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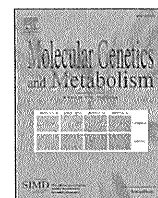
Molecular Genetics and Metabolism 104 (2011) 560–565

Contents lists available at SciVerse ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme



Newborn screening for Pompe disease in Japan

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ARTICLE INFO

Article history:
Received 14 July 2011
Received in revised form 5 September 2011
Accepted 5 September 2011
Available online 10 September 2011

Keywords:
Pompe disease
Acid alpha-glucosidase
Dried blood spots
Newborn screening
Genetic polymorphism

ABSTRACT

Pompe disease is caused by a deficiency of acid alpha-glucosidase (GAA) that results in glycogen accumulation, primarily in muscle. Newborn screening (NBS) for Pompe disease has been initiated in Taiwan and is reportedly successful. However, the comparatively high frequency of pseudodeficiency allele makes NBS for Pompe disease complicated in Taiwan. To investigate the feasibility of NBS for Pompe disease in Japan, we obtained dried blood spots (DBSs) from 496 healthy Japanese controls, 29 Japanese patients with Pompe disease, and five obligate carriers, and assayed GAA activity under the following conditions: (1) total GAA measured at pH 3.8, (2) GAA measured at pH 3.8 in the presence of acarbose, and (3) neutral glucosidase activity (NAG) measured at pH 7.0 without acarbose. The % inhibition and NAG/GAA ratio were calculated. For screening, samples with GAA<8% of the normal mean, % inhibition>60%, and NAG/GAA ratio>30 were considered to be positive. Two false positive cases (0.3%) were found, one was a healthy homozygote of pseudodeficiency allele (c.1726G>A). The low false-positive rate suggests that NBS for Pompe disease is feasible in Japan.

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1. Introduction

Pompe disease (glycogen storage disease type II) is an autosomal recessive lysosomal storage disorder characterized by deficiency of lysosomal enzyme acid alpha-glucosidase (GAA, EC3.2.1.20). Pompe disease is progressive, with massive accumulation of glycogen in lysosomes. The clinical manifestations are variable, depending on age at onset, range of organ involvement, and rate of progression. Patients with infantile-onset Pompe disease have severe cardiomegaly and generalized skeletal muscle weakness, and die before the age of 2 years old. Patients with juvenile- and adult-onset Pompe disease have mainly skeletal muscle involvement and often progress to respiratory failure [1,2].

Enzyme replacement therapy with recombinant human GAA can be used to treat patients with Pompe disease and has been shown to prolong survival, reverse cardiomyopathy, and improve motor function [3–5]. The best motor function outcomes have been achieved after early initiation of enzyme replacement therapy, which underscores the need for early diagnosis [4,6–8]. However, early diagnosis of Pompe disease is difficult because of the low index of suspicion and the lack of specificity in early symptoms. Until recently, demonstration of deficient GAA activity in dried blood spots (DBSs) was not possible because of interference from the isoenzyme maltase glucoamylase (MGA), which is abundant in neutrophils. However, the identification of acarbose as an effective inhibitor of MGA has permitted evaluation of GAA activity in blood samples, including DBSs, on filter paper [5,9–13]. These methods were shown to reliably identify patients with Pompe disease in a large pilot program in Taiwan [13].

It is reported that pseudodeficiency allele reduces GAA activity but causes no symptoms of the disease [14,15]. Pseudodeficiency alleles are known as c.1726G>A (p.G576S) variant in cis with c.2065G>A (p.E689K), also as c.[1726A; 2065A]. The alleles almost segregate together. Substitution c.2065G>A (p.E689K) reduces GAA activity by 50% at most. On the contrary, substitution c.1726G>A (p.G576S) reduces the activity to such extent that it falls into the patient range. Pseudodeficiency allele is more frequent in Japanese population than in other populations studied to date [14], and this may complicate issue in NBS of Pompe disease with Japanese population. According to the article of NBS in Taiwan, the presence of the GAA pseudodeficiency alleles made NBS in Taiwan complicated [15]. Therefore, NBS may require modification to distinguish a healthy homozygote of pseudodeficiency allele from Pompe disease patients by using a new cut-off value in the screening procedure in Japanese population. Herein, we report the GAA activity and presence of the sequence variant c.1726G>A (p.G576S) in the GAA gene in 520 DBSs collected from 400 healthy Japanese newborns, 96 healthy adults, 29 patients with Pompe disease, and 5 obligate carriers.

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doi:10.1016/j.ymgme.2011.09.002

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2. Materials and methods

2.1. Samples

DBSs (Advantec®, ToyoRoshi Kaisha Ltd., Japan) were obtained from 29 Japanese patients with Pompe disease (two infantiles, 14 juveniles and 13 adult phenotypes; age range: 3 months–67 years old), 496 healthy Japanese controls (400 newborns aged 3–5 days old, 96 adults aged 18–62 years old) and 5 obligate carriers (33–46 years old). DBSs were dried at room temperature and stored at −20 °C in plastic bags until analysis. Informed consent was obtained from all subjects or family members. All samples were prepared and analyzed in accordance with the protocols approved by the ethics committee of the National Center for Child Medical Health and Development.

2.2. Reagents

A Big Dye Terminator kit was purchased from Applied Biosystems (Foster City, CA, USA). Acarbose, 4-methylumbelliferone (4MU) and 4-methylumbelliferyl-αD-glucopyranoside (4MUG) were purchased from Sigma-Aldrich (St. Louis, MO). Ampdirect™ Plus was obtained from Shimadzu (Kyoto, Japan) and rTaq DNA polymerase was purchased from Takara (Shiga, Japan). Other chemicals (all from Sigma-Aldrich or Wako, Osaka, Japan) were of reagent grade.

2.3. Frequency of pseudodeficiency alleles

Both pseudodeficiency alleles c.[1726A; 2065A] almost segregate together. Substitution c.2065G>A (p.E689K) reduces GAA activity by 50% at most. On the contrary, substitution c.1726G>A (p.G576S) reduces the activity to such extent that it falls into the patient range. Therefore, we tested for the presence of the s sequence variant only c.1726G>A (p.G576S).

A 1.2-mm diameter disk from each DBS was obtained using Uni-Core™ and placed in a sample tube, into which 10 μL Ampdirect™ Plus (including polymerase chain reaction (PCR) buffer and dNTPs), 0.5 units rTaq DNA polymerase, and a set of specific primers (each 1.0 μmol/L) were added to produce a reaction mixture of total volume 20 μL. The sequences 5′-AGG GAG GGC ACC TTG GAG CCT G-3′ and 5′-GCA GAG GCC CCA ACC TTG TAG G-3′ were designed as forward and reverse primers for amplification of the single nucleotide polymorphism (SNP) c.[1726G] of the *GAA* allele. PCR was performed using denaturation at 95 °C for 10 min; 30 cycles of amplification with denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s; and final extension at 72 °C for 7 min. DNA sequencing analysis was performed on amplified products by direct sequencing with the Big Dye Terminator kit on an ABI PRISM 3100 DNA Genetic Analyzer (Applied Biosystems).

The DBSs were also analyzed for the sequence variant c.1726G>A (p.G576S) in the GAA gene using PCR-RFLP (Restriction Fragment Length Polymorphism). Sample preparation and PCR was performed as described above, using the sequences 5′-AGG GAG GGC ACC TTG GAG CCT G-3′ and 5′-GGG AGG CGA TGG CTT CGG TCA AG-3′ as the forward and reverse primers, respectively, for amplification of the SNP c.[1726G] of the *GAA* allele. For PCR-RFLP, the PCR-amplified product (5 μL) was placed in a sample tube, into which 14 μL of Neb buffer (50 nM potassium acetate, 20 mM tris-acetate, 10 mM magnesium acetate, 1 mM DTT; pH 7.9) and 1 μL of *AluI* enzyme were added to give a reaction mixture of total volume 20 μL. This mixture was then incubated for 4 h at 37 °C.

2.4. GAA activity

For determination of GAA activity, a modified protocol based on the fluorometric assay developed by Chamoles et al. [9] was used. Briefly, a disk of 3.0 mm in diameter obtained from each DBS by Uni-Core™ was placed in a sample tube and 360 μL of water was added, followed by gentle mixing for 1 h at 4 °C on a rocking platform. A 70 mM stock solution of the synthetic substrate 4MUG in dimethyl sulfoxide (Wako) was prepared in advance. Substrate solutions consisting of 1.4 mM 4MUG at pH 3.8 and pH 7.0 were prepared in 40 mM aqueous sodium acetate buffer and 40 mM sodium acetate buffer, respectively. Enzyme reactions at pH 3.8 and pH 7.0 were carried out in 50 μL of substrate solution, 10 μL of deionized water, and 40 μL of DBS extract. For enzyme reactions in the presence of an inhibitor, the water was replaced with 10 μL of 2.7 μM acarbose. These reagents were incubated for 20 h at 37 °C with covering with sealing film (Sumitomo Bakelite Co.). The DBS extract for blanks was incubated separately and combined with the other reagents at the end of the incubation period, immediately followed by addition of 200 μL of 150 mM ethylene diamine triacetic acid (EDTA) (pH 11.5) to all wells.

A 4MU standard curve was prepared on every plate by mixing aqueous standards (100 μL per well) in the range 0.00 to 3.13 μM with EDTA solution (200 μL per well). Eight different standards per curve were used in duplicate. The relative fluorescence (excitation 355 nm, emission 460 nm) of each well was measured using a Wallac 1420-011 Multilevel Counter (PerkinElmer, Turku, Finland). The fluorescence readings were corrected for the blank and the results were compared with the fluorescence of a 4MU calibrator. The absolute amount of whole blood per spot cannot be determined accurately, but is comparable among samples. Therefore, the enzymatic activities are expressed as nmol of substrate hydrolyzed per punch per hour.

For Pompe disease screening, three assays were performed: (1) total GAA activity (tGAA) measured at pH 3.8, (2) GAA activity measured at pH 3.8 in the presence of acarbose, and (3) total neutral glucosidase activity (NAG) measured at pH 7.0 without acarbose. The % inhibition [(tGAA–GAA)/tGAA] and NAG/GAA ratio were calculated using data from these assays.

