

Mitochondrial Acetoacetyl-CoA Thiolase (T2) Deficiency: T2-Deficient Patients with “Mild” Mutation(s) Were Previously Misinterpreted as Normal by the Coupled Assay with Tiglyl-CoA

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ABSTRACT

Mitochondrial acetoacetyl-CoA thiolase (T2) deficiency is an inborn error of metabolism that affects the catabolism of isoleucine and ketone bodies. This disorder is characterized by intermittent ketoacidotic episodes. Recently, we diagnosed T2 deficiency in two patients (GK45 and GK47) by the absence of potassium ion-activated acetoacetyl-CoA thiolase activity, whereas these patients were previously misinterpreted as normal by a coupled assay with tiglyl-CoA as a substrate. This method has been widely used for the enzymatic diagnosis of the T2 deficiency in the United States and Europe. We hypothesized that some residual T2 activity showed normal results in the assay. To prove this hypothesis, we analyzed these two patients together with three typical T2-deficient patients (GK46, GK49, and

GK50) at the DNA level. Expression analysis of mutant cDNAs clearly showed that GK45 and GK47 had “mild” mutations (A132G, D339-V340insD) that retained some residual T2 activity, at least one of two mutant alleles, whereas the other three patients had null mutations (c.52–53insC, G152A, H397D, and IVS8+1g>t) in either allele. These results raise the possibility that T2-deficient patients with mild mutations have been misinterpreted as normal by the coupled assay with tiglyl-CoA. (*Pediatr Res* 56: 60–64, 2004)

Abbreviations

SCOT, succinyl-CoA: 3-oxoacid CoA transferase
T2, mitochondrial acetoacetyl-CoA thiolase

Mitochondrial acetoacetyl-CoA thiolase (T2; EC 2.3.1.9) deficiency is an autosomal recessive disorder that affects the catabolism of isoleucine and ketone bodies (1,2). This disorder, commonly known as the β -ketothiolase deficiency (McKusick catalogue no. 203750), is characterized by intermittent ketoacidotic episodes. The urinary organic acid profiles of T2 deficiency are typically characterized by massive excretion of tiglylglycine, 2-methyl-3-hydroxybutyrate, and 2-methylacetoacetate in both ketoacidotic and stable conditions (2,3). We previously carried out mutation analysis on 26 patients and evaluated missense mutations by transient expression analysis of the mutant cDNAs (3). On the basis of the residual T2

activity in the expression analysis of mutant cDNAs, we divided T2-deficient patients into two groups: patients with null mutations in either allele (patients with “severe” mutations) and patients with mutation(s) that retain some residual T2 activity in at least one of two mutant alleles (patients with “mild” mutations). We found no apparent correlation between clinical severity and genotype (3). However, 7 of the 26 did not excrete any detectable amount of tiglylglycine even during ketoacidotic episodes, and, at that time, 6 of the 7 had mild mutations. We recently showed that urinary organic acid and acylcarnitine profiles during nonepisodic conditions were different between patients with mild mutations and those with severe mutations (4). Hence, there is a clear correlation between biochemical phenotype and genotype.

We recently diagnosed T2 deficiency in two patients who were previously misinterpreted as normal by the coupled assay with tiglyl-CoA (5). We hypothesized that the presence of residual T2 activity in cells from these patients might lead to a

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normal result in this assay and that T2-deficient patients with mild mutation(s) had been incorrectly interpreted as normal by the coupled assay. We herein analyzed these two patients together with three typical T2-deficient patients at the molecular level to prove the hypothesis.

METHODS

Patients. Brief clinical descriptions for all of the patients are as follows. GK45 is an Armenian boy who was born to consanguineous parents. He experienced the first ketoacidotic episode at the age of 15 mo. Blood pH was 7.2, with P_{CO_2} of 13 mm Hg and HCO_3^- of 4 mM. After he obtained i.v. fluids, he recovered promptly. However, another severe episode occurred 20 d later. Urinary organic acid analysis during the crisis revealed the presence of 2-methyl-3-hydroxybutyrate and 2-methylacetoacetate but no tiglylglycine. He was put on a moderate protein-restricted diet ($2 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$). He had no further crises and developed normally. One of us performed the coupled assay with tiglyl-CoA, which revealed normal T2 activity. Then the fibroblasts were analyzed again in Gifu University School of Medicine by potassium ion-activated acetoacetyl-CoA thiolase activity and revealed T2 deficiency.

