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Development of a New Enzymatic Diagnosis Method for Very-long-chain Acyl-CoA Dehydrogenase Deficiency by Detecting 2-Hexadecenoyl-CoA Production and its Application in Tandem Mass Spectrometry-based Selective Screening and Newborn Screening in Japan

GO TAJIMA, NOBUO SAKURA, KENICHIRO SHIRAO, SATOSHI OKADA, MIYUKI TSUMURA, YUTAKA NISHIMURA, HIROAKI ONO, YUKI HASEGAWA, IKUE HATA, ETSUO NAITO, SEIJI YAMAGUCHI, YOSUKE SHIGEMATSU, AND MASAO KOBAYASHI

Department of Pediatrics [G.T., N.S., K.S., S.O., M.T., M.K.], Hiroshima University Graduate School of Biomedical Sciences, Hiroshima 734-8551, Japan; Department of Pediatrics [Y.N.], National Hospital Organization Kure Medical Center, Kure 737-0023, Japan; Department of Pediatrics [H.O.], Hiroshima Prefectural Hospital, Hiroshima 734-8530, Japan; Department of Pediatrics [Y.H., S.Y.], Shimane University, Izumo 693-8501, Japan; Department of Pediatrics [I.H.], Department of Health Science [Y.S.], University of Fukui, Fukui 910-1193, Japan; Department of Pediatrics [E.N.], University of Tokushima, Tokushima 770-8503, Japan

ABSTRACT: The introduction of tandem mass spectrometry (MS/MS) has made it possible to screen for very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency. To confirm the diagnosis in cases with an abnormal profile of blood acylcarnitines, we developed a new enzymatic assay method for determining dehydrogenase activity toward palmitoyl-CoA (C16:0) in lymphocytes. Using this method, the production of 2-hexadecenoyl-CoA (C16:1) by crude cell lysates can be directly quantified using high performance liquid chromatography (HPLC). We applied the assay to 7 myopathic patients, 7 hypoglycemic patients, and 2 presymptomatic newborns with elevated levels of tetradecenoylcarnitine (C14:1 AC) in blood, and found impaired VLCAD activity in all of the 7 myopathic patients and both of the 2 newborns. All of the 7 hypoglycemic patients had normal level of the enzyme activity. Results of the *ACADVL* gene analysis were in consistent with the enzymatic diagnosis. These results suggest that MS/MS-based screening for VLCAD deficiency using blood C14:1 AC as the indicator may show a considerably high false-positive rate in selective screening of symptomatic patients. Our practical enzymatic assay can be a useful test for the accurate diagnosis of VLCAD deficiency cases screened by MS/MS. (***Pediatr Res* 64: 667–672, 2008**)

Acylcarnitine (AC) analysis by tandem mass spectrometry (MS/MS) has made it possible to screen for fatty acid oxidation disorders (FAODs), including very-long-chain acyl-CoA dehydrogenase (VLCAD; EC 1.3.99.-) deficiency (McKusick 201475). Since it was first identified in 1993 (1–3), VLCAD deficiency has been the most frequently diagnosed FAOD in Japan (Yamaguchi S *et al.,* Survey of mitochondrial fatty acid disorders (FAODs) in Japanese, 6th International Congress on Fatty Acid Oxidation, June 2005, Edmond aan Zee, The Netherlands, Abstract), although this may be due in part to the introduction of AC analysis in 1997; (4) the number of patients diagnosed with VLCAD deficiency was 7 by the end of 2000 and had increased to 34 in the following 4 y (Yamaguchi S *et al.,* Survey of mitochondrial fatty acid disorders (FAODs) in Japanese, 6th International Congress on Fatty Acid Oxidation, June 2005, Edmond aan Zee, The Netherlands, Abstract). With respect to clinical phenotypes, 27 of these 34 patients were diagnosed with the myopathic form of the disease.

As MS/MS-based screening becomes more common, rapid confirmatory tests are needed for the various target disorders. One possibility would be a test for mitochondrial acyl-CoA dehydrogenases, which produce 2-enoyl-CoA species in coordination with a reduction of electron transfer flavoprotein (ETF). Recently, we reported enzymatic diagnosis methods for isovaleric acidemia and medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, which detect the production of 3-methylcrotonyl-CoA or 2-octenoyl-CoA, respectively, using the crude lysates of lymphocytes and high performance liquid chromatography (HPLC) (5,6). The diagnosis of VLCAD deficiency can also be confirmed by a similar HPLC-based assay of 2-enoyl-CoA production. Wanders *et al.* (7) reported their method of this type and suggested its superiority to conventional spectrophotometric assays of reduced electron acceptors and radioisotope-dependent methods. In recent reports, their method was applied to presymptomatic patients with VLCAD deficiency identified by MS/MS-based screening (8–10). This method does not measure 2-hexadecenoyl-

