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Japan Elaprase® Treatment (JET) study: Idursulfase enzyme replacement therapy in adult patients with attenuated Hunter syndrome (Mucopolysaccharidosis II, MPS II)

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ABSTRACT

This open-label clinical study enrolled 10 adults with attenuated Mucopolysaccharidosis II and advanced disease under the direction of the Japan Society for Research on Mucopolysaccharidosis Disorders prior to regulatory approval of idursulfase in Japan. Ten male patients, ages 21–53 years, received weekly intravenous infusions of 0.5 mg/kg idursulfase for 12 months. Significant reductions in lysosomal storage and several clinical improvements were observed during the study (mean changes below). Urinary glycosaminoglycan excretion decreased rapidly within the first three months of treatment and normalized in all patients by study completion (−79.9%). Liver and spleen volumes also showed rapid reductions that were maintained in all patients through study completion (−33.2% and −31.0%, respectively). Improvements were noted in the 6-Minute Walk Test (54.5 m), percent predicted forced vital capacity (3.8 percentage points), left ventricular mass index (−12.4%) and several joint range of motions (8.1–19.0 degrees). Ejection fraction and cardiac valve disease were stable. The sleep study oxygen desaturation index increased by 3.9 events/h, but was stable in 89% (8/9) of patients. Idursulfase was generally well-tolerated. Infusion-related reactions occurred in 50% of patients and were mostly mild with transient skin reactions that did not require medical intervention. Two infusion-related reactions were assessed as serious (urticaria and vasovagal syncope). One patient died of causes unrelated to idursulfase. Anti-idursulfase antibodies developed in 60% (6/10) of patients. In summary, idursulfase treatment appears to be safe and effective in adult Japanese patients with attenuated MPS II. These results are comparable to those of prior studies that enrolled predominantly pediatric, Caucasian, and less ill patients. No new safety risks were identified.

Introduction

Mucopolysaccharidosis type II (MPS II, Hunter syndrome, OMIM #309900) is an X-linked recessive, lysosomal storage disorder caused by a deficiency of iduronate-2-sulfatase (IDS, EC3.1.6.13). This lysosomal enzyme catalyzes the first step in the degradation of the glycosaminoglycans (GAG), dermatan sulfate and heparan sulfate [1]. Iduronate-2-sulfatase deficiency leads to the accumulation of GAG within the lysosomes of virtually every cell in the body and is excreted in excessive amounts in the urine. MPS II encompasses a wide phenotypic spectrum that includes severe and attenuated forms. The severe form has onset of symptoms by 2–4 years old, progression of somatic symptoms and severe cognitive impairment during childhood, and death by 10–15 years of age. The attenuated form has a later onset in childhood, slower and milder progression of somatic disease, little to no cognitive impairment, and survival into adulthood. (Fig. 1) Common clinical features include coarse faces, upper airway obstruction, cardiac valve regurgitation, restrictive lung disease, hepatosplenomegaly, hernias, joint contractures, poor endurance, and reduced quality of life [2,3]. *IDS* gene mutations are heterogeneous, but some show genotype–phenotype correlations: deletions and gross rearrangements of the *IDS* gene are associated with the severe form, whereas missense

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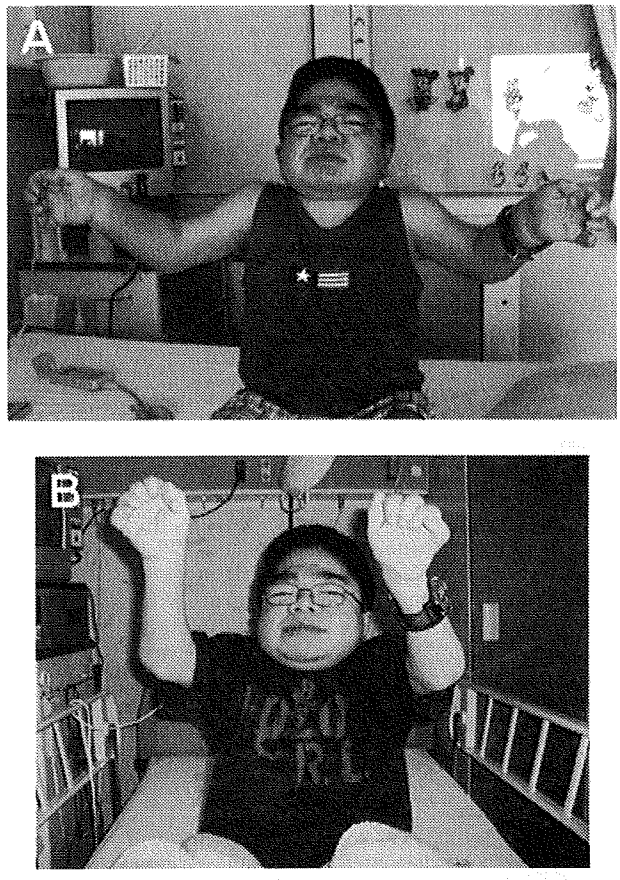

Fig. 1. A 23-year-old Japanese male study patient with MPS II. (A) Before treatment. (B) After 12 months of idursulfase treatment. Note the coarse facial features characteristic of MPS II. At baseline, the patient had severely limited shoulder range of motion (flexion and abduction), which improved following treatment.

mutations are more often associated with attenuated disease [4–10]. No racial or geographic differences have been observed. Females are only rarely affected, most often through skewed X-inactivation [1]. MPS II is the most prevalent MPS disorder in Asia, accounting for >50% of all MPS patients in Japan [10]. The annual incidence of all MPS disorders in Japan is estimated to be 1/50,000–1/60,000, and approximately half of the cases are due to MPS II. The estimated birth incidence of MPS II in Japan is, therefore, 1/90,000–1/100,000 [11], similar to the 1/92,000 to 1/162,000 incidences reported for predominantly Caucasian countries [12–15].

Until recently, treatment of MPS II was mainly palliative and focused on alleviating clinical symptoms through a variety of surgeries, medical devices, therapies, and medications. Several patients have undergone hematopoietic stem cell transplant (HSCT) as a source of iduronate-2-sulfatase, but unlike for MPS I, cognitive decline is not halted and the long-term effects on somatic disease are not well-documented [16,17]. Therefore, most centers consider the risk–benefit profile unfavorable and do not recommend HSCT for patients with MPS II.

Idursulfase (Elaprase®, Shire Human Genetic Therapies, Inc., Cambridge, MA, USA) is a recombinant human form of iduronate-2-sulfatase that is produced in a human cell line. Preclinical studies carried out in an MPS II knockout-mouse model [18] and in a Phase 1/2 dose-ranging study of MPS II patients [19] indicated that idursulfase was effective at reducing lysosomal GAG. The safety and efficacy of idursulfase was confirmed in a Phase 2/3 double-blind, placebo-controlled clinical study that randomized 96 MPS II patients to one of three treatment arms for 52 weeks: 0.5 mg/kg idursulfase weekly, 0.5 mg/kg idursulfase alternating with placebo every other week, or placebo weekly [20]. The primary efficacy endpoint was a composite of changes in percent predicted forced vital capacity (FVC) and the 6-Minute Walk Test (6MWT). Patients who received weekly idursulfase showed a greater difference in the composite endpoint compared to placebo ($p = 0.005$) than did the every other week idursulfase group ($p = 0.042$). The weekly idursulfase arm showed a mean 44.3 m increase in 6MWT distance (37 m difference from placebo, $p = 0.013$) and a mean 3.45 percentage point increase in percent predicted FVC (2.7 percentage point difference from placebo, $p = 0.065$). These clinical changes were associated with significant reductions versus placebo in urinary GAG level (−52.5%, $p < 0.0001$), liver volume (−25.3%, $p < 0.0001$), and spleen volume (−25.1%, $p < 0.0001$). Idursulfase was well-tolerated, with infusion-related reactions being the most common drug-related related adverse events, occurring in 69% (22/32) of patients in the weekly idursulfase arm.

Idursulfase was approved for the treatment of MPS II by the United States Food and Drug Administration (FDA) in July 2006 and by the European Medicines Agency (EMEA) in January 2007. Due to the life-threatening nature of the disease and the small number of patients, the Japanese Ministry of Health, Labour, and Welfare (MHLW) Committee for the Use of Unapproved Drugs recommended that idursulfase be approved based on ethical grounds and the results of overseas clinical trials, which included four Japanese patients. The committee also requested that idursulfase be made available to the most seriously ill MPS II patients prior to approval, which occurred in October 2007. Consequently, the Japan Elaprase Treatment (JET) study was initiated under the direction of the Japan Society for Research on MPS Disorders. Here, we present the results of this study.

Materials and methods

Patients

To be eligible for the study, patients had to meet all of the following inclusion criteria: (1) Documented deficiency of iduronate-2-sulfatase enzyme activity of <10% of the lower limit of normal with a normal enzyme activity level of one other sulfatase. (2) Male and above 20 years of age. (3) Clinically advanced disease status with <80% predicted FVC and New York Heart Association Class II–IV. (4) Capable of showing improved quality of life. (5) Able to complete study assessments.

Patient exclusion criteria included: (1) Previous bone marrow or cord blood transplant. (2) Known hypersensitivity to one of the components of idursulfase. (3) Previous treatment with idursulfase. (4) Unable to receive weekly infusions of idursulfase at the patient's local hospital. All patients provided signed informed consent prior to enrollment.

Study design

This was a multi-center, open-label study that enrolled 10 adult males with MPS II at 5 clinical sites in Japan. The study adhered to the guidelines set forth in the Declaration of Helsinki. Idursulfase was manufactured by Shire Human Genetic Therapies, Inc. and distributed by Genzyme Corporation (Cambridge, MA, USA). Genzyme Corporation performed all statistical analyses, and Genzyme Japan KK (Tokyo, Japan) provided data management support.

Idursulfase

Patients were administered 0.5 mg/kg idursulfase diluted in saline to a final volume of 100 cc intravenously over 3 h on a weekly

basis (±3 days) for up to 12 months. Infusions rates were ramped up over the first hour as described in the Phase 2/3 study [20]. Patients were monitored during each infusion and were discharged 1 h after completing the infusion, if clinically stable.

