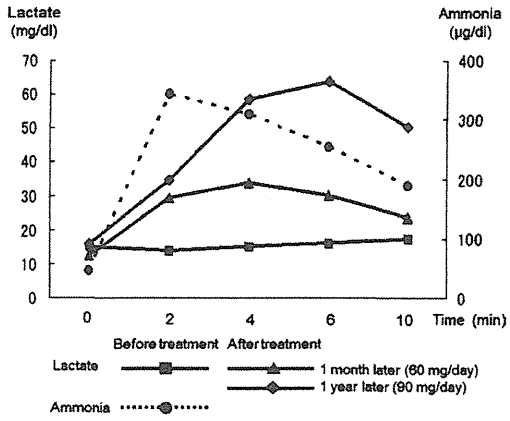

FIGURE 1. Forearm exercise test. For this test, rhythmic (1-Hz) handgrip exercise at maximal voluntary contraction was performed for 2 minutes. Results showed no increase in serum lactate levels before treatment with vitamin B_6 was started. An increase in serum lactate levels was seen 1 month after starting oral vitamin B_6 supplementation at 60 mg/day (1.1 mg/kg/day). Moreover, lactate markedly increased 1 year after treatment with 90 mg/day (1.6 mg/kg/day). Plasma ammonia concentrations were only measured before treatment with vitamin B_6. Time = 0: before exercise. Normal values (at rest): lactate 5.0–20.0 mg/dl; ammonia 9–45 μg/dl.

brachii, which was performed 40 days after the severe rhabdomyolysis, showed variation of muscle fiber size and frequent internal nuclei on hematoxylin and eosin (H&E) staining (Fig. 2A). When muscle phosphorylase activity is preserved, muscle fibers are stained brown or violet with phosphorylase because of their reaction to the iodine–potassium iodide solution used for phosphorylase staining. The higher the activity of phosphorylase, the deeper violet the muscle fibers are stained. However, his muscle fibers did not show phosphorylase staining (Fig. 2B). Periodic acid–Schiff (PAS) staining revealed many glycogen deposits under the sarcolemma of muscle fibers. Under histochemical staining for ATPase activity at pH 4.4, the proportion of type 1 and type 2 fibers was 44% and 56%, respectively. Muscle phosphorylase activity was 3.8 nmol/min/mg protein [control: 58.9 ± 17.5 nmol/min/mg protein (mean ± SD)]. He was found to be homozygous for a single-codon deletion at codon 708/709 in exon 17, which is the most common mutation of muscle phosphorylase among Japanese patients with McArdle disease.[4]

In August 2008, treatment with oral vitamin B_6 supplements (60 mg/day, 1.1 mg/kg/day) was started and, 1 month later, the forearm exercise test showed an increase in lactate levels (Fig. 1). Neurological examination revealed muscle strength improvement (MMT = −0.5). Serum CK levels were normal (146 IU/L) (Fig. 3). In November 2008, we increased the dosage of vitamin B_6 from 60 to 90 mg/day (1.6 mg/kg/day) because serum CK had increased due to more severe physical stress associated with his job. Serum CK levels normalized 1 month after administration of 90 mg/day of vitamin B_6. Subsequently, his muscle weakness gradually improved.

In July 2009, 1 year after starting treatment, the patient's lactate levels markedly increased on the forearm exercise test (Fig. 1). In October 2009, a follow-up muscle biopsy of his right biceps brachii was performed with his informed consent. The fiber size variation was minimized, and 70–80% of the muscle fibers were stained brown with phosphorylase (Fig. 2C and D). The proportions of type 1 and type 2 fibers in the posttreatment sample were 31% and 69%, respectively. Little accumulation of glycogen was observed in the muscle fibers by PAS staining. Muscle phosphorylase activity was 14.4 nmol/min/mg protein, which was 3.8 times higher than before treatment.

The patient's serum CK levels ranged from 120 to 2,093 IU/L (mean 576 IU/L), depending on his physical activities (Fig. 3). However, his clinical condition was stable regardless of the heavy labor he performed during his daily work as a fish dealer. Furthermore, there had been no adverse effects caused by vitamin B_6, including sensory neuropathy.[5–7]

DISCUSSION

Oral vitamin B_6 supplementation (60–90 mg/day) in this patient led to improvements in both muscle weakness and inadequate anaerobic glycolysis; a follow-up muscle biopsy confirmed the presence of increased muscle phosphorylase activity after treatment. Our patient has continued to be engaged in his work for 2 years and 2 months. Although the phosphorylase activity after vitamin B_6 treatment is not completely normal, it is sufficient for him to maintain his regular work activities.

McArdle disease is transmitted as an autosomal recessive trait. The gene for muscle phosphorylase is localized on chromosome 11q13. Deficiency of this enzyme results in inability to metabolize skeletal muscle glycogen during anaerobic metabolism, followed by clinical symptoms such as muscle weakness. There is almost no detectable muscle phosphorylase activity in the majority of affected individuals,[1,4] but some residual activity (i.e., up to 10% of normal values) has been observed in some cases.[1] Our patient had 6.5% of normal muscle phosphorylase activity before treatment, 40 days after the severe rhabdomyolysis in March 2008. The residual activity may have been caused by several regenerating muscle fibers expressing the fetal isoform of muscle phosphorylase.[8,9] In addition, the difference in the proportion of muscle fiber types before and after vitamin B_6 treatment might have been caused by regeneration of skeletal

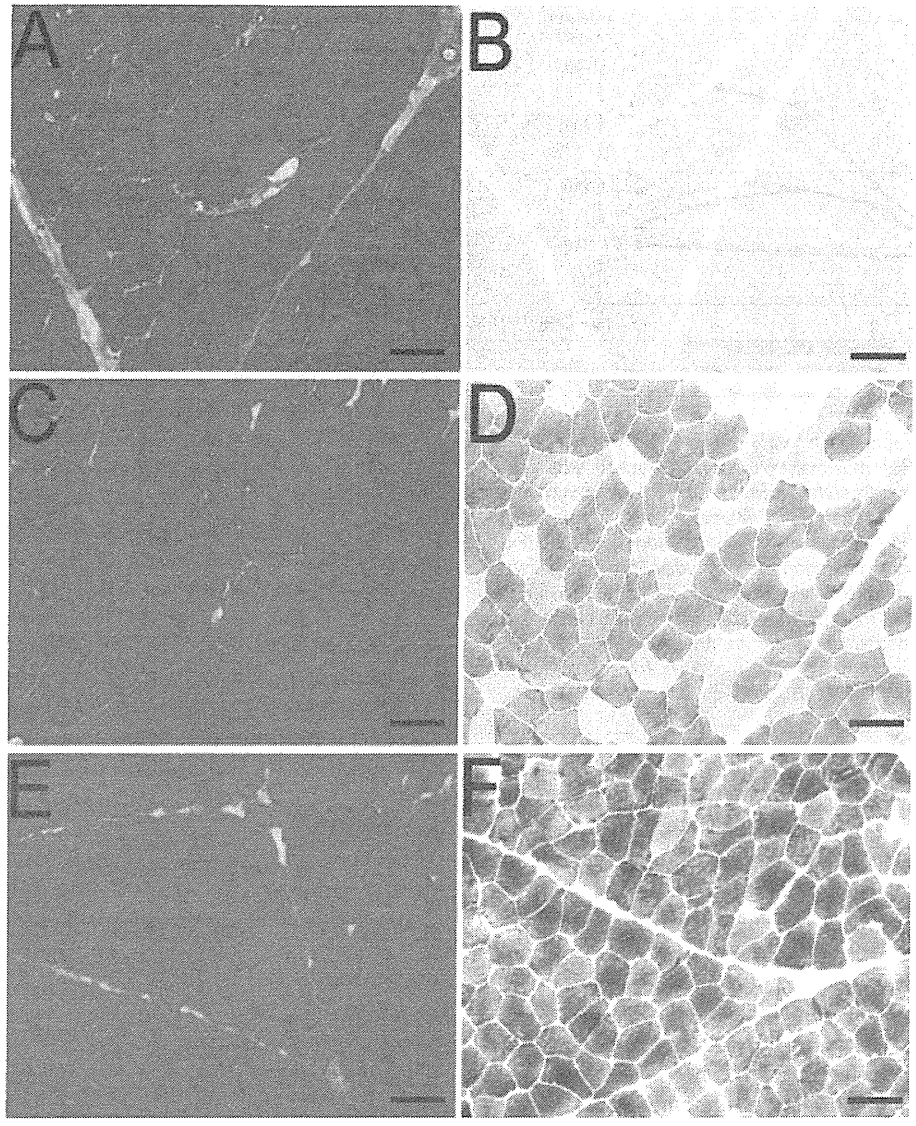

FIGURE 2. Muscle biopsy samples from the patient and a control subject. **(A, B)** Before treatment with vitamin B_6: **(A)** a sample stained with H&E; and **(B)** a sample stained with phosphorylase. Samples showed variation in muscle fiber size and frequent internal nuclei. No muscle fibers were stained with phosphorylase. **(C, D)** After treatment with vitamin B_6: **(C)** a sample stained with H&E; and **(D)** a sample stained with phosphorylase. The muscle fibers stained with phosphorylase increased markedly and muscle fiber size was almost uniform. **(E, F)** Control: **(E)** a sample stained with H&E; and **(F)** a sample stained with phosphorylase. Muscle biopsy samples before and after treatment were stained at the same time as the control sample. Bars = 100 μm.

muscle fibers, especially type 2C fibers. However, we believe that the increased muscle phosphorylase activity of the follow-up muscle biopsy at 1 year and 3 months after treatment was due to treatment with vitamin B_6 because enough time had passed since the episode of severe rhabdomyolysis, and there had been only mildly increased CK levels during treatment.

