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False positive cases of elevated tetradecenoyl carnitine in newborn mass screening showed significant loss of body weight

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ABSTRACT

Very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, a condition in which the body is unable to break down long-chain fatty acids properly, is the most common fatty acid oxidation disorder in Japan. Tandem mass spectrometry has been used in newborn screening (NBS), allowing the detection of patients with VLCAD deficiency even before symptoms manifest. However, tandem mass spectrometry has a high false positive rate. We investigated the clinical characteristics of patients with false positive results for tetradecenoyl acylcarnitine (C14:1). This case-control study used data collected between the 1st of January 2014 and the 31st of March 2019. The case group was defined as patients having levels of both C14:1 and C14:1/C2 ratio higher than cut-off levels in the first newborn mass screening, who were eventually diagnosed as false positives by attending doctors at Kobe University Hospital, Palmore Hospital, or Kakogawa Central City Hospital in Japan. The control group comprised 100 patients randomly selected from the three facilities. The false positive group included 17 cases, and the control group contained 300 patients. The demographics of each group did not show any significant differences in sex, body weight at birth, Cesarean section rate, complete breastfeeding rate, or the number of feedings per day. However, the change in body weight at the sampling day of NBS in the false positive and control groups was −10.2%, and − 4.6%, respectively, showing a statistically significant difference ($p < 0.01$). In addition, body weight gain at the one-month medical checkup was 38.9 g/day in the false positive group and 44.1 g/day in the control group ($p < 0.05$). An elevation of C14:1 carnitine has been reported in situations involving the catalysis of fatty acid. Therefore, patients with severe body weight loss might be associated with poor sucking or poor milk supply, which might cause a false positive elevation of C14:1 and C14:1/C2. In suspected VLCAD deficiency, attending doctors should pay attention to body weight changes recorded during newborn mass screening.

1. Introduction

Very-long-chain acyl-CoA dehydrogenase deficiency (VLCADD, OMIM 201475) is a rare congenital metabolic disorder of fatty acid beta-oxidation, which is inherited in an autosomal recessive manner. It has a prevalence of 1:93000 births in Japan [1]. VLCAD (EC 1.3.8.9) is one of a family of acyl-CoA dehydrogenases catalyzing the dehydrogenation of long-chain fatty acids in order to produce important sources of energy, such as acetyl-CoA, which feed into the TCA cycle [2,3]. Typical clinical symptoms in patients with VLCADD include severe hypoketotic hypoglycemia, liver dysfunction, cardiac involvement, rhabdomyolysis due to the accumulation of toxic metabolites, and shortage of energy [2,4], which are provoked during catabolic situations such as exercise, illness, or fasting [5]. The prevention of catabolism by avoiding fasting is believed to reduce the risk of metabolic decompensation in patients with VLCADD [6,7]. Since early diagnosis is essential for improving the prognosis of this condition, the assessment of VLCADD has been widely incorporated into newborn screening (NBS).

The recent development of electrospray ionization tandem mass spectrometry (MS/MS), a new diagnostic instrument with high specificity and sensitivity [8], could facilitate the quantitative assessment of the amount of long-chain acylcarnitine metabolites of fatty acid beta-oxidation. Routine neonatal screening for VLCADD was instituted in

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Japan in 2014. Several metabolites, including tetradecenoylcarnitine (C14:0), hexadecanoyl-carnitine (C16:0), hexadecenoyl-carnitine (C16:1), and octodecenoyl-carnitine (C18:1) accumulate in patients with VLCADD, but tetradecenoylcarnitine (C14:1) has been reported as the most specific biomarker for VLCADD [9,10]. A combination of C14:1 and several acylcarnitines has been reported to improve the sensitivity of the detection of patients with VLCADD [11,12]. In Japan, the value of C14:1 and the ratio of C14:1/ acetylcarnitine (C14:1/C2) are used as diagnostic markers for VLCADD [13].

Even in healthy individuals, C14:1 and C14:1/C2 ratios may be increased to the same level as in affected patients [13,14], but the etiology of this false positive group is not completely clear. False positive results from NBS can be extremely stressful to patients and their families and adversely affect patient-family relationships [15,16].

