supernatant [16]. Our study in patients with classic type Fabry disease demonstrated that approximately 90% of the GL-3 in whole urine was recovered in the sediment and supernatant fractions and that the concentrations of total GL-3 in both urinary fractions were elevated in Fabry patients. The total GL-3 was distributed between the sediment and supernatant fractions in a ratio of approximately 4:1.

Although all the isoforms of GL-3 were elevated in the urine from classic type Fabry patients, C24-GL-3 was the most abundant isoform of GL-3 in the urine sediments. It has been reported that the pattern of the individual GL-3 isoform levels in the sediments is similar to those of GL-3 accumulated in patient's kidney [24–26].

According to our study, the patterns of GL-3 isoform in sediments and whole urine were not very different, but the pattern in the urinary supernatant was somewhat different.

It is possible that GL-3 in the urine supernatant comes not only from the kidney, but also from other tissues, such as plasma, because it is known that C16-GL-3 is the most abundant isoform in plasma from Fabry disease patients [11].

From our results on the diagnosis and biochemical monitoring of enzyme replacement therapy for Fabry disease, the clinical significance of the measurement of GL-3 in whole urine samples may not be very different from the measurement of GL-3 in the sediment. Moreover, the measurement of total urinary GL-3 using the direct method is much faster than the extraction method.

The mean total concentration of GL-3 in the urine from hemizygotes with classic type Fabry disease was significantly higher than that in controls, although a wide range of urinary GL-3 levels was observed in the patients. This level of GL-3 may depend not only on the nature of the gene mutation in individual patients [23], but also on differences in ABO blood type [27] and secretor status [19].

The mean concentration of total GL-3 in the urine from symptomatic heterozygote carriers was significantly higher than that in controls. However, the GL-3 level in urine from 2 out of 8 heterozygotes with classic type Fabry disease could not be distinguished from control levels. Therefore, the biochemical detection of some heterozygote carriers would be difficult using our method. Random X-chromosomal inactivation at the α-galactosidase A locus [28] might be one of the factors causing the variation in clinical manifestations as well as variable levels of urinary GL-3 in heterozygotes.

Recently, the clinical phenotype of Fabry disease in males was divided into three types, the classic type and patients presenting predominantly with either renal or cardiac manifestations [13,29,30]. It has been reported that mutations of the α-galactosidase A gene expressing a less severe defect of enzyme function [30,31], including the M296I mutation [13], are pathogenically associated with the late onset cardiac variant. In these patients the accumulation of GL-3 is restricted to the heart and GL-3 storage is not detected in the kidney [32]. Renal biopsies have revealed little, if any, glycosphingolipids deposition in the vascular endothelium, mesangial cells or interstitial cells [33], which was found in case d in this paper. Patients with classic type Fabry disease show a general vasculopathy and have a gene mutation that leads to no detectable enzyme activity [23,29].

In our study, a low concentration of urinary GL-3 was found in a pre-symptomatic, 14-year-old, obligate hemizygote in the family of a cardiac variant, who has the M296I mutation, while the pre-symptomatic 3-year-old hemizygote in the family of classic type Fabry disease showed an elevated urinary GL-3 level.

These results are consistent with a previous report [22]. Therefore, screening for the classic type as well as probably renal variant of Fabry disease should be possible by measuring urinary GL-3. The early detection of the patients with the cardiac variant will not be possible using our procedure.

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Enzymatic diagnosis of medium-chain acyl-CoA dehydrogenase deficiency by detecting 2-octenoyl-CoA production using high-performance liquid chromatography: A practical confirmatory test for tandem mass spectrometry newborn screening in Japan

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Abstract

Many of the previously described enzymatic assay methods for the diagnosis of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency have been dependent upon the measurement of radioisotope-labeled co-products or reduction of electron acceptors. We have developed a direct assay method to detect 2-enoyl-CoA production using high-performance liquid chromatography (HPLC). Crude cell lysate prepared from lymphocytes were incubated with *n*-octanoyl-CoA and ferrocenium hexafluorophosphate. The detection of 2-octenoyl-CoA was significantly reproducible. We applied the assay to samples from four infants suspected to have MCAD deficiency by tandem mass spectrometry (MS/MS) newborn screening conducted in the Hiroshima area of Japan. Three of them were proved to have pathologically reduced residual enzyme activities, although they were associated with various clinical and biochemical phenotypes. In addition, another symptomatic Japanese patient and her presymptomatic sibling who were detected by MS/MS selective screening were successfully diagnosed by our enzymatic assay. These results indicate that the method can be a useful confirmatory test for MS/MS screening of MCAD deficiency.
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Keywords: Medium-chain acyl-CoA dehydrogenase; Enzymatic assay; 2-Octenoyl-CoA; High-performance liquid chromatography; Tandem mass spectrometry; Newborn screening; Japanese

1. Introduction

Tandem mass spectrometry (MS/MS) has enabled screening of inborn errors of fatty acid oxidation, including medium-chain acyl-CoA dehydrogenase (MCAD; EC 1.3.99.3) deficiency. In order to aid the timely follow-up of screening results that suggest abnormalities in MCAD, rapid and simple confirmatory tests for the enzyme activity and/or gene mutation analysis should be available. For the enzymatic study, the assay which uses the reduction of electron transfer flavoprotein (ETF) has been utilized as a standard method [1–3]. However, since this method requires purification of pig liver ETF and must be performed under strictly anaerobic conditions, it is disadvantageous and other artificial electron acceptors, such as ferrocenium ion [3,4], and phenazine methosulfate (PMS) in combination with dichlorophenol indophenol (DCIP) [5], have been utilized. The assay for

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tritium release from [2,3-^{3}H]acyl-CoA was described as a radioisotope-dependent method [6]. In addition, intact-cell oxidation assays using the $^{14}CO_2$ release [1,4,5] and the tritium release from [9,10-^{3}H]fatty acids [7–9] were frequently utilized in previous studies.

Despite being the main product of the enzymatic reaction in the assays mentioned above, 2-enoyl-CoA production is not directly detected in these methods. There were several reports on the product formation in such assays. Kølvraa et al. detected the production of 3-hydroxyfatty acid using gas chromatography mass spectrometry (GC/MS) by coupling 2-enoyl-CoA production with crotonation and alkaline hydrolysis [10], and this method was utilized in other studies [11,12]. Wanders [13] and Oey [14] referred to their method that detects the enoyl-CoA species produced by dehydrogenation of 3-phenylpropionyl-CoA using high-performance liquid chromatography (HPLC); however, the details of the method have not been described. In order to realize a practical method for enzymatic diagnosis of MCAD deficiency, we have developed another HPLC-based assay wherein *n*-octanoyl-CoA is used as substrate and 2-octenoyl-CoA production is directly detected. In this report, we will also demonstrate the application of our method to confirming MCAD deficiency in patients found through an MS/MS newborn screening program conducted in Japan.

2. Experimental

2.1. Reagents

n-Octanoyl-CoA (MW 893.7) and flavin adenine dinucleotide (FAD) were purchased from Sigma Chemical (St. Louis, MO, USA). Ferrocenium hexafluorophosphate ($FcPF_6$) was purchased from Aldrich (St. Louis, MO, USA). Acyl-CoA oxidase (ACO) was purchased from Wako Pure Chemical Industries (Osaka, Japan). All the other chemicals used were of the highest purity commercially available.

2.2. Preparation of crude cell lysate

Human lymphocytes were isolated from venous blood samples. Informed consent was acquired prior to blood sampling. Heparinized blood was diluted 1:1 with saline and layered over SEPARATE-L lymphocyte isolation medium (Muto Pure Chemicals, Tokyo, Japan). After centrifugation at 25 °C for 30 min at 400 × *g*, lymphocytes were isolated and washed twice with saline, each time followed by centrifugation at 4 °C for 10 min at 200 × *g*. The number of lymphocytes obtained was counted, and the cells were washed again with saline followed by centrifugation at 4 °C for 10 min at 400 × *g*. Subsequently, the lymphocytes were transferred to other tubes and centrifuged at 4 °C for 5 min at 7200 × *g*. The saline was discarded, and the final cell pellets were either used directly or kept at −80 °C. Immediately before analysis, distilled water was added to the pellets to achieve a cell density of 10^6 lymphocytes/50 μl, and the cells were lysed by pulsed sonic disruption (1 cycle/s with 30% duty cycle of sonic burst at 45 W, using SONICATOR W-225R; Misonics, New York, USA) that took 2 min under ice bath conditions. Complete disruption of the cells was ascertained by microscopic examination. Immediately following sonication the crude cell lysate was used for the reaction.

2.3. Enzymatic reaction

n-Octanoyl-CoA and $FcPF_6$ were dissolved in distilled water just prior to each analysis. The reaction mixtures were comprised of 80 mmol/l K_2HPO_4 (pH 7.0), 2 mmol/l *n*-octanoyl-CoA, 1 mmol/l $FcPF_6$, and a 50 μl aliquot of the crude cell lysate, resulting in a total volume of 100 μl. FAD was added to some samples and omitted in some other samples. The mixture was incubated at 37 °C, and the reaction was terminated by adding 100 μl of acetonitrile. Control blanks, to which acetonitrile was added before incubation, were prepared for each assay. 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 by HPLC.