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Correspondence: Go Tajima, M.D., Ph.D., Department of Pediatrics, Hiroshima University Graduate School of Biomedical Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan; e-mail: acadugo@mac.com

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Abbreviations: AC, acylcarnitine; **ACAD9,** acyl-CoA dehydrogenase-9; **ACO,** acyl-CoA oxidase; **C14:1 AC,** tetradecenoylcarnitine; **CK,** creatine kinase; **DBS,** dried blood spots; **ETF,** electron transfer flavoprotein; **FAD,** flavin adenine dinucleotide; **FAODs,** fatty acid oxidation disorders; **LCAD,** long-chain acyl-CoA dehydrogenase; **MS/MS,** tandem mass spectrometry; **PMS,** phenazine methosulfate; **TDC,** taurodeoxycholic acid; **VLCAD,** very-long-chain acyl-CoA dehydrogenase

CoA (C16:1) production, but does detect hexadecenoylcarnitine (C16:1 AC) and hydroxypalmitoylcarnitine (OH-C16 AC). To the best of our knowledge, however, the details of the method have not yet been fully described.

We describe here a newly developed practical enzymatic diagnosis method that detects 2-hexadecenoyl-CoA production from palmitoyl-CoA (C16:0), which we have applied to Japanese patients suspected of having VLCAD deficiency based on MS/MS-based selective screening and newborn screening.

MATERIALS AND METHODS

Reagents. Palmitoyl-CoA lithium salt (MW 1005.9 for free acid, Li content 2 mol/mol) and flavin adenine dinucleotide (FAD) were purchased from Sigma Chemical Co. (St. Louis, MO). Phenazine methosulfate (PMS) was purchased from Nacalai Tesque (Tokyo, Japan). Acyl-CoA oxidase (ACO; from *Candida* sp.) was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals used were of the highest purity commercially available.

Enzymatic reaction. The initial diagnosis of VLCAD deficiency was confirmed by an enzymatic assay for dehydrogenase activity toward palmitoyl-CoA (hereafter VLCAD activity) in lymphocytes. The method used was a modified version of the enzymatic assay method for isovaleryl-CoA dehydrogenase activity that we described previously (5). Human lymphocytes were isolated from heparinized peripheral blood using SEPARATE-L lymphocyte isolation medium (Muto Pure Chemicals, Tokyo, Japan), and 0.4% taurodeoxycholic acid (TDC) solution was added to the cell pellets to achieve a cell density of 10^6 lymphocytes/50 μL. The cells were then lysed by pulsed sonic disruption (1 cycle/s with 30% duty cycle of sonic burst at 45 W) for 2 min under ice-bath conditions. The crude cell lysate containing 10^6 lymphocytes was mixed with palmitoyl-CoA, PMS, and FAD in a tube containing 80 mM K_2HPO_4 buffer (pH 7.0), resulting in a final volume of 100 μL. The mixture was incubated at 37°C, and the reaction was terminated by adding 100 μL of acetonitrile. Denatured protein and other insoluble constituents were precipitated by centrifugation at 7200 × *g* at 4°C for 10 min, and a 20-μL aliquot of the supernatant was analyzed using an HPLC system (LC-10AD; Shimadzu, Kyoto, Japan) equipped with a reverse-phase octadecylsilane column of 150 × 6.0 mm (STR ODS-II; Shinwa Chemical Industries, Kyoto, Japan). The mobile phase was composed of 100 mM NaH_2PO_4 (pH 4.0) and 49% vol/vol acetonitrile, with the flow rate set at 1.5 mL/min (solvent system A). The CoA-derivatives were detected at 260 nm using an UV-spectrophotometric detector (SPD-6A; Shimadzu).