GK46 was a boy who was born to nonconsanguineous Vietnamese parents in France. He was well until 18 mo of age, when he developed a ketoacidotic episode. Blood gas data in the episode was as follows: pH 6.92, P_{CO_2} 10 mm Hg, HCO_3^- 2 mM. At that time, urinary organic acid analysis showed massive excretion of tiglylglycine and 2-methyl-3-hydroxybutyrate. He recovered after fluid therapy. He was put on a low-protein diet for only 1.5 y. He never had another ketoacidotic episode, but urinary organic acid analysis revealed massive excretion of tiglylglycine and 2-methyl-3-hydroxybutyrate when he was clinically normal. His growth and development are within the normal range.

GK47's case report has been published as a curious case. He is a boy who was born to unrelated white parents and was clinically suspected of having T2 deficiency but normal activity in the coupled assay with tiglyl-CoA (6). He experienced two severe ketoacidotic episodes at the ages of 19 mo and 49 mo. During the first crisis, blood pH was 6.97, with P_{CO_2} of 10.5 mm Hg and HCO_3^- of 2.3 mM. Clinical improvement was obtained by i.v. fluid therapy. Nine months later, he was hospitalized for mental disorientation and speech difficulties. Urinary acylcarnitine analysis showed the presence of tiglylcarnitine and 2-methyl-3-hydroxybutyrylcarnitine. Treatment with L-carnitine ($3 \times 25 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) was administered without any particular low-protein diet and avoided prolonged fasting. He has subsequently been healthy but still excretes tiglylcarnitine and 2-methyl-3-hydroxybutyrylcarnitine in his urine.

GK49 was a boy of consanguineous parents. He experienced the ketoacidotic episode at the age of 15 mo. Blood gas pH was 6.5. Analysis of acylcarnitine in the urine showed the presence of tiglylcarnitine and 2-methyl-3-hydroxybutyrylcarnitine. Clinical improvement took place when he was put on i.v. fluids and a moderately protein-restricted diet. He experienced no further crisis and developed normally.

GK50 is a boy of nonconsanguineous parents. He experienced a ketoacidotic episode at the age of 2 d with polypnea and hypotonia. The blood pH was 7.2, and the anion gap was 35. Urinary organic analysis revealed the presence of tiglylglycine, 2-methyl-3-hydroxybutyrate, and 2-methylacetoacetate. After correcting pH with i.v. fluids, prompt recovery was obtained and milk was reintroduced into his diet at day 4. His development is within the normal range.

Fibroblasts. Informed consent was obtained from the patients' parents to perform skin biopsy, enzyme assay, and molecular analyses. This study was approved by the review board of Gifu University School of Medicine.

Fibroblasts from GK45, GK46, GK47, GK49, and GK50 were grown in Eagle's minimal essential medium that contained 10% FCS.

Enzyme assay and immunoblot analysis. T2 activity was measured as potassium ion-activated acetoacetyl-CoA thiolase activity as described (7). The coupled assay with tiglyl-CoA as a substrate was done according to Gibson *et al.* (5). Immunoblot analysis was performed with a mixture of an anti-T2 antibody and anti-succinyl-CoA:3-oxoacid CoA transferase (SCOT) antibody as the first antibody. SCOT protein served as positive control (8).

Mutation detection. RNA was isolated using ISOGEN kits according to the manufacturer's instructions (Nippon Gene, Tokyo, Japan). Genomic DNA was prepared using Sepa gene kits (Sanko Junyaku, Tokyo, Japan) from fibroblasts. The oligonucleotide primers for cDNA synthesis and PCR amplification of T2 cDNA were described previously (9). The PCR fragments were extracted, subcloned, and sequenced as described (9). Mutations were confirmed by direct sequencing of amplified genomic fragments (8).