Lactate increased more dramatically on the forearm exercise test after treatment with higher doses of vitamin B_6 (90 mg/day, 1.6 mg/kg/day) than with lower doses (60 mg/day, 1.1 mg/kg/day). On the other hand, we decreased the dosage of vitamin B_6 from 90 to 60 mg/day in April 2009, because we believed that the toxicity of vitamin B_6 (90 mg/day) resulted in transient exacerbation of muscle weakness and a reduction in regular work activities in this period. However, we determined that the worsening was due to more severe physical work, and we returned the dosage to 90 mg/day in May 2009. Except for this episode, he has been in good condition under treatment with 90 mg/day of vitamin B_6. Thus, these results suggest that the effects of vitamin B_6 may depend on the dosage.

Many trial treatments other than oral vitamin B_6 supplementation have been used for McArdle

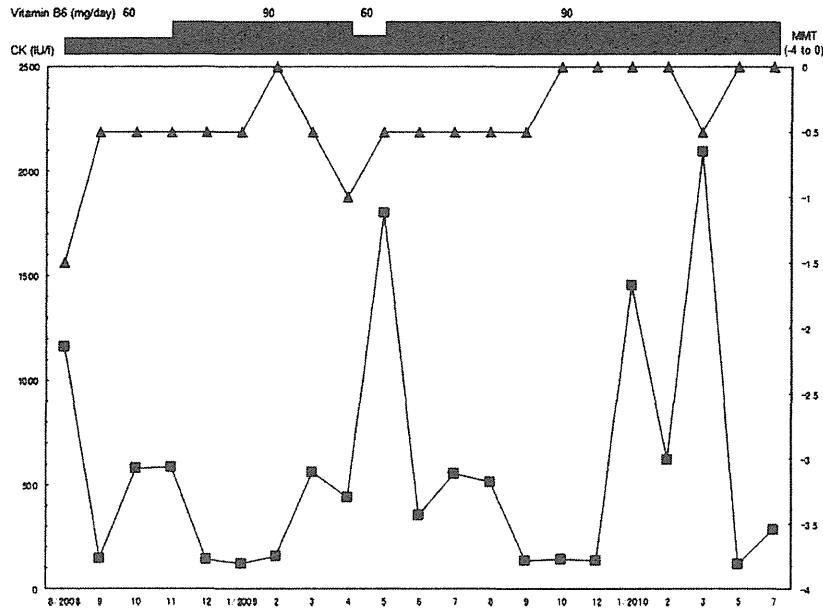

FIGURE 3. Clinical course of the patient. Muscle strength was evaluated by mean MMT of the neck flexors, deltoid muscles, and iliopsoas muscles. The dosage of vitamin B_6 was 60 mg/day from August 2008 to November 2008, 90 mg/day from November 2008 to April 2009, 60 mg/day from April 2009 to May 2009, and 90 mg/day from May 2009 onward. Squares: CK; triangles: MMT. Normal value of CK: 55–290 IU/L.

disease, such as high oral doses of ribose, a fat-rich diet, glucagon, verapamil, a high-protein diet, branched-chain amino acid supplementation, dantrolene sodium, low- or high-dose creatine, oral sucrose, intravenous gentamicin, a ketogenic diet, a high-carbohydrate diet, and ramipril. However, there has been no definitive evidence of any significant benefit from these treatments.[3] On the other hand, the withdrawal of vitamin B_6 supplementation from a patient after 2 years of daily administration resulted in decreased exercise tolerance and increased muscle cramps,[10] which suggested the efficacy of therapy with vitamin B_6 supplements. In addition, a Japanese patient with a very mild case of McArdle disease was treated with vitamin B_6 supplementation (90 mg/day) for 3 months, and the forearm exercise test showed improved glycogenolysis, as in our patient.[11]

In normal individuals, skeletal muscle contains at least 80% of the total body pool of vitamin B_6, bound as pyridoxal 5′-phosphate (PLP) to muscle phosphorylase. One molecule of PLP covalently bound to a lysine residue of each muscle phosphorylase subunit is essential for enzyme activity.[12,13] The decreased phosphorylase in McArdle disease substantially diminishes PLP in skeletal muscle.[12,13] The action of vitamin B_6 supplementation may require the presence of some residual muscle phosphorylase, as in our patient, and probably would not be seen in patients with null mutations, including the R50X mutation, which is most common among Caucasians.[1,14,15]

As noted earlier, most patients lack detectable muscle phosphorylase, as detected by sodium dodecylsulfate–polyacrylamide gel electrophoresis, immunoblot, and enzyme-linked immunosorbent assay.[1,4] This may result from rapid decay of unstable proteins. Thus, we hypothesize that vitamin B_6 supplementation can restore some stability to the mutant enzyme and enhance the residual phosphorylase activity in skeletal muscle of patients, followed by improvement in insufficient anaerobic glycolysis of skeletal muscle. However, other mechanisms are also possible.

Our study suggests that supplementation of vitamin B_6 may be an effective therapy for McArdle disease, especially for patients who have some residual muscle phosphorylase activity, although further studies, including a double-blind, placebo-controlled study, are necessary to draw firm conclusions about the effects of vitamin B_6 supplementation.

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RELAPSED ACUTE MYELOGENOUS LEUKEMIA OF BRACHIAL PLEXUS AFTER MARROW TRANSPLANT

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ABSTRACT: We present a detailed description of brachial plexus infiltration by acute myelogenous leukemia (AML) in the setting of a remission bone marrow biopsy, without evidence of leukemia by flow cytometric analysis. This case illustrates the possibility of dormant leukemic cells in the peripheral nervous system (PNS) in a patient in apparent clinical remission. In patients with an unexplained brachial plexopathy and a history of AML, leukemic infiltrate of the PNS must be considered.

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Metastatic tumors to the brachial plexus are a relatively rare disease entity. Involvement of the brachial plexus by metastatic tumors occurs in most instances via direct extension of the tumor or by means of lymphatic or hematogenous spread.[1] Primary tumors with reported metastases to this region of the peripheral nervous system most frequently include carcinomas of the breast and lung, lymphomas, and melanoma.[2] Although involvement of peripheral nerves by a leukemic infiltrate has been reported rarely, this is a detailed description of brachial plexus pathology by a leukemic infiltrate based on immunohistochemical studies. We describe a patient who had a peripheral nervous system (PNS) relapse of acute myelogenous leukemia (AML) manifested by brachial plexopathy. Of particular interest is that the patient had received a gender-mismatched bone marrow transplant 6 years earlier. The relapse occurred in the setting of a remission bone marrow biopsy with a normal female donor karyotype and with no evidence of leukemia by flow cytometric analysis. A normal complete blood count (CBC) had been present on multiple tests over 6 years.

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; CBC, complete blood count; CLL, chronic lymphocytic leukemia; CNS, central nervous system; CSF, cerebral spinal fluid; EMG,

Key words: brachial plexus, myelogenous leukemia, peripheral nerve metastasis, transplant

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CASE REPORT

History and Neurological Examination. A 33-year-old man was diagnosed with AML when he presented with a hemoglobin of 8.9 g/dl, hematocrit of 26%, leukocytosis [55,000 white blood cells (WBC)/μl], and thrombocytopenia (121,000 platelets/μl). Peripheral blood smear evaluation revealed an abnormal white cell differential with 91% blasts. A bone marrow aspirate and biopsy showed AML with maturation, based on the World Health Organization (WHO) classification.[3] Flow cytometric analysis of the blasts revealed immunophenotypic features indicative of myeloblasts (CD34, CD117, CD33, HLA-DR, CD15, and CD13 positive). Cytogenetic analysis of the bone marrow revealed a trisomy 8 karyotype.

The patient went into remission after chemotherapy, which consisted of daunorubicin and cytarabine (Ara-C), but 1 year later he had a relapse followed by leukemic meningitis. He received intrathecal Ara-C and high-dose intravenous Ara-C (2 g/m^2) and later underwent a gender-mismatched allogeneic bone marrow transplant. His chemotherapeutic regimen for the transplant consisted of ^{131}I monoclonal antibody and fludarabine in addition to low-dose total body radiation. He again went into remission, but his course was complicated by graft-versus-host disease

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Case report

Acid phosphatase-positive globular inclusions is a good diagnostic marker for two patients with adult-onset Pompe disease lacking disease specific pathology

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Abstract

Diagnosis of adult-onset Pompe disease is sometimes challenging because of its clinical similarities to muscular dystrophy and the paucity of disease-specific vacuolated fibers in the skeletal muscle pathology. We describe two patients with adult-onset Pompe disease whose muscle pathology showed no typical vacuolated fibers but did show unique globular inclusions with acid phosphatase activity. The acid phosphatase-positive globular inclusions may be a useful diagnostic marker for adult-onset Pompe disease even when typical vacuolated fibers are absent.

Keywords: Pompe disease; *GAA*; Globular inclusion; Acid phosphatase

1. Introduction

Pompe disease (glycogen storage disease type 2; acid maltase deficiency; OMIM #232300) is an autosomal recessive disease caused by mutations in the gene encoding acid α-glucosidase (GAA, OMIM #606800), a lysosomal enzyme involved in glycogen degradation [1]. Based on age of onset and clinical severity, which depends on residual GAA activity, the disease can be classified into infantile, childhood-onset, and adult-onset forms.