Detection of 2-hexadecenoyl-CoA production. We synthesized 2-hexadecenoyl-CoA from palmitoyl-CoA using ACO in a modified version of the method described by Dieuaide-Noubhani *et al.* (11). The reaction mixture, which contained 50 mM K_2HPO_4 buffer (pH 7.4), 0.1 mg ACO, and 0.2 mM palmitoyl-CoA, was incubated at 37°C for 15 min. The reaction was terminated by adding 100 μL of acetonitrile, and the denatured enzyme was precipitated by centrifugation at 7200 × *g* at 4°C for 10 min. The product was separated by reverse-phase HPLC; the mobile phase was composed of 100 mM CH_3COONH_4 (pH 4.0) and 60% vol/vol acetonitrile, with the flow rate set at 1.5 mL/min (solvent system B). The fraction of the product was collected and dried at 25°C in a centrifugal vaporizer (CVE-200D; Tokyo Rikakikai, Tokyo, Japan). The resultant material was dissolved in 2 mM CH_3COONH_4 with 50% vol/vol acetonitrile and was introduced into a time-of-flight mass spectrometer equipped with an electrospray ion source (ESI-TOFMS; QSTAR XL; Applied Biosystems, Foster City, CA). The scan range was m/z 50–1500 in the negative ion mode.

MS/MS-based screening for VLCAD deficiency in Japan. Blood samples were analyzed by MS/MS following the protocol described by Shigematsu *et al.* (4). Approval for the MS/MS-based screening for inborn errors of metabolism and for the enzymatic and genetic studies as confirmatory tests was obtained from the Ethics Committee of Hiroshima University. Informed consent was obtained from the patients and/or their parents in advance of any procedures.

Selective screening of symptomatic patients. For selective screening, serum specimens were collected from patients who presented with clinical symptoms suggestive of metabolic disorders, and those with elevated levels of tetradecenoylcarnitine (C14:1 AC) in their serum (cut-off, 0.2 nmol/mL) were suspected of having VLCAD deficiency.

Within the period from January 2002 to December 2006, we applied the VLCAD assay to 14 symptomatic patients with elevated levels of C14:1 AC in serum. They were divided into two groups; 7 patients with myopathic symptoms, and the other 7 patients with hypoglycemic symptoms (Table 1).

All of the 7 myopathic patients showed extraordinary elevation of creatine kinase (CK) in serum intermittently, which was accompanied by myalgia, muscle weakness, and rhabdomyolytic syndrome, except for one patient who lacked subjective signs. The age of clinical onset ranged from 2 y into late adolescence. Concerning hypoglycemic patients, their age ranged from 2 to 5 y. Ketosis was documented in six of them.

Newborn screening. In MS/MS-based newborn screening for inborn errors of metabolism, dried blood spots (DBS) were generally collected on the fifth day after birth from newborns whose parents had given written informed consent in advance; the DBS were collected from Fukui, Hiroshima, Tokushima, and other selected areas of Japan. Newborns with elevated levels of C14:1 AC in DBS (cut-off, 0.4 nmol/mL) were suspected of having VLCAD deficiency, and the same analysis was done on their serum at approximately 2 wk of life. As a result, two newborns with positive result were found in our screening of 346,905 newborns from May 1997 to December 2006 (Table 1).

Table 1. *Clinical and biochemical profiles of patients and the results of enzymatic assay for VLCAD activity*

Patient		Clinical symptoms	Creatine kinase (IU/L)	C14:1 AC (nmol/mL)	Ratio of C14:1 AC to C2 AC	Ratio of C14:1 AC to C12 AC	VLCAD activity* (pmol/min/10^6 cells)
1	Male, 0 mo	(Newborn screening)	Not elevated	0.24	0.021	1.043	6.66
2	Female, 0 mo	(Newborn screening)	Not elevated	3.42	0.489	9.243	6.26
3	Male, 5 y	Vomit with ketosis Muscle weakness	18,658	6.49	0.411	27.042	2.89
4	Female, 9 y	Rhabdomyolysis	78,550	0.82	0.117	5.256	3.34
5	Male, 14 y	Reye-like syndrome at 1 y Intermittent CK elevation	10,000	2.62	0.708	13.100	5.08
6	Male, 17 y	Rhabdomyolysis	300,000	0.44	0.250	5.840	3.48
7	Female, 17 y	Intermittent myalgia	Highly elevated	0.51	0.060	2.670	13.58
8	Male, 24 y	Rhabdomyolysis	27,000	1.41	0.175	4.457	8.99
9	Male, 29 y	Rhabdomyolysis	33,552	0.97	0.137	12.278	6.92
10–16, (n = 7)	M:F = 1:6, Age: 2–5 y	Hypoglycemia	Not elevated	0.36–1.26 (0.66 ± 0.35)†	0.010–0.076 (0.031 ± 0.021)†	1.100–2.100 (1.581 ± 0.363)†	37.50–79.38 (54.34 ± 14.86)†

AC, acylcarnitine.

* VLCAD activity in 31 normal subjects (mean ± SD) was 54.49 ± 17.52.

† Mean ± SD.