Efficacy assessments

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) or at Osaka City University Hospital. Liver and spleen volumes were quantitated by computerized tomography (CT), with the upper limits of normal being 2.5% and 0.2% of body weight, respectively. Percent predicted FVC and the 6MWT were performed according to American Thoracic Society guidelines [21,22]. Cardiac structure and function were evaluated by echocardiography (two-dimensional and M-mode). Left ventricular mass index (LVMI) was calculated as the left ventricular mass normalized for body surface area, with normal values defined as <131 g/m^2. Active joint range of motion was measured by goniometry, and included the shoulder (flexion, extension, and abduction), elbow (flexion and extension), hip (flexion and extension), and knee (flexion and extension). Left and right joint ranges of motion for each were averaged for each patient. The sleep study oxygen desaturation index (ODI) was assessed by pulse oximetry and defined as the number of desaturations (<89% oxygen saturation or ⩾4% decrease in oxygen saturation from baseline lasting ⩾10 s) per hour of sleep. A normal ODI was considered to be <5 events/h [23].

Safety assessments

Safety evaluation included continuous monitoring of adverse events and periodic clinical laboratory and physical examination evaluations. Adverse events were reported by severity (mild, moderate, severe, life-threatening) and by relatedness to idursulfase. An infusion-related reaction was defined as any adverse event occurring during or following an infusion (i.e., within 24 h of infusion initiation) that was reported by the investigator as related to idursulfase. Antibodies to idursulfase were measured by an enzyme-linked immunosorbant assay (ELISA; Shire Human Genetic Therapies).

Statistics

Efficacy results are reported as the mean ± standard error of the mean (SEM). For missing data at 12 months, the last observation carried forward method was used for values obtained at 6 months or later. The number of evaluable patients was at least nine for each endpoint, except for LVMI ($n = 6$, primarily due to missing baseline data) and the 6MWT ($n = 7$, primarily due to the inability to perform the test). The Wilcoxon signed rank test was used to evaluate changes in efficacy endpoint from baseline to 12 months, and p-values <0.05 were considered statistically significant. Percent change was tested for pharmacodynamic parameters (i.e., urinary GAG level and liver and spleen volumes), whereas absolute change was tested for clinical endpoints.

Results

Patient disposition

Ten adult Japanese males with attenuated MPS II were enrolled in the study and received idursulfase treatment. Nine patients completed the 12-month study; one patient died of causes unrelated to idursulfase after receiving 41 of 44 scheduled infusions (see Safety Section). Compliance with treatment was excellent, with all 10 patients receiving >93% of scheduled infusions; 80% (8/10) of patients did not miss a single scheduled infusion.

Patients

The mean patient age was 30.1 years (range 21.1–53.9). All patients had been diagnosed during mid-childhood or adolescence with MPS II (mean age 7.9 years), and all had advanced disease burden at the time of enrollment into the study. All patients had short stature (height <3rd percentile for Japanese adult males). Past medical history was significant for the following MPS II-related features (n = number of patients): valvular heart disease consisting mainly of aortic and/or mitral valve insufficiency (10), joint contractures (7), hepatomegaly (7), deafness (6), retinal degeneration (5), sleep apnea (5), otitis media

Table 1
Summary of efficacy changes after 12 months of treatment with idursulfase.

	N	Baseline	12 months	Change	% Change	p-Value
Urinary GAG (mg/g creatinine)	9	106.4 ± 7.8	21.2 ± 2.9	−85.2 ± 7.1	−79.9 ± 2.2	0.004[†]
Liver volume (cc)	10	1491.2 ± 92.9	993.2 ± 75.0	−498.0 ± 70.2	−33.2 ± 4.0	0.002[†]
Spleen volume (cc)	10	210.2 ± 22.5	138.1 ± 12.5	−72.1 ± 15.7	−31.0 ± 5.5	0.002[†]
6-Minute Walk Test (m)	7	286.0 ± 53.4	340.5 ± 49.6	54.5 ± 27.0	37.4 ± 18.1	0.109
Forced vital capacity (% predicted)	9	39.9 ± 6.6	43.7 ± 6.0	3.8 ± 2.8	15.0 ± 8.0	0.250
Forced vital capacity (L)	9	1.4 ± 0.3	1.5 ± 0.2	0.1 ± 0.1	16.3 ± 8.0	0.250
Left ventricular mass index (g/m^2)	6	139.9 ± 25.1	133.2 ± 38.9	−6.7 ± 15.5	−12.4 ± 11.1	0.563
Left ventricular ejection fraction (%)	10	67.0 ± 5.2	64.3 ± 6.0	−2.8 ± 2.5	−6.1 ± 5.7	0.244
Joint range of motion (degrees)					NA	
Shoulder flexion	10	93.8 ± 4.9	109.8 ± 7.1	15.0 ± 7.3		0.066
Shoulder extension	10	44.1 ± 4.1	43.8 ± 3.8	−0.3 ± 4.1		0.945
Shoulder abduction	10	76.3 ± 3.9	95.3 ± 8.1	19.0 ± 8.8		0.125
Knee flexion	9	103.7 ± 8.5	114.4 ± 5.2	10.7 ± 10.3		0.461
Knee extension	9	−11.1 ± 4.5	−10.3 ± 5.0	0.8 ± 2.5		0.875
Hip flexion	9	89.2 ± 8.1	103.3 ± 7.6	14.2 ± 5.1		0.031
Hip extension	9	3.1 ± 5.0	1.9 ± 6.7	−1.3 ± 1.8		0.750
Elbow flexion	10	120.9 ± 4.0	121.8 ± 3.7	0.9 ± 2.5		0.828
Elbow extension	10	−43.1 ± 4.2	−35.0 ± 4.2	8.1 ± 3.4		0.063
Oxygen desaturation index (events/h)	9	18.5 ± 6.1	22.3 ± 7.4	3.9 ± 3.5	NA	0.426

The last observation carried forward (LOCF) method was used to replace a missing value at the 12-month timepoint.
All values are the observed means ± SEM. All p-values are based on the Wilcoxon signed rank test for change from baseline to the 12-month timepoint.
NA, not applicable. Some patients had values of 0 at baseline that precluded calculation of percent change.
[†] The p-value is based on the Wilcoxon signed rank test for % change from baseline to the 12-month timepoint.

(4), macroglossia (3), umbilical hernia (2), carpal tunnel syndrome (2), heart failure (2), and left ventricular hypertrophy (1).

Urinary glycosaminoglycan (GAG)

All nine evaluable patients had elevated urinary GAG levels at baseline (mean 106.4 mg/g creatinine, approximately 8 times the upper limit of normal); one patient lacked an appropriate baseline value (Table 1). Following idursulfase treatment, urinary GAG levels decreased rapidly within the first three months of treatment and remained low for the remainder of the study (Fig. 2A). There was a statistically significant mean decrease in the urinary GAG level of −79.9 ± 2.2% from baseline to 12 months ($p = 0.004$). All nine evaluable patients showed a >70% decrease in urinary GAG levels and had normal values by the end of the study.

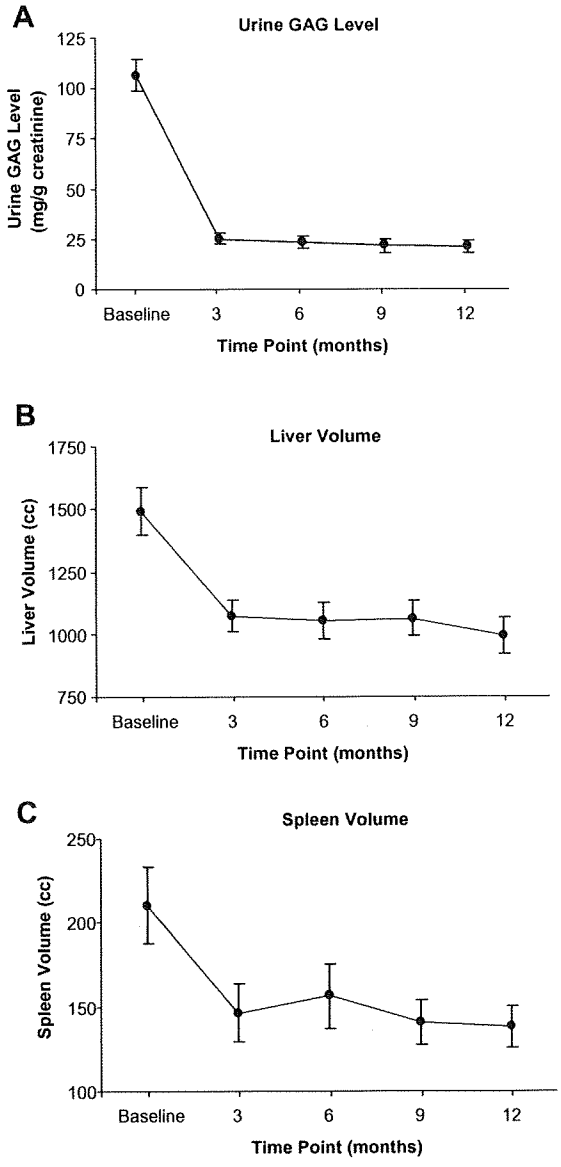

Fig. 2. The effects of idursulfase treatment on lysosomal storage over 12 months. (A) Urinary GAG level. (B) Liver volume. (C) Spleen volume. All changes are reported as mean ± SEM.

Liver and spleen volumes

At baseline, 9 (90%) patients had hepatomegaly (mean 1.3 MN, multiples of normal) and all 10 (100%) patients had splenomegaly (mean 2.4 MN) by CT. After 12 months of treatment, mean liver volume decreased by −33.2 ± 4.0% and mean spleen volume decreased by −31.0 ± 5.5% (Fig. 2B and C; Table 1), and both changes were statistically significant ($p = 0.002$). Most of the reductions occurred within the first three months of treatment. By the end of the study, all patients had liver volumes within the normal range and spleen volumes that were <2 MN, demonstrating efficient reduction of lysosomal GAG storage.