In this research, we investigated the clinical characteristics of patients with elevated C14:1 acylcarnitine and C14:1/C2 ratios, to reduce the incidence of the unnecessary diagnostic workup for unaffected newborns, and hence decrease the physical and mental burdens imposed upon the parents and the infants.

2. Materials and methods

2.1. Patients

This case-control study was performed using data collected from the 1st of January 2014 to the 31st of March 2019. The case group was defined as patients with high ratios of both C14:1 and C14:1/C2 in NBS, but who were diagnosed as false positives by attending doctors at Kobe University Hospital, Palmore Hospital, or Kakogawa Central City Hospital in Japan. Attending doctors diagnosed false positives based on the retesting of acylcarnitine analyses and clinical course. Enzyme activity and molecular analysis were not routinely performed in all cases. A control group with normal levels of acylcarnitine at NBS comprised 100 patients selected at random from the three facilities. Patients who chose not to participate in this study, infants with low body birth weight, preterm infants, and infants with other metabolic disorders or congenital diseases were excluded from this study.

2.2. Clinical information

All clinical data, including situation at birth, gestational age, birth weight, Cesarean section rate, body weight change from birth to the day of taking samples of NBS, number of patients receiving glucose infusion at sampling day to prevent hypoglycemia, complete breastfeeding rate, number of feeds per day, and body weight gain at the one-month medical checkup were collected from medical records. Cesarean section ratio is acquired by collecting the data on the delivery style of each group. Complete breastfeeding is defined as not using any nutrition other than breast milk.

2.3. Acylcarnitine analysis

The acylcarnitine level of dried blood spots collected at Kobe university and Palmore hospital were analyzed using tandem mass spectrometry (MS/MS) (API-3200; Applied Biosystems, California), while those collected at Kakogawa Central City Hospital were analyzed using (Xevo TQD; Waters, Massachusetts). Different cut-off values of NBS were used because these hospitals located far apart, and use different analytical facilities. The cut-off levels of C14:1 and C14:1/C2 were 0.4 μM and 0.013 for Kobe University and Palmore Hospital, while those used at Kakogawa Central City Hospital were 0.27 μM and 0.013.

2.4. Statistical analysis

Shapiro-Wilk tests were used to determine if a data set followed a normal distribution. When the *P*-value was < 0.05, the data set was considered to have a non-normal distribution. Student's *t*-tests were used to compare findings between two groups if the data were distributed normally; otherwise, Mann-Whitney *U* tests were used. Additionally, chi-square tests were used for comparing the ratios of these factors. Receiver operating characteristic (ROC) curves were generated by plotting sensitivity vs. 1-specificity, and the area under the curve (AUC) was calculated. All statistical analyses were performed using GraphPad PRISM 8 (GraphPad Software, Inc., San Diego, California).

Table 1
Clinical characteristics of cases and control groups.

	Case: False positive	Control	P-value
Number of cases	17	300	–
Gestational age (weeks)	39	39	0.805
Sex (male to female ratio)	41:59	57:43	0.205
Birth weight (g)	3015	3077	0.607
Cesarean section (%)	23.5	26.3	> 0.999
Number of patients receiving glucose infusion	0	2	> 0.999
Complete maternal feeding (%)	47.0	53.7	0.316
Number of feeds per day	10 (n = 15)[*1]	10	0.575

[*] 1: Two cases in the case group lacked information about the number of feeds.

2.5. Ethics

Informed consent was obtained in the form of opt-out on the website. The study protocol was approved by the ethics committee of each hospital (approval number 170027, Kobe University).

3. Results

In this study, 17 cases were diagnosed as false positives, while three hundred infants were assigned to the control group. The clinical characteristics of each group are listed in Table 1. The parameters sex, body weight at birth, the ratio of cesarean section, taking glucose infusion, ratio of complete breastfeeding, and the number of feedings per day showed no significant differences between the false positive group and the control group. However, body weight change at the sampling day of NBS and body weight gain at the one-month medical checkup was significantly different.

As shown in Fig. 1, the body weight change of NBS (mean ± SD) was −10.2% ± 2.6% in the false positive group and − 4.6% ± 3.7% in the control group. Body weight change in the false positive group was significantly lower than that of the control group ($p < 0.01$). The distribution of body weight change rate in the control group ranged from −13.4% to 7.2%, but was narrower in the false positive group, ranging between −14.1% and − 5.2%. Body weight gain at the one-month medical checkup was 38.9 g/day in the false positive group and 44.1 g/day in the control group (Fig. 1). The body weight gain at the one-month medical checkup in the false positive group was significantly lower than that in the control group ($p < 0.05$).