2.4. HPLC analysis

The product of the enzymatic reaction and the other constituents were separated by an HPLC system (LC-10AD; Shimadzu, Kyoto, Japan) equipped with a reverse-phase octadecylsilane column of 150 mm × 6.0 mm (STR ODS-II; Shinwa Chemical Industries, Kyoto, Japan). The mobile phase was composed of 100 mmol/l NaH_2PO_4 (pH 4.0) and 28% (v/v) acetonitrile, and it was pumped at a flow rate of 1.5 ml/min. The CoA-derivatives were detected at 260 nm using an ultraviolet spectrophotometric detector (SPD-6A, Shimadzu). The peak area was quantified using a data processing system (C-R4A chromatopac, Shimadzu). Lacking a commercially available authentic standard for 2-octenoyl-CoA, we performed the identification and quantification as follows.

2.5. Identification of 2-octenoyl-CoA

Since the amount of product in the assay for dehydrogenase activity toward *n*-octanoyl-CoA (hereafter MCAD activity) was limited for further analysis, we utilized the ACO activity toward *n*-octanoyl-CoA, using modifications of a previous report [15]. The product, theoretically considered to be 2-octenoyl-CoA, was separated by HPLC. The mobile phase was composed of 100 mmol/l CH_3COONH_4 (pH 4.0) and 30% (v/v) acetonitrile, with the flow rate set at 1.5 ml/min. The fraction of the product was collected and introduced into a time of flight mass spectrometer equipped with an electrospray ion source (ESI-TOFMS; Q-STAR XL;

Applied Biosystems, Foster City, CA, USA). Scan range was m/z 100–1000 in the negative ion mode.

2.6. Quantification of 2-octenoyl-CoA

The collected fraction of the product of the ACO reaction was dried at 25 °C in a centrifugal vaporizer (CVE-200D; Tokyo Rikakikai, Tokyo, Japan). The resultant material was dissolved in distilled water, and the absorbance at 258 nm was quantified using a spectrophotometer (Hitachi 557, Tokyo, Japan). The concentration of 2-octenoyl-CoA was calculated based upon a previous report that determined the extinction coefficient of 2-octenoyl-CoA to be 20.4 $mM^{-1} cm^{-1}$ at 258 nm [16]. The same samples were re-analyzed by HPLC under the condition used for the assay of MCAD activity (hereafter MCAD assay) to determine the correlation between the peak area and the concentration of 2-octenoyl-CoA.

2.7. Identification of mutations in the MCAD gene

Genomic DNA was isolated from white blood cells by standard laboratory procedures. Polymerase chain reaction (PCR) was used to amplify all twelve exons, including part of the flanking intron sequences, of the human MCAD gene, with intron-located primers as previously described [17], under standard conditions in an automated thermal cycler (DNA Thermal Cycler 480; Perkin-Elmer, Wellesley, MA, USA). PCR products were separated on 2% agarose gel and were purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ, USA). Cycle sequencing was performed using DNA BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on an automated sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems).

3. MS/MS newborn screening in the Hiroshima area

We have conducted a pilot study of MS/MS newborn screening in the Hiroshima area of Japan since 1999. All newborns in the area whose parents gave written informed consent in advance were enrolled in this study. Generally, dried blood spots (DBS) were collected on the fifth day after birth, and were analyzed by MS/MS. Details of the MS/MS protocol were described elsewhere [18]. Newborns showing elevated levels of octanoylcarnitine (C8-carnitine) in DBS (cut off < 0.3 nmol/ml) associated with the ratio of octanoylcarnitine to decanoylcarnitine (C8/C10) higher than 1.5 were suspected to have MCAD deficiency.

In a period from April 1999 through October 2004, 101,020 out of 154,998 newborns (65.2%) in the Hiroshima area consented to participate in this screening. Octanoylcarnitine levels were abnormally elevated in DBS obtained from four newborns. One of them was an extremely-low-birth-weight (ELBW) infant who was supplemented with medium-chain triglyceride oil; the C8-carnitine concentration and the C8/C10 ratio in DBS were 0.62 nmol/ml and 5.17, respectively. He was proved to have normal MCAD activity afterward. Profiles of the other three suspected cases are summarized in Table 1. There was no consanguinity in any of the three families.

Table 1
Profiles of the patients diagnosed with MCAD deficiency

	Patient 1	Patient 2	Patient 3
Year of birth	2000	2001	2003
Sex	Male	Female	Male
Ethnicity	Father: Japanese–Peruvian Mother: Japanese–European	Japanese	Japanese
C8 in neonatal DBS[a] (C8/C10 ratio)[b]	0.62 (3.65)	5.92 (11.38)	0.43 (1.87)
Highest C8 in serum[c] (C8/C10 ratio)[b]	5.97 (3.49)	19.56 (11.93)	1.21 (2.24)
Hexanoylglycine in urine	Elevated (in acute metabolic failure)	Elevated (in neonatal period)	Not elevated
Hypoketotic dicarboxic aciduria	Detected (in acute metabolic failure)	Detected (in neonatal period)	Not detected
MCAD activity[d]	10.3%	2.6%	13.2%
MCAD gene mutation (paternal/maternal allele)	449–452delCTGA/157C > T	Unknown/unknown	Unknown/449–452delCTGA
Clinical presentation	Acute metabolic failure (cardiopulmonary arrest) at 8 months old	Asymptomatic as of 42 months old	Asymptomatic as of 16 months old

[a] Concentration of octanoylcarnitine in dried blood spots for newborn screening, expressed as nmol/ml (cut off < 0.3).
[b] The ratio of octanoylcarnitine to decanoylcarnitine in dried blood spots or in serum. The ratio higher than 1.5 associated with the elevation of octanoylcarnitine is indicative of MCAD deficiency.
[c] Concentration of octanoylcarnitine in serum, expressed as nmol/ml (cut off < 0.2).
[d] n-Octanoyl-CoA dehydrogenase activity, expressed as percentage of the mean of those in samples from 15 normal subjects.

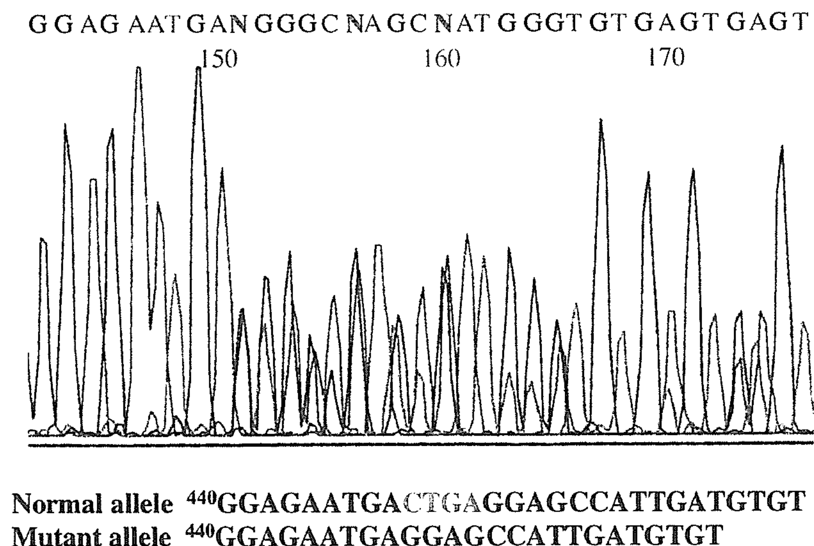

Fig. 1. A part of the sequence of exon 6 in a sample collected from patient 3. Direct sequencing identified a heterozygous deletion of four base pairs (CTGA) at the sites 449–452.

4. Case reports

Patient 1 was a healthy 8-month-old boy of Japanese–Peruvian–European descent born in 2000, who abruptly became ill and rapidly fell into cardiopulmonary arrest associated with liver dysfunction and hyperammonemia (AST 286 IU/l, ALT 257 IU/l, CK 8702 IU/l, $NH_3 > 400\,\mu g/dl$). Plasma glucose at the onset was not measured. Fortunately, resuscitation and intensive care were started early, so that he recovered without any sequelae. The concentration of C8-carnitine and the C8/C10 ratio in serum collected during the acute symptomatic period were 5.97 nmol/ml (cut off < 0.2) and 3.49, respectively, and those in DBS for newborn screening were 0.62 nmol/ml and 3.65, respectively. The organic acid profile of urine was analyzed by GC/MS according to a previously reported method [19]; hypoketotic dicarboxylic aciduria and elevated hexanoylglycine were evident. The concentration of C8-carnitine in serum collected during the period of recovery from the metabolic failure decreased to a level slightly above the cut off value (0.41 nmol/ml). We did not follow this patient any longer because he moved away from the Hiroshima area soon after the recovery.

Patient 2 was a Japanese girl born in 2001 as a mature infant without any familial history suggestive of metabolic disorders. The concentration of C8-carnitine in DBS was 5.92 nmol/ml, with the C8/C10 ratio being 11.38. When she came to our clinic on the 25th day after birth for further examination, there was no abnormal finding in her clinical presentation, blood gas analysis, or in the levels of blood glucose, plasma ammonia, and serous transaminases. Analysis of urine collected on her first visit by GC/MS revealed non-ketotic dicarboxylic aciduria and elevation of hexanoylglycine. She has been asymptomatic until 42 months, though the profiles of acylcarnitines in serum have always been highly abnormal; the concentrations of C8-carnitine reached 19.56 nmol/ml at highest, when the C8/C10 ratio was 11.93.

Patient 3 was a Japanese boy born in 2003. There was no particular problem in his familial, fetal, or perinatal history. The concentration of C8-carnitine in DBS was 0.43 nmol/ml, and the C8/C10 ratio was 1.87. Organic acid profile of urine collected at the age of 1 month was normal. He has been asymptomatic until 16 months old, with the concentration of C8-carnitine always slightly above the cut off value (1.21 nmol/ml at highest with the C8/C10 ratio being 2.24).