3. Results

3.1. Frequency of pseudodeficiency allele

The presence of the sequence variant c.1726G>A (p.G576S) in the GAA gene was examined in DBSs using DNA sequencing analysis and PCR-RFLP. The results were as follows: among Pompe disease patients (n = 29), 16 (55.1%) had sequence c.1726G/G (G/G), 10 (35%) had c.1726G/A (G/A), and 3 (10%) had c.1726A/A (A/A); among obligate carriers (n = 5), 3 (60%) had G/G, 2 (40%) had G/A, and among 496 healthy controls, 332 (67%) had G/G,149 (30%) had G/A, and 15 (3.0%) had A/A. These data are similar to those in previous reports. The A/A allele occurred at a significantly higher frequency (10%) in the patients than in controls (3%).

3.2. GAA activity

GAA activities measured with 4MU in the presence of acarbose in DBSs were as follows. The average GAA activity in the 496 healthy controls was 21.6 pmol/punch/h (range 1.3–63.4, Fig. 1A). The distribution of GAA activity based on the frequency of the three alleles is shown in Fig. 1B. A cut-off point for the GAA activity of 8% of the normal average (1.7 pmol/punch/h) identified 29 patients (100%), 5 healthy homozygotes of pseudodeficiency allele (33%), 1 obligate carrier (20%), and no healthy control without pseudodeficiency allele.

3.3. NAG/GAA ratio

The averages (range) of the NAG/GAA ratios were 5.0 (0.9–30.8) for healthy control without pseudodeficiency allele, 25.2 (14.6–57.6) for healthy homozygotes of pseudodeficiency allele, 22.3 (11.1–39.5) for obligate carrier, and 89.4 (41.9–222.3) for patients with Pompe disease. A cut-off value of 30 identified 29 patients (100%), 12 healthy

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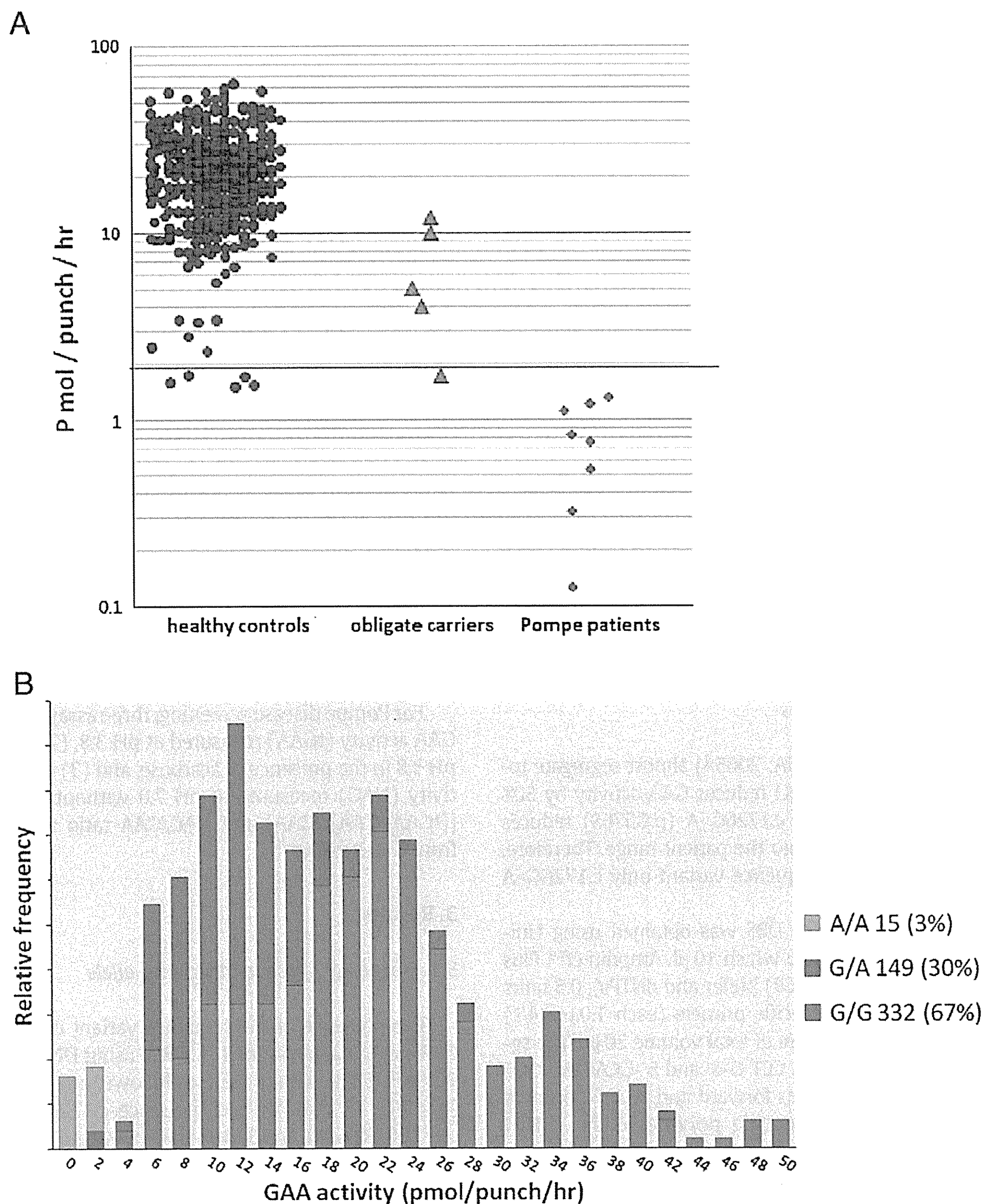

Fig. 1. Correlation between GAA activity (pmol/punch/h) and genotype. (A) A log scale of GAA activity (pmol/punch/h) in healthy controls, obligate carriers, and patients with Pompe disease. A cut-off point shows with red bar (1.7 pmol/punch/h). (B) GAA activity (pmol/punch/h) in G/G (no sequence variant of c.1726G>A in the GAA gene) G/A (heterozygote sequence variant), and A/A (homozygote sequence variant) cases.

Table 1
Positive results in screening for healthy controls, obligate carriers, and patients with Pompe disease.

Cut-off point		Healthy controls (with pseudodeficiency allele)	Obligate carriers	Pompe patients
Single screening	GAA (% of nM/mean)<8%	1.0% 5/496 (5)	20% 1/5	100% 29/29
	NAG/GAA>30	2.4% 12/496 (10)	40% 2/5	100% 29/29
	% inhibition>60	2.8% 14/496 (12)	20% 1/5	100% 29/29
Combination screening (double marker screening)	% inhibition>60 NAG/GAA>30 Meet the above condition	1.0% 5/496 (5)	20% 1/5	100% 29/29
Combination screening (triple marker screening)	GAA (% of nM/mean)<8 % NAG/GAA>30 % inhibition>60 Meet the above condition	0.20% 1/496 (1)	20% 1/5	100% 29/29

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Molecular Genetics and Metabolism 104 (2011) 597–602

Contents lists available at SciVerse ScienceDirect

Molecular Genetics and Metabolism

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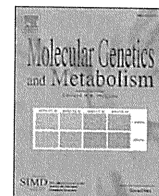

Enzyme replacement therapy attenuates disease progression in two Japanese siblings with mucopolysaccharidosis type VI

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ARTICLE INFO

Article history:
Received 1 August 2011
Received in revised form 26 August 2011
Accepted 26 August 2011
Available online 28 August 2011

Keywords:
Mucopolysaccharidosis type VI (MPS VI)
N-Acetylgalactosamine-4-sulfatase (ASB)
Enzyme replacement therapy (ERT)
Glycosaminoglycan (GAG)
Galsulfase

ABSTRACT

Mucopolysaccharidosis type VI (MPS VI) is a progressive, multisystem autosomal recessive lysosomal disorder resulting from deficient N-acetylgalactosamine-4-sulphatase (ASB) and the consequent accumulation of glycosaminoglycan (GAG). Preclinical and clinical studies had demonstrated clinical benefits of early initiation of systemic therapies in patients with MPS. In this case report, two siblings with MPS VI started enzyme replacement therapy (ERT) with weekly infusions of recombinant human ASB (Galsulfase) at 1 mg/kg. Sibling 1 started ERT 5.6 years of age and Sibling 2 was 6 weeks old. The disease status in these two siblings prior to and for no less than 36 months of ERT was followed up and compared. The treatment was well tolerated by both siblings. During 36 months of ERT, symptoms typical of MPS VI including short stature, progressive dysmorphic facial features, hepatosplenomegaly, hearing impairment, corneal clouding, and dysostosis multiplex were largely absent in the younger sibling. Her cardiac functions and joint mobility were well preserved. On the other hand, her affected brother had typical MPS VI phenotypic features described above before commencing ERT at the equivalent age, of 3 years. There was significant improvement in the shoulder range of motion and hearing loss after 36 months of treatment and cardiac function was largely preserved. His skeletal deformity and short stature remained unchanged. The results showed that early ERT initiated at newborn is safe and effective in preventing or slowing down disease progression of MPS VI including bone deformities. These observations indicate that early diagnosis and treatment of MPS VI before development of an irreversible disease is critical for optimal clinical outcome.

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1. Introduction

Mucopolysaccharidosis (MPS) type VI, or Maroteaux–Lamy syndrome, is the autosomal recessive lysosomal disorder in which deficient N-acetylgalactosamine 4-sulfatase (arylsulfatase B, or ASB) impairs the stepwise degradation of the glycosaminoglycan (GAG) dermatan sulfate (DS) [1]. Partially degraded GAG accumulates in lysosomes and in a wide range of tissues, causing a chronic progressive disorder characterized by short stature and dysostosis multiplex, as well as characteristic facial features, corneal clouding, cardiac and pulmonary manifestations, which subsequently results in significant functional impairment and a shortened lifespan. Similar to other MPS disorders, MPS VI is a clinically heterogeneous condition. Case studies reported in the literature have identified subjects who presented with marked disease in the first year of life and those who presented with slowly advancing disease that progressed over many decades [2,3].