6-Minute Walk Test (6MWT)

At baseline, the mean 6MWT distance was 286.0 m for the seven patients who could perform the test (Table 1). All but one patient walked <399 m, the lower limit of normal for healthy adult men in the United States [24]. Three patients could not perform the 6MWT: one patient broke his leg just prior to the start of the study; one patient was wheelchair-bound secondary to shortness of breath and muscle weakness; and one patient was obese and could only walk a few steps with assistance. By the end of the study, the mean 6MWT distance had increased by 54.5 ± 27.0 m (Fig. 3A). This change represents a relative increase of 37.4%, and included one patient whose 6MWT distance increased by 131%. Four patients (57%) showed a clinically meaningful improvement of ⩾54 m [25], while the one patient with a normal 6MWT at baseline showed a decline (−71 m).

Percent predicted forced vital capacity (FVC)

Nine patients underwent spirometry at baseline and all showed a restrictive lung disease pattern: three were classified as having a severe defect (<50% predicted FVC) and five had a very severe defect (<34% predicted FVC) [26]. At baseline, mean percent predicted FVC was 39.9% (Table 1), and after 12 months it increased by 3.8 ± 2.8 percentage points (Fig. 3B). This improvement corresponds to a relative increase of 15.0% over baseline, which is considered clinically meaningful (⩾15% relative change) [25] and was achieved by four (44%) patients. Similarly, mean FVC increased by 16.3% over the baseline of 1.4 L. The mean forced expiratory volume in 1 s (FEV_1):FVC ratio remained unchanged at 0.70 during the study.

Cardiac

All patients had valve disease that remained stable during the study. The mean ejection fraction (EF) was normal at baseline and showed little change over 12 months (67.0–64.3%, change of −2.8 ± 2.5%) (Table 1). One patient with pre-existing cardiac failure showed gradual worsening during the study (EF 27–14%). At baseline, mean LVMI was slightly elevated at 139.9 g/m^2 (normal <131 g/m^2), and 50% (3/6) of evaluable patients had an elevated LVMI. After 12 months, mean LVMI decreased by −12.4%, with four patients showing a clinically meaningful improvement of >10% [27]. The patient with the largest LMVI at baseline showed a further increase (254.1–312.9 g/m^2).

Joint range of motion

Fig. 4 and Table 1 show the changes in joint range of motion observed during the study. At baseline, patients had significant joint contractures involving the shoulder (flexion, extension, and abduction), knee (flexion and extension), hip flexion and extension), and elbow (flexion and extension). Following 12 months of treatment, several joints showed increased range of motion, including mean

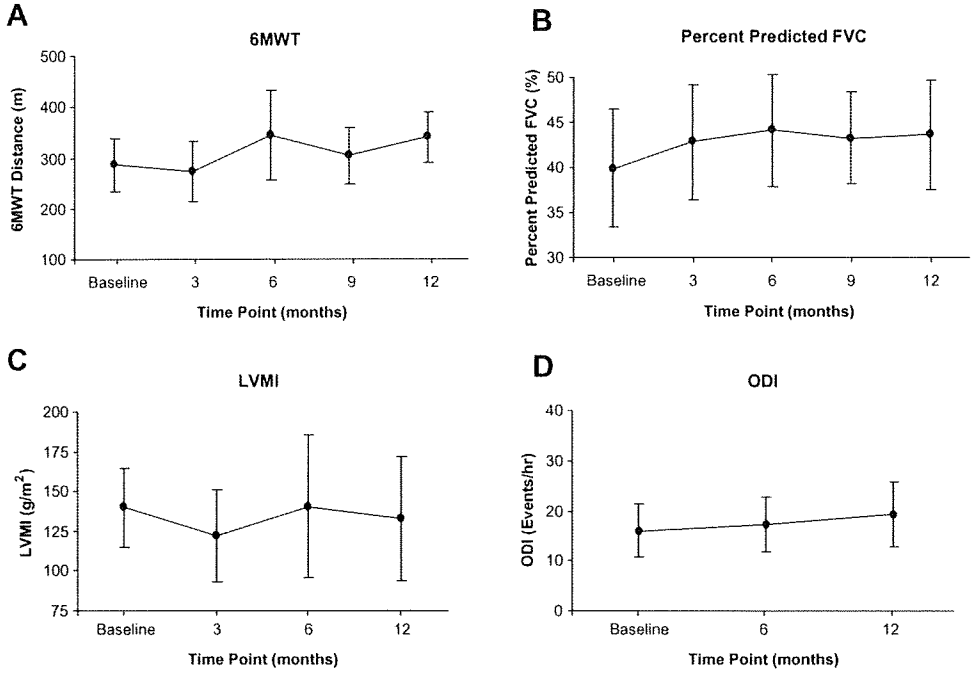

Fig. 3. The effects of idursulfase treatment on clinical endpoints over 12 months. (A) 6-Minute Walk Test. (B) % Predicted forced Vital Capacity. (C) Left Ventricular Mass Index. (D) Oxygen Desaturation Index. All changes are reported as mean ± SEM.

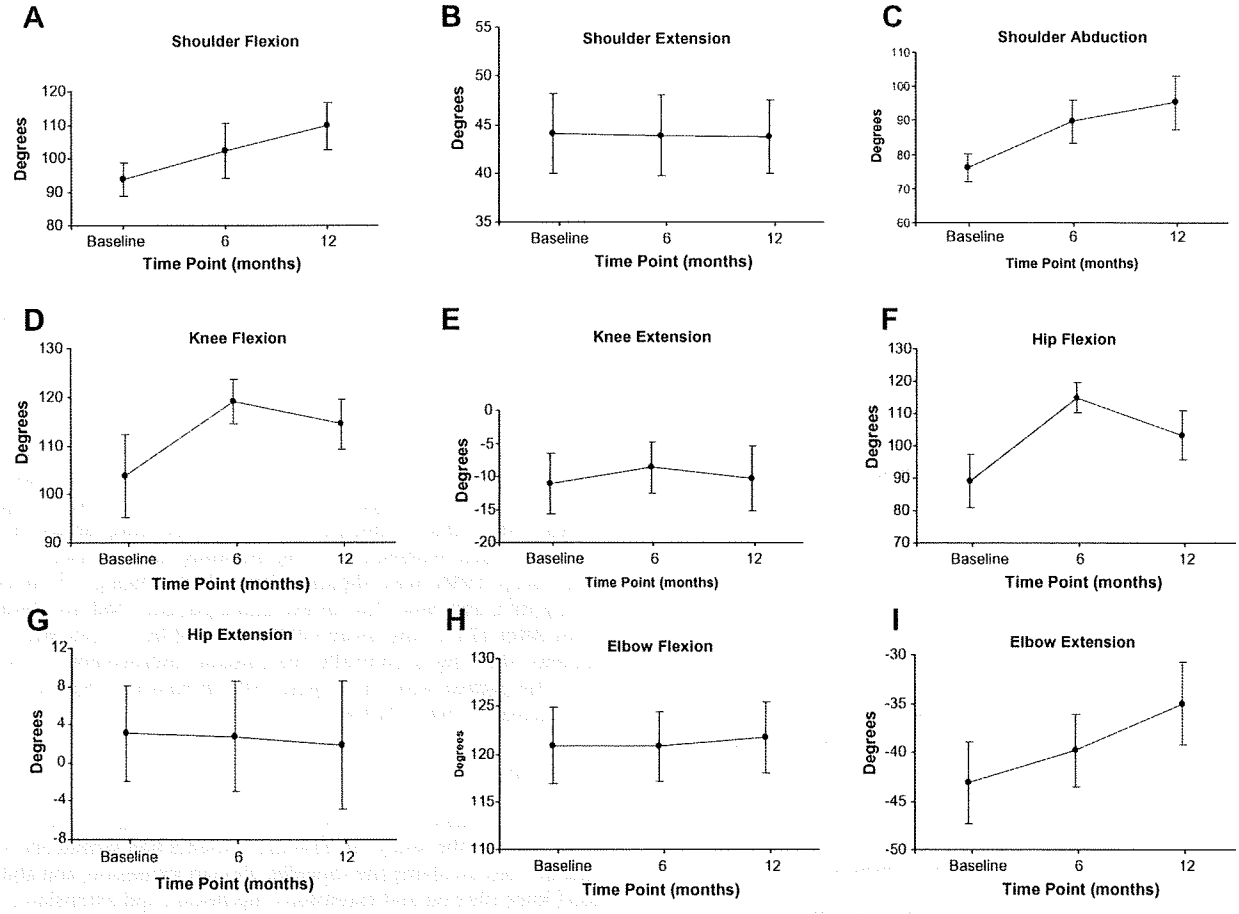

Fig. 4. The effects of idursulfase treatment on joint range of motion over 12 months. (A) Shoulder flexion. (B) Shoulder extension. (C) Shoulder abduction. (D) Knee flexion. (E) Knee extension. (F) Hip flexion. (G) Hip extension. (H) Elbow flexion. (I) Elbow extension. All changes are reported as mean ± SEM.

shoulder flexion (15.0 ± 7.3 degrees), shoulder abduction (19.0 ± 8.8 degrees), knee flexion (10.7 ± 10.3 degrees), hip flexion (14.2 ± 5.1 degrees; $p = 0.031$), and elbow extension (8.1 ± 3.4 degrees). However, most of the changes did not achieve statistical significance. Shoulder extension (−0.3 ± 4.1 degrees), elbow flexion (0.9 ± 2.5 degrees), knee extension (0.8 ± 2.5 degrees), and hip extension (−1.3 ± 1.8 degrees) showed little change during the study. Fig. 1 shows a 23 year-old study patient with severely limited shoulder range of motion (abduction and flexion), which improved following one year treatment with idursulfase.

Oxygen desaturation index (ODI)

At baseline, the mean oxygen desaturation index (ODI) was 18.5 events/h ($n = 9$), which is moderately abnormal [23]. Three patients had a normal ODI (<5 events/h), two had a mildly abnormal ODI (5–15 events/h), and four had a moderately to severely abnormal ODI (>15 events/h). During the study, the mean ODI increased by 3.9 ± 3.5 events/h, which was largely due to a single patient with an increase of 26.8 events/h. The other seven patients had stable ODI values (changes ⩽ 10 events/h).