We analyzed whether body weight change in NBS sampling day or at the one-month medical checkup could distinguish between the false positive group and the control group. The AUC of the ROC curve of the body weight change in NBS sampling day obtained from the false positive group and the control group was 0.89 ($p < 0.0001$), while the AUC of the ROC curve of the body weight gain at one-month checkup obtained from the two groups was 0.66 ($p < 0.05$). In the group with −6.1% body weight change at NBS, we could differentiate the false positive group from the control group with a sensitivity of 63.0% and a specificity of 94.1% (Fig. 2).

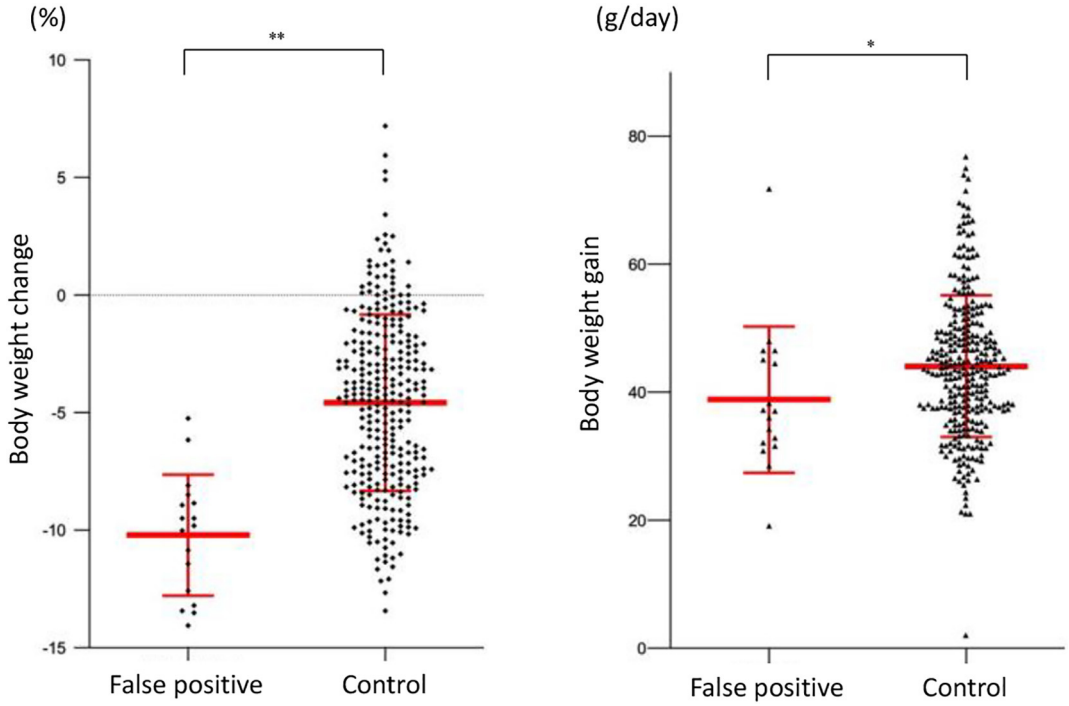

Fig. 1. Scatter plot of body weight change at sampling day of NBS and body weight gain at one-month medical checkup in false positive group and control group. Each data point is represented by a diamond. Red line indicates the mean ± SD. Dotted line is zero% line; *$p < 0.05$, ** $p < 0.01$.