Phase 1/2, phase 2 and phase 3 double-blind, placebo-controlled clinical studies using recombinant ASB for enzyme replacement therapy (ERT) have demonstrated that weekly infusions of 1 mg/kg Galsulfase were well tolerated, producing a rapid reduction in urinary GAG levels and improved endurance in patients with rapidly advancing MPS VI disease [4–6]. However, these studies excluded young patients with age under 5 years old.

Severely affected MPS VI patients are usually diagnosed by 2 or 3 years of age, and develop gross abnormalities of the skeletal and heart, joint stiffness, and corneal clouding. Some of these pathological changes might not be reversed by ERT. Previous studies of Galsulfase administration in MPS VI animal models from birth [7,8] have indicated that very early initiation of ERT before development of signs and symptoms of the disease leads to better long-term outcomes. We have shown in earlier studies that skeletal deformities can be normalized and the articular cartilage and growth plate can be corrected pathologically by single injection of adenovirus vector into neonatal MPS VII mice [9]. Recently, a sibling study in MPS VI patients from as young as approximately 8 weeks of age demonstrated a clear benefit of early initiation of ERT to slow or prevent the development of significant pathological changes of MPS VI [10].

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doi:10.1016/j.ymgme.2011.08.029

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In this report, we evaluated the safety and efficacy of ERT in two Japanese siblings with MPS VI. ERT was initiated in a newborn girl at as early as 6 weeks after birth and her affected older brother. The two siblings were followed up for 3 years. We are especially interested in whether skeletal deformities and other systemic manifestations of MPS VI could be prevented, normalized or slowed down by the treatment.

2. Materials and methods

The pair of siblings was born to consanguineous Japanese parents. Both of the parents had a missense mutation p.Y85H (c. 252T>C). Both siblings had homozygous missense mutation Y85H in the *ARSB* gene.

2.1. Sib 1 (older brother)

The boy experienced learning difficulties at 2.6 years of age; at age 3 years he was noted to have coarse facial features, dysostosis multiplex, joint contracture, hearing loss, recurrent otitis media, corneal clouding, glaucoma, chronic rhinitis, mitral and tricuspid valve insufficiency, adenoid hypertrophy, and hepatosplenomegaly. MPS VI was diagnosed at age 3.3 years by elevated urine GAG, urinary uronic acid with significantly increased dermatan sulfate. Urinary GAG level was 370 μg/mg creatinine (normal range (NR): 47.8 ± 11.6 μg/mg creatinine at 3 to 12 years of age). Urinary uronic acid was 216 mg/g creatinine with dermatan sulfate (DS) 61%, heparan sulfate (HS) 9%, and chondroitin sulfate (CS) 30%. ASB activity was 9 nmol/mg protein/hour (NR 107–495) in leukocytes and 24 nmol/mg protein/hour (NR 154–545) in skin fibroblasts. The boy started ERT since November 2006 when he was 5.6 years of age and his body weight was 17.8 kg (−0.37 SD) and height was 99.1 cm (−2.4 SD). The boy has no past history of bone marrow transplantation (BMT) or surgery before starting ERT.

2.2. Sib 2 (younger sister)

The girl is the younger sister of sib 1. The parents received genetic counseling during the pregnancy of the mother. The girl was born by vaginal delivery at 39 weeks of gestation with birth weight of 2.95 kg and length of 49.50 cm. After her delivery, umbilical cord blood was obtained from the girl for genetic test and she had the same mutation as her brother. By this genetic test, she was diagnosed with MPS VI. Her urine was collected at 10 days after birth and blood was collected at 12 days after birth. Urinary GAG and uronic acid test and ASB enzyme activity were also measured. At 1 month of age, her weight was 4.32 kg and length 55.80 cm. Urinary GAG level was 1093 μg/mg creatinine. Urinary uronic acid was 448 mg/g creatinine with DS 54%, HS 5%, and CS 41%. ASB activity was less than 9 nmol/mg protein/hour in leukocytes. The girl started ERT at 6 weeks of age, May 2007. Slightly-abnormal mitral valve regurgitation was observed by ECHO before starting ERT.

2.3. ERT treatment and clinical assessment

The two siblings received recombinant human ASB Galsulfase (BioMarin Pharmaceuticals Inc.) diluted with physiological saline solution (JP) at 1 mg/kg/week infused over a 4-hour period. Vital signs and laboratory tests were evaluated every 12 weeks. Urinary GAG level was determined as the concentration of uronic acid normalized for creatinine (mg/g creatinine) and was measured using the carbazole reaction at a central laboratory (SRL Medisearch, Tokyo, Japan).

Evaluation of endurance by 6-minute walk test (6MWT) was performed every 12 weeks for sib 1. For sib 2, 6MWT was only conducted when she was on ERT for 30 months. There was no baseline measurement for both siblings. Pulmonary function test was not successful in both siblings.

Height and weight were measured before ERT and every 12 weeks. Joint range of motion (ROM) test, skeletal X-ray and audiology examination were performed at baseline and every 24 weeks during ERT. Active joint ROM was measured by goniometry, and included shoulder (flexion, extension, and abduction), elbow (flexion and extension), hip (flexion and extension), and knee (flexion and extension).

Standard 12-lead electrocardiogram (ECG) was performed at baseline and every 12 weeks during ERT. Cardiac structure and function were evaluated by echocardiography (ECHO, two-dimensional and M-mode). Liver and spleen volumes were estimated by palpation.

Safety evaluation included continuous monitoring of adverse events (AE) and periodic clinical laboratory and physical examination. Serum was collected every 6 weeks and sent to a central laboratory (BioMarin Pharmaceutical Inc.) for evaluation of immunoglobulin G anti-rhASB antibody levels as determined by antibody assays.

3. Results

By May 2010, the older sibling (sib 1) has been on ERT for 3.5 years and the younger sibling (sib 2) 3 years. The treatment was generally well tolerated. No infusion associated reaction (IAR) was observed in the sib 1. For sib 2, urticaria was developed at the 9th dose at 2 hours and 15 minutes after the initial administration of Galsulfase and was recovered after intravenous administration of 1 mL Hydrocorton. The treatment was reassumed and no other drug-related AE was reported in the following doses. Both siblings developed antibody to rhASB after 6 weeks of treatment in sib 1 and after 80 weeks in sib 2 (data not shown).

There was no sufficient 6MWT data to evaluate the endurance. By initiating ERT, the baseline urinary uronic acid level in sib 1 decreased rapidly and had subsequently remained below 100 mg/g creatinine during months 3–42. Sib 2 had much higher baseline urinary uronic acid level that had quickly decreased and remained below 100 mg/g creatinine during months 6–36 (Fig. 1). Although urinary uronic acid levels were significantly decreased in both siblings, urinary uronic acid level had not been further reduced to normal level of <10 mg/g creatinine.

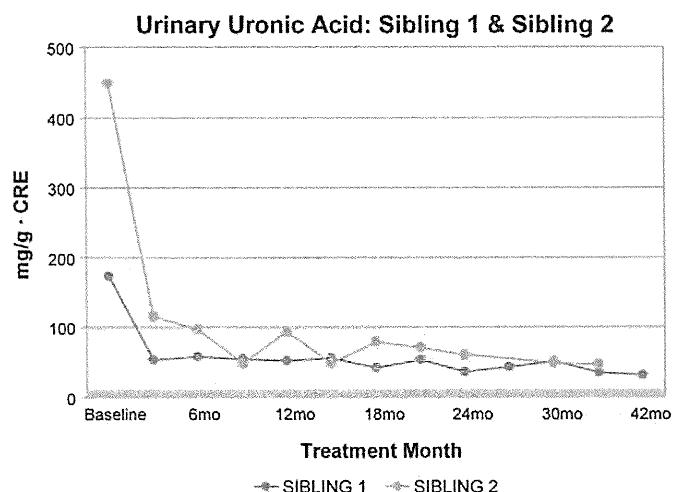

Fig. 1. The effect of Galsulfase treatment on urinary uronic acid levels. Decrease in urinary uronic acid levels from baseline to month 42 in sib 1 and month 36 in sib 2. Normal range of uronic acid concentration is age-dependent. 43.4 ± 12.9 μg/mg creatinine (<1 year of age), 36.0 ± 10.1 μg/mg creatinine (1 year of age), 29.7 ± 13.3 μg/mg creatinine (2 years of age), 30.0 ± 12.8 μg/mg creatinine (3 years of age), 26.8 ± 5.9 μg/mg creatinine (4 years of age), 26.4 ± 8.1 μg/mg creatinine (5 years of age), 28.1 ± 8.5 μg/mg creatinine (6 years of age), 22.9 ± 5.8 μg/mg creatinine (7 years of age), 19.8 ± 6.5 μg/mg creatinine (8 years of age), 19.4 6.1 μg/mg creatinine (9 years of age), 19.4 ± 6.8 μg/mg creatinine (10 years of age). Data shown as mean ± standard deviation.

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Sib 1 demonstrated typical MPS VI facial changes at age of 9 years and sib 2 had normal facial appearance at 3 years of age (Fig. 2). Sib 1 also had clawing hands at age of 9 years and sib 2 had mild claw hands deformity at 3 years of age (Fig. 3). Radiographs of the hands in sib 1 showed point-shaped metacarpal bones and in sib 2 showed mild point-shaped metacarpal bones (Fig. 4). The radiological findings of the spine are characteristic of MPS VI (Fig. 4). Defective development of the vertebral bodies with abnormal egg-shape and anterior beaking were present in sib 1 and there were no significant changes prior to and after ERT (Fig. 4). In sib 2, there were milder egg-shaped vertebral bodies with anterior beaking prior to and after ERT (Fig. 4).

Joint ROM of the shoulders, elbows, knees and hips was measured. The older sibling demonstrated the most significant improvement in shoulder ROM after 36 months of ERT. Right shoulder flexion increased from 105 degree before ERT to 180 degree after 36 months of ERT and abduction from 85 degree to 180 degree. The ROM in other joint was preserved. For the younger sibling starting ERT at age of 6 weeks, ROM of the shoulders (Fig. 5), elbows, knees and hips remained largely normal after 36 months of ERT except that there was a slight decrease in the hip flexion (data not shown).

Over 3 years of ERT, height and weight increased over time in both siblings (Fig. 6). Sib 1's growth rate continued to decelerate prior to ERT and showed catch-up growth after initiation of ERT. However, heights and weights in both siblings were lower than the corresponding average age-related heights and weights in a normal Japanese population.