Safety

Idursulfase was well-tolerated over the course of the study. Adverse events were mainly mild, unrelated, and attributable to expected symptoms of MPS II disease. Fifty percent (5/10) of patients experienced a total of 11 drug-related adverse events. Urticaria was the most frequent event (five events in two patients), followed by erythema (two events in the same patient). Similarly, 50% (5/10) of patients experienced infusion-related reactions (i.e. adverse events assessed as drug-related and occurring within 24 h of the infusion). The highest patient incidence involved skin reactions, i.e. urticaria and erythema (three patients each), while dyspnea, abdominal pain, and vasovagal syncope also were observed in one patient each. Except for one patient who experienced several episodes of urticaria between 9 and 12 months, the other four patients had infusion-related reactions only once or twice during the first three months of treatment. Management of infusion-related reactions included antihistamine therapy and temporary interruption of the infusion, and all events were followed by a successful patient recovery. There were no clinical laboratory abnormalities reported as related to idursulfase.

Two patients experienced serious adverse events, including one death, in the study. A 26 year-old male experienced an infusion-related reaction involving diffuse urticaria, flushing, and numbness of the tongue 1 h after initiation of the fifth infusion. The patient was pre-medicated with antihistamines without further events. A 42 year-old male had an infusion-related reaction reported by the investigator as vasovagal syncope, which consisted of hypotension, vomiting, weak pulse, and decreased consciousness and occurred 30 min into the first infusion. Subsequent infusions were preceded by corticosteroid pre-medication administration without further infusion-related reactions. The patient had a history of cardiac valve incompetence and cardiac failure requiring medications, including furosemide. Later in the study, he experienced an increase in leg edema secondary to worsening congestive heart failure. He was depressed and attempted suicide by drug overdose (not idursulfase). Upon arrival at the hospital, the patient went into cardiac arrest. Subsequent resuscitation measures were unsuccessful, and he died due to hypoxic encephalopathy, pneumonia and renal failure.

Antibodies

Anti-idursulfase IgG antibodies were detected in 60% (6/10) of patients, two of who became seronegative later in the study. No IgE antibodies were detected in patients who underwent testing for infusion-related reactions. The mean reductions in urinary GAG levels did not differ between patients who were seropositive at any time (−80.9% ± 3.8%; $n = 5$) and those who remained seronegative throughout the study (−78.6% ± 1.8%; $n = 4$). Although hypersensitive reactions or infusion-related adverse reactions tended to occur in the antibody-positive patients (four antibody-positive patients versus one antibody-negative patient), there was no correlation between the presence of antibodies and other adverse events. Furthermore, the frequency of hypersensitivity reactions did not correlate with antibody titer.

Discussion

The most remarkable difference between this and previous clinical studies of idursulfase [19,20] relates to the patient demographics and characteristics. The purpose of the JET study was to provide access to treatment for the most seriously ill MPS II patients while awaiting regulatory approval of idursulfase in Japan, which occurred in October 2007. Patients in the JET study had a mean age of 30.1 years, all were Japanese, and all were seriously ill (mean percent predicted FVC 39.9% and mean 6MWT distance 286.0 m). By comparison, MPS II patients in the Phase 1/2 and Phase 2/3 studies of idursulfase were younger (mean ages 13.9 years and 14.2 years), predominantly Caucasian (100% and 83%, respectively), and less severely affected (mean percent predicted FVC 55.1% and 55.4%; mean 6MWT distance 397 m and 395 m) [19,20]. Despite these patient differences, the JET study has shown that idursulfase is a safe and effective (Table 1) treatment for Japanese patients with MPS II and its risk–benefit profile is similar to that reported in previous studies.

In this study, idursulfase efficiently reduced GAG storage, as evidenced by the statistically significant reductions in urinary GAG levels ($p = 0.004$) and hepatosplenomegaly ($p = 0.002$) (Fig. 2; Table 1). These pharmacodynamic changes appeared to translate into clinical benefit, as evidenced by trends towards improvement in functional capacity (mean 54.5 m increase in 6MWT), respiratory function (mean 15.0% relative increase in percent predicted FVC), joint range of motion (mean increases ranging from 8.1–19.0 degrees for several joints), and LVMI (mean −12.4% decrease). Cardiac EF and valve disease remained mostly stable, although one patient with severe congestive heart failure showed progressive worsening and one patient with a greatly elevated LVMI showed a further increase. The mean ODI increased slightly by 3.9 events/h, but importantly 89% (8/9) of patients showed no clinically significant changes.

The safety profile of idursulfase in the JET study was similar to that of previous studies with no new or unexpected adverse events despite the older and more seriously ill patient population. Most adverse events were considered by investigators to be disease-related and unrelated to idursulfase. The most common drug-related adverse events were infusion-related reactions, occurring in 50% of patients. The most common infusion-related reactions were skin reactions consisting of urticaria and erythema. There were two related serious adverse events that occurred during the infusions—one involving urticaria, flushing, and numbness of the tongue, and the other involving vasovagal syncope. The one patient death was attributed to suicide from a drug overdose and was not related to idursulfase.

MPS II is a progressive and debilitating multisystem disease that is associated with a shortened lifespan, primarily from cardiorespiratory compromise [28]. Therefore, it is noteworthy that in this one-year study, cardiac and respiratory functions were improved or stable in most patients. Decreasing lung volumes are known to be associated with increased morbidity and mortality [26];

given the low percent predicted FVC values at baseline in study patients (mean 39.9%), a relative increase of 15% is of particular importance. The American Thoracic Society defines a >15% relative change in FVC occurring over a one-year period as being clinically meaningful [26]. Similarly, the 54.5 m mean increase in 6MWT distance also is considered to be a clinically meaningful improvement, based on a study of adult men with chronic obstructive pulmonary disease [25]. The 6MWT is a sub-maximal exercise test that is a composite assessment of cardiac, respiratory, and musculoskeletal function. Because all three of these organ systems are involved in the MPS disorders, walking tests have been widely used as primary efficacy endpoints in clinical trials of enzyme replacement therapy for other MPS disorders, including MPS I [29,30] and MPS VI [31].

We observed no evidence for an effect of race on immunogenicity or safety. IgG antibodies were detected in 60% (6/10) of patients treated with idursulfase, which is similar to the 49.6% rate seen in the Phase 2/3 study that enrolled predominantly Caucasian and other non-Asian patients [20]. In addition, the adverse event profile was similar in all respects; infusion-related reactions occurred in 50% of patients in the current study compared to 69% of patients receiving weekly idursulfase in the Phase 2/3 study [20].

Limitations of this study include its open-label treatment, lack of control group, and small sample size. Other aspects of the study design, however, including the treatment dose and regimen, study duration, and efficacy and safety assessments were identical or very similar to those used in the Phase 2/3 study [20]. A placebo effect in this study cannot be excluded, especially for effort-dependent assessments such as the 6MWT and active joint range of motion. Nevertheless, the magnitude of change in the 6MWT distance was similar to those observed in previous studies of idursulfase [19,20]. Determination of FVC by spirometry is less susceptible to a placebo effect given the requirement for test–retest reproducibility at each assessment [21]. This study enrolled only 10 patients, which may not have had sufficient power to detect a statistically significant clinical response even if clinical improvements were present. On the other hand, the biomarkers of lysosomal GAG clearance, i.e. liver and spleen volumes and urinary GAG level, did have sufficiently large effect sizes (change/standard deviation of change) to show statistically significant differences. Finally, the study involved only adult males, all of whom had a substantial pre-existing disease burden. This study shows that many disease features of seriously ill patients, including diminished cardiorespiratory function, restricted joint range of motion, and hepatosplenomegaly can improve with idursulfase treatment. An even better response is expected in young children prior to final organ maturation and the development of chronic tissue damage. In this regard, a study in MPS II patients $\leqslant$5 years of age is underway.

Conclusions

Idursulfase was generally well-tolerated and produced clinical improvements in adult Japanese patients with attenuated MPS II treated with the labeled dose, 0.5 mg/kg administered intravenously once weekly. Treatment with idursulfase also resulted in substantial reductions in hepatosplenomegaly and urinary GAG excretion, indicating efficient clearance of lysosomal GAG. The safety profile and immunogenicity of idursulfase appear to be similar between Japanese and previously studied Caucasian patients.

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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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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.

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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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.

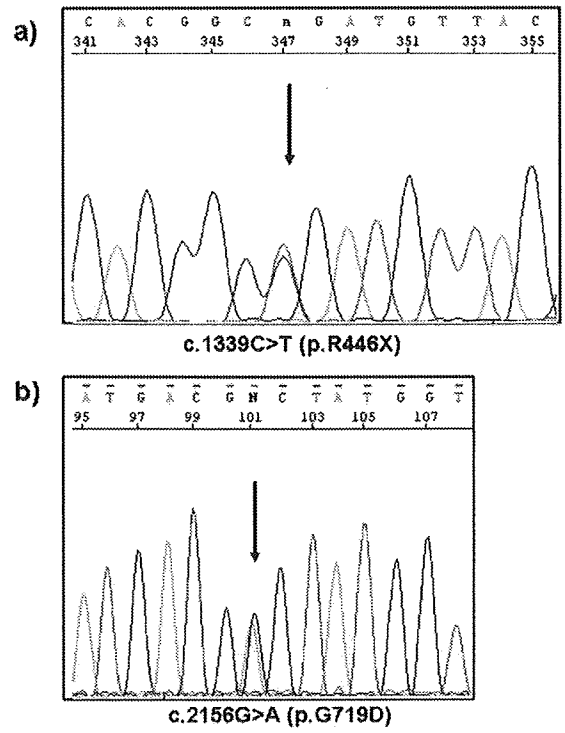

Fig. 1. (a) A C-to-T substitution at c.1139 in exon 11 was detected in a heterozygous pattern. This c.1339C > T substitution created a stop codon (p.R446X). (b) A G-to-A substitution at c.2156 in exon 18 was found in a heterozygous pattern. The c.2156G > A substitution changed the codon of glycine at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

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Brief Communication

A novel molecular aspect of Japanese patients with medium-chain acyl-CoA dehydrogenase deficiency (MCADD): c.449-452delCTGA is a common mutation in Japanese patients with MCADD

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ABSTRACT

We studied 11 Japanese patients with medium-chain acyl-CoA dehydrogenase deficiency (MCADD) and found a common mutation, c.449-452delCTGA, which accounted for 45% of the mutations. Seven of 10 independent patients carried at least one copy of this mutation. Phenotypes of homozygous patients with the c.449-452delCTGA mutation varied from asymptomatic to life-threatening metabolic decompensation in Japanese patients with MCADD, similar to the phenotypic variations in Caucasians. This study suggests the genotypic difference between those of Caucasians and Japanese regarding MCADD.

