapiens*) CPT1A (P50416) with rat (*Rattus norvegicus*) CPT1A (P32198), mouse (*Mus musculus*) CPT1A (P97742), trout (*Oncorhynchus mykiss*) CPT1A (D9Z8Q8), roundworm (*Caenorhabditis elegans*) CPT1A (Q9U2F2), human (*Homo sapiens*) CPT1B (Q92523), and human (*Homo sapiens*) CPT1C (Q8TCG5) using protein sequences downloaded from the UniProt database (http://www.ebi.ac.uk/uniprot/index.html) and aligned using the ClustalW alignment tool (http://www.clustal.org/). Conserved residues are marked in *dark grey* and similar residues in *light grey*. Transmembrane domain 1 (TMD 1) is indicated with a *black arrow* and the c.167C>T/p.Pro56Leu change is highlighted in *red*

c.167C>T variation in exon 3. Both parents and a healthy brother with normal plasma carnitine levels were heterozygous for the c.167C>T variation. The variation is not found in the SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) and also not in 114 Danish control alleles (data not shown). We therefore suggest that the c.167C>T variation is responsible for the abnormal biochemical findings observed in the patient. Most likely, the c.167C>T variation results in an abnormal and less active protein in which the highly conserved proline-56 is replaced by a leucine (p.Pro56Leu) (Fig. 1). In addition to the c.167C>T variation we found the patient to be homozygous for three intronic variations (c.556 −16C>T, c.1741 −28G>A and c.2142 +93C>T). These variations are most likely polymorphic and neutral since they are all present in the SNP database with allele frequencies that are more common than the prevalence of CPT1A deficiency.

CPT1 and CPT2 activity in skin fibroblasts was measured essentially as described (Bennett et al. 2004). CPT1 activity was determined as the malonyl-CoA inhibited fraction in the absence of detergent, and CPT2 activity was determined in the presence of malonyl-CoA and *n*-octyl-β-D-glucopyranoside. We found an average CPT1 activity of 0.18 nmol/min/mg protein [ref. (n = 14): 0.40–1.58, mean 0.86, median 0.72] and CPT2 activity of 0.33 (ref.: 0.16–0.57, mean 0.27, median 0.26), with a CPT1/CPT2 ratio of 0.56 (ref.: 1.9–5.1, mean 3.3, median 3.1). These values reflect a relatively mild CPT1A enzyme defect with a decreased residual enzyme activity of 17–25%.

Discussion

Since 2002, the Danish newborn screening program has included screening for disorders of fatty acid oxidation using tandem mass spectrometry, and more than 500.000 newborn children have been tested. Out of these children, one was diagnosed with CPT1A deficiency. According to results from newborn screening programs in Australia, Germany, and the USA the incidence of CPT1A deficiency may be as low as 1:750,000 to 1:2,000,000 (Lindner et al. 2010).

Patients with CPT1A deficiency do not present with symptoms until there is increased energy demands such as during fasting or febrile illness. During acute illness, typical laboratory findings are hypoketotic hypoglycaemia, elevated serum concentrations of liver transaminases, ammonium, and total carnitine (Bennett 2010).

Our patient had clinical symptoms during episodes of acute illness. She had increased plasma carnitine but high plasma glucose, and normal ketones and liver transaminases.

High plasma glucose levels suggest that the acute illness and the slight motor retardation when the patient was stable at age 12 months may have been due to the viral infection, but most likely the reduced CPT1A activity has predisposed to the illness.

She had the newborn screening done at age 7 days and had no symptoms. The test showed increased free carnitine and an increased C0/(C16 + C18) ratio as did a repeat sample at day 20 consistent with a diagnosis of CPT1A deficiency. However, follow-up samples of plasma-free carnitine, acylcarnitines, and of urine organic acids at age 20 days were within the control range. When the patient was 8 months old, acutely ill and laboratory tests once again gave suspicion of CTP1A deficiency with slightly increased plasma-free carnitine and an increased C0/C16 + C18 ratio, the diagnosis of CPT1A deficiency was confirmed by *CPT1A* gene sequence analysis and enzyme

activity measurements revealing homozygosity for a c.167C>T/p.Pro56Leu variation and reduced CPT1A enzyme activity (17–25% of normal controls).

The current case illustrates that also milder cases of CPT1A deficiency are detected through MS/MS-based newborn screening programmes, and that such cases can be difficult to diagnose due to the lack of clear-cut abnormalities on standard metabolic test, particularly during periods when the patient is anabolic and clinically stable.