The older sibling had a palpable liver and spleen edge prior to ERT and the distance of the liver edge below the right costal margin was 4 cm and the spleen edge below the left costal margin was 1 cm. The liver edge disappeared after 12 months of ERT and spleen edge disappeared after 9 months of ERT and both remained impalpable at 36 months of ERT. There was no palpable liver and spleen edge in the younger sibling prior to and during ERT treatment.

Auditory test was conducted every 6 months. An improvement in the hearing was observed in sib 1, in which threshold right moved from 45 dB before ERT to 20 dB after 36 months of treatment and threshold left moved from 45 to 25 dB. The hearing was normal in the sib 2 prior to ERT and remained normal. No otitis media occurred in the sib 2.

ECG was normal during treatment in both siblings. There was no ECG data in sib 2 at baseline. No axis deviation, conduction abnormalities, and ST-wave abnormalities were observed in both siblings at any point of assessment. ECHO in sib 1 revealed aortic valve thickening, mitral valve thickening, mild mitral valve and tricuspid valve regurgitation. Left ventricular posterior wall thickness (LVPWth) and left ventricular end diastolic dimension (LVDd) were moderately increased and ejection fraction (EF) was normal. His LV functions were preserved and there were no significant changes in other parameters during the 36 months of treatment. The younger sibling developed aortic valve thickening at the age of 6 months while she was on ERT, whereas LV size and function remained normal and no significant abnormalities were found in mitral and tricuspid valves.

Table 1 summarized the comparison data in both siblings before and after 36 months of ERT. As compared to sib 1, who had received no therapy at 3 years of age, sib 2 had normal facial appearance, minimal joint involvement and cardiac involvement.

4. Discussion

In this study, weekly infusions of Galsulfase into two MPS VI Japanese siblings, one starting at 6 weeks of age and the other at 5.6 years of age, were well tolerated. Only one episode of urticaria was observed in the younger sibling. After starting ERT, uronic acid levels were found to have quick and significant reduction as compared to the baseline in both siblings, which indicates effective clearance of accumulated GAG substrates. However, urinary uronic acid was not further reduced to normal level in both siblings. It is unclear whether increasing Galsulfase dosage can further reduce urinary uronic acid level.

ERT shows a clear benefit in preserving normal organ function and preventing disease progression normally seen in MPS VI patients. Growth of the younger sister is almost normal, and improvement of growth velocity is observed in older sibling after ERT. In the younger sibling, cardiac functions, normal facial morphology, and joint mobility were well preserved. Unlike her affected brother, most of typical MPS VI symptoms were absent in the younger sibling when she was 3 years old. Clear clinical benefits of ERT were also observed in the older sibling, including significant improvement in the shoulder range of motion and hearing loss. Our observation is consistent with the Australian sibling study, in which ERT was well tolerated in neonatal patient starting treatment at 8 weeks and ERT was associated with the absence of the development of scoliosis and preserved joint movement, cardiac valves and facial morphology [10]. The younger sibling developed mild claw hand deformity and slight decrease in the right hip flexion. Whether increase of Galsulfase dosage can prevent or further delay such skeletal manifestations merits further investigation.

Cardiac dysfunction is a major cause of morbidity and mortality across the spectrum of MPS VI patients. In the younger sibling, with enzyme treatment left ventricle size and function remained normal and valvular functions were well preserved. In the older sibling, valvular insufficiency was presented prior to and during treatment. His cardiac condition was stable and did not progress. The result suggests that earlier initiation of ERT might limit development or progression of cardiac valve disease. In a recent study of cardiac ultrasound in 53 patients with MPS VI, left ventricular dimensions and function remain normal and left ventricular septal wall thickness decreases

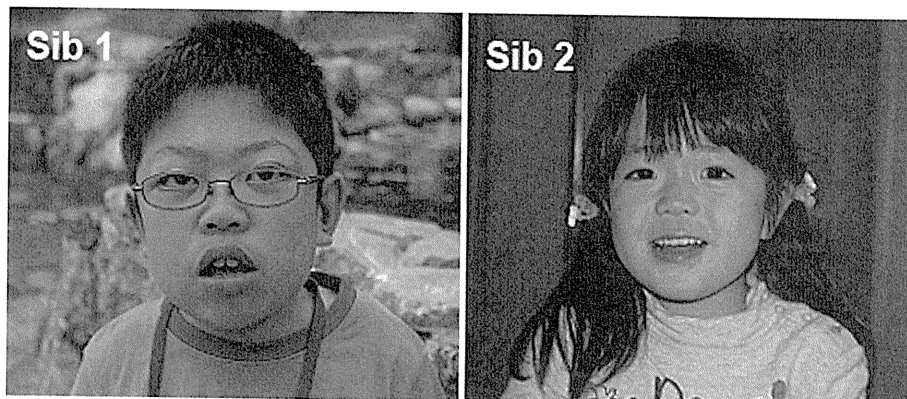

Fig. 2. Typical facial changes of MPS VI in sib 1 at age of 9 years and normal facial features in sib 2 at 3 years of age.

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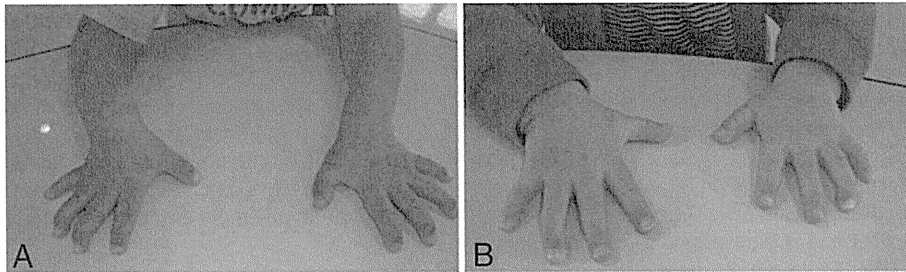

Fig. 3. Photograph showing the clawed hands in sib 1 at age of 9 years (A). Sib 2 had mild claw hands deformity at 3 years of age (B).

significantly after 96 weeks of ERT, possibly related to removal of GAG. However, ERT does not appear to improve valve disease. Earlier initiation of ERT might be necessary to prevent occurrence and progression of valve disease [11].

MPS VI often involves hearing impairment due to GAG deposits in the post-nasal space, eustachian tubes and middle ear [12]. Tokic et al. described improved hearing in a patient with MPS I receiving ERT after rejecting BMT at an early stage of the disease [13]. In this report, the auditory function of the older sibling was dramatically improved during the treatment. No auditory abnormalities were noted in the younger sibling. These data indicate that hearing impairment can be prevented in patients with MPS VI if enzyme replacement therapy is initiated early in life and can be partially normalized if the patient had developed such deficits.

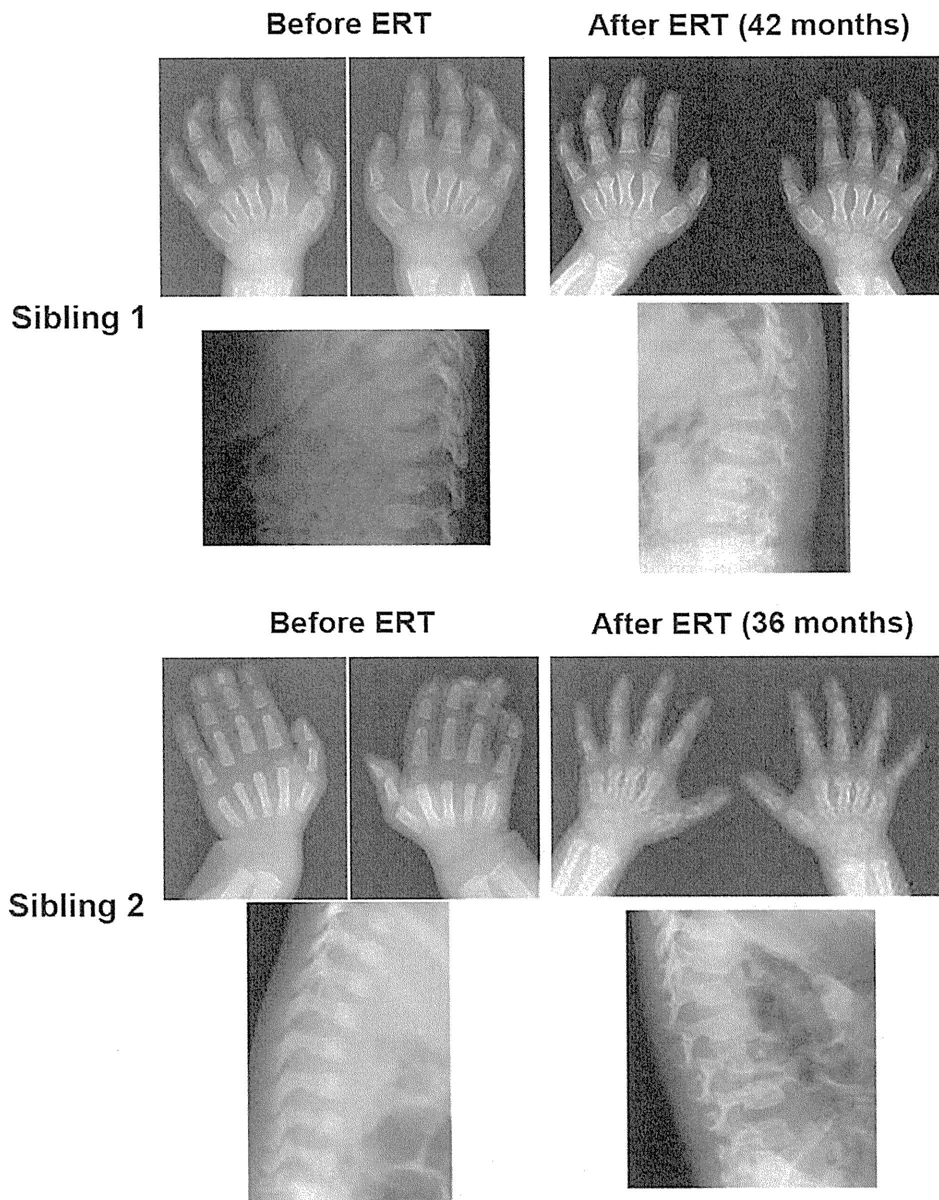

Fig. 4. Radiograph of hands and spinal column at baseline and after ERT in two siblings.