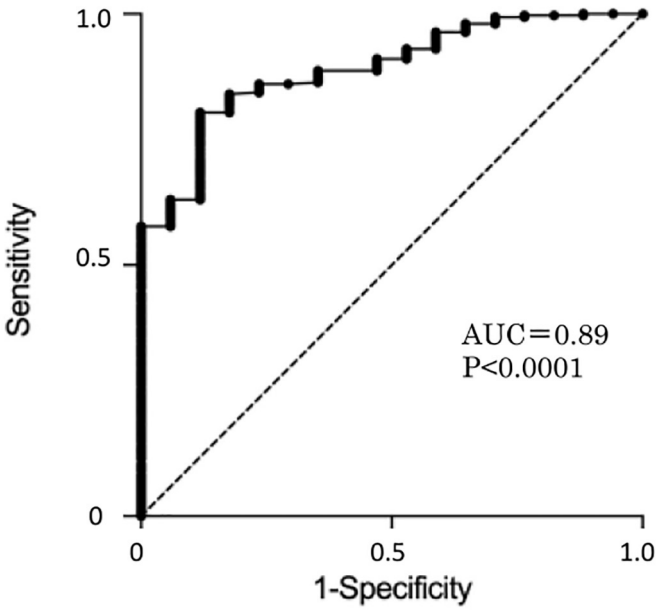

Fig. 2. Receiver operating characteristic (ROC) curves of body weight change on the NBS sampling day. Dotted line is the reference line.

4. Discussion

In this study, we found that a false positive group with elevated C14:1 acylcarnitine showed significant body weight loss at NBS sampling, and poor body weight gain at the one-month medical checkup.

NBS for VLCAD deficiency is challenging because the levels of long-chain acylcarnitines can be affected by several conditions, resulting in the misdiagnosis or overdiagnosis of VLCADD. False negatives are often associated with medical treatment, such as glucose infusions, which could normalize plasma acylcarnitine levels in subjects with VLCADD [3], while false positives have been identified in heterozygous carriers of VLCADD, and even in healthy individuals, under catabolic situations like prolonged fasting between maternal feeding [11,13,14,17]. Under prolonged fasting, as a physiological response to lipolysis, a large amount of stored fatty acids are transported into the mitochondria and undergo beta-oxidation, which elevates the amount of long-chain acylcarnitines, including C14:1 acylcarnitines, even in healthy individuals [18]. Poor weight gain results from poor sucking or poor maternal milk production, so patients in the false positive group may have been starving and in catabolic status when undergoing NBS.

In order to reduce the false positive rate for VLCAD, raising the cut-off value of C14:1 and C14:1/C2 is not sufficient, because the numbers of false-negative patients then increase since some patients with levels of ACs which overlap with healthy controls have already been reported [19]. To prevent false positive identification of VLCAD deficiency, the use of other combinations of biomarkers, such as C14:1/ C12:1 or C14:1/C16, has been suggested [13,20]. However, no conclusion has been reached as to which combination is the best, because these new combination markers could not effectively distinguish affected subjects with VLCADD from healthy individuals when using data from dried blood spots [13].

Expanded NBS is necessary to detect a wider range of congenital metabolic disorders, but the optimal timing of sampling is different for each disease. To detect VLCADD, sampling using dried blood spots has been recommended to be performed in the catabolic period, 36 to72 hours after birth [8]. In Japan, NBS is recommended 4–6 days after birth for the sensitive detection of other congenital metabolic disorders. Since Japanese NBS is performed after the catabolic period, the delay in sampling can lead to an increased number of false negatives. However, in this study, many patients with false positive diagnoses were detected, suggesting the cut-off of C14:1 and C14:1/C2 ratios may have been set too low in an effort to reduce false negatives. In this setting, we could not distinguish healthy controls with false positives without changing the sampling time. It is time to reconsider accelerating the timing of sampling for NBS to decrease the false positive rate of VLCADD.

Since the analysis of acylcarnitine is time-consuming, the results of which cannot be returned to the attending doctors within a few days, so the situation may be different when patients are examined at

specialized medical institutions. Attending doctors should focus on the body weight change at NBS sampling day and the one-month medical checkup. Targeted cut-off levels should be reconsidered for babies having severe body weight loss at NBS.

One limitation of this study was that we did not perform molecular analysis and enzymatic tests of the patients. Therefore, a mild form of the condition or heterozygous carriers of VLCADD might have been assigned to the false positive group. Although the sample size was large enough to estimate statistical significance, a larger cohort study, including molecular analysis, would be valuable.

5. Conclusions

Newborns with elevated C14:1 carnitine and C14:1/C2 ratios at NBS are generally regarded as being at risk for VLCADD. However, our results indicate that the use of this metric might lead to high levels of false positives, and should not be regarded as a reliable diagnostic measure. When assessing these patients, the attending doctor should pay attention to the body weight changes recorded at newborn mass screening and the one-month medical checkup.

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