RESULTS

Conditions of the enzymatic assay. Figure 1*A* shows a representative chromatogram of the VLCAD assay using lymphocytes of a normal control subject; the peak thought to be the product of crude enzymatic reaction was eluted after a 15-min retention time by HPLC of solvent system A, and was well resolved from the other sample constituents. In the ACO reaction, 0.2 mM of palmitoyl-CoA contained in the reaction mixture was completely converted to a product that appeared at 15 min of retention by HPLC of solvent system A (Fig. 1*B*); this retention time was identical to that of the product peak in the VLCAD assay. The product of the ACO reaction, which was analyzed by ESI-TOFMS after the isolation by HPLC of solvent system B, presented a peak of m/z 500.7, corresponding to doubly deprotonated molecular ions ($[M\text{-}2H]^{2-}$; Fig. 2*A*). As a reference, the authentic palmitoyl-CoA lithium salt showed three peaks of deprotonated molecular ions ($[M\text{-}H]^{-}$) at m/z 1016.4, 1010.4, and 1004.4; and two peaks of $[M\text{-}2H]^{2-}$ at m/z 504.7 and 501.7 (Fig. 2*B*). These results demonstrate that the product peak of the VLCAD assay was indicative of 2-hexadecenoyl-CoA. The amount of 2-hexadecenoyl-CoA produced by the complete oxidation of 0.2 mM palmitoyl-CoA by ACO correlated linearly with its peak area on HPLC of solvent system A within the range of 10–80 pmol/20 μL of sample introduced into HPLC (Fig. 3). Based on the method described in the International Union of Pure and Applied Chemistry provisional draft (12), the detection and quantification limits of 2-hexadecenoyl-CoA were determined to be 2.1 and 6.4 pmol, respectively.

The production of 2-hexadecenoyl-CoA by the crude lysates of lymphocytes increased linearly within the following range of each assay factor: 0.25–3 mM of palmitoyl-CoA ($R^2 = 0.9825$); 2–20 min of incubation ($R^2 = 0.9853$); and 0.25×10^6–2×10^6 cells in crude enzyme solution ($R^2 = 0.9967$). The function between 2-hexadecenoyl-CoA production and PMS concentration was logarithmic within the range of

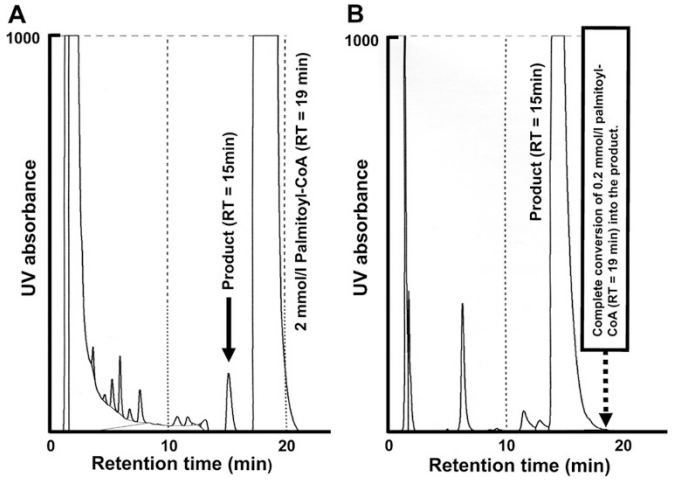

Figure 1. *A*, Representative chromatogram of the assay for palmitoyl-CoA dehydrogenase activity in lymphocytes from a normal subject (analyzed by HPLC of solvent system A). The product of the reaction was eluted at a retention time of approximately 15 min, and was well resolved from the peak for palmitoyl-CoA appearing after 19 min of retention, and from those of other sample constituents. *B*, This retention time was identical to that of a compound generated from palmitoyl-CoA by the reaction of ACO. In this chromatogram (also analyzed by HPLC of solvent system A), 0.2 mM palmitoyl-CoA contained in the reaction mixture did not appear at 19 min, and a huge product peak was observed at 15 min, indicating complete conversion of the substrate by ACO activity.