Most of the infantile and childhood-onset forms exhibit disease-specific skeletal muscle pathology, which shows fibers occupied by huge vacuoles that contain basophilic amorphous materials. However, diagnosis of the adult-onset form is sometimes challenging due to clinical similarities to muscular dystrophy and the paucity of typical vacuolated myofibers. We diagnosed 37 patients with Pompe disease including 11 infantile, 16 childhood-onset, and 10 adult-onset forms in the muscle repository of the National Center of Neurology and Psychiatry (NCNP), Japan, based on a deficiency of GAA enzyme activity assayed using biopsied muscles, as previously described [2]. Among these 37 patients, two unrelated Japanese patients did not have disease-specific vacuolated muscle fibers but did have unique cytoplasmic inclusions. Here, we report the diagnostic utility of acid phosphatase (ACP)-positive globular inclusions for adult-onset Pompe disease.

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2. Case report

2.1. Clinical summary

Patient 1: A 44-year-old man had been well until the age of 41 years when he started having difficulty in running. He was admitted to the hospital because of progressive muscle weakness. His parents were first cousins, but there was no family history of neuromuscular disorders. He was clinically suspected to suffer from muscular dystrophy because of slowly progressive muscle weakness and elevated creatine kinase levels of around 800 IU/L (normal, <171 IU/L). On examination, he had grade 4-muscle weakness on medical research council (MRC) scale and marked atrophy in his thighs. He did not have apparent respiratory impairment. Electromyography (EMG) showed myopathic changes with fibrillation and increased polyphasic motor unit potentials (MUPs).

Patient 2: A 62-year-old woman first noticed difficulty in climbing stairs at the age of 35 years, and needed a stick to walk at 45 years. Muscle weakness gradually worsened predominantly in her proximal limbs, and she became wheelchair-bound at 55 years. A muscle biopsy was performed at the age of 61 years. On examination, she had muscle weakness and atrophy predominantly in the proximal upper and lower limbs at the grade 3–4 on MRC scale. Serum CK level was 70 IU/L (normal, <142 IU/L). An EMG showed myopathic changes with increased polyphasic MUPs and myotonic-like repetitive discharges. She had been on non-invasive positive-pressure ventilation since the age of 62 years when the respiratory insufficiency appeared.

2.2. Skeletal muscle pathology

The skeletal muscle pathology from the vastus lateralis of patient 1 and from the biceps brachi of patient 2 showed nonspecific myopathic changes with moderate fiber size variation, mild endomysial fibrosis, and some fiber splitting (Fig. 1A). No necrotic or regenerating fibers were seen. No vacuoles containing amorphous materials were observed. Importantly, both muscles contained red–purple globular inclusions on modified Gomori-trichrome (mGT) stain (Fig. 1A and B). The average percentages of fibers with globular inclusions in the whole mGT-stained section were 0.5% in patient 1 and 2% in patient 2. These inclusions were invariably highlighted by ACP stain but not stained by periodic acid Schiff (PAS) (Fig. 1C). Inclusions were stained only faintly on menadione-linked α-glycerophosphate dehydrogenase (MAG) without substrate (Fig. 3A). Fibers with ACP-positive globular inclusions were also found in 15 of 16 childhood-onset and seven of eight adult-onset patients with disease-specific pathology in varying proportions (0.1–10%). The rate of fibers with inclusions was not significantly different between the childhood-onset and adult-onset forms. Fibers carrying inclusions did not have typical vacuoles with amorphous materials inside. In the infantile cases, more than 90% of the fibers were vacuolated, whereas non-vacuolated fibers with inclusions were hardly recognizable.

Double immunostaining was performed using primary antibodies against a lysosomal marker, lysosomal associated membrane protein-2 (LAMP-2; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA) and an autophagosomal marker, microtubule-associated protein 1 light chain 3 (LC3; Novus Biologicals, Littleton, CO, USA). In fibers with ACP-positive inclusions, immunoreactivity for LAMP-2 and LC3 were accumulated focally in inclusions and surrounding area (Fig. 1D). We also examined another samples from adult-onset patients with typical vacuoles. Fibers with typical vacuoles were entirely positive for LAMP-2 and LC3 (data not shown).

On PAS staining, performed on epon-embedded sections (Epon-PAS) to detect glycogen more sensitively, PAS was negative in globular inclusions but positive in the surrounding area (Fig. 1E).

Electron micrography was performed as previously described using a Tecnai spirit transmission electron microscope (FEI, Hillsboro, OR, USA) [3]. The inclusions consisted of homogeneous electron-dense globules surrounded by increased glycogen particles and autophagic vacuoles (Fig. 1F). The globules contained neither dotted glycogen particles nor a filamentous structure.

2.3. GAA enzymatic analysis and genetic analysis

Presence of globular inclusions led us to suspect Pompe disease, and GAA enzymatic activity analyses revealed 7.5% of normal control activity in patient 1 and 12.3% in patient 2.

Genomic DNA was extracted from peripheral lymphocytes or biopsied muscle using a standard protocol for mutational analysis of *GAA*. All exons and their flanking intronic regions of *GAA* were amplified by PCR and directly sequenced with an ABI PRISM 3100 Automated Sequencer (Applied Biosystems, Foster City, CA, USA). Both patients carried the homozygous *GAA* mutation at the last codon of exon 2 (c. 546G > T). RT-PCR and direct sequencing were performed using RNA extracted from biopsied muscles. This novel mutation causes aberrant splicing by skipping exon 2 (Fig. 2). This homozygous c. 546G > T mutation was also found in another patient with the adult-onset form, whose muscle pathology showed typical skeletal muscle pathology with vacuolated fibers.

3. Discussion

ACP-positive globular inclusions were a good diagnostic marker for the two patients with adult-onset Pompe disease lacking typical vacuolated fibers. Among 12,103 muscle biopsies in the NCNP repository from 1979 to 2010, ACP-positive globular inclusions were not reported, except for Pompe disease.

The globular inclusions are most likely the same as "reducing body-like globular inclusions in late-onset Pompe disease" reported by Sharma et al., as the pathological features are

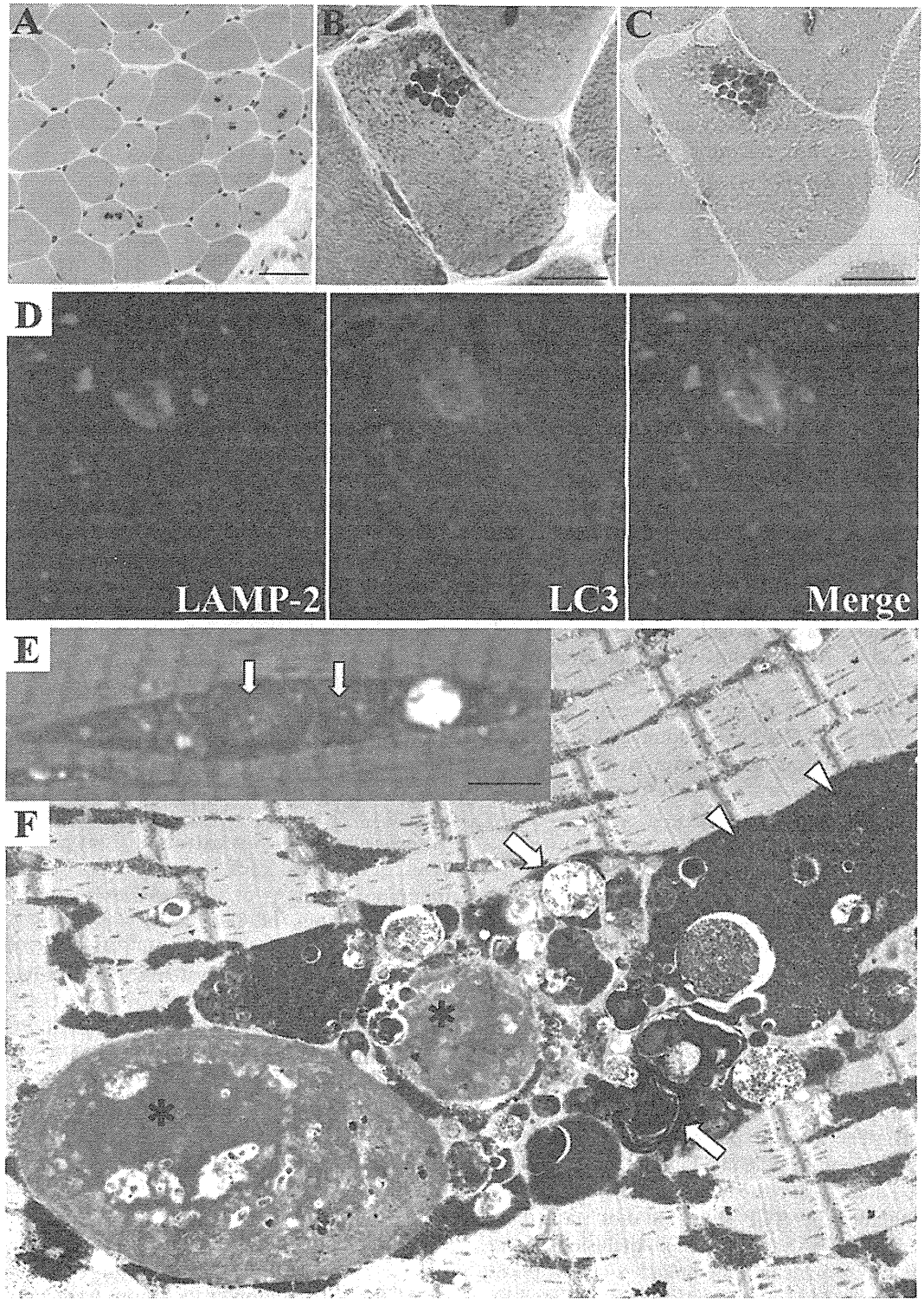

Fig. 1. Acid phosphatase-positive globular inclusions in patient 2. (A and B) Biopsied skeletal muscle showed nonspecific myopathic changes with scattered red–purple colored globular inclusions on modified Gomori-trichrome stain. (C) The inclusions have intense activity on acid phosphatase stain. Bar = 20 μm. (D) Double immunostaining for LAMP-2 (green) and LC3 (red) demonstrates colocalization of positive immunoreactions in the inclusions and surrounding area (B–D; serial sections). (E) On epon-embedded section, periodic acid Schiff stain is negative in inclusions (arrows). Bar = 5 μm. (F) On electron microscopy, globular inclusions (asterisks) lack Z-line structure, which differs from cytoplasmic bodies. Autophagic vacuoles (arrows) and glycogen particles (arrow heads) are seen in the vicinity of globular inclusions (12000×).