Introduction

Medium-chain acyl-CoA dehydrogenase deficiency (MCADD) (MIM #201450) is an autosomal recessive inherited metabolic disorder of mitochondrial fatty acid oxidation. Clinical symptoms are hypoketotic hypoglycemia, lethargy, seizure, coma, and sudden infant death syndrome (SIDS)-like illness triggered by catabolic stress such as infection and prolonged fasting. Up to 20% of these patients die during their first metabolic decompensation [1]. MCADD is the most common fatty acid oxidation disorder among Caucasians, especially those of Northern European descent. The disease frequency has been estimated to range between 1:4900 and 1:25,000 based on newborn screening programs worldwide [2–5]. The common missense c.985A > G mutation reportedly occurs in 80–90% of Caucasian patients with MCADD [6–11], whereas this disease has rarely been reported in Asian countries. However, after acylcarnitine analysis became available in Japan, MCADD has been identified more frequently. The disease frequency was estimated to be approximately 1:51,000 in Japan based on a newborn screening pilot study [12]. Herein, we report the genetic aspects of 11 Japanese patients with MCADD.

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Subjects and methods

Subjects

Eleven Japanese patients with MCADD from 10 unrelated families, including four previously-reported patients, were studied (Table 1). No families showed consanguineous marriage. Patients' diagnoses were made by urinary organic acid and/or blood acylcarnitine analyses. Six of them were symptomatic patients. One was asymptomatic sibling case, and four of them were diagnosed in a newborn screening pilot study. The diagnoses of Cases 1–7 were confirmed by an enzyme assay and *in vitro* assay of β-oxidation. In this study, we performed mutation analysis in Cases 1–7 and 10. Informed consent to perform DNA analysis was obtained from the parents of the patients. This study was approved by the Ethical Committee of the Shimane University Faculty of Medicine.

Methods

Genomic DNA was extracted from the patients' fibroblasts and blood filter papers using the QIAamp DNA Micro Kit (Qiagen GmbH, Hilden, Germany) and from peripheral blood lymphocytes using the DNA Quick II kit (Dainippon Pharmaceuticals, Osaka, Japan). We designed 12 sets of primers for the amplification of

Table 1
Clinical and molecular features of Japanese patients with MCADD.

Cases	Age at onset	Age at diagnosis	Clinical symptoms	Allele 1	Allele 2
Case 1	1 y 3 m	1 y 3 m	Unconsiousness, apnea, vomiting	**Deletion of exons 11 and 12**	**Deletion of exons 11 and 12**
Case 2	1 y 4 m	2 y 2 m	Respiratory tract infection, enterocolitis, hypoglycemia, (neonatal transient hypoglycemia)	**449-452delCTGA**	**449-452delCTGA**
Case 3*	1 y 4 m	1 y 4 m	Gastroenteritis, seizures, hypoglycemia, ketonuria, (neonatal transient hypoglycemia)	**449-452delCTGA**	**449-452delCTGA**
Case 4	1 y 7 m	1 y 7 m	Unconciousness, fever, hypoglycemia, acidosis, hyperammonemia	**c.275C>T (P67L)[a]**	**c.157C>T (R28C)**
Case 5	–	5 d	Neonatal mass screening (asymptomatic)	**449-452delCTGA**	**c.134 A>G (Q20R)**
Case 6	–	5 d	Neonatal mass screening (asymptomatic)	**449-452delCTGA**	**c.820A>G (M249V)[a]**
Case 7*	–	5 y 5 m	Sibling of case 2 (asymptomatic)	**449-452delCTGA**	**449-452delCTGA**
Case 8[1]	7 m	3 y	Respiratory tract infection, lethargy, fever, vomiting, fatigue, hypoglycemia	449-452delCTGA	c.1189T>A (Y397N)
Case 9[2]	8 m	8 m	Cardiopulmonary arrest, liver dysfunction, hyperammonemia	449-452delCTGA	c.157C>T (R28C)
Case 10[2]	–	5 d	Neonatal mass screening (asymptomatic)	**c.1085G>A (G337E)[a]**	**c.843A>T (R256S)**
Case 11[2]	–	5 d	Neonatal mass screening (asymptomatic)	449-452delCTGA	Unknown

[1]Yokoi et al. (2007) and [2]Tajima et al. (2005) reported, respectively.
* Siblings; Age: y, year; m, month; d, day; –, asymptomatic patients detected by neonatal screening. Common mutations are underlined. Mutations in bold were identified in this study.
[a] Novel mutations.

each exon including 5′ and 3′ splice sites of the acyl-CoA dehydrogenase, medium-chain (ACADM) gene (Supplementary data 1). Exons were amplified for 35 cycles using the polymerase chain reaction (PCR) as follows: denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s with the AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using the iCycler (Bio Rad Laboratories Inc., Hercules, CA, USA). All PCR-amplified fragments of ACADM were then directly sequenced using ABI Big Dye Terminator Cycle Sequencing FS Ready Reaction Kits and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Results

In this study, we performed mutation analysis in Cases 1–7 and 10. Their clinical presentation and mutations are summarized in Table 1, together with information about three cases (Cases 8, 9, and 11) whose mutations were reported previously [13,14]. Three novel missense mutations, c.275C > T (P67L), c.820A > G (M249V) and c.1085G > A (G337E), three reported missense mutations, c.134A > G (Q20R) [15], c.157C > T (R28C) [16], and c.843A > T (R256S) [17], and two null mutations, c.449-452delCTGA [13–14] and a large deletion including exons 11 and 12 [17], were identified in this study (Table 1). Among them, c.449-452delCTGA was identified in 11 alleles including that of siblings (Cases 3 and 7). Therefore, frequency of c.449-452delCTGA was calculated as 45% of mutant alleles (nine out of 20 independent alleles) in the Japanese patients with MCADD since mutant alleles of siblings were derived from same origin. This mutation introduces a frameshift and premature termination at codon 128 (P128X), indicating that this common mutation is a null mutation. The novel mutations, c.275C > T (P67L), c.820A > G (M249V) and c.1085G > A (G337E), were not detected in 120 chromosomes from unaffected Japanese individuals, suggesting that they are not polymorphisms. In Case 1, no genomic fragments for exons 11 and 12 were amplified, indicating a large gene deletion including exons 11 and 12, as previously-reported [17].

Discussion

It remained unknown why there were no Japanese patients with MCADD until Tajima et al. reported three cases in 2005 [14]. After acylcarnitine analysis became available in Japan in 2002, the detection of MCADD has been increasing, and several acute encephalopathic patients with hypoglycemia and hyperammonemia were identified as having MCADD. We have noted 11 patients with MCADD from 10 families in the Japanese population thus far.

Our study clearly showed that there is a common mutation, c.449-452delCTGA, in Japanese patients with MCADD. c.449-452delCTGA was identified in 45% of mutant alleles in Japanese patients with MCADD. In the Caucasian population, a well-known common c.985A > G missense mutation represents 80–90% of mutant alleles [6–11]. The c.985A > G missense mutation was not detected in any Japanese patient in this study and in a survey of c.985 A > G mutation in patients with SIDS as well as a healthy Japanese population [18–19]. The prevalence of heterozygous carriers of c.985A > G missense mutations in Caucasians of northwestern Europe is reported to be 1/68–1/101 in newborns [20]. Although the frequency of c.449-452delCTGA mutation in Japanese patients with MCADD is less than that of the c.985A > G mutation in Caucasian patients, it is of interest to determine the prevalence of heterozygous carriers of c.449-452delCTGA in a Japanese population. Recently, two Korean patients with MCADD were also identified as heterozygotes for this mutation [15,17]. These findings suggest that the c.449-452delCTGA mutation may be common not only in Japanese but also in other Mongoloid populations. Namely, it is likely that this study shows the genotypic difference between those of Caucasian and Mongoloid ethnicity in MCADD.

As discussed above, c.449-452delCTGA is a null mutation. In this study, the age at onset varied among the patients with the same genotype of 449-452delCTGA. Two patients (Cases 2 and 3) with homozygous c.449-452delCTGA mutation showed neonatal transient hypoglycemia and developed life-threatening metabolic decompensation upon respiratory tract infection and enterocolitis within 2 years. However, Case 7 (sibling of Case 3) was asymptomatic until 5.5 years old, even though he had the same homozygous mutation. Patients generally become symptomatic upon infection, starvation, or in the presence of other stresses when catabolism becomes active. Depending on the time and degree of exposure to the stress, some patients develop MCADD attack earlier than others. These phenotypic variations among Japanese patients with homozygous null mutations are similar in MCADD patients in a Caucasian population [1,7,9].

In summary, this study indicates that c.449-452delCTGA represents a common mutation in Japanese patients with MCADD, in

contrast to the common c.985A > G mutation found in Caucasians, and adds three novel missense mutations, c.275C > T (P67L), c.820A > G (M249V) and c.1085 G > A (G337E), to the ACADM gene catalogue. The mutational spectrum of MCADD is still unknown across all ethnicities; however, there are different common mutations in Caucasians and Japanese.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymgme.2008.10.012.