Two different mutations in the MCAD gene were identified in the samples collected from the three patients; a novel mutation of 449–452delCTGA (exon 6) in one allele of patients 1 and 3 each (Fig. 1), and a single base transition of 157C > T (exon 3) in the other allele of patient 1. Regarding patient 1, the del449–452 mutation derived from his father of Japanese–Peruvian descent, and the 157C > T mutation from his mother of Japanese–European descent. Patient 3 inherited the allele harboring the four-base deletion from his mother. No mutation was identified in the other allele of patient 3, or in either allele of patient 2.

5. Results

Kinetic studies on the MCAD activity were initially performed using crude cell lysate prepared from 10^6 lymphocytes. Product formation linearly increased within the range of *n*-octanoyl-CoA concentration from 0.5 to 4 mmol/l ($n=4$, Fig. 2a). Product formation with regard to $FcPF_6$ concentration and incubation time exhibited an increase with a tendency to reach a plateau within the range tested; 0.25–4 mmol/l of $FcPF_6$ ($n=3$, Fig. 2b), and 5–30 min of incubation ($n=3$, Fig. 2c). Adding FAD to the reaction mixture at concentrations of 0.1, 1, and 10 mmol/l did not

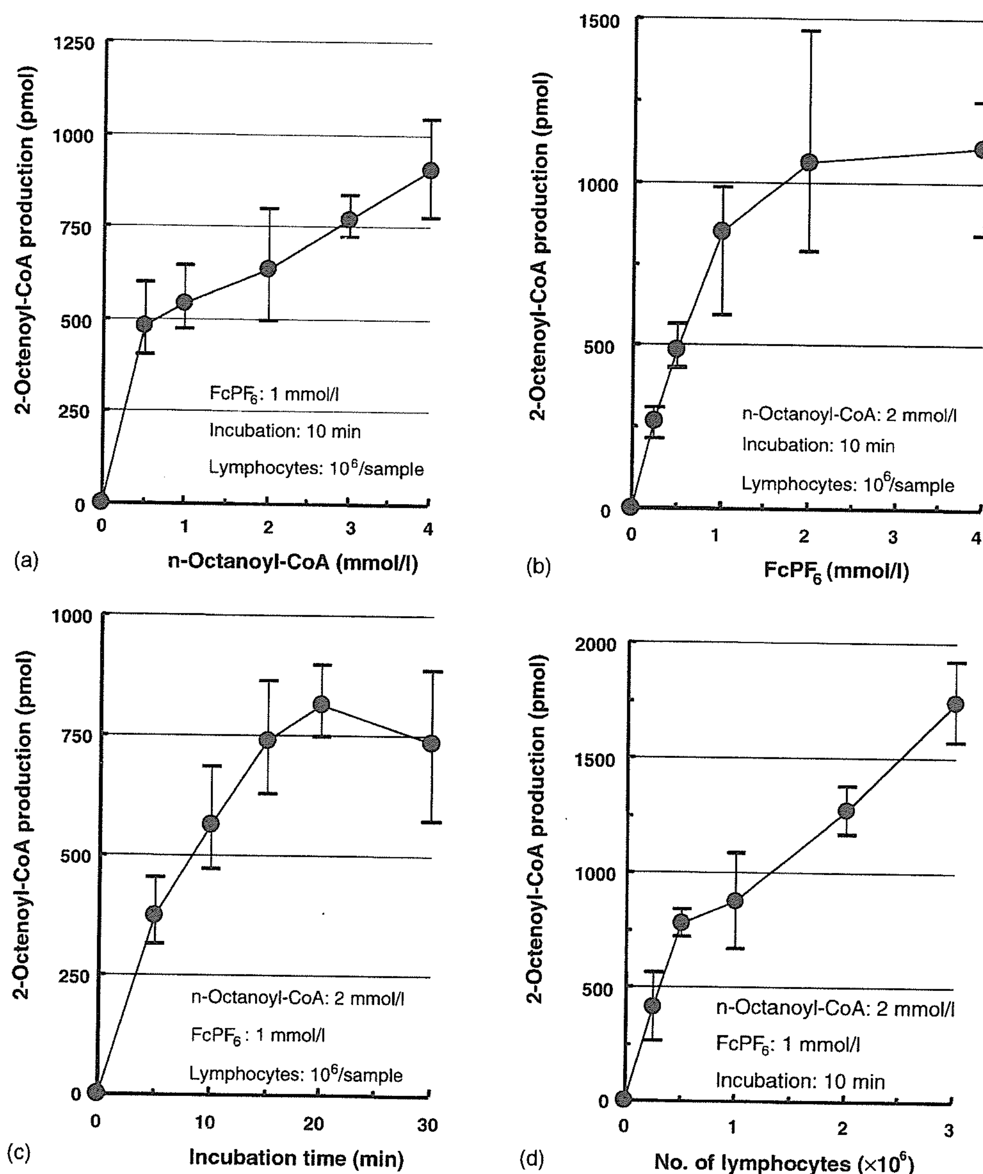

Fig. 2. Rate of 2-octenoyl-CoA production by crude cell lysates prepared from lymphocytes of normal subjects, as a function of (a) *n*-octanoyl-CoA concentration ($n=4$); (b) $FcPF_6$ concentration ($n=3$); (c) incubation time ($n=3$); and (d) density of lymphocytes in the crude cell lysate ($n=2$). Each point of the data represents the mean and the range of the values.

affect product formation (data not shown). When the concentrations of *n*-octanoyl-CoA and $FcPF_6$ in the reaction mixture were kept at 2 mmol/l and 1 mmol/l, respectively, and the incubation time was kept 10 min, product formation increased linearly depending on the initial cell density in the crude cell lysate. This cell density was within the range of 0.25×10^6–3×10^6 lymphocytes/50 μl ($n=2$, Fig. 2d). Based on these results, we determined the assay condition as follows; the reaction mixture should contain the crude cell lysate prepared from 10^6 lymphocytes, 2 mmol/l *n*-octanoyl-CoA, and 1 mmol/l $FcPF_6$, with the incubation for 10 min.

Fig. 3a shows a representative chromatogram of the MCAD assay in a sample from a normal subject. The peak of the product that was assumed to be 2-octenoyl-CoA was eluted after an 8-min retention time and was well resolved from the other constituents. The retention time was compatible to that of the product of the ACO reaction. Analyzed by ESI-TOFMS, this compound presented a base peak of *m/z* 444.5, while the mass spectrum of the base peak for authentic *n*-octanoyl-CoA was *m/z* 445.5, both corresponding to doubly-deprotonated molecular ions ($[M-2H]^{2-}$; Fig. 3b and c). These results demonstrated that the peak obtained in the chromatogram of the MCAD assay was indicative of 2-octenoyl-CoA. The concentration of 2-octenoyl-CoA dissolved in distilled water that was calculated from the absorbance at 258 nm correlated linearly with its peak area on HPLC within the range of 1.03–102.94 pmol per 20 μl of samples introduced into HPLC (Fig. 4). Based on the method from IUPAC provisional draft (Currie LA, 1994), detection limit and quantification limit of 2-octenoyl-CoA

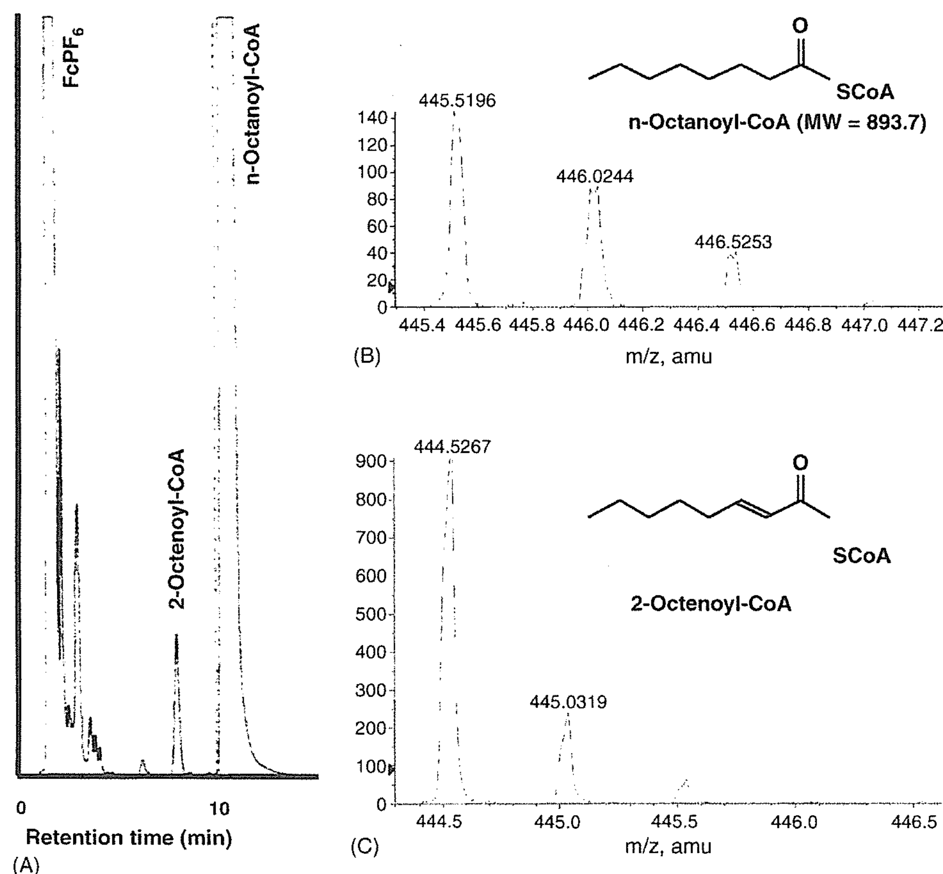

Fig. 3. (a) A representative chromatogram of the assay for the MCAD activity in lymphocytes from a normal subject. The product of the reaction was eluted at a retention time of approximately 8 min. The retention time was compatible to that of the product of the ACO reaction. Analyzed by ESI-TOFMS, (b) authentic *n*-octanoyl-CoA presented a base peak of *m/z* 445.5, while (c) the fraction of the product of the ACO reaction contained a compound presenting a base peak of *m/z* 444.5. Both mass spectra corresponded to $[M - 2H]^{2-}$ molecular ions.