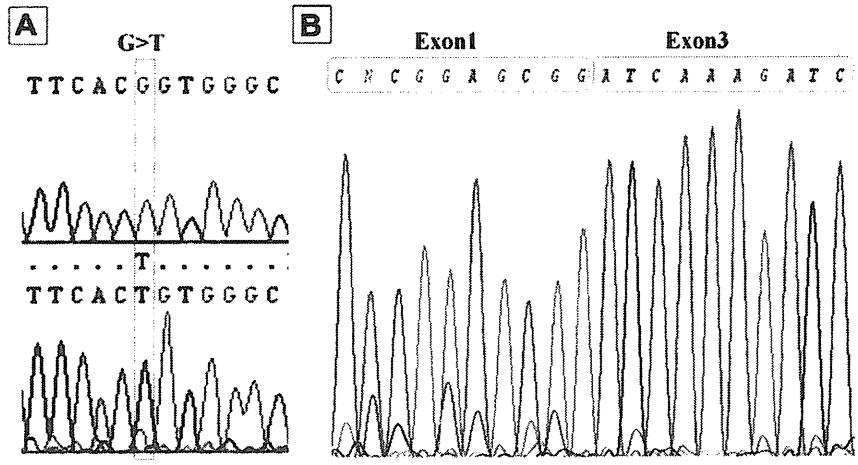

Fig. 2. Mutational analysis of *GAA*. Both patient have a homozygous c. 546G > T mutation at the last codon of exon2 (A upper: control, lower: patient), which creates mRNA with skipping exon 2 (B).

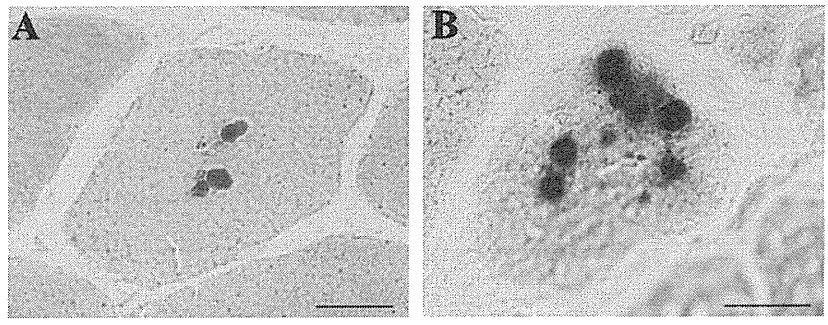

Fig. 3. Inclusions on menadione-linked α-glycerophosphate dehydrogenase (MAG) without substrate. Globular inclusions in Pompe disease (A) are only faintly stained comparing reducing bodies in reducing body myopathy with *FHL1* mutation (B). Bar = 20 μm.

rather similar [4]. However, globular inclusions showed much fainter staining on MAG without substrate than genuine reducing bodies seen in reducing body myopathy with *FHL1* mutations (Fig. 3). More importantly, ACP positivity has not been clearly described previously.

These globular inclusions are reminiscent of cytoplasmic bodies, which are nonspecific findings reflecting degeneration of the Z-disk in various neuromuscular diseases, particularly myofibrillar myopathies. However, the nature of the globular inclusions differs essentially from cytoplasmic bodies because of positive ACP staining and the lack of associated Z-disk components. Although it remains unclear how the ACP-positive globular inclusions are formed, the absence of glycogens in the globular inclusions suggest that they differ from glycogen accumulations in lysosomes. Fibers with typical vacuoles were diffusely positive for both lysosomal and autophagosomal markers as shown previously [5,6]. On the other hand, immunoreactivities of these markers accumulated more focally in fibers with inclusions. Further study should be needed to clarify what causes these pathological differences.

In conclusion, ACP-positive globular inclusions may be a hallmark of Pompe disease and a useful diagnostic marker for adult-onset Pompe disease lacking typical vacuolated fibers. Since enzyme replacement therapy is effective, albeit not fully, in adult-onset patients, early diagnosis is necessary for a better prognosis.

Ethical approval

All clinical materials used in this study were obtained for diagnostic purposes with written informed consent approved by the Ethical Committee of NCNP.

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CASE REPORT

Three Japanese Patients with Beta-Ketothiolase Deficiency Who Share a Mutation, c.431A>C (H144P) in *ACAT1*: Subtle Abnormality in Urinary Organic Acid Analysis and Blood Acylcarnitine Analysis Using Tandem Mass Spectrometry

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Abstract Mitochondrial acetoacetyl-CoA thiolase (T2) deficiency affects both isoleucine catabolism and ketone body metabolism. The disorder is characterized by intermittent ketoacidotic episodes. We report three Japanese patients. One patient (GK69) experienced two ketoacidotic episodes at the age of 9 months and 3 years, and no further episodes until the age of 25 years. She had two uncomplicated pregnancies. GK69 was a compound heterozygote of the c.431A>C (H144P) and c.1168T>C (S390P) mutations in T2 (*ACAT1*) gene. She was not suspected of having T2 deficiency during her childhood, but she was diagnosed as T2 deficient at the age of 25 years by enzyme assay using fibroblasts. The other two patients were identical twin siblings who presented their first ketoacidotic crisis simultaneously at the age of 3 years 4 months. One of them (GK77b) died during the first crisis and the other (GK77) survived. Even during severe crises, C5-OH and C5:1 were within normal ranges in their blood acylcarnitine profiles and trace amounts of tiglylglycine and small amounts of 2-methyl-3-hydroxybutyrate were detected in their urinary organic acid profiles. They were H144P homozygotes. This H144P mutation has retained the highest residual T2 activity in the transient expression analysis of mutant cDNA thus far, while the S390P mutation did not retain any residual T2 activity. The "mild" H144P mutation may result in subtle profiles in blood acylcarnitine and urinary organic acid analyses. T2-deficient patients with "mild" mutations have severe ketoacidotic crises but their chemical phenotypes may be subtle even during acute crises.

Abbreviations

SCOT Succinyl-CoA:3-ketoacid CoA transferase
T2 Mitochondrial acetoacetyl-CoA thiolase

Introduction

Mitochondrial acetoacetyl-CoA thiolase (T2, gene symbol ACAT1) deficiency (OMIM 203750) is an autosomal recessive inborn error of metabolism that affects the catabolism of isoleucine and ketone bodies. This disorder, first described by Daum et al. (1971), is characterized by intermittent episodes of metabolic ketoacidosis associated with vomiting and unconsciousness often triggered by infections (Fukao et al. 2001). There are no clinical symptoms between episodes. Typical T2 deficiency is easily diagnosed by urinary organic acid analysis, characterized by massive excretion of tiglylglycine, 2-methyl-3-hydroxybutyrate and 2-methylacetoacetate both during ketoacidotic episodes and between episodes (Fukao et al. 2001, 2003). Diagnosis is confirmed by measurement of T2 activity on cultured skin fibroblasts (Robinson et al. 1979; Zhang et al. 2004). T2 deficiency is caused by mutations in the *ACAT1 (T2)* gene located on chromosome 11q22.3-q23.1 (Fukao et al. 1990; Kano et al. 1991). T2 deficiency is very heterogeneous at the genotype level, with at least 50 different mutations described (Fukao et al. 1995, 1997, 1998, 2001, 2002, 2003, 2007, 2008, 2010a, b; Wakazono et al. 1995; Nakamura et al. 2001; Zhang et al. 2004, 2006; Sakurai et al. 2007).

Some T2-deficient patients with mutations which retain some residual activity do not show typical urinary organic acid profiles (Fukao et al. 2001, 2003). We herein describe three Japanese patients with T2 deficiency whose H144P mutation retains significant residual activity. Their urinary organic acid and blood acylcarnitine profiles were atypical and subtle even during severe ketoacidotic crises.

Materials and Methods

Case Reports

GK69

This Japanese woman (GK69), born in 1984, developed severe metabolic acidosis at the age of 9 months. On admission to a third-level hospital, she was semicomatose, polypneic (48/min), and hypotonic. Laboratory values were: blood glucose 6.8 mmol/L, NH_3 92 μmol/L, blood pH 7.225, pCO_2 7.2 mmHg, bicarbonate 3 mmol/L, base excess −21.3, Na 153 mEq/L (normal range: 139–146), BUN 28.5 mg/dL (normal range: 10–18), and creatinine 1.1 mg/dL (normal range: 0.18–0.46). Metabolic acidosis was refractory to sodium bicarbonate therapy. Peritoneal dialysis was performed for 2 days. On the second hospital day, polypnea and unconsciousness disappeared and the blood gas data improved. Urinary organic acid analysis showed massive amounts of acetoacetate and 3-hydroxybutyrate with dicarboxylic aciduria. No increases in 2-methyl-3-hydroxybutyrate or tiglylglycine were noted, although this analysis was performed in an outside laboratory and no urine samples were available for reanalysis. At that time, T2 deficiency was excluded from differential diagnosis based on this organic acid data and the tentative diagnosis was succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency. However, an enzyme assay for SCOT was not performed. At the age of 3 years, the patient had a similar but milder episode. Subsequently, she had no further ketoacidotic episodes. Growth and development were normal. She had two uncomplicated pregnancies.