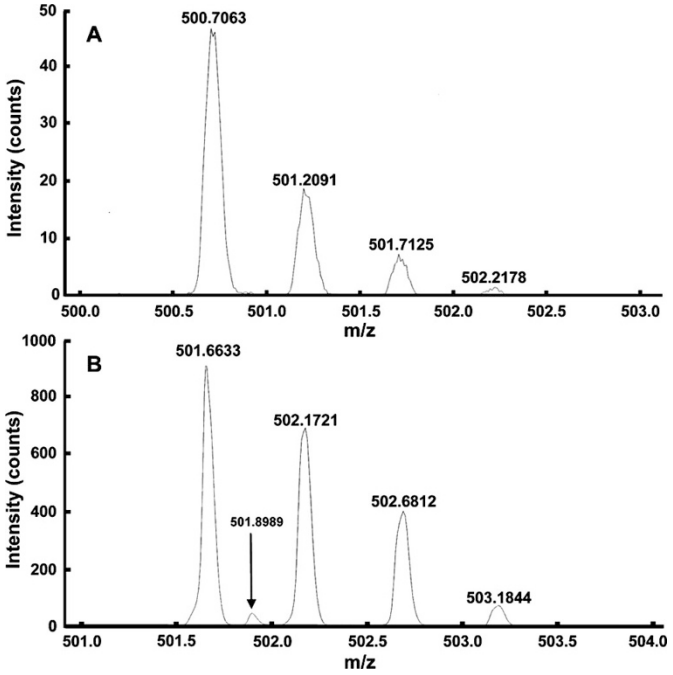

Figure 2. *A*, The product of ACO incubated with palmitoyl-CoA was isolated by HPLC of solvent system B and was analyzed by ESI-TOFMS in the negative ion mode. Other than a peak of m/z 59.0 derived from the solvent (not shown), there was a peak of m/z 500.7, corresponding to $[M\text{-}2H]^{2-}$ molecular ions. *B*, In comparison, the authentic palmitoyl-CoA showed several peaks of molecular ions (not shown), among which a $[M\text{-}2H]^{2-}$ peak of m/z 501.7 was observed. These data indicate that the ACO product should be 2-hexadecenoyl-CoA.

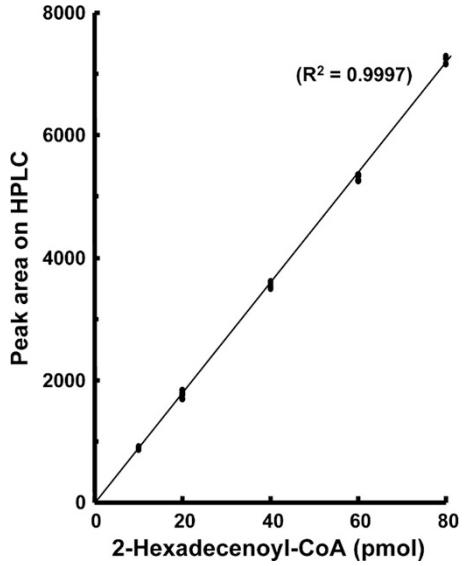

Figure 3. Regression line between the amount of 2-hexadecenoyl-CoA and the peak area on HPLC. 2-Hexadecenoyl-CoA completely converted from 0.2 mM palmitoyl-CoA by ACO was diluted and introduced into the HPLC (solvent system A) at five different concentrations ranging from 10 to 80 pmol/20 μL of sample, and the process was repeated five times for each concentration.

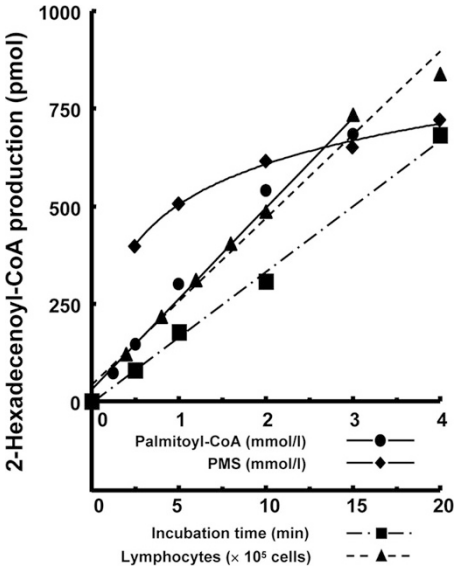

Figure 4. Rate of 2-hexadecenoyl-CoA production by crude cell lysates prepared from the lymphocytes of a normal subject, as a function of palmitoyl-CoA concentration (*circles*), PMS concentration (*diamonds*), incubation time (*squares*), and number of lymphocytes in the crude cell lysate (*triangles*). Within the ranges studied, the function was linear with respect to palmitoyl-CoA concentration, incubation time, and number of lymphocytes, whereas it was logarithmic with respect to PMS concentration.