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Clinical and molecular aspects of Japanese patients with mitochondrial trifunctional protein deficiency

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ABSTRACT

Mitochondrial trifunctional protein (MTP) deficiency is a rare inherited metabolic disorder of mitochondrial fatty acid oxidation. We newly characterized three novel mutations in 2 Japanese patients with MTP deficiency, and investigated the clinical and molecular aspects of 5 Japanese patients including 3 previously reported cases. Herein, we describe the characterization of four missense mutations, R214C, H346R, R411K, and V422G, in the HADHB gene, which have been identified in Japanese patients, employing a newly developed, sensitive transient expression analysis. Co-transfection of wild-type HADHA and HADHB cDNAs in SV40-transfected fibroblasts from a MTP-deficient patient yielded sufficient enzyme activity to evaluate low-level residual enzyme activity, using two incubation temperatures of 30 °C and 37 °C. At 30 °C, residual enzyme activity was higher than that at 37 °C in V422G, R214C, and R411K. However, H346R, which was seen in the most severe case, showed no enzyme activity at both temperatures. Our results demonstrate that a defect of HADHB in MTP deficiency is rather common in Japanese patients, and the mutational spectrum is heterogeneous. The present findings showed that all missense mutations in this study were disease-causing. Although the number of patients is still limited, it is suggested that the phenotype is correlated with the genotype and a combination of two mutant alleles of the HADHB gene in MTP deficiency.

Introduction

Mitochondrial trifunctional protein (MTP), bound to the inner mitochondrial membrane, plays a significant role in the last three steps of the β-oxidation cycle of long-chain acyl-CoAs [1,2]. MTP is an α4β4 hetero-octamer encoded by two different genes of HADHA (MIM *600890) and HADHB (MIM *143450), which are both located on chromosome 2p23 [2–4]. The ⟨alpha⟩-subunits (HADHA) encode *trans*-2,3-long-chain enoyl-CoA hydratase (LCEH) and long-chain 3-OH-acyl-CoA dehydrogenase (LCHAD) activities, while ⟨beta⟩-subunits (HADHB) encode long-chain 3-ketoacyl-CoA thiolase (LCKT) activity [1].

MTP deficiency is clinically characterized by cardiomyopathy, hypoketotic hypoglycemia, metabolic acidosis, sudden infant death, metabolic encephalopathy, liver dysfunction, peripheral neuropathy, exercise-induced myoglobinuria, or rhabdomyolysis, and is classified into three clinical phenotypes: (1) lethal phenotype with neonatal onset (severe), (2) hepatic phenotype with infant onset (intermediate), and (3) myopathic phenotype with late-adolescent onset (mild) [5,6].

Isolated LCHAD deficiency (MIM #609016) was initially reported in 1989 [7], and it is usually caused by a common 1528 G–C transversion in the HADHA gene in Caucasians. Over 60 cases with this mutation have been described to date [8,9]. The first case of complete MTP deficiency (MIM #609015) with the decreased activity of all three enzymes was biochemically characterized in 1992 [10,11], and molecularly described in 1995 [12]. Since the initial case report, the molecular basis of MTP deficiency has been reported in more than 50 cases [6,12–23]. Twenty-six of them showed mutations of the ⟨beta⟩-subunit, suggesting that the frequency of mutations of the HADHA and HADHB genes in MTP deficiency is similar. Clinical and biochemical findings do not appear to differ between patients with HADHA and HADHB gene mutations [5,6].

In this report we newly characterized three novel mutations in 2 Japanese patients with MTP deficiency, and investigated the clinical and molecular aspects of 5 Japanese patients including three

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previously reported cases. Further, we employed a sensitive transient expression analysis to determine the residual activities of four missense mutations, R214C, H346R, R411K, and V422G, in the HADHB gene identified in the 5 Japanese patients with MTP deficiency.

Materials and methods

Subjects

The clinical features of these 5 Japanese pediatric patients (3 boys and 2 girls) are summarized in Table 1. The age at onset of the patients ranged from 0 days to 15 years. Of the 5 patients, 2 each showed lethal (severe form) and hepatic (intermediate form) phenotypes, respectively, and 1 had a myopathic phenotype (mild form). Data on patient 1, with a unique intronic mutation leading to deep intronic sequence exonization, were published separately [24]. Clinical data and mutation analyses of patients 4 and 5 were previously reported [25,26].

Patient 1: A female neonate. The detailed medical history of the patient has been described previously [27,28]. Briefly, the baby was found to have metabolic acidosis, hypoketotic hypoglycemia, and hyperammonemia soon after birth, and died of cardiac and renal failure on the 8th day of life. Her sibling showed a similar clinical course, and died on the 5th day of life.

Patient 2: A male neonate, who was born of consanguineous (second-degree cousins) parents, had dyspnea and cyanosis on the 5th day of life. Laboratory tests revealed hyperammonemia (172 μg/L), metabolic acidosis (pH 7.227, BE −4.1), and liver dysfunction (AST 255 IU/L, ALT 86 IU/L, and LDH 2208 IU/L). The level of creatine kinase (CK) was 9635 IU/L on the 5th day, and increased to 24,707 IU/L on the 7th day of life. At 3 months of age, he died of sudden cardiac arrest. Pathological examination revealed cardiomyopathy.

Patient 3: A 9-month-old girl, who was born of a mother with Basedow–Graves disease and type 1 diabetes, showed delayed motor development. Routine blood and thyroid function tests at 1 month of age showed liver dysfunction (AST 139 IU/L, ALT 110 IU/L, LDH 4685 IU/L, and CK 7543 IU/L) and the mild elevation of TSH (TSH 10.11 μU/ml). When she was at 9 months of age, she presented with unconsciousness and lactic acidemia. ESI-MS/MS analysis of blood acylcarnitines suggested MTP or LCHAD deficiency. In 2 year after diagnosis, she hospitalized 19 times with symptoms of rhabdomyolysis triggered by infection and exercise. She had a mild mental retardation.

Patient 4: A 13-month-old boy initially presented with lethargy, hypotonia, and developed respiratory failure after an upper respiratory tract infection. Cardiac arrest occurred after convulsion, but he was successfully resuscitated. Laboratory studies revealed an increase in CK, AST, and LDH. The patient was re-hospitalized frequently until 26 months of age because of recurrent hypotonia and respiratory failure when he had an upper respiratory tract infection. His psychomotor development has been delayed, which is likely related to the hypoxic encephalopathy resulting from cardiac arrest [25].

Patient 5: A 15-year-old boy born as the second child of consanguineous parents (second-degree cousins), who had shown normal growth. From the age of 15 years, he experienced intermittent acute muscle pain, weakness, and passed dark-brown urine suggesting rhabdomyolysis after prolonged exercise and fasting. He developed severe pain in his leg muscles after a 20 km hike without eating, and showed difficulty in walking at the age of 21 years. He showed moderate weakness of the proximal muscles of both lower extremities but no signs of muscle atrophy. CK and LDH were as high as 121,800 and 3921 U/L, respectively, during attacks. Muscle biopsy histochemistry of the left quadriceps femoris showed slight lipid accumulation in type I fibers [26].

Cell culture

Fibroblasts from the patients 1, 2 and 3, and SV40-transformed fibroblasts were cultured in Eagle's minimum essential medium containing 10% fetal calf serum and antibiotics (100 μg/mL each of penicillin and streptomycin, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan).

Enzyme assay

Mitochondrial long-chain 3-ketoacyl-CoA thiolase (LCKT) activity was determined in patients' fibroblasts and SV40-transformed fibroblasts according to the previously reported techniques [10]. Briefly, LCKT activity was measured in: 100 mM Tris–HCl (pH 8.3), 50 mM KCl, 25 mM $MgCl_2$, 0.1% (w/v) Triton X-100, and 0.2 mg/ml BSA, with 10 μM 2-ketopalmitoyl-CoA as a substrate. Reactions were started by the addition of coenzyme-A followed by absorbance measurements at 303 nm using a Shimadzu UV-1600 Spectrometer (Shimadzu, Kyoto, Japan).

Cytosolic acetoacetyl-CoA thiolase (CT) activity in transfected fibroblasts was determined to examine the transfection efficiency

Table 1
Clinical and molecular features of 5 Japanese patients with MTP deficiency.

Patient	Age at onset	Consanguinity	Phenotype	Clinical course	Laboratory findings	Mutation (aminoacid)		Outcome
						allele 1	allele 2	
P1[a]	0 days	No	Lethal	Cardiomyopathy, respiratory distress	Hypoglycemia, hyperammonemia, metabolic acidosis, liver dysfunction	c.1136A > G (H346R)	g.33627A > G or IVS7 + 614A > G (Truncation at c.159)	Death (8 days)
P2	5 days	Yes		Cardiomyopathy, respiratory distress	Hypoglycemia, hyperammonemia	c.1364T > G (V422G)	c.1364T > G (V422G)	Death (3 months)
P3	9 months	No	Hepatic	Coma, convulsion, developmental delay	Lactic acidemia, liver dysfunction	c.739C > T (R214C)	c.817 del G (Truncation at c.259)	Delayed development
P4[b]	13 months	No		Respiratory failure, hypotonia	Liver dysfunction	c.1331G > A (R411K)	c.777insT (G226-P237 del)	Delayed development
P5[b]	15 years	Yes	Myopathic	Muscle pain, weakness	Elevation of CK, myoglobinuria	c.1331G > A (R411K)	c.1331G > A (R411K)	Normal development

P, patient. P1 through 5 represent the same individuals in all tables and figures. Mutations are designated by the nucleotide number from the start codon and the amino acid number from the mature N-terminus.

[a] Published in J. Purevsuren et al. Mol. Genet. Metab. [24].