Transient expression analysis. Transient expression analysis of T2 cDNAs was done using a pCAGGS eukaryote expression vector (10) as described (11,12). The expression constructs were transfected into SV40-transformed T2-deficient fibroblast cell lines (GK03) with Lipofectamine 2000 (Invitrogen, San Diego, CA, U.S.A.). After transfection, the cells were further cultured at either 37°C or 30°C for 72 h, and then they were harvested. The cells were freeze-thawed and sonicated in 50 mM sodium phosphate (pH 8.0) and 0.1% Triton X-100. After centrifugation at $10,000 \times g$ for 10 min, the supernatants were used for enzyme assay and for immunoblot analysis. Heat instability for enzyme activity was tested using 30°C expression samples, as described (8,13).

RESULTS

Enzyme assay and immunoblot analysis. As shown in Table 1, enzyme assay data using the coupled assay with tiglyl-CoA were within the normal range in fibroblasts from GK45 and GK47, whereas potassium ion-activated acetoacetyl-CoA thiolase activity was hardly detected in the fibroblasts from all the patients. Immunoblot analysis using 30 μg of protein showed that T2 protein was hardly detected in fibroblasts from all five patients, but the T2 protein in the control fibroblast was clearly detected even when 3.7 μg of protein was applied to SDS-

Table 1. Enzyme assays in fibroblasts

Case	Coupled assay with tiglyl-CoA	Acetoacetyl-CoA thiolase			SCOT
		-K ⁺	+K ⁺	+K ⁺ /-K ⁺	
GK45	35	2.7 ± 0.1	3.0 ± 0.1	1.1 ± 0.1	4.2 ± 0.1
GK46	0.6	5.2	5.4	1.0	9.7
GK47	23	4.3 ± 0.8	5.2 ± 1.0	1.2 ± 0.1	6.5 ± 4.1
GK49	1.8	5.3	5.5	1.0	6.7
GK50	0	4.2	5.5	1.3	4.6
Normal (n = 19)	46 ± 24				
Normal (n = 16)		4.9 ± 1.1	9.9 ± 2.5	2.1 ± 0.3	6.5 ± 2.5

In the cases of GK45 and GK47, acetoacetyl-CoA thiolase and SCOT activities were assayed three times and the average and SDs are shown.

PAGE (Fig. 1). These data confirmed that all five patients had the T2 deficiency.

Mutations in the five patients. We amplified the entire coding region of T2 cDNA in all five patients. Mutations were screened at the cDNA level first after subcloning RT-PCR fragments and then confirmed at the genomic level by direct sequencing. We found six mutations in the five T2-deficient patients. GK45 was a homozygote of c.395C>G (A132G). GK46 was a compound heterozygote of c.52-53insC and c.455G>C (G152A). GK47 was also a compound heterozygote of G152A and c.1018-1019insATG (D339-V340insD). GK49 and GK50 were homozygotes of c.1189C>G (H397D) and IVS8+1g>t, respectively. C.52-53insC causes a reading frame shift and produces a premature termination. IVS8+1g>t causes exon 8 skipping and was proved to produce no T2 protein in the previous study (9). To confirm the other mutations as being causative mutations, we performed a transient expression analysis of mutant cDNAs.

Transient expression analysis of mutant cDNAs. Transient expression of mutant cDNAs (A132G, D339-V340insD, G152A, and H397D) was done with a mild mutant (T297M) as a positive mutant for retaining significant residual T2 activity (14). After expression of cDNAs in the recipient T2-deficient cells for 72 h at 37°C and 30°C, enzyme assays and immunoblots were done (Fig. 2). In the case of the transfection of wild-type T2 cDNA, T2 activity in the expression at 30°C was higher than that in the expression at 37°C. This temperature-sensitive character was more evident in the transfection of mutant cDNAs. A132G and D339-V340insD mutant T2 cDNAs retained 10% residual T2 activity in the expression at 37°C and higher residual activity in the expression at 30°C. D339-V340insD mutant T2 protein migrated slower than the