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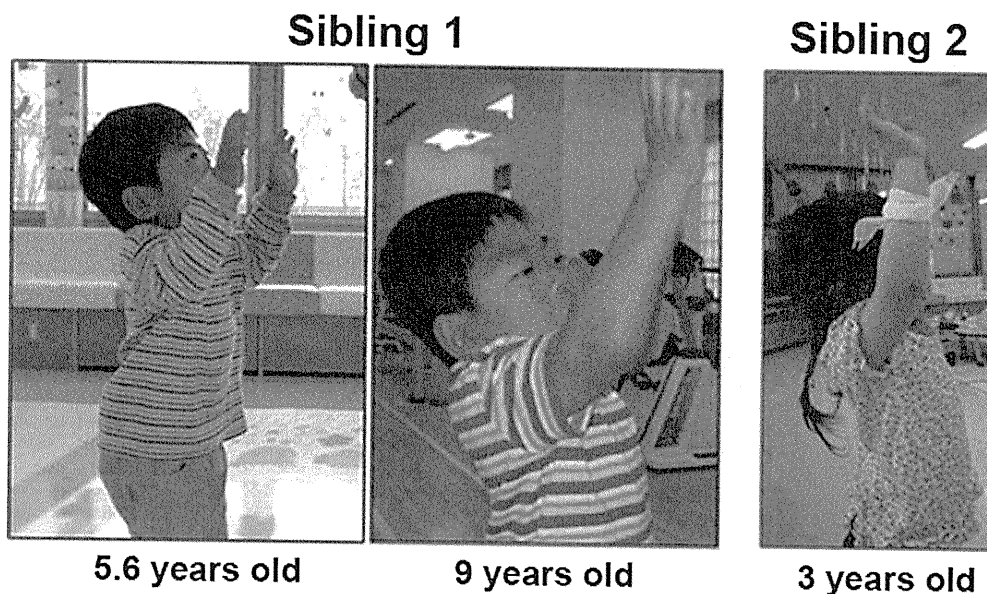

Fig. 5. Joint range of motion (ROM) in two siblings. Significant improvement in shoulder ROM after 36 months of ERT was observed in sib 1. Should ROM is normal in sib 2 at 3 years of age.

Growth failure is characteristic of untreated MPS VI. Growth plate failure in MPS disease may be related to abnormal GAG storage in chondrocytes leading to inflammatory response, cell dysfunction, and death [14,15]. A recent study has shown significant increase in height and growth rate in MPS VI patients receiving long-term ERT [16]. This impact was greatest in patients aged below 16 years. Height increase may result from bone growth and/or reduction in joint contractures. In the report, the growth rate of older sibling continued to decelerate prior to ERT and showed catch-up growth after initiation of ERT. However, the data is limited to 3 years post-ERT. Longer-

Fig. 6. Growth charts for height (cm) and weight (kg) of the two siblings at baseline and during ERT. Growth was expressed as height-for-age and weight-for-age *z* scores using the cross-sectional chart for Japanese. Height and weight in these charts are presented as a standard deviation of the mean.

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Table 1
Comparison of clinical signs and symptoms between sib 1 and sib 2 at baseline and 36 months after ERT.

	Sib 1		Sib 2	
	Baseline	36 Months after ERT	Baseline	36 Months after ERT
Facial appearance	Coarse face	Coarse face	Normal	Normal
Echocardiogram	EF not measured; normal aortic valve; mild mitral valve and tricuspid valve insufficiency	EF 66–67%; aortic valve thickening appeared at 12 months of ERT and remained unchanged; mitral and tricuspid valve thickening, level 1 mitral regurgitation and slight tricuspid regurgitation developed	EF 64%; normal aortic valve; slightly-abnormal mitral valve regurgitation and tricuspid valve regurgitation	Aortic valve thickening appeared at 6 months of ERT and then became normal; mitral and tricuspid valves normal
Ophthalmology	Moderate corneal clouding, glaucoma	Moderate corneal clouding	Normal	Mild corneal clouding
Audiology	Moderate hearing loss (around 45 dB)	Normal ABR with hearing improvements after ERT (hearing declined at 30 months of ERT but was improved with ear tube insertion; hearing improved further at 36 months of ERT)	Normal	Hearing tests remained normal at most assessments (except at 24 months of ERT when ABR test was abnormal and left ear 35 dB V-wave slightly extended)
Hand	Claw-hand deformity	Claw-hand deformity	Normal	Mild claw-hand deformity
Gait	Toe-walking	Toe-walking	Normal	Normal
ROM	Significant limitation in the shoulders, elbows, knees and hips	Significant improvement in shoulder, no deterioration in other joints	Normal	Slight decrease in the right hip flexion, no significant changes in other joints

term data and larger patient number will be needed to further demonstrate the effect of ERT on growth over time in the MPS VI population.

Both siblings in this report developed antibodies to Galsulfase. Sib 2 experienced only one episode of urticaria at 9th week of ERT before development of antibody and no drug-related AE and infusion-related reaction reported in sib 1. Therefore, there seems no correlation between antibody levels and IAR in this case. Despite the presence of antibody to the protein, both patients exposed to drug experienced positive clinical benefit. This is consistent with the finding from Galsulfase phase III study [6]. Therefore, there are no clinically significant safety concerns regarding the antibody.

ERT is not curative and may not improve all affected organs and systems in individuals when irreversible changes have developed. Thus, early diagnosis and treatment of MPS VI before development of an irreversible disease is critical for long-term favorable clinical outcome. The observations in this sibling report shows clear evidence that initiation of Galsulfase at very early age is effective in preventing disease progression of MPS VI. Dysostosis multiplex appeared milder, growth remained normal and joint mobility remained normal with early therapy. Moreover, facial dysmorphology was largely prevented. The clinical use of Galsulfase was demonstrated to be safe and effective in young Japanese patients with MPS VI including newborn.

Acknowledgments

We thank the following members of the Japanese MPS-VI Study Group:

Tadao Orii, Orii Clinic
Yoshikatsu Eto, The Tokyo Jikei University School of Medicine
Akemi Tanaka, Osaka City University
Yasuyuki Suzuki, Gifu University School of Medicine
Toya Ohashi, The Tokyo Jikei University School of Medicine
Toju Tanaka, National Center for Child Health and Development

This work was supported by the grants from the ministry of health, welfare and labor in Japan.

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Carnitine Palmitoyltransferase 2 Deficiency: The Time-Course of Blood and Urinary Acylcarnitine Levels during Initial L-Carnitine Supplementation

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Carnitine palmitoyltransferase 2 (CPT2) deficiency is one of the most common mitochondrial beta-oxidation defects. A female patient with an infantile form of CPT2 deficiency first presented as having a Reye-like syndrome with hypoglycemic convulsions. Oral L-carnitine supplementation was administered since serum free carnitine level was very low (less than 10 μmol/L), indicating secondary carnitine deficiency. Her serum and urinary acylcarnitine profiles were analyzed successively to evaluate time-course effects of L-carnitine supplementation. After the first two days of L-carnitine supplementation, the serum level of free carnitine was elevated; however, the serum levels of acylcarnitines and the urinary excretion of both free carnitine and acylcarnitines remained low. A peak of the serum free carnitine level was detected on day 5, followed by a peak of acetylcarnitine on day 7, and peaks of long-chain acylcarnitines, such as C16, C18, C18:1 and C18:2 carnitines, on day 9. Thereafter free carnitine became predominant again. These peaks of the serum levels corresponded to urinary excretion peaks of free carnitine, acetylcarnitine, and medium-chain dicarboxylic carnitines, respectively. It took several days for oral L-carnitine administration to increase the serum carnitine levels, probably because the intracellular stores were depleted. Thereafter, the administration increased the excretion of abnormal acylcarnitines, some of which had accumulated within the tissues. The excretion of medium-chain dicarboxylic carnitines dramatically decreased on day 13, suggesting improvement of tissue acylcarnitine accumulation. These time-course changes in blood and urinary acylcarnitine levels after L-carnitine supplementation support the effectiveness of L-carnitine supplementation to CPT2-deficient patients.

Keywords: carnitine palmitoyltransferase 2; CPT2; L-carnitine; acylcarnitine profile; carnitine administration
Tohoku J. Exp. Med., 2010, **221** (3), 191-195. © 2010 Tohoku University Medical Press

Carnitine palmitoyltransferase 2 (CPT2) deficiency (EC 2.3.1.21, OMIM 600650) is one of the most common disorders of mitochondrial fatty acid oxidation. CPT2 deficiency has several clinical presentations (Bonnefont et al. 1999). The adult form is characterized by episodes of rhabdomyolysis triggered by prolonged exercise. The infantile form presents as severe attacks of hypoketotic hypoglycemia, occasionally associated with sudden infant death or a Reye-like syndrome (Demaugre et al. 1991; Hug et al. 1991). The most severe kind, the neonatal form, is almost always lethal during the first month of life.

Secondary carnitine deficiency, characterized by low levels of total and free carnitines associated with an increase in the long-chain acylcarnitine fraction, is observed in the infantile form of CPT2-deficient patients (Bonnefont et al. 2004; Longo et al. 2006). Hence, L-carnitine supply might be useful in severe CPT2 deficiencies (Bonnefont et al. 2004), although supplementation with L-carnitine in patients with beta-oxidation defects of long-chain acyl-CoA has long been a matter of controversy (Costa et al. 1998;

Received March 23, 2010; revision accepted for publication May 17, 2010. doi:10.1620/tjem.221.191
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Liebig et al. 2006; Primassin et al. 2008).

In this report, we describe a CPT2-deficient patient who presented as having a Reye-like syndrome with secondary carnitine deficiency. We focused on time-dependent changes in the serum and urinary acylcarnitine profiles after intial L-carnitine supplementation.