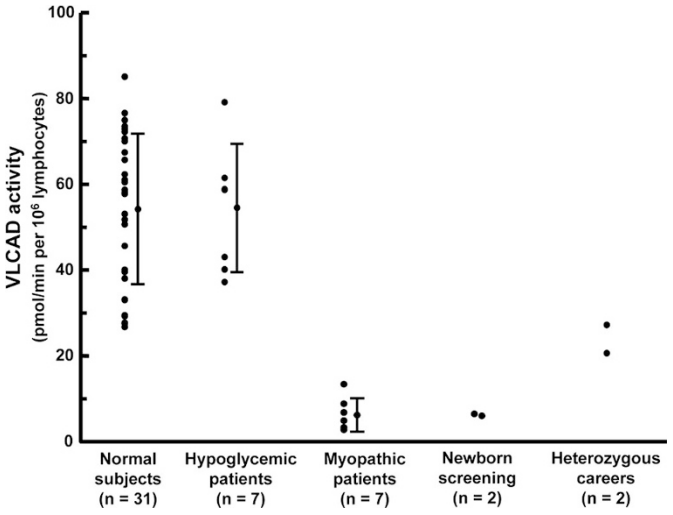

Figure 5. The VLCAD activities in samples from normal subjects ($n = 31$), myopathic patients ($n = 7$), hypoglycemic patients ($n = 7$), presymptomatic newborn patients ($n = 2$), and heterozygous careers ($n = 2$), measured under the defined assay conditions. The distribution of the enzyme activities of the myopathic patients and the newborns was clearly distinct from that of normal subjects and from that of the hypoglycemic patients. The enzyme activities of the heterozygous careers ranged between those of normal subjects and those of VLCAD-deficient patients.

0.5–4 mM ($R^2 = 0.9928$) (Fig. 4). The addition of FAD to the reaction mixture at a concentration of 0.1, 1, or 10 mM did not affect product formation (data not shown).

Based on these data, we determined that the reaction mixture should contain 2 mM palmitoyl-CoA, 2 mM PMS, 0.1 mM FAD, and crude lysate of 10^6 lymphocytes and that it should be incubated for 10 min. Under these conditions, VLCAD activity was determined as a mean value in duplicated assays with subtraction of a blank value, expressed as pmol 2-hexadecenoyl-CoA/min per 10^6 lymphocytes. The coefficient of variation of the intraassay ($n = 5$) was 8.4%, and that of the interassay conducted on five separate days ($n = 5$) was 5.0% (data not shown).

In the test for the stability of VLCAD activity, the relative enzymatic activities in lymphocytes isolated after a 24- or 48-h delay (stored at 25°C after blood aspiration) were 89 or 94% of the basal value, respectively (data not shown).

In the lymphocytes of 31 normal subjects, VLCAD activity ranged from 27.0 to 85.4 picomoles of 2-hexadecenoyl-CoA/min/10^6 lymphocytes with a mean ± SD value of 54.5 ± 17.5. Therefore, the detection limit of 2-hexadecenoyl-CoA was approximately as low as 3.9% of the mean product formation in samples from normal subjects.

Enzymatic diagnosis of the patients. We diagnosed all of the seven myopathic patients with VLCAD deficiency (patient nos. 3–9; Table 1); their VLCAD activities ranged from 2.9 to 13.6 pmol/min/10^6 lymphocytes, with a mean ± SD value of 6.3 ± 3.9. All of the seven hypoglycemic patients (patients No. 10–16 in Table 1) were judged to have normal VLCAD activity, ranging from 26.3 to 79.4 pmol/min/10^6 lymphocytes with a mean ± SD value of 44.0 ± 15.9. Both of the two suspected cases in newborns (patient nos. 1 and 2; Table 1) were shown to have impaired VLCAD activity (6.7 and 6.3 pmol/min/10^6 lymphocytes, respectively). These results are illustrated in Figure 5. The distribution of the enzyme activities of the seven myopathic patients and the two newborns were clearly distinct from that of normal subjects and from that of hypoglycemic patients. On the other hand, there was an apparent overlap of C14:1 AC levels in serum among these groups (Fig. 6*A*). Although the ratios of C14:1 AC to acetylcarnitine (C2 AC) or to lauroylcarnitine (C12 AC) may be helpful for more accurate diagnosis, there nevertheless remained a certain amount of overlap after these ratios were applied (Fig. 6*B* and *C*).