The reliability of urine organic acids has been discussed. Usually, C12 dicarboxylic acids (Bennett et al. 2004; Brown et al. 2001; Stoler et al. 2004) and according to a single report also 3-OH glutaric acids are elevated in acute cases of CPT1A deficiency (Korman et al. 2005), but as seen in our patient and reported by others urine organic acids may be normal when the patient is well (Bennett 2010; Tsuburaya et al. 2010). Plasma C0/C16 + C18 ratios and to a lesser extent the level of free carnitine are, on the other hand, considered reliable diagnostic markers for CPT1A deficiency even when the patient is stable and biochemical markers of metabolic decompensation are within the control range (Fingerhut et al. 2001; Sim et al. 2001). However, in the current case the C0/C16 + C18 ratios were rather low (two to threefold increase) as compared to the values reported by Fingerhut and co-workers (6–60 fold increases) (Fingerhut et al. 2001) and the repeat C0 values were within the control range or only slightly increased (Fingerhut et al. 2001; Sim et al. 2001). The data most likely reflect the relatively mild enzyme defect in the current case (17–25% of normal controls) as compared to the severe enzyme deficiencies (<7% of normal controls) reported in previously published cases (Fingerhut et al. 2001; Sim et al. 2001).

Our case illustrates that diagnostic levels of plasma-free carnitine and acylcarnitines may depend on the metabolic state of the patient being abnormal only during acute crisis. Newborn screening programs, which allow early detection of metabolic markers in dried whole blood spots when the newborn is catabolic, are therefore very important. However, to avoid delayed or false-negative diagnoses, it is important to supplement biochemical confirmation of an abnormal newborn screen with analysis of the *CPT1A* gene and/or enzyme activity measurements. There is almost complete genetic heterogeneity of disease causing *CPT1A* variations with each affected family demonstrating novel variation(s) of *CPT1A* (Bennett et al. 2004). Therefore, analysis of the entire *CPT1A* gene is required to confirm an abnormal newborn screen and the disease-causing nature of the abnormal genotype needs to be carefully interpreted. The c.167C>T variation is not observed in DNA from healthy control individuals and the variation segregates with the disease in the family. The c.167C>T variation results in replacement of the evolutionary conserved proline-56 with leucine. Proline-56 is located in an α-helical hydrophobic segment (amino acids 48–75), which makes up one of two transmembrane domains that anchor the CPT1A enzyme into the outer mitochondrial membrane (Fraser et al. 1997). It is likely that p.Pro56Leu affects membrane insertion and stability of the variant CPT1A enzyme, which gives rise to the mildly decreased residual enzyme activity and impaired conversion of long-chain acyl-CoAs to their acylcarnitine derivates as reflected in the slightly increased C0/(C16 + C18) ratio in samples from the patient. Excluding the present all disease-causing variations identified to date are located in the C-terminal domain (amino acids 123–773) making up the catalytic core of the protein and most are associated with CPT1A enzyme activities between 0 and 10% and onset of hepatic encephalopathy in infancy or early childhood (Bennett et al. 2004; Brown et al. 2001; Fingerhut et al. 2001; Korman et al. 2005; Sim et al. 2001; Stoler et al. 2004). Exception is the enigmatic p.Pro479Leu Inuit variation, which has residual enzyme activity of 20% (Bennett et al. 2010) and the p.Gly719Asp variant, which was recently identified in a presymptomatic case of CPT1A detected through newborn screening in Japan and with residual activity of 11–26% of controls (Tsuburaya et al. 2010).

The case reported by Tsuburaya et al. and the current case illustrate very well the ethical considerations associated with any screening program for pre-symptomatic genetic diseases, where children with borderline biochemical tests and milder variants of uncertain clinical significance will be identified. Careful follow-up programs and evaluation of new genetic variants are required to establish biochemical cut-off values that allow a reasonable balance between the clinical benefits and the psychosocial risks of screening. Another essential component of neonatal screening for inherited diseases is to have a diagnostic algorithm for each disorder to be able to establish if it is a true or a false positive case. In the present case, the diagnosis relied entirely on the measurement of free carnitine in plasma and to a lesser extent on the ratio between free carnitine to long chain acylcarnitines (C0/C16 + C18). A finding of a carnitine value in plasma within the control range led to a delayed diagnosis. The correct diagnosis was first established when the patient later on developed symptoms that could be consistent with CPT1 deficiency. As a consequence, CPT1 deficiency was excluded from the Danish newborn screening program in 2008. After establishment of *CPT1A* gene analysis and CPT1A enzyme activity measurements, we are currently deliberating on whether to re-include screening for CPT1A deficiency in Denmark.

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Synopsis

Findings of normal levels of plasma-free carnitine and acylcarnitines in follow-up samples from a presymptomatic case of carnitine palmitoyl transferase 1 (CPT1) deficiency detected through newborn screening in Denmark emphasize the importance of *CPT1A* gene testing and/or enzyme assay to avoid delayed diagnoses.

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