were determined to be 0.62 and 1.87 pmol, respectively. The detection limit was lower than approximately 2% of the mean 2-octenoyl-CoA production in samples from normal subjects. In the above conditions, the MCAD activity was determined as a mean of duplicated assays with subtraction of a blank value, expressed as pmol 2-octenoyl-CoA/min per 10^6 lymphocytes. Thus, activity that is as low as 0 pmol/min per 10^6 lymphocytes can theoretically be determined. Coefficient of variation of the intra-assay ($n = 5$) was 6.6% and that of the inter-assay conducted on five consecutive days ($n = 5$) was 4.0%.

The MCAD activities in the samples collected from normal subjects ($n = 15$) ranged from 24.96 to 40.92 pmol/min per 10^6 lymphocytes, with a mean ± S.D. value of 32.96 ± 5.47 pmol/min per 10^6 lymphocytes, whereas those in the samples collected from patients 1, 2, and 3 were 3.40, 0.85, and 4.34 pmol/min per 10^6 lymphocytes, respectively. Residual activities of the three patients were calculated to be 10.3%, 2.6%, and 13.2% of the mean of those in normal subjects, respectively (Fig. 5). The MCAD activity in the lymphocytes of the ELBW infant, who showed abnormal results in the MS/MS screening, was normal (107.4% of the mean of those in normal subjects).

In addition, we applied the enzymatic assay to another symptomatic Japanese girl, who was the first confirmed patient of MCAD deficiency in Japan and was referred to in our previous report [18] (patient 4), and her younger brother who was presymptomatically found to have elevated levels of C8-carnitine (patient 5). The MCAD activities in their lymphocytes were 3.2% and 2.4% of the mean of those in normal subjects, respectively. More detailed information about these siblings will be described elsewhere (Ohtake et al., in preparation).

The MCAD activity of the mother of patient 3, who had the same deletion in one allele of the MCAD gene, was 12.97 pmol/min per 10^6 lymphocytes (39.4% of the mean of those in normal subjects), while that of his father was slightly below the range of the normal control value (23.18 pmol/min per 10^6 lymphocytes, or 71.9% of the mean of those in normal subjects). Comparatively, the enzyme activities in lymphocytes of the mother of patient 2, and the father and the mother of patients 4 and 5, were 32.2%, 36.5%, and 27.0% of the mean of those in normal subjects, respectively. Distribution of the MCAD activities of these carriers is also presented in Fig. 5.

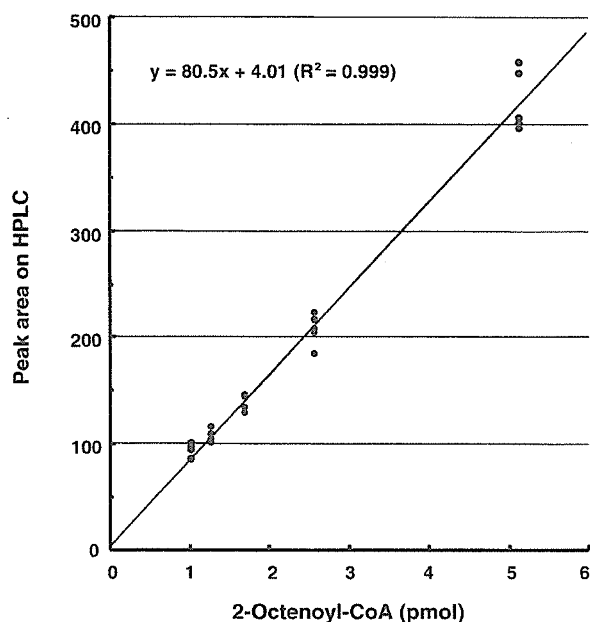

Fig. 4. A regression line between the amount of 2-octenoyl-CoA and the peak area on HPLC is described within the lower part of the range studied. 2-Octenoyl-CoA dissolved in distilled water was introduced into the HPLC at eight different concentrations ranging from 1.03 to 102.94 pmol per 20 μl of sample and repeated five times for each concentration.

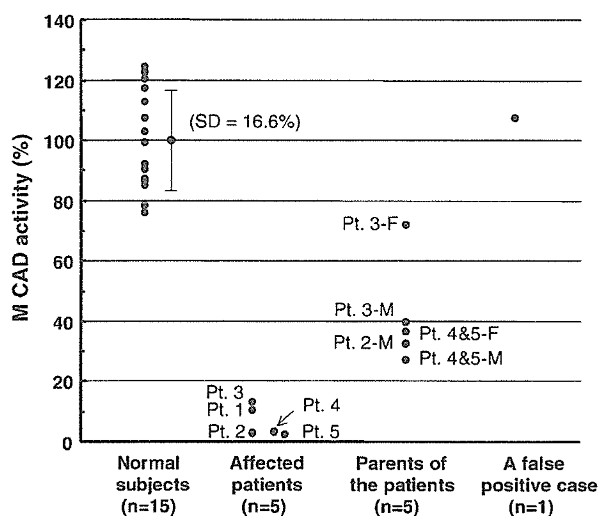

Fig. 5. The MCAD activities in samples from normal subjects ($n = 15$), patients with MCAD deficiency ($n = 5$), their parents ($n = 5$), and a false positive case, measured under the determined assay condition, are shown as percentage of the mean of those in normal subjects. Residual MCAD activities of the patients ranged from 2.4% to 13.2%. The enzymatic activities of their parents ranged between those of the patients and the normal subjects (27.0–71.9%). The very-low-birth-weight neonate supplemented with medium-chain triglyceride oil had normal level of MCAD activity (107.4%).

6. Discussion

The introduction of acylcarnitine analysis by MS/MS has enabled mass screening of fatty acid oxidation disorders including MCAD deficiency. As the application of this technique to newborn screening prevails, there will be increasing need for simpler tests to confirm the abnormal results indicative of MCAD deficiency, especially in cases that do not have the common 985A > G mutation in the MCAD gene. Thus, it is worth establishing a rapid and simple method of enzymatic diagnosis. Various methods have been described in previous reports and are summarized in Table 2. In a previous review [13], Wanders et al. pointed out the following demerits of these conventional methods: use of radioisotopes ($^{14}CO_2$ release, tritium release), requirement of commercially unavailable reagents (tritium release from [2,3-^{3}H]acyl-CoA, ETF reduction), demand of anaerobic conditions (ETF reduction), high background activity ($FcPF_6$ reduction, PMS/DCIP reduction), or need for derivatization (GC/MS-based assay). They suggested that their HPLC-based product formation assay was superior, and using this method Oey et al. reported some data on the activities of MCAD and other fatty acid oxidation enzymes in human placenta [14]. However, based on the information referred by us, neither details of their method, nor data obtained by applying the method to patients with MCAD deficiency have been described.

We also intended to develop an MCAD assay method for directly detecting 2-enoyl-CoA formation. Since it was previously reported that lymphocytes could be used for enzymatic assay of MCAD [1,4,13], we have shortened the assay process by using peripheral lymphocytes as a crude enzyme source. Although the dehydrogenase activity toward *n*-octanoyl-CoA in a crude cell lysate can be influenced by the similar enzymes located in peroxisomes [20], Wanders et al. showed that such peroxisomal contribution should be only minimal [4]. The analytical procedure was simplified by adopting HPLC, abolishing the need of radioisotopes and further derivatization of the product. Thus, it only takes several hours from sampling of blood to the end of the assay. The amount of the blood required of newborns is a few milliliters of whole blood, which is usually adequate to isolate sufficient number of lymphocytes that are required for repeated assays. Concerning the substrate, Wanders et al. used 3-phenylpropionyl-CoA [13], which was shown to be highly specific to MCAD, least influenced by overlapping chain length specificities of other acyl-CoA dehydrogenases [21]. However, the superiority of this compound diminishes, because it has to be synthesized in each laboratory. For practical purpose, it is desirable to establish a reliable assay method by using *n*-octanoyl-CoA, which has been utilized in many of the previous studies (Table 2), and is commercially supplied.