Twin Siblings (GK77b and GK77)

GK77b is a twin Japanese boy. He was born at 36 weeks gestation weighing 2,400 g. His parents had no known consanguinity but both were from a small island in Amami islands in Japan. He experienced several febrile illnesses without ketoacidosis. However, at 3 years 4 months of age, after a 3-day history of fever, cough, and vomiting, he developed anorexia, lethargy, and polypnea. He was admitted to a local hospital. His blood glucose level was 2.3 mmol/L. Blood gas analysis was not performed. Hypoglycemia was corrected with intravenous glucose injection of 20 ml of 20% glucose solution followed by continuous infusion of a 2.6% glucose solution. About 30 h after admission, his condition worsened. Blood gas analysis revealed severe metabolic acidosis showed pH 6.88, pCO_2 6.1 mmHg, and bicarbonate 1.1 mmol/L. He was transferred to a regional hospital. On arrival at the hospital, he was unconscious with a heart rate of 168/min and respiratory rate of 39/min. Blood laboratory data were: WBC 19,050/μL, CRP 0.2 mg/dL (normal values: <0.15), BUN 36.2 mg/dL (normal range: 10–18) creatinine 0.5 mg/dL (normal range: 0.25–0.49), NH3 33.5 μmol/L, glucose 3.8 mmol/L, pH 7.17, pCO_2 20 mmHg, bicarbonate 6.3 mmol/L, base excess −22.4 mmol/L, and total ketone bodies 16.3 mmol/L. He received continuous infusion of 5% glucose solution at 3.4 mg/kg/min and sodium bicarbonate at 0.4–0.47 mEq/kg/h. However, unconsciousness and metabolic acidosis did not improve. On the fifth hospital day, he died before being transferred to a third-level hospital.

GK77 is the twin brother of GK77b. Pyloric stenosis was diagnosed at the age of 1 month and corrected surgically;

thereafter, he was well until 3 years 4 months of age. Two days after the onset of his twin brother, he developed frequent repeated vomiting after cough and nasal discharge. Therefore, he was admitted to the regional hospital at the same time as his twin. On admission, he was lethargic. Laboratory findings were: WBC 7,760/μL, CRP 0.5 mg/dL (normal values: <0.15), BUN 20.2 mg/dL (normal range: 10–18), creatinine 0.4 mg/dL (normal range: 0.25–0.49), glucose 3.7 mmol/L, NH3 25 μmol/L, blood pH 7.135, pCO_2 19.5 mmHg, bicarbonate 6.3 mmol/L, base excess −22.4 mmol/L, and total ketone bodies 10.1 mmol/L. He received a continuous infusion of 5% glucose solution at 3.4 mg/kg/min and sodium bicarbonate at 0.3 mEq/kg/h. On the third hospital day, his condition worsened and he was transferred to a third-level hospital. On admission, the blood gasses were pH 7.372, pCO_2 21.6 mmHg, bicarbonate 12.2 mmol/L, and base excess −11.2 mmol/L. A glucose infusion rate was further increased to 6.5 mg/kg/min with 10% glucose solution. Acidosis normalized with 9 h (pH 7.399, bicarbonate 21.7 mmol/L, base excess −2.6 mmol/L). Two days later, the urinary ketones became negative and he started eating.

GK77 is now 4 years 8 months and has experienced no further ketoacidotic episodes. The family has been advised to avoid fasting and to come to the local hospital if he has a high fever or appetite loss. His growth and development are within normal ranges.

Urinary Organic Acid Analysis and Acylcarnitine Analysis

Urine samples containing 0.2 mg of creatinine were used for our high risk screening of organic acids. As internal standards, 20 mg each of tropate (TA, C9), margarate (MGA, C17), and tetracosane (C24) were added to these samples. Trimethylsilylated samples were analyzed using capillary gas chromatography-mass spectrometry (QP 5050A, Shimadzu Co. Ltd., Kyoto, Japan), as described earlier (Kimura et al. 1999). The values of organic acids were expressed as the peak area (%) relative to IS-1 (margarate) on the mass chromatogram. Quantification of 2-methyl-3-hydroxybutyrate and tiglylglycine in urine samples from GK77b and GK77 was kindly done by Dr. Sass (Freiburg University) (Lehnert 1994). For comparison, quantification was also done in urine samples from T2-deficient patients whose urinary screening profiles had typical T2 deficient ones. We used urine sample in stable condition from GK01 who is a compound heterozygote of A333P and c.149delC (Fukao et al. 1998) and samples in acute and stable conditions from T2-deficient patients from India (GK(Ind)) in our high-risk screening. Blood spot and serum acylcarnitine analysis using tandem mass spectrometry was also done, as described (Kobayashi et al. 2007), and blood spot samples from GK75 and GK79, who are R208X homozygotes (Fukao et al. 2010b) were used as positive controls.

Enzyme Assay and Immunoblot Analysis Using Fibroblasts

Control and patients' fibroblasts were cultured in Eagle's minimum essential medium containing 10% fetal calf serum. Acetoacetyl-CoA thiolase activity was assayed, as described (Robinson et al. 1979; Zhang et al. 2004). We assayed acetoacetyl-CoA thiolase activity in the presence and absence of potassium-ion, since T2 is the only thiolase which is activated by the ion. Immunoblot analysis was done, as described (Fukao et al. 1997). In the cases of the controls, twofold serial dilution samples from 30 to 3.75 μg were electrophoresed together with samples (30 μg) of GK68 and GK77 to determine the amount of T2 protein in the patients' fibroblasts relative to that in the control fibroblasts.

Mutation Analysis

This study was approved by the Ethical Committee of the Graduate School of Medicine, Gifu University. Genomic DNA was extracted from fibroblasts using a SepaGene kit (Sanko Junyaku, Tokyo, Japan). Mutation screening was performed by PCR and direct sequencing of genomic fragments that included each exon and its surrounding intron sequences (Fukao et al. 1998). For GK77b and the parents, exon 5 was amplified from a dried blood spot 1.25 mm in diameter, which was used for tandem mass spectrometry, using Amplidirect Plus (Shimadzu Biotech, Tsukuba, Japan).

Restriction Enzyme Assay to Detect c.431A>C (H144P)

The c.431A>C (H144P) mutation creates a new BmgT120 I site (GGACC). DNAs from 110 Japanese controls were examined using a restriction enzyme assay, as follows.

A fragment (314 bp), including exon 5 and its surrounding introns, was amplified using the following primers:

In4 as (in intron, −69 to −48)5′-CATGCTCTATTAAG-TTCTGCAG-3′

In5 as (in intron, +137 to +119) 5′-ATCCAGACACTCT-TGAGCA-3′

An aliquot of the resulting amplicon was digested with BmgT120 I, then resolved on a 5% polyacrylamide gel. The c.431A fragment (wild-type) is 314-bp long and the c.421C fragment is cut into 162-bp and 152-bp fragments.

Transient Expression Analysis of Mutant cDNAs

Transient expression of T2 cDNAs was performed using a pCAGGS eukaryote expression vector (Niwa et al. 1991), as described (Sakurai et al. 2007). After transfection, cells were cultured at 37°C or 40°C for 48 h, then harvested and kept at −80°C until use. Cells were freeze-thawed and sonicated in 50 mM sodium phosphate (pH 8.0) and 0.1% Triton X-100. After centrifugation at 10,000 × g for 10 min, the supernatant was used in an enzyme assay for acetoacetyl-CoA thiolase activity and for immunoblot analysis.

Results and Discussion

Confirmation of the Diagnosis

GK69's fibroblasts were assayed for SCOT activity to confirm the diagnosis in 2008, when GK69 was 24 years old. As shown in Table 1, she was diagnosed as having T2 deficiency but not as having SCOT deficiency.

SCOT deficiency was first suspected in GK77 and GK77b, based on the following facts (1) Two of the four SCOT deficient Japanese families were from the Amami islands, the population of which is about 120,000. They were T435N homozygotes (Fukao et al. 2004). (2) The acylcarnitine profiles and urinary organic acid analysis during acute ketoacidotic crisis in both patients had no typical profile for T2 deficiency, as discussed below. As shown in Table 1, GK69's and GK77's fibroblasts had normal SCOT activity and a higher ratio (1.3) of acetoacetyl-CoA thiolase activity in the presence to the absence of potassium ions than typical T2-deficient fibroblasts (the ratio was around 1.0). Immunoblot analysis also showed a clearly detectable amount of T2 protein in GK77's fibroblasts, and a lower amount in GK69's fibroblasts. Densitometric analysis showed that the amounts of T2 protein in GK77 and GK69 were estimated to be 50% and 25% of control, respectively (Fig. 1).