wild T2 protein on the 10% polyacrylamide gel. The G152A mutant retained ~5% of residual activity only in the expression at 30°C. Mutant H397D retained no residual T2 activity in the expression at either 37°C or 30°C. In immunoblot analysis, the amount of mutant T2 protein was estimated by comparison with 2-fold serial diluted samples of wild-type T2. In mock cDNA transfection, T2 protein was not detected in the expression at either 37°C or 30°C. In the expression of A132G and D339-V340insD mutant cDNAs, mutant proteins were detected and the accumulation of these mutant proteins was temperature sensitive. In the case of G152A, mutant protein was faintly detected in the expression at 37°C, but T2 protein was clearly detected in the expression at 30°C (~15% of control). The H397D mutant protein was not detected even in the expression at 30°C. The heat stability for the enzyme activity of A132G, G152A, and D339-V340insD mutant proteins was almost the same as that of wild-type T2 protein (data not shown).

DISCUSSION

In this article, we presented two T2-deficient patients (GK45 and GK47) whose fibroblasts showed normal activity in the coupled assay with tiglyl-CoA but defective potassium ion-activated acetoacetyl-CoA thiolase activity for the first time. We confirmed T2 defects in GK45 and GK47 in immunoblot analysis followed by mutation analysis. The coupled assay was first described in 1992 (5) and had been used to diagnose T2-deficient patients until 2001 in the United States and Europe. GK47 was previously reported as a curious case whose clinical presentation, including biochemical phenotype, accorded with T2 deficiency but had normal enzyme activity using the coupled assay (6). As shown in the case reports, there is no apparent clinical difference between these two patients and the other patients.

Three enzyme assays have been used for the diagnosis of the T2 deficiency (5,7,15). Potassium ion-activated acetoacetyl-CoA thiolase activity (7) and 2-methylacetoacetyl-CoA thiolase activity are specific for T2 activity (15), but the coupled assay examines the pathway of isoleucine catabolism from tiglyl-CoA to methylmalonyl-CoA (5). Hence, a defect in tiglyl-CoA hydratase, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase, T2, or propionyl-CoA carboxylase is theoretically detectable. If any of these enzymes is completely defective, then the total activity measured by the assay is expected to be very low. However, it is possible that some residual enzyme

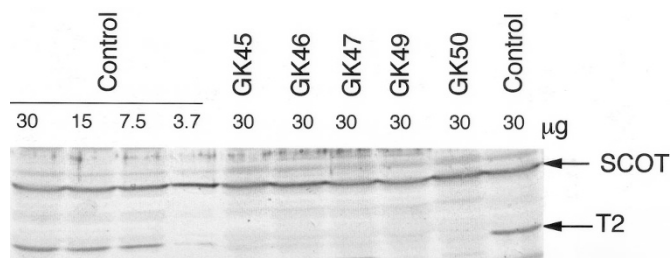


Figure 1. Immunoblot analysis in fibroblasts. A mixture of anti-T2 and anti-SCOT antibodies was used as the first antibody. Positions of SCOT and T2 are indicated with arrows. Thirty micrograms of protein was applied in the lanes of patients. In the lanes of the control, 2-fold serially diluted samples were applied, as indicated above the lanes.