The levels of residual MCAD activity in our three patients seem to be either consistent with or superior to those in previous reports (Table 2). Patient 1 abruptly developed severe metabolic decompensation associated with elevated levels of medium-chain acylcarnitines in serum, and the diagnosis of MCAD deficiency was confirmed both enzymatically and genetically. Compared with this case, patient 2 had always shown severer biochemical abnormalities, and the residual MCAD activity in her lymphocytes was also clearly lower

Table 2
Methods for enzymatic diagnosis of MCAD deficiency described in previous reports

Author	Method	Source of enzyme (crude cell lysate)	Substrate	Number of patients	Mean residual activity (%)
Coates [1]	ETF reduction	Fibroblasts	Octanoyl-CoA	10	9.2
		Lymphocytes		6	9.0
Frerman and Goodman [2]	ETF reduction	Fibroblasts	Octanoyl-CoA	2	(1) 9.2
					(2) <3.3
Lehman [3]	ETF reduction	Fibroblasts	Octanoyl-CoA	5	7.6
Lehman [3]	$FcPF_6$ reduction	Fibroblasts	Octanoyl-CoA	5	4.5
Wanders [4]	$FcPF_6$ reduction	Fibroblasts	Octanoyl-CoA	4	9.8
Rhead [5]	PMS/DCIP reduction	Mitochondria of fibroblasts	Octanoyl-CoA	3	5.2
Amendt [6]	Tritium release	Fibroblasts	[2,3-^{3}H]Octanoyl-CoA	19	7.8
Kølvraa [10]	3-OH-Fatty acid formation (GC/MS)	Fibroblasts	Octanoyl-CoA	1	25.0
Niezen-Koning [11]	3-OH-Fatty acid formation (GC/MS)	Fibroblasts	Hexanoyl-CoA	2	35.7
Duran [12]	3-OH-Fatty acid formation (GC/MS)	Lymphocytes	Octanoyl-CoA	5	2.3–18.8
Wanders [13]	2-Enoyl-CoA formation (HPLC)	Fibroblasts	3-Phenylpropionyl-CoA	ND	ND
		Lymphocytes			
Tajima	2-Enoyl-CoA formation (HPLC)	Lymphocytes	Octanoyl-CoA	5	6.3 (2.4–13.2)

ND: not described.

than that of patient 1. Nevertheless, no mutation was detected within the entire coding region of the MCAD gene, including exon–intron junctions. On the other hand, profiles of acylcarnitines in DBS and serum from patient 3 have been mild, and only one mutant allele has been clarified; these observations suggest that this case could be a heterozygous carrier. However, the concentrations of C8-carnitine in DBS and serum of patient 1 during non-symptomatic periods were as low as those of patient 3, and the residual MCAD activity of patient 3 was also at approximately the same level as that of patient 1. In addition, the MCAD activity of his mother, who was shown to have the same deletion in one allele, was compatible with the carrier status and was definitely higher than that of patient 3. Similar levels of residual activity were also observed in the mother of patient 2 and the parents of patients 4 and 5. These results indicate that patient 3 should also be a true patient with MCAD deficiency. Taking it into consideration that the father of patient 3 had much higher MCAD activity than the other carriers, patient 3 is supposed to have another mutation in paternal allele of the MCAD gene that should disturb the enzymatic function in a very mild fashion. According to a review by Chace et al. [22], there are cases of newborns with less than 1 nmol/ml of C8-carnitine who have become metabolically decompensated. Therefore, the severity of MCAD deficiency should not be predicted solely on the basis of C8-carnitine concentration.

Genetic heterogeneity observed in our patients suggests that it may be difficult to utilize a common mutation-specific assay as a confirmatory test for MCAD deficiency among Japanese. Although at least one allele was found to harbor the 985A > G mutation in more than 90% of Caucasian patients [23,24], previous studies on the prevalence of this mutation failed to identify any mutant allele among Japanese newborns [24–26]. To our knowledge other than 985A > G, 49 sporadic mutations have been reported [17,27–39], only two of which were definitely documented in non-Caucasian subjects [37,39]. None of these various mutations was observed in the three patients in this study, except 157C > T in one allele of patient 1, which had been identified in two symptomatic European patients [28]. Instead, 449–452delCTGA, which provokes premature stop codon at the sites 479–481, is the first pathological mutation documented in Japanese patients. Though there were cases where no mutation could be detected [29], the remaining genetic abnormalities in the patients in this study are under further investigation.

These facts and speculations highlight the importance of enzymatic diagnosis of MCAD deficiency, especially among non-Caucasian populations, and indicate that our MCAD assay method can diagnose patients with mild deficiency as well as those with severe clinical and/or biochemical phenotypes. By using this method as a confirmatory test in the MS/MS newborn screening program, we have revealed unexpectedly high frequency of MCAD deficiency in the Hiroshima area. It is estimated to range from 1:51,666 to 1:33,673, which may not be so much less than that in the Western countries; recent reports demonstrated that the frequencies of MCAD deficiency in the United States, Germany, and Australia were 1:15,000 [29], 1:20,800 [40], and 1:21,300 [41], respectively. In conclusion, our practical enzymatic diagnosis method can be a useful confirmatory test for MCAD deficiency found through MS/MS newborn screening.

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タンデム質量分析法による新生児マススクリーニング対象疾患の検討

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要　旨

現在わが国では，質量分析法を用いる新生児代謝異常マススクリーニングの方法として，GC/MS法とMS/MS法がある．今回我々は，両法において，個々のスクリーニング対象疾患に対する診断精度およびスクリーニングへの適用性について比較検討した．新生児期発症型メチルマロン酸血症や新生児期発症型プロピオン酸血症およびPKUなどは，MS/MS法によりスクリーニングが可能であったが，GC/MS法にて容易に発見される良性型，軽症型，ビタミンB_{12}反応型のメチルマロン酸血症およびOTC欠損症などは，MS/MS法でのスクリーニングは困難であった．GC/MS法とMS/MS法とでは，スクリーニング診断においてアプローチしやすい疾患と，しにくい疾患があり，わが国における新生児スクリーニングへのMS/MS法の導入においては，その対象とする疾患は，エビデンスに基づき適切に選択することが重要であると考えられた．

キーワード

新生児スクリーニング，GC/MS，MS/MS

緒　言

現在，新生児代謝異常症マススクリーニングにおいて，欧米をはじめわが国においても質量分析法の導入が行われようとしている．この質量分析法には，血液濾紙を分析するタンデム型質量分析（MS/MS）法と尿を分析するガスクロマト質量分析（GC/MS）法[1)]がある．欧米ではフェニルケトン尿症（PKU）や脂肪酸β-酸化異常症，特に中鎖アシルCoA脱水素酵素（MCAD）欠損症の頻度が高い[2)]という実情から，いち早くMS/MS法を用いたマススクリーニングが開始されている．わが国においても血液濾紙を作成し分析する現行のシステムがそのまま利用できるというメリットもあり，MS/MS法の導入が検討されている．しかし，代謝異常症の発生頻度は国や人種により大きく異なっている[3)]ため，欧米でのシステムをそのまま適用するにはその根拠が必要と考えられる．一方我々は1996年からGC/MS法を用いて新生児スクリーニングを開始し，メチルマロン酸血症をはじめ多くの疾患を発見している[4)]．今回，MS/MSの新規導入を契機に，GC/MS法とMS/MS法の両質量分析法について，それぞれのスクリーニング対象疾患に対する診断精度，ならびにわが国におけるマススクリーニングへの適応性について比較検討した．

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方 法

既に診断のついたメチルマロン酸血症 11 例（新生児期発症型 4 例，ビタミン B_{12} 反応型 2 例，いわゆる良性型および軽症型 5 例の 3 タイプ），新生児期発症型プロピオン酸血症 2 例，SCAD 欠損症疑いを含むエチルマロン酸尿症 2 例，オルニチントランスカルバミラーゼ（OTC）欠損症 2 例，古典型フェニルケトン尿症 3 例の患者検体，計 20 例を用いて，両質量分析法により検討した．血液濾紙を用いる MS/MS 法は，17 生日から 50 才 1 ヶ月までの血液濾紙を重松らの方法[5]に従い試料調製したものを Waters Quattro Premier ESI-LC/MS/MS により分析した．尿を用いる GC/MS 法は，3 生日から 50 才 1 ヶ月までの尿を松本，久原らのウレアーゼ処理法[6]により試料調製したものを Agilent 5973 GC/MS を用いて分析した．個々の疾患の分析結果にもとづき，両法の診断精度およびスクリーニングへの適用性について比較検討した．今回の比較検討に用いた各法における診断マーカーを Table 1 に示した．なお，MS/MS 法におけるアシルカルニチンおよび各アミノ酸値による判定は，重松や Hoffmann[7]のカットオフ値を参考に行った．なおメチルマロン酸血症は，現在，酵素活性や complementation study などによる生化学的分類は知られているが，臨床分類は確立された報告はない．本稿では新生児期発症型，ビタミン B_{12} 反応型，それら以外をいわゆる良性型および軽症型[8)-10)]とした．

結果および考察

MS/MS 法におけるフェニルケトン尿症の判定は，血中フェニルアラニン値（Phe）およびフェニルアラニン／チロシン比（Phe/Tyr）を用いる[7]が，今回の PKU 患児血液濾紙の MS/MS 分析ではいずれのマーカーもカットオフ値以上の高値を示し，スクリーニングが十分に可能であると考えられた．GC/MS 法においても診断マーカーであるフェニルアラニン，フェニル乳酸，フェニルピルビン酸および 2-ヒドロキシフェニル酢酸いずれの物質も尿中排泄増加を示し，感度よく化学診断が可能であった．

新生児期発症型プロピオン酸血症の MS/MS 分析では，血中プロピオニルカルニチン値（C3），プロピオニルカルニチン／アセチルカルニチン比（C3/C2）およびプロピオニルカルニチン／フリーカルニチン比（C3/C0）とも著明に増加し，感度良くスクリーニング可能であった．ただし，プロピオン酸血症とメチルマロン酸血症の診断マーカーは同じであるため両者の判別は困難であった．