[b] Published in K.E. Orii et al. Hum. Mol. Genet. [13].

in 15 μM of acetoacetyl-CoA and 50 μM of coenzyme-A in 0.1 M Tris–HCl (pH 8.0), 25 mM $MgCl_2$, and 0.5 mM DTT [29,30]. Enzyme activity was monitored based on a decrease of acetoacetyl-CoA at 303 nm. The protein concentration of the lysates was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

Western blot analysis

For Western blot analysis, each cell extract was subjected to 7.5% or 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS/PAGE). The blot was performed as described by Towbin et al. [31] using rabbit polyclonal antibody binds to both ⟨alpha⟩- and ⟨beta⟩-subunits of MTP as the primary antibody (generously provided by Dr. T. Hashimoto, Professor Emeritus, Shinshu University, Matsumoto, Japan) and visualized using the Immuno-Pure NBT/BCIP Substrate Kit™ (Promega, Madison WI, USA). Anti-human VLCAD or anti-human CT antibodies were used as positive controls. The quantity of mutant protein was estimated densitometrically, comparing it to the signal intensities of the wild-type MTP protein.

Mutation screening

The protocol for DNA studies was approved by the Ethical Committee of Shimane University Faculty of Medicine. Informed consent to perform DNA analysis was obtained from the parents of patients. Genomic DNA was extracted from the patients' fibroblasts and peripheral blood lymphocytes using the QIAamp DNA Micro Kit (Qiagen GmbH, Hilden, Germany) and DNA Quick II kit (Dainippon Pharmaceuticals, Osaka, Japan), respectively. First-strand cDNA was prepared from total RNA isolated using the ISOGEN kits according to the manufacturer's instructions (Nippon Gene, Tokyo, Japan).

We designed 20 and 16 sets of primers to amplify each exon including 5′ and 3′ splice sites of HADHA (not shown) and HADHB genes, respectively. In the replicate experiments, four overlapping fragments for the entire coding region of HADHB cDNA were amplified [24]. PCR was performed as follows: 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s with AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using the iCycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). Amplified PCR products were purified using QIAquick PCR Purification Kits (Qiagen K.K., Tokyo, Japan), and then directly sequenced using ABI Big Dye Terminator Cycle Sequencing FS Ready Reaction Kits and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, CA, USA). Nucleotide and amino acid numbering was carried out according to the HADHB cDNA sequence deposited in GenBank.

Construction of eukaryote transient expression vectors

cDNA synthesis was performed using total RNA from control fibroblasts with a mixture of the HADHA-specific antisense primer 5′-^{2387}CGTTACTCTGATAAATCTAGACACC-3′ and HADHB-specific antisense primer 5′-^{1529}CTAAGAGGAGCTAGGAACTACAAAT-3′. Wild-type (WT) HADHA cDNA including the full coding sequence was amplified using the sense primer 5′-AGGCCTCGAG^{-66}AG AAAAG TCCTCCGCTCGG^{-49}-3′ and antisense primer 5′-CGGCCTCGA^{2329}GGG TTAGTGCACTGACTGAG^{2310}-3′. A WT HADHB cDNA with the full coding sequence was amplified using the sense primer 5′-GAGCCTCG AG^{-60}ACTTGGACCTGAACCTTGCT^{-41}-3′ and antisense primer 5′-CAT TCTCG^{1470}AGTGTGAGTGTTGCACAGAAAC^{1449}-3′. Underlined sequences were tag sequences with the XhoI site. Nucleotides were numbered with A of the initiator methionine codon being + 1. Amplified HADHA and HADHB cDNAs were ligated into the pUC118 *Hinc* II/BAP vector using the Mighty Cloning Kit (Takara Bio Inc., Japan). After confirming the sequences, they were designated as pUC118-WTα and pUC118-WTβ, respectively. Four mutant HADHB cDNAs, H346R, V422G, R214C, and R411K, were introduced into the pUC118-WTβ by mutagenesis using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA, http://www.stratagene.com) according to the manufacturer's instructions. After the mutations were confirmed by sequencing, pUC118-WTα, pUC118-WTβ cDNA, and pUC118-mutant HADHB cDNA fragments were subcloned into the pCAGGS eukaryote expression vector [32], and designated as pCAGGS-WTα, pCAGGS-WTβ, and pCAGGS-mutant β, respectively.

Transient expression analysis of mutant cDNAs

Recipient cells, SV40-transformed fibroblasts, from patient 1 with the severe form were prepared as described [33]. Wild-type (pCAGGS-WTβ) or pCAGGS-mutant β expression vectors (4 μg) were co-transfected with 4 μg of pCAGGS-WTα and 1 μg of cytosolic acetoacetyl-CoA thiolase (CT) cDNA-expressing vector (pCAGGSct) into ~5×10^5 recipient cells employing Lipofectamine 2000 (GIBCO BRL Invitrogen Inc., Carlsbad, CA, USA). In mock cells, 4 μg of pCAGGS-WTα and pCAGGS(−) vector together with 1 μg of pCAGGSct were used to transfect the same cell line. Cells were incubated at 37 °C for 24 h, followed by additional 48-h incubation at 30 °C or 37 °C. The cell pellets were stored at −80 °C until LCKT and CT activities were assayed.

Statistics

The values are expressed as mean ± SD (standard deviation), and statistical significance was evaluated using Student's *t*-test in Microsoft Excel.

Results

Enzyme assay

Detection limit of mitochondrial LCKT activity using 3-keto-palmitoyl-CoA as a substrate was around 1.25 nmol/min/mg protein in fibroblast. The LCKT activity was under the detection limit in fibroblasts from patients 1, 2, and 3, while that of healthy control fibroblasts ($n = 3$) was 13.97 ± 0.15 nmol/min/mg protein. In this study, LCKT activity could not be measured in patients 4 and 5, since their fibroblasts were not available.

Western blot analysis

Western blot analysis showed very faint bands for ⟨alpha⟩- and ⟨beta⟩-subunits of MTP in cultured fibroblasts from patients 1 and 2, whereas the expression of both subunits was faint in patient 3. In contrast, both subunits were clearly detected in control fibroblasts (Fig. 1). Additionally, a faint non-specific extra band was detected even in the control. VLCAD protein, used as positive control, was equivalent in all patients to that of controls.

Mutations of HADHA and HADHB genes

It is known that many mutations of either ⟨alpha⟩- or ⟨beta⟩-subunit destabilize the entire MTP. Hence, we sequenced both HADHA and HADHB genes. Molecular data on Japanese patients with MTP deficiency are summarized in Table 1. Patient 1 was a compound heterozygote of maternal missense c.1136A–G transition (H346R) and a paternal deep intronic mutation, g.33627A–G (IVS7 + 614A–G), resulting in intronic sequence exonization of

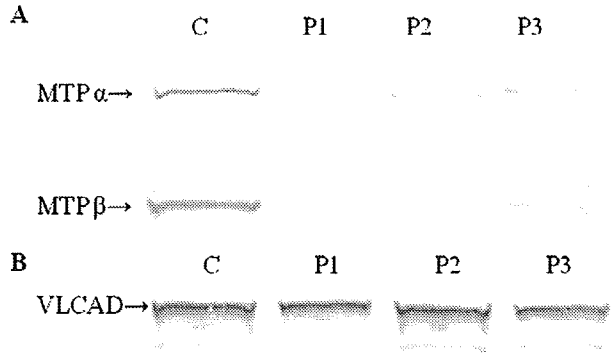

Fig. 1. Western blot analysis of fibroblasts in patients with MTP deficiency. Ten micrograms of protein from indicated sources were applied. MTPα and MTPβ represent alpha- and beta-subunits of MTP, respectively. VLCAD: very long-chain acyl-CoA dehydrogenase. Lane C: control fibroblasts; lanes P1, P2, and P3: patient 1, 2, and 3 fibroblasts, respectively. The positions of the alpha- and beta-subunits of MTP and VLCAD proteins are indicated by arrows. The values of LCKT activity are expressed as means ± SD of three experiments.

the HADHB gene, as described previously [24]. Patient 2 had a novel homozygous missense c.1364T–G transversion (V422G) in HADHB gene, and was of consanguineous parents. Patient 3 was a compound heterozygote of a paternal c.739C–T transition (R214C) and a maternal single c.817G deletion (240 frameshift) in HADHB gene. This single deletion caused a short termination at codon 259 of the mature N-terminal. However, cDNA screening failed to detect c.817delG, strongly suggesting that mRNA for c.817delG does not exist. Since nonsense mediated decay is an mRNA surveillance mechanism to detect nonsense mutations and prevent the expression of truncated or erroneous proteins [34], it is likely that c.817delG results in nonsense mediated mRNA decay that destabilizes truncated mRNA. Mutations in patients 4 and 5 were previously reported [13]. Patient 4 was a compound heterozygote of c.1331G–A missense mutation (R411K) and aberrant splicing, which was caused by exonic single T insertion (c.777insT). Patient 5 was a homozygote of c.1331G–A (R411K) missense mutation of consanguineous parents. All 5 Japanese patients with MTP deficiency had a defect in ⟨beta⟩-subunits of MTP. None of the mutations were found in 150 alleles from normal Japanese controls.

Transient expression analysis of mutant cDNAs

To investigate the biological significance of the missense mutations, R214C, H346R, R411K, and V422G, which were identified among the 5 Japanese patients, we performed transient expression analysis of the mutant cDNAs. Firstly, we expressed the pCAGGS-WTβ and pCAGGS-mutant β expresson vectors without pCAGGS-WTα in SV40-transformed fibroblasts from patient 1. However, LCKT activity was barely detectable using pCAGGS-WTβ alone (data not shown). Hence, it was difficult to characterize the mutants in this system. We considered that the transient expression of pCAGGS-WTβ alone was insufficient to achieve significant LCKT activity since MTP forms an α4β4 hetero-octamer. Mutations of either ⟨alpha⟩- or ⟨beta⟩-subunits destabilize the entire MTP, and the enzyme is neither fully active nor stable unless an intact α4β4 hetero-octamer is formed [15,19]. We performed transient co-expression of pCAGGS-WTα and pCAGGS-WTβ or pCAGGS-mutant β expression vectors in the same cell line. As shown in Fig. 2, co-transfection of pCAGGS-WTα and pCAGGS-WTβ yielded sufficient LCKT activity at both 37 °C and 30 °C. The same experiment was repeated 3 times for wild-type and mutants transfected cells. In Western blot analysis, both ⟨alpha⟩- and ⟨beta⟩-subunits were clearly detected.