Further confirmation of the diagnosis by genetic analysis. Mutation analysis of the *ACADVL* gene was performed in 5 of the 9 patients diagnosed enzymatically with VLCAD defi-

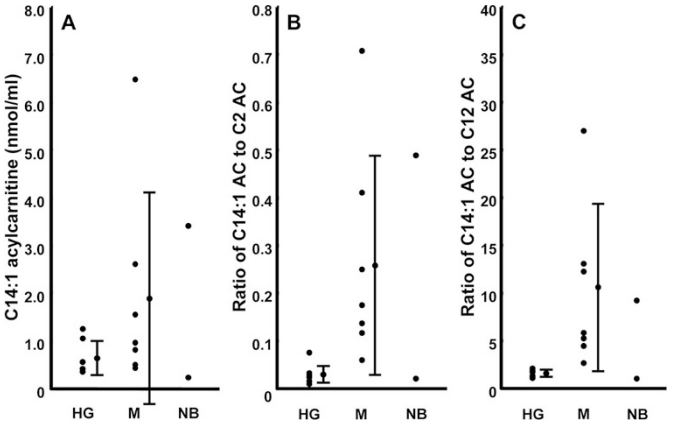

Figure 6. *A*,Distribution of the concentrations of C14:1 acylcarnitine (AC) in the serum of 7 hypoglycemic patients (HG), 7 myopathic patients (M), and 2 newborns (NB). The values of 5 of the 9 affected patients (M and NB) overlapped with those of false-positive cases (HG). (*B*,*C*) This overlap was reduced by applying the ratios of C14:1 AC to C2 AC and to C12 AC, but full differentiation was not achieved.

Table 2. *Results of the genetic study of patients diagnosed enzymatically with VLCAD deficiency*

Patient no.	Residual VLCAD activity (%)*	Mutation analysis of the *ACADVL* gene	
1	12.2	C237R (exon 8)	R511Q (exon 16)
2	11.5	T258M (exon 8)	997insT (exon 10)
3	5.3	(Compound heterozygous mutations were detected)†	
4	6.1	995insT (exon 10)	V547M (Ex17)
5	9.3	V174M (exon 7)	R450H (exon14)
10–16	68.8–145.7	No mutant alleles were detected	
Father of patient 2	38.1	T258M (exon 8)	
Mother of patient 2	50.3	997insT (exon 10)	

* Relative enzyme activities are presented as a percentage of the mean of those in lymphocytes from normal subjects.

† These data will be described elsewhere by the doctor in charge of the patient.

ciency following a somewhat modified version of the method described in our previous report (13). Compound heterozygous mutations were detected in all of them (Table 2). The parents of patient 2 were shown to be heterozygous careers whose VLCAD activities ranged between those of normal control subjects and those of VLCAD-deficient patients (Table 2, Fig. 5). We also performed the *ACADVL* gene analysis in all of the seven hypoglycemic patients, but no mutant alleles were detected in any of them.

DISCUSSION

We have presented a new enzymatic assay method that directly detects the production of 2-hexadecenoyl-CoA. In previous reports, several types of enzymatic assay methods for VLCAD activity have been described. As in the case of other acyl-CoA dehydrogenases, an assay that measures the reduction of ETF was used as a standard method for VLCAD (1,14). However, this method had disadvantages, as it required the purification of pig liver ETF and it must be performed under strictly anaerobic conditions. Measurement of the reduction of an artificial electron acceptor, ferrocenium ion, has been used as an alternative method (15). Assays for the oxidation of radioisotope-labeled substrates by intact cells have been used in several studies, such as the $^{14}CO_2$ release assay (14,15) and the tritium release assay (14,16), though these assays did not show the enzymatic defect directly. It has been suggested that these methods have potential disadvantages in specificity and sensitivity, because none of them detect the generation of the enoyl-CoA species that are the main product of the enzyme reaction. For comparison, the mean of the relative residual activities in our nine patients was calculated to be 11.7%. This value seems to be consistent with, or rather superior to, those in previous reports; 7.9% ($n = 22$) (14) and 31.1% ($n = 2$) (1) by ETF reduction assay; 22.8% ($n = 7$) (15) by ferrocenium reduction assay; 25.6% ($n = 7$) (15) and 32.1% ($n = 28$) (14) by $^{14}CO_2$ release assay; 14.8% ($n = 8$) (16) and 31.7% ($n = 7$) (14) by tritium release assay.