Table 1 Diagnostic markers and the disease

	GC/MS (urine)	MS/MS (blood spot)
PKU	PLA, PPA, 2-OH-PAA, Phe	Phe, Phe/Tyr
PA	3-OH-PA, MCA	C3, C3/C0, C3/C2
MMA	MMA, MCA, 3-OH-PA	C3, C3/C0, C3/C2
VB_{12}responsible	MMA, (3-OH-PA)	C3, C3/C0, C3/C2
benign	MMA, (3-OH-PA)	C3, C3/C0, C3/C2
SCADD	ethylmalonic acid	C4
OTCD	orotic acid, uracil	Cit, Gln

PKU=Phenylketonuria, PA=Propionic acidemia, MMA=Methylmalonic acidemia, SCADD=Short-chain acyl-CoA dehydrogenase deficiency, OTCD=Ornithine transcarbamylase deficiency
PLA=phenyllactic acid, PPA=phenylpyruvic acid, 2-OH-PAA=2-hydroxy phenylacetic acid
3-OH-PA=3-hydroxy propionic acid, MCA=methylcitric acid, MMA=methylmalonic acid
Phe=phenylalanine, Tyr=tyrosine, Cit=citrulline, Gln=glutamine
C0=free carnitine, C2=acetyl carnitine, C3=propionyl carnitine, C4=butyryl carnitine

メチルマロン酸血症における GC/MS 法と MS/MS 法の病型による指標値の比較を Fig.1 に示した．メチルマロン酸血症の中で新生児期発症型メチルマロン酸血症患児の MS/MS 分析では，血中プロピオニルカルニチンの増加が確認でき，十分スクリーニング可能であった．しかし，上述と同様にプロピオン酸血症との判別は困難であった．他のホモシスチン排泄を伴ったビタミン B_{12} 反応型と良性型および軽症型メチルマロン酸血症の MS/MS 分析では，血中プロピオニルカルニチンの増加は見られず C3, C3/C0, C3/C2 いずれのマーカーもカットオフ値内であり，スクリーニング判定は陰性となった．一方，GC/MS 法によるメチルマロン酸血症のスクリーニングは，メチルマロン酸，3-ヒドロキシ-プロピオン酸，メチルクエン酸の尿中排泄量およびホモシスチン検出の有無により，スクリーニングだけではなく各タイプ別の診断も可能である（Fig.2）．このように，新生児期発症型メチルマロン酸血症以外のプロピオニルカルニチンの増加を伴わないタイプのメチルマロン酸血症は，MS/MS 法で見逃される可能性が非常に高いと考えられる．以上のことからプロピオン酸血症，メチルマロン酸血症に関する MS/MS 法によるスクリーニングは，プロピオニルカルニチンのみをマーカーとする限りスクリーニングは可能なものの診断における感度および特異性は低く両疾患の判別は困難である．

SCAD 欠損症疑いのエチルマロン酸尿症については，4 生日尿の GC/MS スクリーニングでエチルマロン酸の異常高値を認めたため，再検の必要を指摘した症例であった．その後，他施設の MS/MS 測定でブチリルカルニチン（C4）の異常値が検出され短鎖アシル CoA 脱水素酵素（SCAD）欠損症の可能性が高いと指摘されたが，その後診断の確定はなされていない．最近，ブチリルカルニチンをマーカーとするスクリーニングでは SCAD 欠損症はスクリーニングできないと報告されている[11]．

Fig.3 に早期診断により救命できたオルニチントランスカルバミラーゼ（OTC）欠損症患児の発症前（5 生日尿）と発症後（8 生日尿）の GC/MS 測定結果を示した．発症前（上段）の段階において，オロト酸はカットオフ値（11.07 mmol / mol Cr.）の約 2 倍（23.19 mmol / mol Cr.），ウラシルはカットオフ値（3.27 mmol / mol Cr.）の約 8 倍（25.73 mmol / mol Cr.）の排泄が確認され，発症直後（下段）では，オロト酸（4556 mmol / mol Cr.），ウラシル（66.23 mmol / mol Cr.）ともさらに増加し，とりわけオロト酸が著増している．この症例は GC/MS 法で発症前の OTC 欠損症のスクリーニングが可能であることを示唆しているが，一方自験例の一例だけですべてスクリーニング可能と結論付けるのはあまりにも早急であろう．これらのマーカーは疾患特異性は低いものの，OTC 欠損症のような尿素サイクル異常症で認められる重要なマーカーでもあり，注意深くその後の経過をチェックすることが必要と考えられた．一方，MS/MS 法では，シトルリンあるいはアルギニンの低下などにより，スクリーニングの可能性が報告[5] されているが，OTC 欠損症に特異的な診断マーカーがないため，スクリーニングは難しいと考えられる．

新生児期発症型メチルマロン酸血症やプロピオン酸血症および PKU などは，MS/MS 法により診断することは容易であるとされており[12]，今回のわれわれの比較検討においても一致するものであった．しかし海外でも報告されているように[13] GC/MS 法にて容易に発見されるいわゆる良性のメチルマロン酸血症，軽症型メチルマロン酸血症，ビタミン B_{12} 反応型メチルマロン酸血症，OTC 欠損症などは，MS/MS 法でスクリーニングすることは困難であった．また新生児期の血中診断マーカーのレベルは新生児以降同様，飢餓状態，栄養状態，腎障害など[14] 各種疾患で変動する．このように血中診断マーカーの大きな変動要因なども考慮すると MS/MS によるスクリーニングでは軽症型メチルマロン酸血症や OTC 欠損症は見逃される可能性が大きいものと考えられ，両疾患を MS/MS 法の対象から除外することも含め更なる検討が必要である．

今回の検討は既に診断のついたサンプルを使

用しており，後方視的研究であるので，厳密なコントロールスタディではない．しかし，今回の結果および従来の報告などにより，血液濾紙による MS/MS 法と尿による GC/MS 法とでは，スクリーニング診断においてアプローチしやすい疾患としにくい疾患があることがわかった．新生児マススクリーニングに質量分析法を適用する場合，これらの点を明確に把握しておく必要がある．とりわけわが国において当面の課題となっている現行法への MS/MS 法の導入に関し[15), 16)]，その対象とする疾患は慎重に検討し選択することが重要であると考えられる[17), 18)]．

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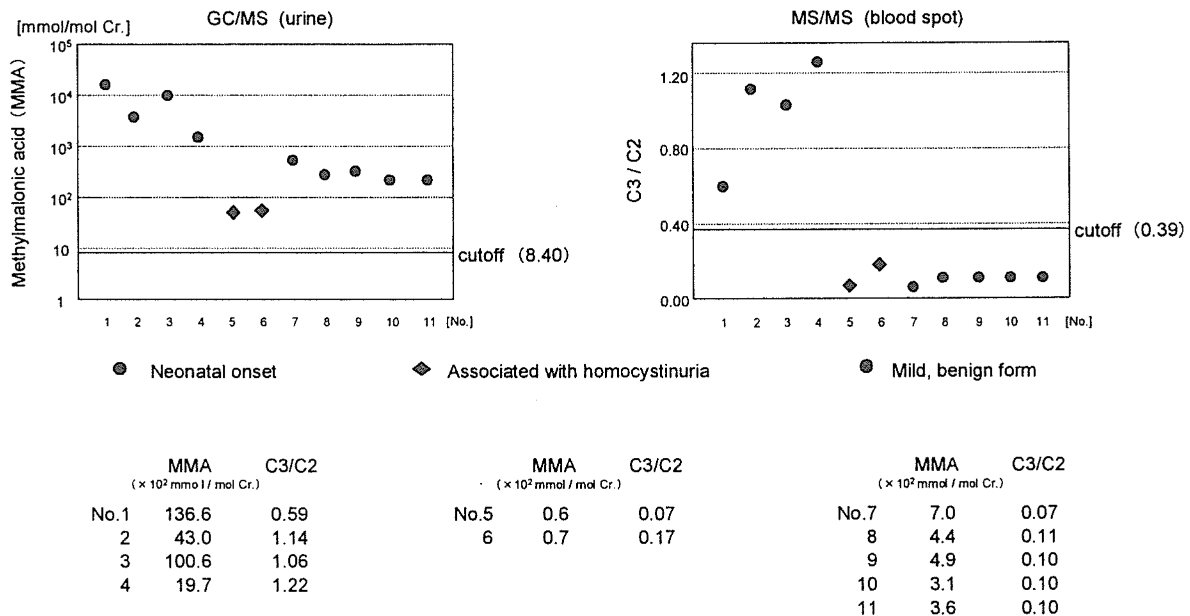

	MMA (×10² mmol / mol Cr.)	C3/C2
No.1	136.6	0.59
2	43.0	1.14
3	100.6	1.06
4	19.7	1.22

	MMA (×10² mmol / mol Cr.)	C3/C2
No.5	0.6	0.07
6	0.7	0.17

	MMA (×10² mmol / mol Cr.)	C3/C2
No.7	7.0	0.07
8	4.4	0.11
9	4.9	0.10
10	3.1	0.10
11	3.6	0.10