Clinical Report

The patient, a female, was born to nonconsanguineous Japanese parents. She had been well until 15 months of age when she suddenly had tonic-clonic convulsions at 3:00 a.m. for about 30 minutes and became unconscious. Ten days before the convulsions, she had a cold and was given Cefteram pivoxil (CFTM-PI) for four days. When she arrived at another hospital, she had hypoglycemia (blood glucose 1.1 mmol/L), hepatic dysfunction (AST 85 IU/L, ALT 55 IU/L, LDH 402 IU/L), and mild hyperammoniemia (NH3 84 μmol/L). Urinary ketones were not detected. Brain MRI and cerebrospinal fluid were normal. She was suspected of being affected by a Reye-like syndrome and transferred to Gifu University Hospital.

On admission, her height was 72 cm (−1.5S.D.) and her weight was 10 kg (+0.73S.D.). She had a fever (38.3°C) and exhibited lethargy. Physical examination revealed mild hepatomegaly. A laboratory test showed AST 382 IU/L, ALT 441 IU/L, LDH 557 IU/L, PT 31%, NH3 84 μmol/L, and blood glucose 4.7 mmol/L.

We tentatively diagnosed her as having a Reye-like syndrome and treated her with intravenous glucose. Her consciousness level became clear on the 4th hospital day and she started oral intake of food. An abdominal CT scan still showed hepatomegaly and a fatty liver (20HU) on the 6th hospital day. The finding of cardiac ultrasonography was normal. Urinary organic acid analysis during the hypoglycemic condition showed hypoketotic dicarboxylic aciduria. The initial measurements of serum free carnitine and acyl-

Table 1. Time-course of serum and urinary acylcarnitine levels measured by tandem MS.

	Day	-1	3	5	7	9	13
Serum (μmol/L)	range						
C0	10 - 55	2.98	12.70	40.75	24.31	18.49	58.22
C2	4 - 60	2.25	3.85	14.87	20.15	8.37	14.8
C8	- 1.0	0.035	0.024	0.088	0.058	0.073	0.10
C8DC	- 0.25	0.035	0.046	0.12	0.89	0.97	0.063
C10	- 0.8	0.055	0.062	0.25	0.12	0.17	0.21
C10DC	- 0.1	0.063	0.12	0.24	0.33	0.53	0.19
C12:1	- 0.2	0.038	0.038	0.18	0.15	0.15	0.091
C12DC	- 0.05	0.053	0.064	0.19	0.14	0.27	0.054
C14:1	- 0.1	0.075	0.16	0.47	0.58	0.68	0.18
C16	- 0.5	1.01	1.29	2.99	4.45	8.07	2.56
C18	- 0.3	0.49	0.65	1.46	1.67	3.07	0.99
C18:1	- 0.46	1.50	1.84	4.21	6.09	10.03	3.62
C18:2	- 0.3	0.46	0.67	1.47	1.43	2.05	0.98
(C16+C18:1)/C2	- 0.36	1.12	0.81	0.48	0.52	2.16	0.42
C total		12.35	26.74	86.07	84.99	67.46	85.52
Urine (μmol/mmol Cr)	range*						
C0	5.67 - 56.09	0.61	1.31	82.33	37.85	45.95	329.15
C2	6.87 - 60.48	0.56	0.02	25.44	128.00	41.83	53.58
C4	0.07 - 0.74	0.31	0.47	0.92	0.47	1.38	2.32
C6	0.04 - 0.48	0.18	0.09	0.21	0.22	0.61	0.23
C6DC		1.25	1.34	1.63	15.69	83.33	2.93
C8	0.05 - 0.39	0.00	0.02	0.33	0.98	1.33	0.62
C8DC		0.25	0.52	0.83	23.90	122.99	1.11
C10	0.03 - 0.36	0.05	0.06	0.11	2.66	1.76	0.12
C10DC		0.11	0.02	0.10	0.75	4.03	0.08
C12DC		0.00	0.02	0.01	0.23	1.52	0.01
C16	0.05 - 1.55	0.04	0.02	0.02	0.18	0.63	0.08
C total		4.75	6.86	122.16	226.51	344.91	408.34

* Reference values for urine acylcarnitines were obtained from data reported by Mueller et al. (2003) (10th - 90th percentile)

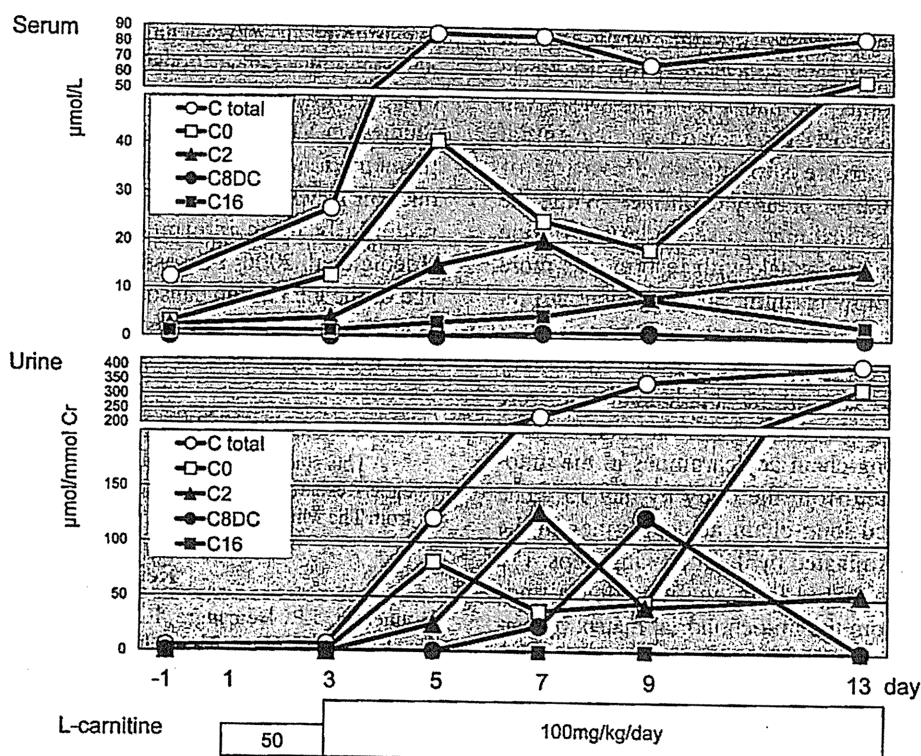

Fig. 1. Time-course of serum and urinary acylcarnitine levels measured by tandem MS. The levels of representative acylcarnitines are shown. The first day of L-carnitine supplementation is designated as day 1. Urinary carnitines were assayed using the first urine in the morning.

carnitine fractions by the enzymatic cycling method were 9.5 and 5.9 μmol/L, respectively. The initial serum acylcarnitine profile (Table 1) showed a very low free carnitine level and relatively high long-chain acylcarnitine levels. This profile was compatible with the secondary carnitine deficiency due to CPT2 or translocase deficiency.

After confirmation of the carnitine deficiency, we supplied her with L-carnitine orally from the 15th hospital day (day 1 in the Table 1 and Fig. 1) at a dose of 50 mg/kg/day for the first two days and 100 mg/kg/day from day 3. Blood and urinary samples were obtained before plus 3, 5, 7, 9 and 13 days after L-carnitine supplementation. During carnitine supplementation, the patient had continuous intravenous glucose infusion of 2.5 mg/kg/min until day 11. We analyzed the serum and urinary acylcarnitines by tandem mass analysis, as previously reported (Mueller et al. 2003; Kobayashi et al. 2007a,b). Table 1 shows details of the analyses. Fig. 1 shows the changing patterns of free carnitine (C0), acetyl-carnitine (C2), C8DC representing medium-chain dicarboxylic acylcarnitines, and C16 representing long-chain acylcarnitines in the serum and urine. Urinary excretion of C0 and acylcarnitines remained at very low levels on day 3. Sequential peaks of free carnitine (day 5), acetylcarnitine (day 7), and long-chain acylcarnitines (day 9) were found in the serum, which corresponded to peaks of free carnitine, acetylcarnitine, and dicarboxylic medium-chain acylcarnitines in the urine.

The fatty liver and hepatomegaly improved as judged by an abdominal CT scan on the 26th hospital day (day 13). Informed consent for a skin biopsy, enzyme assay, and DNA was obtained from the parents. CPT2 activity in the patient's fibroblasts was 0.18 nmol/min/mg of protein (3 controls; 0.82, 1.27, and 1.26 nmol/min/mg of protein), confirming the diagnosis of CPT2 deficiency.

Now the patient is 4 years of age. After carnitine supplementation, she did not experience hypoglycemia at all. She is being treated with 1,000 mg L-carnitine/day (current body weight 19.8 kg). Her growth and development are within normal ranges. She had some rhabdomyolysis attacks (the highest CK recorded was 16,769 IU/L) during a febrile illness even after L-carnitine supplementation.

Discussion

The diagnosis of CPT2 deficiency was first suspected by the data on urinary organic acid analysis and acylcarnitine analysis and was confirmed by enzyme assay using fibroblasts. Our patient is a compound heterozygote of a previously reported E174K mutation from the father and an unknown mutation from the mother which was not detected by exon sequencing. According to an in vitro expression analysis of mutant CPT2 cDNAs carrying E174K, the mutant E174K protein was present as much as a wild type protein and retained 10% residual CPT2 activity (Wataya et al. 1998). This "mild" mutation from the father, together with possible null mutation from the mother, may result in an infantile form of CPT2 deficiency.

Initially, she developed secondary carnitine deficiency. Chronic administration of pivalate-conjugated antibiotics is

a major cause of secondary carnitine deficiency even in healthy children (Stanley 2004). Ten days before the onset of the Reye-like syndrome, she had a cold and was given Cefteram pivoxil (CFTM-PI) for four days. The initial serum acylcarnitine profile showed no elevation of hydroxy-C5 carnitine, nor of pivaloylcarnitine. While the antibiotic might have contributed to secondary carnitine deficiency in part, the acute attack with fasting was more likely the course of the low carnitine in the patient at presentation.

