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Newborn screening for carnitine palmitoyltransferase II deficiency using (C16 + C18:1)/C2: Evaluation of additional indices for adequate sensitivity and lower false-positivity

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ABSTRACT

Background: Carnitine palmitoyltransferase (CPT) II deficiency is one of the most common forms of mitochondrial fatty acid oxidation disorder (FAOD). However, newborn screening (NBS) for this potentially fatal disease has not been established partly because reliable indices are not available.

Methods: We diagnosed CPT II deficiency in a 7-month-old boy presenting with hypoglycemic encephalopathy, which apparently had been missed in the NBS using C16 and C18:1 concentrations as indices. By referring to his acylcarnitine profile from the NBS, we adopted the (C16 + C18:1)/C2 ratio (cutoff 0.62) and C16 concentration (cutoff 3.0 nmol/mL) as alternative indices for CPT II deficiency such that an analysis of a dried blood specimen collected at postnatal day five retroactively yielded the correct diagnosis. Thereafter, positive cases were

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assessed by measuring (1) the fatty acid oxidation ability of intact lymphocytes and/or (2) CPT II activity in the lysates of lymphocytes. The diagnoses were then further confirmed by genetic analysis.

Results: The disease was diagnosed in seven of 21 newborns suspected of having CPT II deficiency based on NBS. We also analyzed the false-negative patient and five symptomatic patients for comparison. Values for the NBS indices of the false-negative, symptomatic patient were lower than those of the seven affected newborns. Although it was difficult to differentiate the false-negative patient from heterozygous carriers and false-positive subjects, the fatty acid oxidation ability of the lymphocytes and CPT II activity clearly confirmed the diagnosis. Among several other indices proposed previously, C14/C3 completely differentiated the seven NBS-positive patients and the false-negative patient from the heterozygous carriers and the false-positive subjects. Genetic analysis revealed 16 kinds of variant alleles. The most prevalent, detected in ten alleles in nine patients from eight families, was c.1148T > A (p.F383Y), a finding in line with those of several previous reports on Japanese patients.

Conclusions: These findings suggested that CPT II deficiency can be screened by using (C16 + C18:1)/C2 and C16 as indices. An appropriate cutoff level is required to achieve adequate sensitivity albeit at the cost of a considerable increase in the false-positive rate, which might be reduced by using additional indices such as C14/C3.

1. Introduction

Carnitine palmitoyltransferase (CPT) II is an enzyme bound to the mitochondrial inner membrane. Long-chain fatty acids are transported into the mitochondria as acylcarnitines of the corresponding chain-length via the sequential function of acyl-CoA synthetase, CPT I, and carnitine-acylcarnitine translocase (CACT). These long-chain acylcarnitines, represented by palmitoylcarnitine (C16), are then turned back into acyl-CoA by CPT II to supply substrates for the β-oxidation system. Since the first case report on this subject [1], CPT II deficiency has been clinically classified into three phenotypes: 1) a lethal, neonatal form associated with cardiomyopathy; 2) a severe, infantile form which provokes hypoglycemia, Reye-like encephalopathy, and in the worst cases, cardiopulmonary arrest mainly during infancy and young childhood; and 3) an adult-onset form presenting recurrent rhabdomyolysis in adolescence or later. Since the severe, infantile form of CPT II deficiency was identified as the cause of sudden infantile death, this potentially fatal disease has become an important target of tandem mass spectrometry (MS/MS)-based newborn screening (NBS).

MS/MS-based NBS was introduced into Japan in 1997, and pilot studies were begun in several research centers. In Hiroshima, where the first author currently works, screening for CPT II deficiency was initiated in January 2004 using C16 (cutoff 6.3 nmol/mL) and C18:1 (cutoff 3.6 nmol/mL). These cutoff values corresponded to the mean + 4SD when they were set. No positive results were achieved until 2010, when a 7-month-old boy presented with acute encephalopathy associated with hypoketotic hypoglycemia, hyperammonemia, and marked elevation of serum creatine kinase resulting in severe neurological sequelae. The diagnosis of CPT II deficiency was confirmed [2]. The patient had apparently passed the regional pilot study on MS/MS-NBS with C16 and C18:1 at 3.45 nmol/mL and 1.68 nmol/mL in a dried blood specimen (DBS) collected on postnatal day 5, respectively. This "false-negative" case motivated us to revise the screening indices for CPT II deficiency.