The purpose of the present enzymatic assay is to rapidly confirm the diagnosis of patients with abnormal results on MS/MS-based screening for VLCAD deficiency. According to a report by Zytkovicz *et al.* (17), four of more than 160,000 newborns were suspected of VLCAD deficiency, and one was diagnosed. Schulze *et al.* (18) reported that they diagnosed one case with VLCAD deficiency and 31 cases had false-positive results in their expanded newborn screening of 250,000 newborns; however, the details of these false-positive newborns were not described. More recently, Liebig *et al.* (10) found 11 out of approximately 1,000,000 neonates with elevated C14:1 AC, and VLCAD deficiency was excluded in four of them by enzymatic and genetic study. In contrast, no false-positive cases were found in the present MS/MS-based newborn screening program, in which sample collection of DBS from newborns was carried out on the fifth day after birth. It is supposed that this timing of the first sampling of DBS should often be late for the screening of FAODs, because biochemical disturbances can be masked when the infant is well fed (9,19).

On the other hand, there has been little information on such false-positive cases in MS/MS-based selective screening of symptomatic patients. To our experience, hypoglycemic patients accounted for a considerably high ratio among cases with abnormally elevated serum levels of C14:1 AC. As is suggested by high false-positive rates in newborn screening, mild elevation of C14:1 AC can also occur in those who are not affected by VLCAD deficiency due to increased β-oxidation after stress. Schymik *et al.* (9) presented a false-positive case of this kind.

The high false-positive rates in newborn screening suggested by these previous reports, and that in selective screening indicated by our experience, require us to prepare a practical confirmatory test as part of a reliable MS/MS-based screening system. In a previous report, we tested the diagnostic ratios of C14:1 AC to octenoylcarnitine (C8:1 AC), to C2 AC, and to C12 AC. The latter two ratios were found to be helpful in reducing the false-positive rate, but the discrimination was not complete (20). Mutation analysis of the *ACADVL* gene may not be a practical test to rule out the false-positive cases because few prevalent mutations have been found among patients with VLCAD deficiency (10,21).

We feel that our enzymatic diagnosis method is a good potential practical test that will reduce the problem of false-positive test results. The time for sample preparation is shortened by the use of lymphocytes in peripheral blood, and the analytical operation is simplified by adopting HPLC. As a result, our method takes only several hours from blood sampling to the end of the assay for one case. Additionally, the amount of blood required of newborns is a few milliliters of whole blood or even less, which is usually adequate to isolate a sufficient number of lymphocytes for repeated assays.

A possible problem in our enzymatic diagnosis method is that there are other acyl-CoA dehydrogenases that catalyze palmitoyl-CoA: long-chain acyl-CoA dehydrogenase (LCAD)

and acyl-CoA dehydrogenase-9 (ACAD9). LCAD is an enzyme localized to the mitochondrial matrix. In assays for mitochondrial matrix enzymes such as MCAD, the use of detergents is not needed (6), whereas we failed to observe the conversion of palmitoyl-CoA into 2-hexadecenoyl-CoA unless TDC was added to the procedure of cell lysis. Therefore, the contribution of LCAD to palmitoyl-CoA dehydrogenation is thought to be undetectable in the present assay method.

ACAD9 is the most recently detected acyl-CoA dehydrogenase (22), and its physiologic functions are not yet well understood (23). Like VLCAD, ACAD9 is an enzyme bound to the mitochondrial membrane (24), and its activity should be contained in the cell lysate prepared using TDC. In our series of patients with genetically confirmed VLCAD deficiency; however, residual activity of palmitoyl-CoA dehydrogenation accounted for approximately 10% or less of the mean of normal control value, suggesting that the contribution of ACAD9 to palmitoyl-CoA dehydrogenase activity in lymphocytes could be estimated to be approximately 10% at most.

Recently, the first three patients with ACAD9 deficiency were reported (25). Their clinical symptoms were not distinct from those of other long-chain FAODs including VLCAD deficiency; a Reye-like episode and cerebellar stroke in the first patient, acute liver failure and hypoglycemia in the second, and cardiomyopathy in the third. They were diagnosed based on the defects in ACAD9 at the protein and mRNA levels. The enzymatic defects in these patients were not proved directly; in one patient, dehydrogenase activity toward palmitoyl-CoA in liver sample, where VLCAD and ACAD9 were shown to be present in equal proportion, was around 50% of control value, whereas the activity was normal in fibroblasts. From the viewpoint of biochemical screening using MS/MS, it was suggested that the most diagnostic abnormalities in blood acylcarnitine profiles should be elevations of C18:1 AC and C18:2 AC. If these findings are true, the risk of misdiagnosis between VLCAD deficiency and ACAD9 deficiency does not seem to be high.

In conclusion, our practical enzymatic diagnosis method can be a useful confirmatory test for MS/MS-based screening for VLCAD deficiency. This method will help to evaluate the efficacy of MS/MS-based screening in the management of VLCAD deficiency from both medical and social viewpoints.

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