Fig.1　Methylmalonic acid and C3/C2 in methylmalonic acidemia

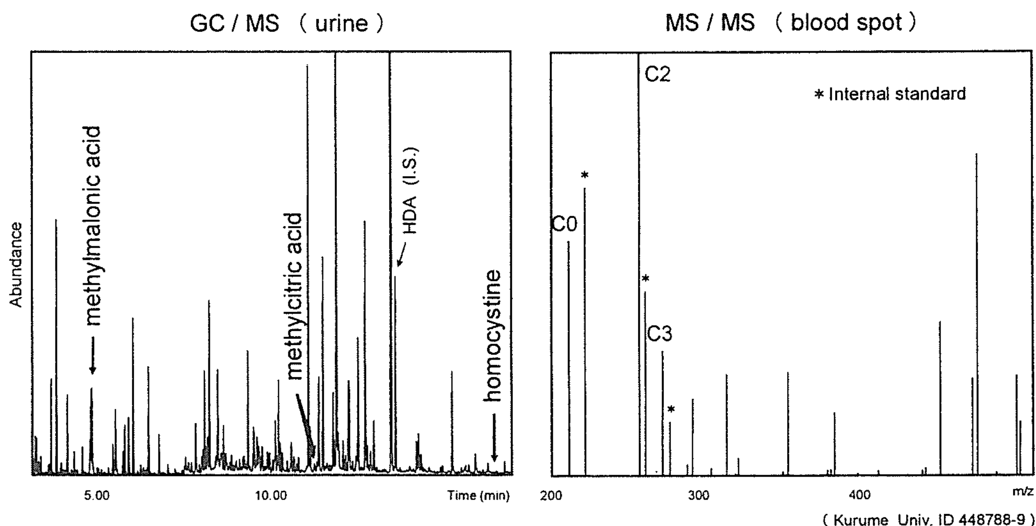

Fig.2　Methylmalonic acidemia associated with homocystinuria

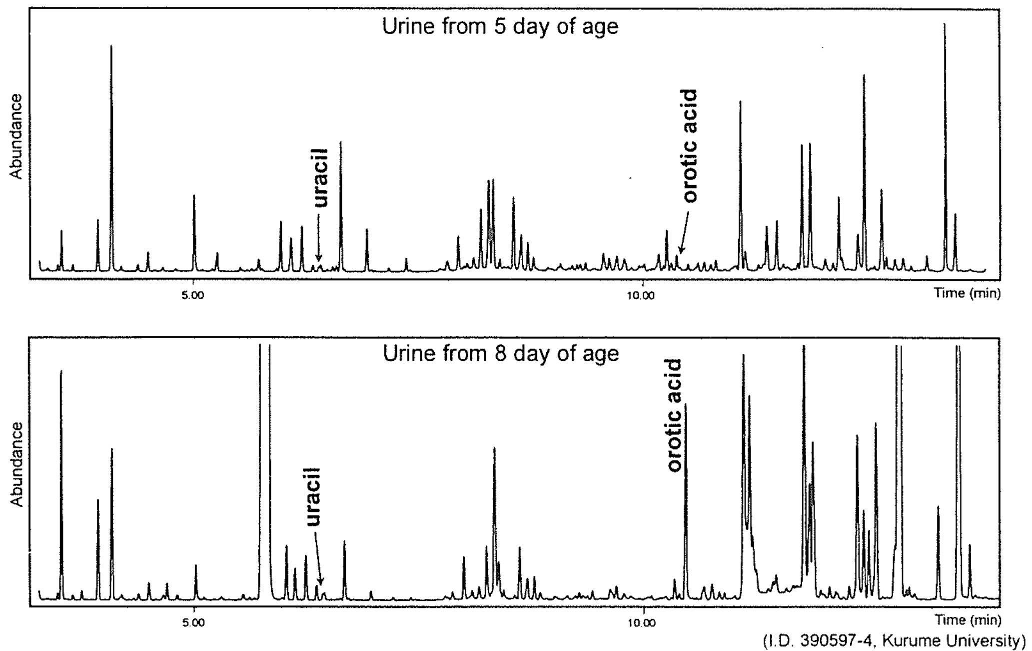

Fig.3 TIC chromatogram of OTC deficiency

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Indication of MS/MS for neonatal metabolic screening

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RESEARCH ARTICLE

Comprehensive Mutation Analysis of *GLDC*, *AMT*, and *GCSH* in Nonketotic Hyperglycinemia

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Nonketotic hyperglycinemia (NKH) is an inborn error of metabolism characterized by accumulation of glycine in body fluids and various neurological symptoms. NKH is caused by deficiency of the glycine cleavage multi-enzyme system with three specific components encoded by *GLDC*, *AMT*, and *GCSH*. We undertook the first comprehensive screening for *GLDC*, *AMT*, and *GCSH* mutations in 69 families (56, six, and seven families with neonatal, infantile, and late-onset type NKH, respectively). *GLDC* or *AMT* mutations were identified in 75% of neonatal and 83% of infantile families, but not in late-onset type NKH. No *GCSH* mutation was identified in this study. *GLDC* mutations were identified in 36 families, and *AMT* mutations were detected in 11 families. In 16 of the 36 families with *GLDC* mutations, mutations were identified in only one allele despite sequencing of the entire coding regions. The *GLDC* gene consists of 25 exons. Seven of the 32 *GLDC* missense mutations were clustered in exon 19, which encodes the cofactor-binding site Lys754. A large deletion involving exon 1 of the GLDC gene was found in Caucasian, Oriental, and black families. Multiple origins of the exon 1 deletion were suggested by haplotype analysis with four *GLDC* polymorphisms. This study provides a comprehensive picture of the genetic background of NKH as it is known to date. Hum Mutat 27(4), 343–352, 2006. © 2006 Wiley-Liss, Inc.

KEY WORDS: GLDC; AMT; GCSH; glycine encephalopathy; nonketotic hyperglycinemia; NKH; glycine cleavage system; mutation spectrum; genotype–phenotype

INTRODUCTION

Nonketotic hyperglycinemia (NKH, MIM# 605899), also termed glycine encephalopathy, is an inborn error of amino acid metabolism characterized by the accumulation of a large amount of glycine in body fluids [Hamosh and Johnston, 2001]. Glycine levels are elevated to a much greater extent in cerebrospinal fluid (CSF) than in plasma; hence, an abnormally high value for the CSF/plasma glycine ratio is observed. NKH is clinically classified (by onset of symptoms) as three types: neonatal, infantile, or late-onset. Later onset appears to be associated with a better prognosis. The vast majority of patients fall into the neonatal category, which involves a stereotypic presentation with severe hypotonia, apnea requiring assisted ventilation, and intractable seizures. Approximately 30% of such patients die in the neonatal period. Survivors often have severe psychomotor retardation, although 15–20% of survivors achieve developmental milestones such as head control, independent sitting, or walking [Hoover-Fong et al., 2004]. Patients with the infantile type of NKH are often asymptomatic in the neonatal period and the phenotype is characterized by mild to moderate psychomotor retardation, behavioral problems, seizures, and chorea. The clinical presentations of late-onset NKH are heterogeneous. In previous studies, two families did not have seizures or mental retardation, but exhibited progressive paraplegia and optic atrophy [Bank and Morrow, 1972; Steiman et al., 1979]. Another family was reported to present with mental retardation and choreoathetosis [Singer et al., 1989].

The fundamental defect lies in the glycine cleavage system (GCS; EC2.1.2.10) [Tada et al., 1969]. The GCS is a mitochondrial complex enzyme system that consists of four

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individual proteins [Kikuchi, 1973]: glycine decarboxylase (also called P-protein), aminomethyltransferase (T-protein), hydrogen carrier protein (H-protein), and dihydrolipoamide dehydrogenase (L-protein). The enzymatic analysis of NKH patients revealed that approximately 80% of patients with NKH have a *GLDC* deficiency and the rest have an *AMT* deficiency [Tada and Hayasaka, 1987]. Mitochondrial precursors of human P, T, H, and L-proteins consist of 1,020, 403, 173, and 509 amino acids, respectively. Dihydrolipoamide dehydrogenase is a housekeeping enzyme that serves as an E3 component of other α-keto acid dehydrogenase complexes, such as pyruvate dehydrogenase. Deficiency of dihydrolipoamide dehydrogenase causes progressive neurological deterioration with lactic acidosis but not hyperglycinemia [Hong et al., 1996]. The three GCS-specific components (P, T, and H-proteins) are encoded by distinct genes: *GLDC* (MIM# 238300) on chromosome 9p24 [Isobe et al., 1994], *AMT* on 3p21.1-21.2 [Nanao et al., 1994], and *GCSH* (MIM# 238330) on 16p24 [Kure et al., 2001], respectively.

To date, a limited number of NKH mutations have been reported (Human Gene Mutation Database, Cardiff; http://www.hgmd.cf.ac.uk). The *GLDC* mutations reported to date include the S564I mutation that is prevalent in Finnish patients [Kure et al., 1992], the R515S mutation found in 5% of Caucasian patients [Toone et al., 2000], microdeletions [Kure et al., 1991], large deletions [Takayanagi et al., 2000; Sellner et al., 2005], one abnormal splicing [Flusser et al., 2005], one nonsense mutation [Sellner et al., 2005], and 10 missense mutations [Toone et al., 2002; Korman et al., 2004; Kure et al., 2004; Boneh et al., 2005; Dinopoulos et al., 2005]. The *AMT* gene (MIM# 238310) mutations identified to date include nine missense mutations [Nanao et al., 1994, 1994a; Kure et al., 1998; Toone et al., 2000, 2001, 2003], one microdeletion [Kure et al., 1998, 1998b], and one splicing mutation [Toone et al., 2000]. In *GCSH* we have found one abnormal splicing in a patient with a transient form of NKH [Kure et al., 2002]. Since multiple genes are responsible for NKH, previous studies screened only a small number of *GLDC* and *AMT* exons and/or a few patients, which hampered elucidation of the genetic background of NKH.