Table 1 Acetoacetyl-CoA thiolase activities in the absence and presence of potassium ions

Fibroblasts	Acetoacetyl-CoA thiolase activity			SCOT activity
	$-K^+$	$+K^+$	$+K^+/-K^+$	
Controls ($n = 5$)	5.0 ± 0.7	10.8 ± 0.9	2.2 ± 0.3	6.7 ± 2.1
GK69	3.6 ± 0.5	4.1 ± 0.9	1.2 ± 0.1	4.7 ± 1.4
GK77	4.2 ± 0.3	5.8 ± 1.5	1.4 ± 0.3	3.9 ± 0.5
T2D	4.5 ± 1.4	4.7 ± 1.6	1.0 ± 0.1	5.6 ± 0.5

Enzyme activity is expressed as nmol/min/mg of protein. In cases of patients, enzyme assay was done three times and shows average ± SD. T2D, A disease control

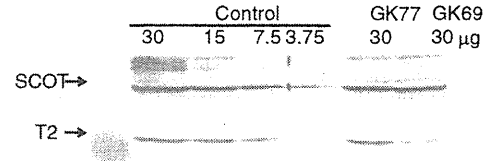

Fig. 1 Immunoblot analysis. In the cases of the controls, serial twofold dilutions from 30 to 3.75 μg were studied together with samples (30 μg) from GK68 and GK77. The first antibody was a mixture of an anti-T2 antibody and an anti-SCOT antibody. The positions of the bands for T2 and SCOT are indicated by *arrows*

Mutations and Their Effects on T2 Protein

Mutation screening revealed that GK69 was a compound heterozygote of c.431A>C (H144P) and c.1168T>C (S390P). Her mother had S390P heterozygously but did not have H144P. The father's DNA was not available for analysis. GK77 had an H144P mutation homozygously, shown by mutation screening at the genomic level. Their parents and GK77b were heterozygous carriers and a homozygote of H144P, respectively. The c.431A>C (H144P) mutation creates a BmgT120I site (GGACA to GGACC). We could not find c.431A>C (H144P) in the 110 Japanese controls using the restriction enzyme assay with BmgT120I.

We performed transient expression analysis of wild-type and mutant cDNAs in T2-deficient SV40-transformed fibroblasts. Following expression of T2 cDNAs for 48 h at 37°C, an enzyme assay and immunoblots were performed (Fig. 2a,b). The transfection of wild-type T2 cDNA produced high potassium ion-activated acetoacetyl-CoA thiolase activity (T2 activity), whereas that of mock cDNA produced no demonstrable enzyme activity at any temperature. The H144P mutant retained a residual T2 activity of ~25% of the wild-type value (Fig. 2a). The S390P mutant did not retain any residual T2 activity. In immunoblot analysis (Fig. 2b), the H144P mutant protein was detected, whereas no S390P protein was detected. The relative amount of the H144P mutant protein, as compared to the wild-type, was estimated to be 50%. Hence, the specific activity (unit/mg of T2 protein) of the H144P mutant protein was estimated to be about 50% of the wild type. Protein-folding and post-folding stability is predicted to vary with the incubation temperature. Hence, we also performed transient expression at 40°C for 48 h. The H144P mutant in expression at 40°C had a similar level of residual activity to that at 37°C.

We reported the tertiary structure of the human T2 tetramer (Haapalainen et al. 2007). Figure 3a shows the positions of the H144P and S390P mutations on the dimer.

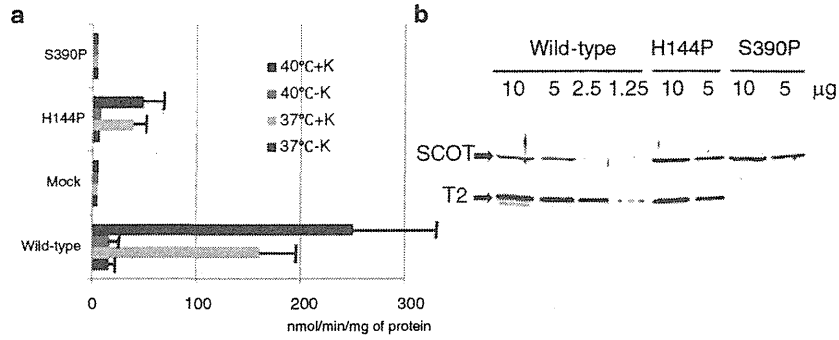

Fig. 2 Transient expression analysis of H144P and S390P mutant cDNAs. Transient expression analysis was performed at 40°C and 37°C. (**a**) Potassium ion-activated acetoacetyl-CoA thiolase assay. Acetoacetyl-CoA thiolase activity in the supernatant of the cell extract was measured. The mean values of acetoacetyl-CoA thiolase activity in the absence (−K) and presence (+K) of potassium ions are shown together with the SD of three independent experiments. (**b**) Immunoblot analysis. The protein amounts applied are indicated above the lanes. The first antibody was a mixture of an anti-T2 antibody and an anti-SCOT antibody. The positions of the bands for T2 and SCOT are indicated by *arrows*

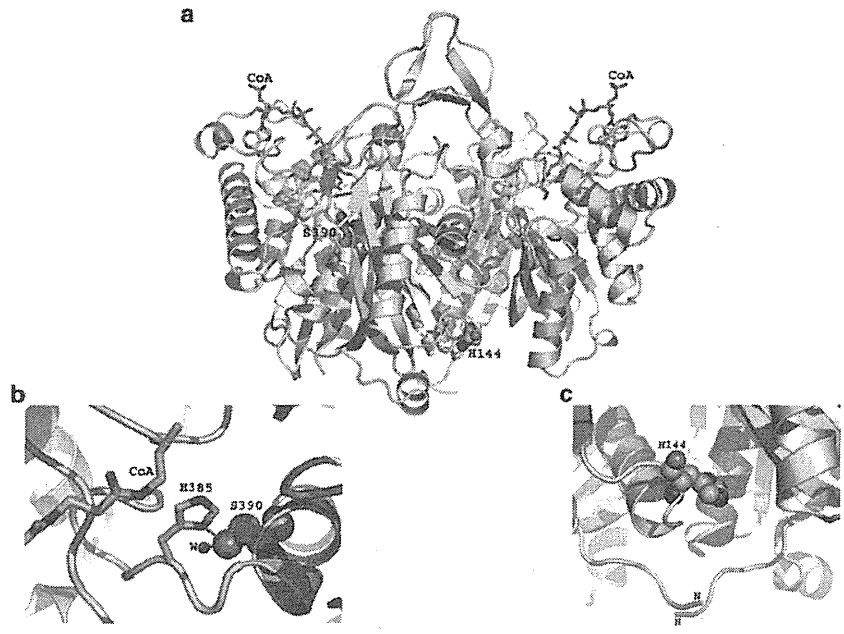

Fig. 3 The positions of H144P and S390P on the tertiary structure of human T2 dimers with substrates of coenzyme A

As seen in the figure, S390 is close to the active site and H144 is at the dimer interface close to the surface of the protein. Figure 3b shows a zoomed-in view around S390. This mutant is located at the active site. S390 is hydrogen-bonded to catalytic histidine, H385; it could be that this serine is needed to orient histidine in a way that the histidine can stabilize the transient negative charge of the substrate optimally. S390 is also hydrogen-bonded to a water molecule that is needed in stabilizing parts of the enzyme. So, if S390 is mutated into proline, these two hydrogen bonds do not exist. Hence, this S390P is expected to bring about a serious change in T2 catalytic cavity. In our expression analysis, this S390P was also too unstable to detect mutant protein. Figure 3c shows a zoomed-in view at the dimer interface. H144 is interacting with the residues of the neighboring subunit. If this residue is mutated into Pro, there is less dimeric interaction, which in turn might destabilize the overall structure. Since this residue is far from the active site and substrate binding site, it is difficult to explain why this H144P mutant had reduced specific activity in transient expression analysis from the viewpoint of structural analysis.

Urinary Organic Acid Analysis

GK69 was first suspected to having T2 deficiency as a probable diagnosis; however, urinary organic acid analysis at the first ketoacidotic crisis indicated no characteristic profile for T2 deficiency such as elevated 2-methyl-3-hydroxybutyrate and tiglylglycine in 1985 (no data was available). The results of the urinary organic acid analysis of our patients are shown in comparison with those of typical T2-deficient patients, GK01 and GK(Ind) (Table 2, Fig. 4). At the age of 24 years when her condition was stable, GK69's urinary organic acid analysis showed that there were only trace amounts of 2-methyl-3-hydroxybutyrate and tiglylglycine (Table 2). In our screening, this low level of tiglylglycine was difficult to detect. Urinary organic acid analysis during the acute crises of GK77 and GK77b showed huge amounts of 3-hydroxybutyrate and acetoacetate with elevated 2-methyl-3-hydroxybutyrate but only trace amounts of tiglylglycine. The levels of 2-methyl-3-hydroxybutyrate and tiglylglycine during a stable condition in GK77 are similar with those in GK69.

In cases of typical T2-deficient patients, it is easy to suspect T2 deficiency based on large amounts of 2-methyl-3-hydroxybutyrate and tiglylglycine as shown in Fig. 4. However, even in cases of trace amounts of tiglylglycine (possibly under the detection limit), T2 deficiency cannot be excluded. An H144P mutation, which retained high residual activity, may contribute to atypical profiles in the presented cases. These findings strengthen our previous observations that some T2-deficient patients with mutations, which retain some residual activity do not show typical urinary organic acid profiles (Fukao et al. 2001, 2003).