In transient expression at 37 °C, pCAGGS-H346Rβ and pCAGGS-V422Gβ transfection resulted in no apparent residual enzyme activity, whereas pCAGGS-R214Cβ and pCAGGS-R411Kβ transfection generated 5% and 14% LCKT activity compared to pCAGGS-WTβ, respectively (Fig. 2). Western blot analysis demonstrated no detectable ⟨alpha⟩- and ⟨beta⟩-subunits for all mutants at 37 °C, except for pCAGGS-R411Kβ, which showed faint expression of ⟨alpha⟩- and ⟨beta⟩-subunits (Fig. 3). However, cells transfected with pCAGGS-R214Cβ or pCAGGS-R411Kβ showed much higher residual enzyme activity at 30 °C compared with 37 °C. Especially, pCAGGS-R411Kβ led to more than 50% residual enzyme activity (Fig. 2). The residual enzyme activity of pCAGGS-V422Gβ was detectable only at 30 °C, whereas pCAGGS-H346Rβ showed no detectable residual activity at both 30 °C and 37 °C. Western blot analysis also detected ⟨alpha⟩- and ⟨beta⟩-proteins due to the presence of pCAGGS-R214Cβ and pCAGGS-R411Kβ at 30 °C (Fig. 3). In mock cells, in which pCAGGS-WTα and pCAGGS(−) with pCAGGSct were transfected, neither ⟨alpha⟩- nor ⟨beta⟩-proteins were detected at both temperatures. Each transfectant showed similar levels of CT activity in the enzyme assay and CT protein in Western blot analysis at both 30 °C and 37 °C, respectively, thereby confirming the transfection efficiency to be similar among each transfected cells.

Discussion

We identified three novel mutations, R214C, V422G, and an exonic c.817G single deletion, in the HADHB gene of 2 Japanese patients with MTP deficiency. We subsequently characterized four missense mutations in the HADHB gene identified in 5 Japanese patients including two missense mutations reported previously using a newly developed and sensitive transient expression analy-

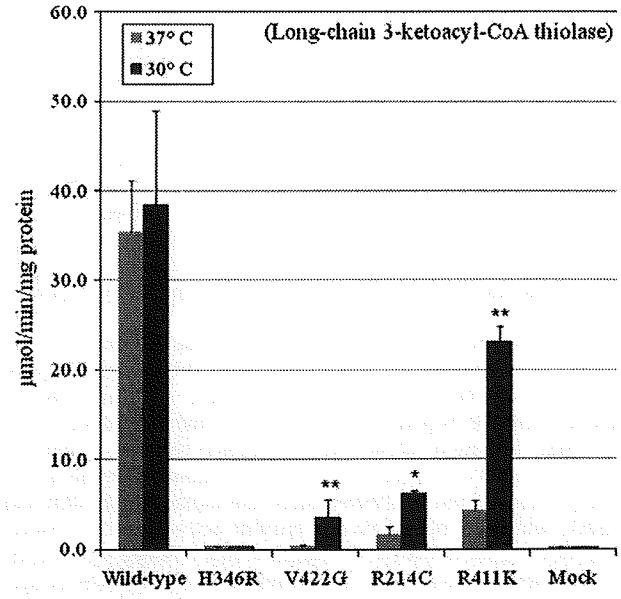

Fig. 2. Long-chain 3-ketoacyl-CoA thiolase activity in lysates of transiently transfected cells. Transient expression analyses were performed at 37 °C and 30 °C. pCAGGS-WTβ (wild-type), pCAGGS-H346Rβ, pCAGGS-V422Gβ, pCAGGS-R214Cβ, or pCAGGS-R411 Kβ (4 μg) and pCAGGS-WTα (4 μg) together with 1 μg of pCAGGSct were used to transfect SV40-transformed fibroblasts of patient 1, as a recipient cell line. Mock indicates transfection using 4 μg each of pCAGGS-WTα and a pCAGGS vector without the insert plus 1 μg of pCAGGSct. $^{*}P < .05$ or $^{**}P < .01$, respectively, compared with transfection at 37 °C determined using Student's *t*-test.

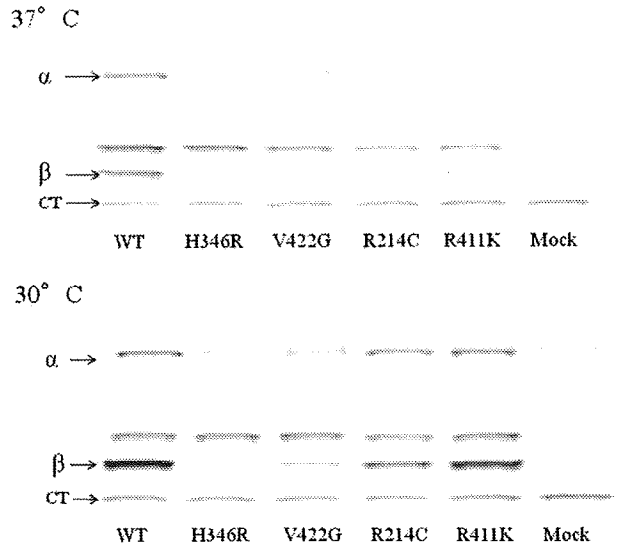

Fig. 3. Western blot analysis of cell extracts in transient expression analysis. Western blot analyses of MTP and CT are shown. Ten micrograms of lysates from indicated sources were applied. The position of the α- and β-subunits of MTP and CT proteins are indicated by arrows.

sis. This allowed us to study the correlation between the clinical phenotype and genotype in Japanese patients. In Japan, only 5 patients with MTP deficiency have been reported thus far. All these patients showed mutations in HADHB but not HADHA, suggesting that beta-subunit mutation may be common in Japan. However, there were no common mutations except for R411K in the HADHB gene, shared by 2 unrelated patients [13].

Co-transfection with wild-type HADHA and HADHB cDNAs increased the enzyme activity 4-fold compared to control fibroblasts in our system. Since we used an SV40-transformed MTP-deficient cell line as recipient cells, the background activity was nearly zero. Hence, this system can evaluate even a low level of residual enzyme activity. Moreover, we used two different incubation temperatures of 30 °C and 37 °C to determine the temperature sensitivity. This clearly showed a higher residual enzyme activity at 30 °C compared to 37 °C for V422G, R214C, and R411K. A lower temperature may stabilize the mutant subunit in protein folding and multimer formation. Previous studies successfully evaluated "mild" temperature-sensitive mutations using this method, which showed thermolabile residual enzyme activity and protein in beta-ketothiolase deficiency, succinyl-CoA: 3-ketoacid CoA transferase deficiency, and very long-chain acyl-CoA dehydrogenase deficiency [35–39].

In the 5 Japanese patients, one had myopathic and two each had hepatic and lethal forms. The patient with the myopathic form (patient 5) was homozygous for the "mildest" mutation, R411K. Patients with the hepatic form (patients 3 and 4) had R411K or the "mild" mutation R214C, which retained residual activity at both 30 °C and 37 °C, at least in one allele. Patients with the severe form (patients 1 and 2) showed missense mutations (H346R and V422G), which did not retain any enzyme activity at 37 °C. However, patient 2 showed a V422G mutation that retained some residual enzyme activity at 30 °C, and survived until 3 months of age, whereas patient 1 died on the 8th day. These findings may explain the longer survival of patient 2 compared to patient 1. Although the number of patients was restricted, our analysis suggests that a phenotype–genotype correlation may exist in MTP deficiency.

The molecular basis of more than 50 patients with MTP deficiency has been characterized worldwide to date [14–25]. Of them, 26 patients showed mutations of the HADHB gene, and clinical presentation of 25 patients with HADHB gene mutations were clearly described. Nine (36%) of the 26 patients with mutations on HADHB gene were associated with lethal, five hepatic (20%), and eleven myopathic (44%) phenotypes [14,15,17–20,40]. Those previous reports emphasized that the myopathic phenotype is relatively common in MTP deficiency. However, only one myopathic patient was found in Japan, whose age at onset was 15 years old. This may represent one of the ethnic differences from other countries. Alternatively, the frequency of the mild phenotype of MTP deficiency may be underestimated in Japan, since patients with the myopathic phenotype may remain asymptomatic for a long period without any diagnosis being made.

From Asian countries, the molecular analysis of 5 Korean cases including monozygotic twins has been reported to date. Out of the 5 Korean cases, 3 patients showed mutations (exon 16 skipping caused by homozygous IVS16 + 2T–G in twins, and a single heterozygous 2 bp c.1793–4del in another case) of the HADHA gene and these cases showed the lethal phenotype. The other 2 patients showed compound heterozygous missense mutations of the HADHB gene, and both showed the myopathic phenotype [18,40]. In Asian countries, molecular basis of LCHAD or MTP deficiency is rarely described. However LCHAD deficiency, which is caused by a common 1528G > C mutation, is more common in Caucasian [9]. This common 1528G > C mutation has not been found in the Japanese or Korean patients so far. Similarly, molecular basis of medium chain acyl-CoA dehydrogenase deficiency (MCADD) was different between Japanese and Caucasian [41]. The c.985A > G missense mutation represents 80–90% of mutant alleles in Caucasian with MCADD, whereas this mutation was not detected in any Japanese patient with MCADD, and c.449–452delCTGA was identified in 45% of mutant alleles in Japanese [41]. These results suggest that ethnic differences may exist in the genes affected in MTP deficiency, as observed in MCADD.

In summary, our results found no HADHA mutations but variety of HADHB mutations among Japanese patients with MTP deficiency, and the mutational spectrum is heterogeneous. Their phenotypes were correlated with the genotypes, and a combination of two mutant alleles. The present findings show that all missense mutations described in this study are disease-causing, and add two missense mutations (R214C and V422G) and an exonic c.817G single deletion (240 frameshift) to the catalog of HADHB gene mutations. Although no common mutation was found in Japanese patients with MTP deficiency, some mutations appeared to be associated with a specific phenotype. A genetic diagnosis may help to predict the potential outcome of patients and provide more accurate diagnostic information for patients and families with MTP deficiency.

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