The purpose of the present study was to establish the mutation spectrum of NKH by performing a comprehensive screening for mutations in *GLDC*, *AMT*, and *GCSH* in 69 families with three different types of NKH. The structure of *AMT* has been determined [Nanao et al., 1994, 1994b]. Also, we previously reported the exon-intron organizations of *GLDC* [Takayanagi et al., 2000] and *GCSH* [Kure et al., 2001], which provided us with basic information to amplify the entire coding regions for the three genes. To increase the sensitivity of mutational screening, we directly sequenced all amplicons without employing prescreening scanning methods such as single-strand conformation polymorphism.

MATERIALS AND METHODS

Patients

Patients were examined in the metabolic disease clinics of a number of referring hospitals. NKH was clinically suspected based on the presentation of symptoms characteristic of each disease type and electroencephalograms (EEG) recordings, and were subsequently confirmed by amino acid analysis. The CSF/serum glycine ratio at diagnosis was >0.04 in all patients, whereas it was <0.03 in normal neonates. Patients were classified into three clinical subtypes (neonatal, infantile, and late-onset) based on the onset of clinical symptoms.

Neonatal type. We studied 56 families with neonatal onset. Initial symptoms, including hypotonia, apnea, and coma, developed within 7 days after birth–in most cases within 3 days. Almost all of the patients showed a burst suppression pattern on EEG within 2 weeks after birth, and hypsarrhythmia thereafter.

Infantile type. Six families were enrolled. Symptoms started at 3–12 months of age. Five of the six patients had no symptoms in the neonatal period. Very mild hypotonia and apnea were observed in patient P107 [Dinopoulos et al., 2005]. Mild mental retardation and abnormal behaviors, such as aggressiveness, developed in adolescence.

Late-onset type. We studied seven patients with late-onset NKH. All seven patients had elevated glycine concentrations in repeated amino acid analysis of CSF and/or plasma. Spastic paraplegia without mental retardation developed in three of the seven patients. These patients resembled those in previously reported families [Bank and Morrow, 1972; Steiman et al., 1979]. The rest of the patients presented with mental retardation after they entered school or during adolescence. There is some confusion in terms of phenotypic classification of the mild form of the infantile type and the late-onset type, since there are some reports of patients in whom developmental delay or mild hypotonia started in the middle or late infantile periods [Flannery et al., 1983; Singer et al., 1989]. We classified such patients as having the infantile type—not the late-onset type. In the present study we classified patients as having the late-onset type when they were free of any symptoms during infancy.

Mutational Screening

Exons and flanking intron sequences of *GLDC* (GenBank NM_000170, NT_008413.16), *AMT* (GenBank NM_00481, NT_086638.1), and *GCSH* (GenBank NM_004483, NC_00016.8) were amplified by PCR from genomic DNA, followed by direct sequencing analysis using the dye-primer sequencing method as previously described [Kure et al., 2001]. The 18-mer oligonucleotides of the M13 and reverse sequencing primers were added to the 5′ end of the forward and reverse PCR primers, respectively. We initially screened for a large deletion involving *GLDC* exon 1 by semiquantitative PCR using the pseudogene of *GLDC* (*GLDCP*) as a gene dose control [Takayanagi et al., 2000]. PCR primer sequences for amplifying *GLDC* exons 1–6 were previously reported [Takayanagi et al., 2000], and those for other exons are described in Supplementary Table S1 (available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat). Each PCR cycle consisted of denaturing at 98°C for 10 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, with repetition for 35 cycles. For PCR amplification of *GLDC* exon 1, 10% dimethylformamide was added to the reaction mixture. Fifty control subjects were subsequently screened for any detected base changes to exclude noncausative polymorphisms. If no mutations were detected in the sequencing analysis of *GLDC*, we screened for mutations in *AMT* by amplifying all of the nine exons. When no mutations were identified in either *GLDC* or *AMT*, we sequenced all of the five exons in *GCSH*. For characterization of *GLDC* and *AMT* missense mutations, each mutated amino acid residue was compared with the corresponding amino acid in rat [Sakata et al., 2001], chicken [Kume et al., 1991], pea [Turner et al., 1992], and *E. coli* [Okamura-Ikeda et al., 1993], as shown in Tables 2 and 3.

Characterization of the *GLDC* Deletion

We identified the minimum deleted region of both alleles of each homozygous patient of the *GLDC* exon 1 deletion. Fifteen sequence tagged sites (STSs) were used for this deletion mapping,

as illustrated in Fig. 1. PCR primer sequences and amplification conditions for D9S281 and RH92434 were obtained from the website of the UCSC Genome Browser (http://genome.ucsc.edu). The PCR primer sequences and amplicon sizes of STSs 1–8 are presented in Supplementary Table S1. STSs 1–7 were located 5′ upstream of the *GLDC* gene, and STS 8 was located in intron 2. Amplification primers for *GLDC* exons 1–5 were also used in the deletion mapping. We amplified these 15 STSs by using genomic DNA of Patients P5 and P36 as a template in order to test whether each STS was involved in the homozygously deleted region. Structural information about the 5′ upstream region of *GLDC* and the location of *Alu* motifs was obtained from the UCSC Genome Browser (Fig. 1).

RESULTS

Mutation Screening

We performed mutational screening in 69 NKH families (56 neonatal type, six infantile type, and seven late-onset type). First, the *GLDC* exon 1 deletion was screened by semiquantitative PCR amplification using the *GLDCP* as a control of the gene copy number. This deletion was found in six families (Table 1). Subsequent extensive sequencing of *GLDC*, *AMT*, and *GCSH* coding exons revealed that 42 of the 56 neonatal-type families (75%) had *GLDC* or *AMT* mutations in at least one of two alleles. *GLDC* mutations were found in 31 (74%) and *AMT* mutations were detected in 11 (26%) of the 42 families. No differences were

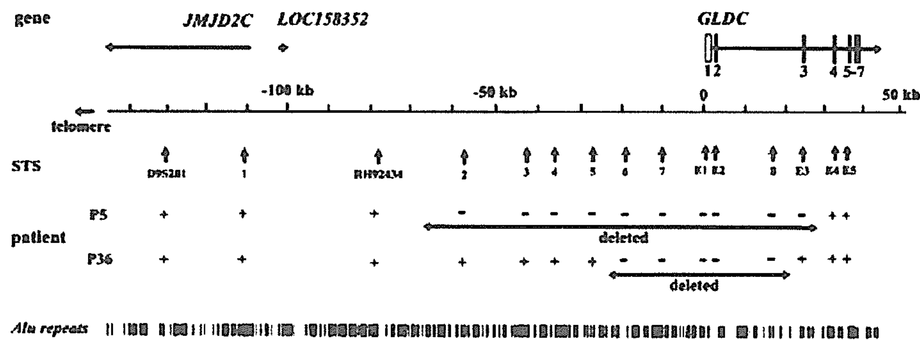

FIGURE 1. **Mapping of the *GLDC* deletions. Genomic regions that were homozygously deleted in patients P5 and P36 were defined by amplification of 13 STS markers. The JMJD2C gene and the gene-like structure, LOC158352, are shown based on the information of the UCSC Genome Browser. E1–5 indicate amplicons including the *GLDC* exons 1–5, respectively. Amplicons (+) indicate that the STS was successfully amplified, while (–) means that the STS failed to be amplified.**

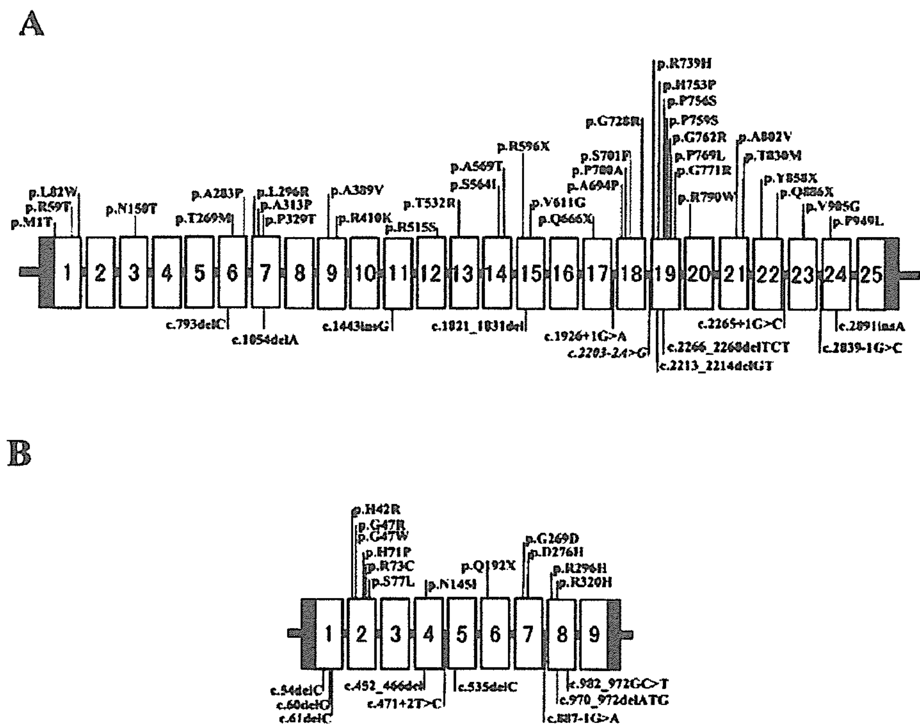

FIGURE 2. **NKH mutations identified in this and previous studies. The *GLDC* (A) and *AMT* (B) exons are indicated by open boxes, and noncoding regions are shaded. Missense and nonsense mutations are shown above the exon boxes, and deletions/insertions and mutations of splicing errors are indicated below the exon boxes.**