The time-course changes in the serum and urinary acylcarnitine levels after L-carnitine supplementation were studied. These changing profiles suggest that accumulated and potentially toxic long-chain acylcarnitines in the mitochondria were eliminated from the body by day 13. The majority of accumulated long-chain acylcarnitines in the mitochondria may be eliminated by the following steps: 1) a large amount of accumulated long-chain acylcarnitines should be transferred from the mitochondrial matrix by carnitine acylcarnitine translocase if there is a sufficient amount of free carnitine outside of the mitochondrial matrix; 2) then peroxisomal beta-oxidation reduces the chain length of such accumulated fatty acids; 3) the resultant medium-chain fatty acids can be catalyzed in the mitochondria, or further ω-oxidized into dicarboxylic acids in the microsomes; 4) these medium-chain DC and their carnitine conjugates can be excreted into the urine efficiently. It took several days for oral L-carnitine administration to increase the serum carnitine levels, probably because the intracellular stores were depleted and it took several days for them to be replenished. Thereafter, the administration increased the excretion of abnormal acylcarnitines, some of which had probably accumulated within the tissues.

It is noteworthy that the acetylcarnitine in both the serum and the urine was a predominant acylcarnitine on day 7 (Fig. 1). Elevation of acetylcarnitine in the serum and urine indicates the presence of enough acetyl-CoA in the mitochondria and the availability of acetyl-CoA for carnitine acyltransferase reactions in the cells, and might account for the increased beta-oxidation rates upon L-carnitine therapy (Fontaine et al. 1996). In general, acetylcarnitine is a major acylcarnitine in healthy controls and is regarded as a marker of undisturbed beta-oxidation (Costa et al. 1998). Since CPT2-deficient patients have beta-oxidation restrictions of long-chain acyl-CoA, L-carnitine supplementation may increase beta-oxidation of medium-chain acyl-CoAs, which could be supplied via peroxisomal beta-oxidation of long-chain acyl-CoA.

Carnitine supplementation in the treatment of long-chain beta-oxidation defects is still controversial. In patients with a defect in the mitochondrial beta-oxidation spiral, when a preceding L-carnitine deficiency is normalized, and transport into the mitochondria of long-chain fatty acids is also normalized, acyl-CoAs accumulate instead of being oxidized by the defective reaction and, consequently, in such cases, free CoA is depleted in the mitochondria (Yoshino et al. 2003). This may be true in beta-oxidation defects such as very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency and trifunctional protein deficiency. Studies on VLCAD-deficient mice suggested carnitine supplementation results in the induction of acylcarnitine production in various tissues and significant accumulation of potentially toxic intermediate acylcarnitines in tissues (Liebig et al. 2006; Primassin et al. 2008). However, blockage of the CPT2 step causes the accumulation of long-chain acylcarnitines but does not primarily cause the accumulation of intermediate CoA esters in beta-oxidation.

Acknowledgment

This study was supported in part by Health and Labour Science Research Grants for Research on Children and Families from The Ministry of Health, Labour and Welfare of Japan.

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Brain & Development 32 (2010) 409–411

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

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Case report

Molecular analysis of a presymptomatic case of carnitine palmitoyl transferase I (CPT I) deficiency detected by tandem mass spectrometry newborn screening in Japan

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Received 4 January 2009; received in revised form 24 February 2009; accepted 5 March 2009

Abstract

Carnitine palmitoyl transferase I (CPT I) deficiency is a rare disorder of long-chain fatty acid oxidation. It is one of the metabolic diseases detectable by tandem mass spectrometry. We report herein a presymptomatic CPT I deficiency detected in a Japanese female newborn by tandem mass spectrometry newborn screening. A mutation analysis of the *CPT1A* gene revealed two novel mutations, p.R446X and p.G719D.
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Keywords: Carnitine palmitoyl transferase I; CPT IA; Tandem mass spectrometry; Newborn screening

1. Introduction

The β-oxidation of long-chain fatty acids is an important source of energy production, especially during times of increased energy demand, such as fasting, illness, or prolonged exercise. Carnitine palmitoyl transferase I (CPT I) is the key enzyme of long-chain fatty acid oxidation. CPT I deficiency generally occurs with febrile or gastrointestinal illness, when energy demands are increased. Clinical symptoms range from recurrent hypoketotic hypoglycemia to Reye-like syndrome and sudden death [1].

More than 20 metabolic diseases, CPT I deficiency among them, can now be screened by tandem mass spectrometry on dried blood spots [2]. CPT I deficiency is characterized by decreased levels of long-chain acyl-carnitines such as palmitoylcarnitine (C16) and stearoylcarnitine (C18), and increased levels of free carnitines (C0). According to a tandem mass spectrometry pilot study in Japan, the deficiency is detected in about 1 out of every 200,000 newborns.

We herein report a patient with presymptomatic CPT I deficiency who was discovered by tandem mass spectrometry newborn screening. The results of sequencing analysis of *CPT1A* gene revealed a novel nonsense mutation (p.R446X) and a novel missense mutation (p.G719D).

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0387-7604/$ - see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.braindev.2009.03.004

2. Case report

The patient is the first child of healthy nonconsanguineous Japanese parents with no family history of metabolic disease or neuromuscular disease. At the late-phase of pregnancy, intrauterine growth retardation was detected. The patient was born by cesarean section because of breech presentation. Her birth weight, height, and head circumference were 2230 g, 48.0 cm, and 32.0 cm, respectively.

The patient was admitted to our hospital at 1 month of age, when tandem mass spectrometry newborn screening disclosed an elevation in free carnitine (C0 140 μM: cutoff, lower than 90) and a decreased level of palmitoylcarnitine (C16 0.03 μM). Hypotonia and hepatomegaly were absent on physical exanimation. Her body weight gain was about 40 g/day, with breast milk feeding. Biochemical testing uncovered no particular abnormal findings. The carnitine profile in dried blood spots revealed an elevation of free carnitine (C0 105 μM) and decreased levels of long-chain acyl-carnitines (C16 0.09 μM, C18 0.043 μM). The ratio of free carnitine to the sum of long-chain acyl-carnitines {C0/(C16 + C18)} was 789, which suggested a diagnosis as CPT I deficiency (cut off <100). No metabolic acidosis (pH 7.357, PCO_2 42.1 mmHg, HCO_3 23.6 meq/L, BE −2), hypoglycemia (blood sugar 105 mg/dl), or renal tubular acidosis was observed. Urine organic acid analysis was normal.

Enzymatic analysis in blood revealed a low level residual CPT I activity of 11–26% of control. Sequencing analysis of 18 exons from exon 2 to exon 19 in the *CPT1A* gene was performed with the written informed consent of her parents. The results showed two novel mutations: c.1339C > T (p.R446X) in exon 11 and c.2156G > A (p.G719D) in exon 18 (Fig. 1). The p.R446X mutation was transmitted from her father; the other mutation (p.G719D) was transmitted from her mother (data not shown).

The patient is now given a low-fat diet with supplementation of medium-chain triacylglycerol (MCT) milk. On earlier occasions when she fell sick, hypoglycemia was prevented by early intervention with glucose infusion. On the latest examination at 3 years, her psychomotor development was appropriate for her age.

3. Discussion

Most CPT I-deficient patients present recurrent episodes of coma and seizure due to hypoketotic hypoglycemia. With tandem mass spectrometry newborn screening, patients in a presymptomatic state can be detected. Our patient seems to have developed normally, without severe metabolic crisis, up to the present. Tandem mass spectrometry screening allows early medical intervention for patients with fatty acid oxidation defect.

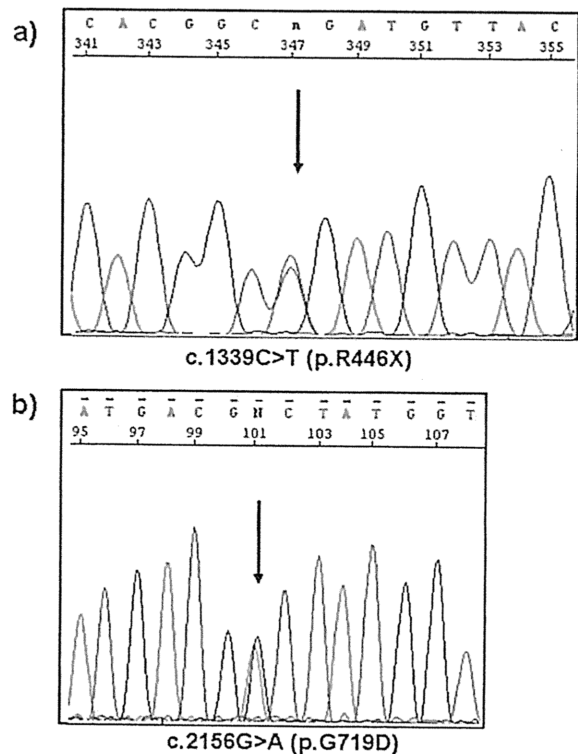

Fig. 1. (a) A C-to-T substitution at c.1139 in exon 11 was detected in a heterozygous pattern. This c.1339C > T substitution created a stop codon (p.R446X). (b) A G-to-A substitution at c.2156 in exon 18 was found in a heterozygous pattern. The c.2156G > A substitution changed the codon of glycine at 719 to aspartic acid (p.G719D).

Enzyme assay and/or mutational analysis are necessary to confirm the diagnosis. In most individuals with CPT I deficiency, residual enzyme activity is 1–5% of control [3]. In contrast, the residual enzyme activity of the myopathic type of the Inuit is 15–25%. Our patient had residual activity of 11–26% of control, a level as high as that of the myopathic type.

More than 20 mutations responsible for the CPT I deficiency have been identified in the *CPT1A* gene [4–6]. Most of the mutations seem to be unique or restricted to only a few pedigrees, except p.G710Q in the Hutterite population and p.P479L in the Inuit population [3,7]. Sequencing for the present patient revealed a novel nonsense mutation (p.R446X) and a novel missense mutation (p.G719D). The p.G719D mutation proved to be absent in 50 unrelated controls (data not shown). The glycine at 719 of CPT I is conserved in mouse, rat, horse, and zebra fish. These data suggest the substitution appears not to be a polymorphism, but a disease-causing mutation. A clear genotype–phenotype correlation has been reported only between the p.P479L mutation (common mutation in Inuit) and adult-onset myopathic presentation with high residual activity. The data on our present patient suggest that the mutant pG719D-CPT I protein might have relatively high residual activity, as the other mutation was a nonsense mutation. An

expression study will be necessary to confirm this hypothesis.

Acknowledgments

This work was partly supported by grant from The Ministry of Health, Labor and Welfare of Japan.