Table 2 Quantitative analysis of urinary organic acid analysis during acute crises and stable conditions

Patients	Acute crises		Stable conditions	
	2M3HB	Tiglylglycine	2M3HB	Tiglylglycine
GK69	NA	NA	14.0	13.3
GK77b	405.7	45.8	NA	NA
GK77	160.2	6.7	27.3	14.8
GK01	NA	NA	399.1	732.1
GK(Ind)	484.6	503.9	195.1	797.6
Controls ($n = 42$)			10.7 ± 7.6	24.6 ± 14.6

Values are expressed as mmol/mol creatinine

NA means that samples were not available for the analysis. GK01 is a compound heterozygote of c.149delC and A333P, which retained no residual activity (Fukao et al. 1998). GK(Ind) indicates a patient with typical T2-deficient profiles of urinary organic acids in our screening

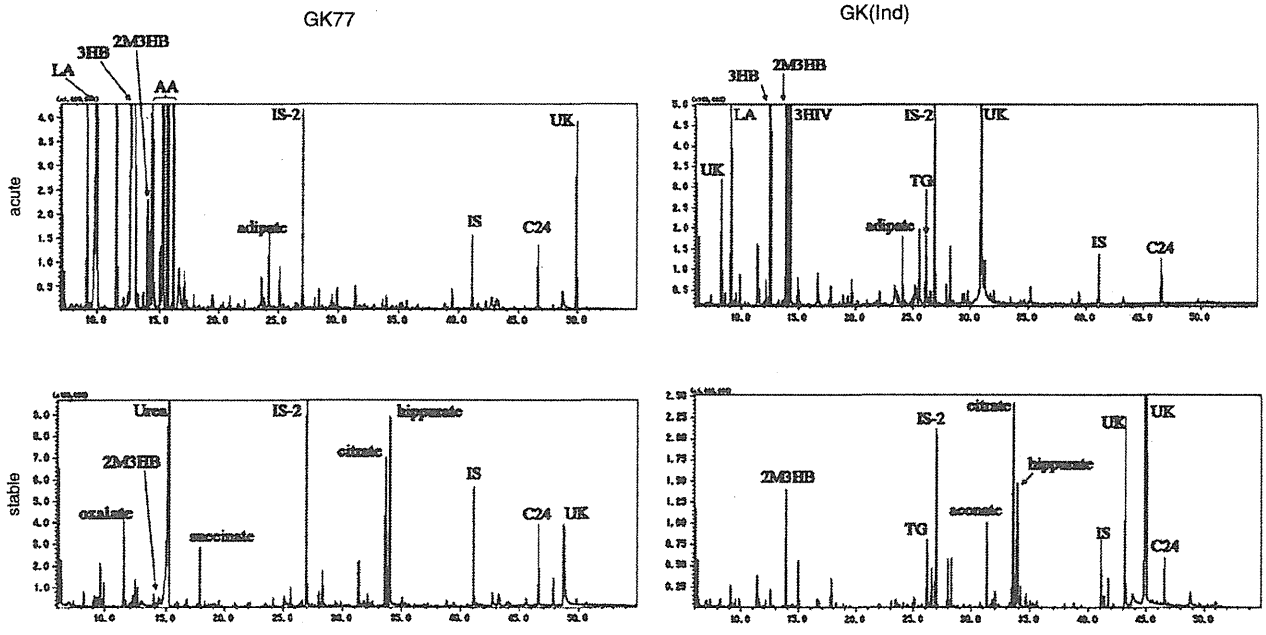

Fig. 4 Urinary organic acid profiles of GK77 during the acute episode and an asymptomatic period in comparison with those of a typical T2-deficient patient (GK(Ind)). *LA* Lactate, *3HB* 3-OH-butyrate, *3HIV* 3-OH-isovalerate, *AA* Acetoacetate, *2M3HB* 2-Methyl-3-OH-butyrate, *TG* Tiglylglycine, *IS-2* and *IS* Internal standards, *UK* Unknown. Since acetoacetate is unstable and samples from GK(Ind) were shipped on filter papers after thoroughly drying, the levels of acetoacetate are likely underestimated

Table 3 C5-OH and C5:1 carnitines in blood filters and serum samples from GK77 and GK77b during acute crises

Patients	Dried blood spots		Serum	
	C5:1	C5-OH	C5:1	C5-OH
GK77b	0.027	0.11	ND	0.12
GK77	0.012	0.11	0.044	0.10
R208X homozygotes				
GK75 (acute)	0.89	2.89	NA	NA
GK79 (stable)	1.20	2.35	NA	NA
Controls (n = 30)				
Average ± SD	0.015 ± 0.016	0.26 ± 0.15	0.015 ± 0.013	0.059 ± 0.024

ND not detected, *NA* not applicable

The values are expressed as μmol/L

GK75 and GK79 are positive controls for T2 deficient patients who are R208X homozygotes (Fukao et al. 2010b)

Blood and Serum Acylcarnitine Analyses

Acylcarnitine analysis was done using samples during the acute crises of GK77 and GK77b. Table 3 shows the results in comparison with those of typical T2-deficient patients (R208X homozygotes) (Fukao et al. 2010b). C5:1 and C5OH elevation in blood spots, characteristic for T2 deficiency, was clearly detected in the samples from the typical T2-deficient patients but was absent in samples from GK77 and GK77b. We previously reported that the abnormality of the acylcarnitine profiles in T2-deficient patients with mutations which retain some residual activity is subtle during nonepisodic conditions (Fukao et al. 2003), but the present study clearly showed that it could be also subtle even during severe ketoacidotic episodes. This means that acylcarnitine analysis using blood spots cannot detect some T2-deficient patients like GK77 and GK77b. Serum acylcarnitine analysis might detect elevation of these compounds to some extent, but we need to analyze more cases to clarify the usefulness of serum acylcarnitine analysis in such T2-deficient patients with mutations which retain some residual activity.

T2 deficiency cannot be excluded even if acylcarnitine profiles during acute episodes are within normal ranges. Careful evaluation of urinary organic acids, especially for the presence of 2-methyl-3-hydroxybutyrate, is necessary not to overlook T2 deficiency.

Clinical Issues

Since they were confirmed as identical twins by DNA analysis (data not shown), their genetic backgrounds were identical and most environmental factors were also very similar between them. One died during the ketoacidotic crisis and the other survived.

In Japan, intravenous infusion therapy for vomiting, appetite loss, and dehydration is commonly performed with commercially available initial infusion solution, such as Solita T1 (2.6% glucose) followed by maintenance solution, such as Solita T2 and T3 (4.3% glucose). These solutions are effective for physiological ketosis. However, in the case of T2 deficiency, a higher concentration of glucose may be necessary. Accordingly, we had the impression that GK77 became much better after the glucose concentration was changed from 5% to 10%. In the case of prolonged ketoacidosis, consideration should be given to increasing the infusion rate of glucose to ensure high normal blood glucose level to suppress ketone body synthesis and isoleucine catabolism via insulin secretion.

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Concise One-Sentence Take-Home Message

Patients with beta-ketothiolase deficiency having a mutation which retains some residual activity showed subtle abnormality in urinary organic acid analysis and blood acylcarnitine analysis even during acute ketoacidotic episodes.

Details of the Contributions of Individual Authors

Toshiyuki Fukao and Naomi Kondo performed the enzyme assays, immunoblot/mutation analysis, and expression analysis of cDNAs. Toshiyuki Fukao mainly wrote this manuscript. Shinsuke Maruyama, Toshihiro Ohura, Mitsuo Toyoshima, Naomi Kuwada, and Mari Imamura are the physicians responsible for the patients. Yuki Hasegawa and Seiji Yamaguchi performed gas chromatography-mass spectrometry and tandem mass spectrometry analyses and first suspected the disorder. Isao Yuasa confirmed GK77 and 77b as identical twins by DNA analyses. Antti M Haapalainen and Rik K Wierenga analyzed the tertiary structural effects of the mutations.

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Alpha-methylacetoacetic acidura, mitochondrial acetoacetyl-CoA thiolase deficiency (OMIM 203750, 607809)

Mitochondrial acetoacetyl-CoA thiolase, acetyl-CoA acetyltransferase 1 (EC 2.3.1.9)

ACAT1 gene (gene ID 38, NM_000019.3)

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Details of Ethics Approval

This study has been approved by the Ethical Committee of the Graduate School of Medicine, Gifu University.

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RESEARCH ARTICLE

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Molecular Basis of Two-Exon Skipping (Exons 12 and 13) by c.1248+5g>a in *OXCT1* Gene: Study on Intermediates of *OXCT1* Transcripts in Fibroblasts

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ABSTRACT: The molecular basis of simultaneous two-exon skipping induced by a splice-site mutation has yet to be completely explained. The splice donor site mutation c.1248+5g>a (IVS13) of the *OXCT1* gene resulted predominantly in skipping of exons 12 and 13 fibroblasts from a patient (GS23) with succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency. We compared heteronuclear RNA (hnRNA) intermediates between controls' and GS23's fibroblasts. Our strategy was to use RT-PCR of hnRNA to detect the presence or absence of spliced exon clusters in RNA intermediates (SECRIs) comprising sequential exons. Our initial hypothesis was that a SECRI comprising exons 12 and 13 was formed first followed by skipping of this SECRI in GS23 cells. However, such a pathway was revealed to be not a major one. Hence, we compared the intron removal of SCOT transcript between controls and GS23. In controls, intron 11 was the last intron to be spliced and the removal of intron 12 was also rather slow and occurred after the removal of intron 13 in a major pathway. However, the mutation in GS23 cells resulted in retention of intron 13, thus causing the retention of introns 12 and 11. This "splicing paralysis" may be solved by skipping the whole intron 11–exon 12–intron 12–exon 13–mutated intron 13, resulting in skipping of exons 12 and 13.
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KEY WORDS: two-exon skipping; splicing order; splice site; heteronuclear RNA; SCOT; succinyl-CoA:3-ketoacid CoA transferase

Introduction

Transcription of mRNA precursors by RNA polymerase II and their splicing by the spliceosome are essential steps in gene expression. Moreover, splicing is cotranscriptional [Maniatis and Reed, 2002]. Splicing is thus ordered but not processive [Gudas et al. 1990; Kessler et al. 1993; Lang and Spritz 1987].