2. Methods

2.1. Screening of CPT II deficiency

Blood samples were analyzed by MS/MS (LCMS-8030, Shimadzu, Kyoto, Japan; API 4000 LC/MS/MS system, AB Sciex, Framingham, MA, USA; ACQUITY TQD, Waters, Milford, MA, USA, etc.) following the protocol described in our previous report [3]. For NBS, dried blood specimens were generally collected on postnatal day 4 or 5. This protocol has been used since NBS for phenylketonuria and other amino acid disorders started in 1977. It is widely accepted that earlier sampling of dried blood is desirable for detecting disorders of fatty acid oxidation, but this method is not yet practiced in Japan. To improve the sensitivity for detecting CPT II deficiency, we adopted (C16 + C18:1)/C2, which had previously been proposed for the screening of symptomatic cases using serum or plasma [4]. We set the cutoff value for this ratio at 0.62, which was as high as the 99.9th percentile in healthy control subjects (n = 5914, mean ± SD = 0.282 ± 0.073) and below the value of the "false-negative" patient's newborn DBS (0.75). In order to avoid excessive false-positive results, we decided to retain C16 as the second index but reduced the cutoff value from 6.3 nmol/mL to 3.0 nmol/mL (79.5th percentile; n = 5914, mean ± SD = 2.37 ± 0.87). These alternative indices have been used in NBS in Hiroshima since April 2011 before being adopted in other areas. For selective screening, serum specimens were collected from patients presenting with suggestive clinical symptoms. Patients with elevated serum levels of C16 (cutoff 0.1 nmol/mL) and C18:1 (cutoff 0.1 nmol/mL) were suspected of having CPT II deficiency.

2.2. Measurement of fatty acid oxidation (FAO) by intact cells

Lymphocytes collected from heparinized whole blood using the Ficoll-Paque solution method were suspended in 1 mL of Dulbecco's phosphate-buffered saline (D-PBS) and incubated at 37 °C for 2 h after adding D-PBS containing L-carnitine and a fatty acid solution containing deuterium-labeled palmitate (d_{31}-palmitate: 0.5 mg/mL in 3% fatty acid-free bovine serum albumin solution). The washed lymphocytes were homogenized in methanol, and the supernatant, spiked with stable isotope-labeled acylcarnitines as internal standards, was analyzed by flow-injection electrospray-ionization tandem mass spectrometry using API 4000 LC/MS/MS system (AB Sciex). Fatty acid oxidation was assessed by the ratio of d_1-acetylcarnitine (d_1C2) to d_{31}-palmitoylcarnitine (d_{31}C16) while the CPT II activity was assessed by the ratio of d_{27}-tetradecanoylcarnitine (d_{27}C14) to d_{31}C16.

2.3. Measurement of CPT II activity

As the revised indices for CPT II deficiency raised the number of positive cases, we developed a simple and rapid enzymatic assay as another confirmatory test. In brief, the production of palmitoyl-CoA from palmitoyl-L-carnitine (C16AC; Sigma Chemical, St. Louis, MO) and coenzyme A trilithium salt ($CoALi_3$; Kohjin, Tokyo, Japan) catalyzed by a crude lysate of peripheral lymphocytes was detected by high-performance liquid chromatography (HPLC). Lymphocytes were sonicated in 1% octyl glucoside (Sigma Chemical, St. Louis, MO) solution so as to abolish the activity of CPT I [5]. The final concentration of each reagent in the reaction mixture was as follows: 100 mmol/L Tris-HCl (pH 7.4), 10 mmol/L C16AC, 10 mmol/L $CoALi_3$, and lysate of 4×10^5 lymphocytes. The mixture was incubated at 37 °C for 10 min, and the reaction was terminated by the addition of acetonitrile. After centrifugation, the supernatant was introduced into an HPLC system