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Molecular Genetics and Metabolism 104 (2011) 556–559

Contents lists available at SciVerse ScienceDirect

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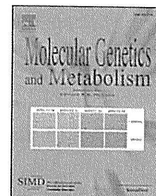

A patient with mitochondrial trifunctional protein deficiency due to the mutations in the *HADHB* gene showed recurrent myalgia since early childhood and was diagnosed in adolescence☆

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ARTICLE INFO

Article history:
Received 5 August 2011
Received in revised form 20 September 2011
Accepted 20 September 2011
Available online 28 September 2011

Keywords:
Rhabdomyolysis
Fatty acid oxidation
Mitochondrial trifunctional protein (MTP)
Long-chain 3-ketoacyl-CoA dehydrogenase (LKAT)
HADHB gene

ABSTRACT

Mitochondrial trifunctional protein (MTP) is a multienzyme complex involved in the metabolism of long-chain hydroxyacyl-CoA, a product of the fatty acid β-oxidation cycle. MTP is an α4β4 hetero-octomer encoded by two different genes: *HADHA* (OMIM 600890) and *HADHB* (OMIM 143450). MTP deficiency induces three different types of presentation: (1) a lethal phenotype with neonatal onset (severe); (2) a hepatic phenotype with infant onset (intermediate); and (3) a neuromyopathic phenotype with late-adolescent onset (mild). While acylcarnitine analysis has revealed increased levels of long-chain hydroxyacylcarnitine in blood when an MTP deficiency exists, the neuromyopathic type is usually asymptomatic and does not always result in an abnormality in acylcarnitine analysis results. We report here the case of a 13-year-old girl with recurrences of intermittent myalgia since her early childhood, for whom the disorder had not been definitely diagnosed. Since she was referred to our hospital because of rhabdomyolysis, we have repeatedly performed blood acylcarnitine analysis and found slight increases in long-chain 3-OH-acylcarnitine levels, on the basis of which we made a chemical diagnosis of MTP deficiency. Immunoblot analysis of skin fibroblasts revealed loss of α- and β-subunits of MTP. In addition, analysis of the *HADHB* gene, which encodes long-chain 3-ketoacyl-CoA thiolase, one of the enzymes constituting MTP, identified compound heterozygous mutations of c.520 C>T (p.R141C) and c.1331 G>A (p.R411K).

MTP deficiency is considered an extremely rare disorder, as only five cases (lethal phenotype, two patients; hepatic phenotype, two patients; and neuromyopathic phenotype, one patient) have thus far been reported in Japan. However, it is likely that the neuromyopathic phenotype of MTP deficiency has not yet been diagnosed among patients with recurrences of intermittent myalgia and rhabdomyolysis, as in our patient reported here.

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1. Introduction

Mitochondrial trifunctional protein (MTP) is a multienzyme complex involved in the metabolism of long-chain hydroxyacyl-CoA, a product of the fatty acid β-oxidation cycle [1]. When an abnormality exists in this complex, the fatty acid β-oxidation cycle fails to supply an adequate amount of energy, resulting in three different types of presentation: a lethal phenotype with neonatal onset, in which hepatic and/or cardiac muscular disturbance occurs early in infancy, causing sudden death; a hepatic phenotype with infant onset, in which non- or low-ketotic hypoglycemia occurs; and a neuromyopathic phenotype with late-adolescent onset, in which muscular symptoms such as intermittent myalgia or rhabdomyolysis occur [2,3]. The diagnosis is based on increased levels of long-chain 3-OH-acylcarnitine, as demonstrated by blood acylcarnitine analysis using ESI-MS/MS. However, the neuromyopathic phenotype is usually asymptomatic and frequently shows no abnormal test results; therefore, its definite diagnosis may require an extended length of time.

A 13-year-old girl with repeated myalgia since early childhood, in whom MTP deficiency had not been diagnosed, was referred to our hospital because of rhabdomyolysis. We repeatedly performed blood acylcarnitine analysis, found slight increases in long-chain 3-OH-acylcarnitine levels, and finally diagnosed MTP deficiency by Western blot and genetic analysis.

☆ Databases: HADHA OMIM:600890, GDB:434026, GenBank:NM_000182HADHB OMIM:143450, GDB:344953, GenBank:NM_000183

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doi:10.1016/j.ymgme.2011.09.025

2. Materials and methods

2.1. Case

The 13-year-old girl was the first child of non-consanguineous parents, and was born at 42 weeks of gestation after an uneventful pregnancy. Her birth weight was 3032 g. One month after birth, an apparent life-threatening event (ALTE) developed; however, she showed normal growth and development. From the age of 3 years, she complained of pain in her leg muscles after walking over a long distance. From the age of 9 years, the frequency of intermittent acute muscle pain increased and even mild exercise occasionally caused severe discomfort in her leg muscles. The symptom was often triggered by infection and menstruation. She experienced bouts of muscle pain after hard exercise such as running on a school field day or hiking on a school excursion. After hard exercise, she felt difficulty in moving because of unusual severe pain in her generalized muscles. Serum acylcarnitine analysis performed at 10 years of age revealed slightly increased levels of long-chain acylcarnitine, on the basis of which very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency was suspected. However, her VLCAD activity was normal and the disorder had not been definitely diagnosed.

At the age of 13 years, she was admitted to Kobe University Hospital with severe myalgia over the whole body. On admission, hematological and biochemical investigations revealed markedly elevated serum creatine kinase (22,885 IU/L), aldolase (62.6 IU/L), and myoglobin (2960 ng/mL). Plasma free and total carnitine and total keton bodies concentration were normal (Table 1). Echocardiography and electocardiography did not reveal any evidence of a cardiomyopathy.

Table 1
Laboratory findings upon hospitalization.

<Blood biochemistry>			Reference range
AST	529	IU/L	13–31
ALT	205	IU/L	8–34
LDH	909	IU/L	115–217
CK	22,885	IU/L	46–168
CK-MB	434	IU/L	0–25
Aldolase	62.6	IU/L	2.2–5.5
Myoglobin	2960	ng/mL	0–60
BUN	11	mg/dL	9–22
Cre	0.34	mg/dL	0.5–1.3
T.chol	167	mg/dL	146–219
TG	58	mg/dL	28–149
Glu	180	mg/dL	61–92
Lactic acid	19.1	mg/dL	3–17
Pyruvic acid	1.47	mg/dL	0.3–0.94
Total ketone bodies	39	μmol/L	26–122
Acetoacetate	24	μmol/L	13–69
β-hydroxy butylate	15	μmol/L	0–76
Total carnitine	57	μmol/L	45–91
Free carnitine	45.7	μmol/L	36–74
Acylcarnitine	11.3	μmol/L	6–23
<Acylcarnitine analysis>			Reference range
(Blood spot)			
C14:1	0.21	μM	<0.4
C14-OH	0.12	μM	<0.12
C16:1	0.15	μM	<0.785
C16:0-OH	0.18	μM	<0.12
C18-OH	0.066	μM	<0.1
C18:1-OH	0.15	μM	<0.07
(Serum)			
C14:1	0.4	μM	<0.1
C14-OH	0.2	μM	<0.1
C16:1	0.2	μM	<0.1
C16:0-OH	0.16	μM	<0.8
C18-OH	0.077	μM	<0.05
C18:1-OH	0.13	μM	<0.7

2.2. Methods

2.2.1. Urine organic acid and acylcarnitine analysis

Urine organic acid and blood acylcarnitine analysis from dried blood spots and serum were performed by GC/MS and ESI-MS/MS, respectively, as described in detail previously [4].

2.2.2. Cell culture

Fibroblasts from the patient were cultured in Eagle's minimum essential medium containing 10% fetal calf serum and antibodies (100 μg/mL each of penicillin and streptomycin; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan).

2.2.3. Western blot analysis

Western blot analysis was performed following 12.5% SDS/PAGE [5] using rabbit polyclonal antibodies raised against purified MTP protein as the primary antibody. Bound antibodies were visualized using the ImmunoPure NBT/BCIP Substrate Kit (Promega, Madison WI, USA). Protein concentrations were determined using the Bio-Rad protein assay protocol (Bio-Rad Laboratories, Hercules, CA).

2.2.4. Mutation analysis

Genomic DNA was extracted from the patient's fibroblasts using a QIAamp DNA Micro Kit (Qiagen GmbH Hilden, Germany). We designed 20 sets of primers for amplification of *HADHA* (one for each exon, including 5′ and 3′ splice sites), and 16 sets for amplification of *HADHB*. Each exon was amplified by polymerase chain reaction (PCR) and directly sequenced as described previously [6].

3. Results

3.1. Urine organic acid and acylcarnitine analysis

Urine organic acid analysis performed at the same time revealed slight ketosis (as indicated by slightly increased excretion of 3-OH-butyrate), low-ketotic dicarboxylic aciduria (as indicated by increased excretion of adipate and suberate), and 3-OH dicarboxylic aciduria (as indicated by increased excretion of 3-OH-sebacate and 3-OH-dodecanedioate).

Acylcarnitine analysis of blood spots at the time of hospitalization revealed increases in the levels of long-chain 3-OH-acylcarnitines (C14-OH, C16-OH, C18-1-OH). The changes indicated an MTP deficiency. On the other hand, elevation of both long-chain acylcarnitines (C14-1, C16-1) and long-chain 3-OH-acylcarnitines (C14-OH, C18-OH) in serum suggested a VLCAD deficiency (Table 1). These findings strongly suggested long-chain fatty acid metabolism disorder, VLCAD or MTP deficiency, however, it was difficult to distinguish between the two. So acylcarnitine analysis was performed another four times, with inconsistent results: slightly increased levels of long-chain 3-OH-acylcarnitine (C16-OH, C18-1-OH), a feature of MTP deficiency, were noted on two occasions, while no abnormality was noted on the other two occasions. On the basis of these results and her clinical manifestations, we made a chemical diagnosis of a mild form of MTP deficiency, in which neither symptoms nor abnormal laboratory findings are noted during attack-free intervals.

3.2. Western blot analysis

Western blot analysis using samples extracted from the patient's skin fibroblasts detected neither α- nor β-subunits of MTP (Fig. 1), whereas both subunits were detected in control fibroblasts. These findings showed that the patient had an MTP deficiency.