Most introns have conserved 5′ (donor) and 3′ (acceptor) sequences that flank the exons, a short polypyrimidine tract adjacent to the acceptor site, and a branch point sequence 18–40 nucleotides upstream from the acceptor dinucleotide [Berget, 1995; Hawkins, 1988]. Mutations in these sites can lead to exon skipping, short deletions, or insertions in the mature mRNA. It is difficult to predict the effects of splice-site mutations and understanding requires analysis of the various mRNA spliced products in cells that express the target gene. Mutations affecting splicing are a major fraction of disease-causing mutations in humans. The mechanisms whereby gene mutations result in aberrant splicing are mostly apparent from current exon-definition models of splicing [reviewed by Berget, 1995].

There are some examples where a single nucleotide substitution at such splice junctions causes the skipping of two exons [Fang et al., 2001; Haire et al., 1997; Hayashida et al., 1994; Schneider et al., 1993; Takahara et al., 2002]. The mechanisms for multiple skipping are not apparent from current exon- and intron-definition models of splicing [Takahara et al., 2002] and not well understood. The unusual occurrence of a two-exon skip may be explained by the order of intron removal in this region such that an intron is removed rapidly, in most transcripts, with respect to adjacent two introns, so that a large exon-like structure can then be skipped [Takahara et al., 2002]. In this article, we designate such an exon-like structure as a spliced exon cluster in RNA intermediates (SECRIs). Splicing order, therefore, may be an important factor in the understanding of outcomes of splice-site mutations and that multiple products are derived from different splicing pathways. Kessler et al. (1993) reported a method to determine the order of intron removal using RT-PCR, followed by several reports that determined the order of intron removal using this method [Attanasio et al., 2003; Schwarze et al., 1999; Takahara et al., 2002].

Succinyl-CoA:3-ketoacid CoA transferase (SCOT, gene symbol *OXCT1*, EC 2.8.3.5, MIM #601424), a mitochondrial homodimer essential for ketone body utilization, catalyzes the activation of acetoacetate to acetoacetyl-CoA in extrahepatic tissues [Mitchell and Fukao, 2001]. SCOT deficiency (MIM #245050), clinically characterized by episodes of severe ketoacidosis, is an autosomal recessive inborn error of metabolism, first described in 1972 [Tildon and Cornblath, 1972]. Since the first description of SCOT deficiency, fewer than 30 affected probands have been reported including personal communications [Baric et al., 2001; Berry et al., 2001; Cornblath et al., 1971; Fukao et al., 1996, 2000, 2004, 2006, 2007, 2010,

Additional Supporting Information may be found in the online version of this article.

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2011; Kassovska-Bratinova et al., 1996; Longo et al., 2004; Merron and Akhtar, 2009; Niezen-Koning et al., 1997; Perez-Cerda et al., 1992; Pretorius et al., 1996; Rolland et al., 1998; Sakazaki et al., 1995; Snyderman et al., 1998; Song et al., 1998; Tildon and Cornblath, 1972; Yamada et al., 2007].

We describe herein the molecular basis of SCOT deficiency in a 7-month-old boy (GS23). We detected a homozygous gene mutation, c.1248+5g>a, and the simultaneous skipping of exons 12 and 13. We compared splicing order in GS23's fibroblasts with that of control cells, to understand the mechanism of two-exon skipping in GS23 cells.

Materials and Methods

Patients

The patient (GS23) was a male infant born to nonconsanguineous parents of Mexican ancestry. He suffered recurrent ketoacidotic episodes in association with intercurrent infection beginning at the age of 7 months. He was suspected to have SCOT deficiency because he had permanent ketosis. The diagnosis was confirmed by enzyme assay. This study was approved by the Ethical Committee of Gifu University School of Medicine. The parent's DNAs were not available for this study.

Fibroblasts and Enzyme Assay

Fibroblasts from GS23 and controls were cultured in Eagle's minimal essential medium containing 10% fetal calf serum (FCS). A SCOT assay was performed as described by Williamson et al. (1971), with modifications [Song et al., 1997]. Briefly, the assay mixture contained 30 μM acetoacetyl-CoA and 50 mM sodium succinate in 50 mM Tris–HCl (pH 8.5), 10 mM MgCl2, and 4 mM iodoacetamide, and SCOT activity was measured spectrophotometrically as a decrease of acetoacetyl-CoA absorption at 303 nm.

Immunoblot Analysis

Immunoblot analysis was performed as described by Fukao et al. (1997), using a mixture of antihuman SCOT antisera and antihuman mitochondrial acetoacetyl-CoA thiolase antisera as the first antibody and 30 μg of fibroblast protein extract.

Mutation Screening at Genomic Level

Genomic DNA was purified using Sepa Gene kits (EIDIA, Tokyo, Japan) from GS23's fibroblasts. Mutation analysis was performed by PCR amplification of each exon and its boundaries (at least 18 bases from the exon/intron boundaries for both directions) with a pair of intronic primers, described in Supp. Table S1, followed by direct sequencing. Genomic sequence was obtained from GenBank NM 000436.3. The identified mutation was submitted to the Leiden Open Variation Database (www.lovd.nl/OXCT1).

Nonsense-Mediated mRNA Decay Inhibition

To determine whether any observed reduction of the transcript was due to nonsense-mediated mRNA decay (NMD) [Maquat, 2005], we inhibited NMD by fibroblasts with cycloheximide (CHX) (Sigma, St. Louis, MO), a general protein translation inhibitor. Fibroblasts were left untreated or cultured in the presence of 200 μg/ml CHX for 5 hr before RNA extraction. This analysis was performed as described by Hernan et al. (2011).

cDNA Analysis

Total RNA was isolated from fibroblasts using ISOGEN kits (Nippon Gene, Tokyo, Japan). Total RNA (5 μg) was reverse transcribed in 20 μl of 50 mM Tris–HCl pH 7.5, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothretiol, 0.5 mM dNTPs, 200 U of M-MLV reverse transcriptase (Life Tech., Rockville, MD) with a primer mixture including 5 pmol each of SCOT-specific antisense primer, SCOT 25 5′-$^{c.1646}$CAATTATGATTATTGATGTCC-3′, GAPDH-specific antisense primer, GAPDH3 5′-^{1040}GTGCTCTTGCTGGGGCTG-3′, and oligo dT primers. The above preparation was incubated at 37°C for 1 hr. One microliters of this cDNA solution served as a PCR template. The positions of the PCR primers were numbered in relation to the adenine of the initiation methionine codon, which was assigned position +1.

The full-coding sequence of SCOT cDNA was amplified (c.–4 – 1586).

Primer SCOT 42 (sense) 5′-$^{c.-4}$GAAGATGGCGGCTCTCAAA-3′

Primer SCOT 24 (antisense) 5′-$^{c.1586}$AGCCTGGTACAAATATCC ATA-3′

To evaluate the amount of total mRNA products, GAPDH cDNA was also amplified with the following sets of primers:

GAPDH1 (sense) 5′-$^{c.-4}$GGGAAGGTGAAGGTC-3′

GAPDH2 (antisense) 5′-^{c1015}AGGGGTCTTACTCCTTGGAG-3′

These cDNA sequences were obtained from GenBank accession number NM 002046.4 for GAPDH and NM 000436.3 for SCOT.

After 30 PCR cycles, amplified fragments were separated by electrophoresis on a 3.5% (w/v) polyacrylamide gel and a 1% (w/v) agarose gel, and extracted using a Geneclean II kit (BIO 101, Vista, CA). Sequences of amplified fragments were confirmed by direct sequencing.

Isolation of Nuclear RNA

Fibroblasts were grown to near confluence in 80 cm^2 flasks in DMEM with 10% FCS. The incubation was terminated by repeated rinsing with ice-cold 1× PBS to inhibit nuclear mRNA export. Nuclear and cytoplasmic fractions were separated by incubating the scraped cell pellets in a Nonidet P-40 buffer (10 mM Tris, pH 7.4; 10 mM NaCl; 3 mM MgCl2; 0.5% Nonidet P-40) for 10 min on ice, followed by centrifugation for 5 min at 1,450 ×*g*, 4°C. The supernatant contained the cytoplasmic components. The nuclear pellet was stirred by pipetting 10 times, washed in the Nonidet P-40 buffer, and centrifuged for 5 min under the same conditions as described above. The supernatant was discarded, and the pellet was used as the nuclear fraction. Nuclear RNA was isolated from the nuclear fraction using ISOGEN kits (Nippon Gene). Nuclear RNA (10 μg) was incubated in 50 μl of 1 U deoxyribonuclease (RT Grade; Nippon Gene) at 37°C for 5 min, then a 5-μl stop solution was added and it was inactivated at 70°C for 10 min.

Analysis of SECRIs by RT-PCR

The isolated nuclear RNA contains a mixture of various stages of splicing intermediates. To investigate the order of intron removal or splicing order, we analyzed the presence or absence of various SECRIs by RT-PCR. Our strategy was to first identify, using specific primer pairs, all possible SECRIs of two adjacent exons (such