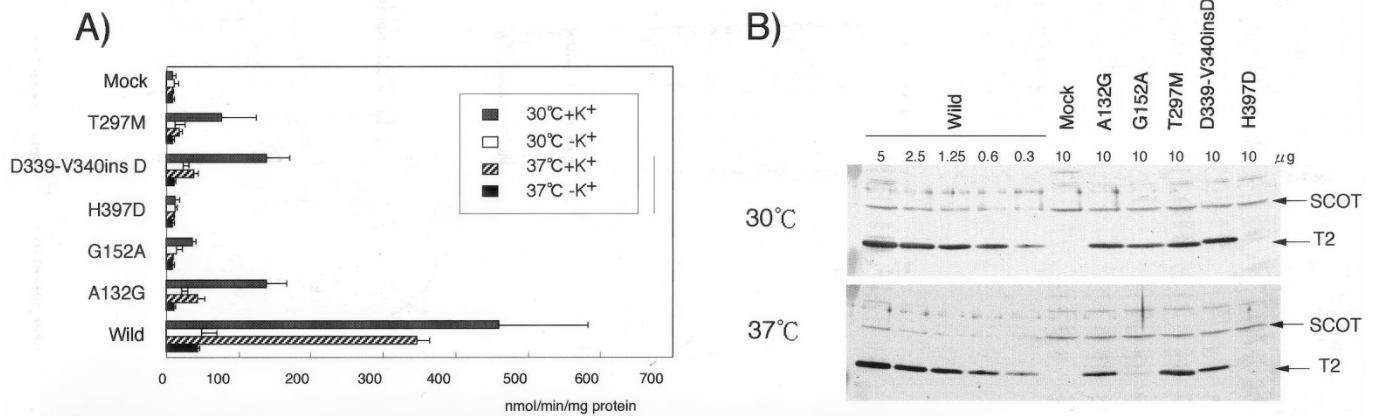


Figure 2. Transient expression analysis of mutant cDNAs. Transient expression analyses were done at both 37°C and 30°C. The T297M mutant is a positive control that retained some residual T2 activity. (A) Potassium ion-activated acetoacetyl-CoA thiolase assay. Acetoacetyl-CoA thiolase activity in the supernatant of the cell extract was measured. The mean values of acetoacetyl-CoA thiolase activity in the absence and presence of potassium ions are shown together with the SD of four experiments. (B) Immunoblot analysis. Protein amounts applied are shown above the lanes. A mixture of anti-rat T2 antibody and anti-human SCOT antibody was used as the first antibody

activity in one of them is enough to present normal enzyme activity in the coupled assay.

We clearly proved our hypothesis that some residual T2 activity shows normal results in the coupled assay with tiglyl-CoA. We demonstrated by mutation analysis followed by transient expression analysis of mutant cDNAs that A132G and D339-V340insD retained ~10% of the residual T2 activity in the expression at 37°C. Because GK45 is a homozygote of A132G and GK47 is a compound heterozygote of D339-V340insD and G152A, their fibroblasts are expected to have ~10 and 5% residual activities, respectively. However, none of the mutations (c.52–53insC, G152A, H397D, and IVS8+1g>t) identified in the other three T2-deficient patients retained any residual activity in the expression at 37°C. These findings have good correlation with the fact that GK45 and GK47 had normal activity and the others were defective in the coupled assay with tiglyl-CoA.

Our findings are supported by the findings in another paper (5). One patient with the T2 deficiency (L.V.; our laboratory number GK03) was reported to have exceptionally high activity ($8.7 \pm 3.7 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$; control values $32 \pm 23 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) compared with other T2-deficient patients ($0.7 \pm 0.5 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) in this assay (5). Middleton *et al.* (15,16) reported that GK03's fibroblasts have low but significant residual T2 activity ($6.6 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$; control values $89 \pm 11 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$; typical T2-deficient fibroblasts $<4.0 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$), using the T2 specific assay with 2-methylacetoacetyl-CoA. Taken together, even very low residual T2 activity may result in much higher residual activity in the coupled assay using tiglyl-CoA.

Among the five patients studied in this article, GK45 and GK47 can be subgrouped into patients with mild mutations and the others with severe mutations. We divided T2-deficient patients into two groups on the basis of the residual T2 activity in the expression analysis of mutant cDNAs (3,4)—patients with null mutations in either allele (patients with severe mutations) and patients with mutation(s) that retain some residual

T2 activity in at least one of two mutant alleles (patients with mild mutations)—and compared several aspects of these groups. The biochemical phenotype, such as blood acylcarnitine and urinary organic acid profiles in a nonepisodic condition, is different between the two groups (3,4). However, present and previous studies showed that T2-deficient patients with mild mutations do not differ from those with severe mutations in 1) the severity of ketoacidotic crises, 2) frequency of ketoacidotic crises, and 3) prognosis. Hence, it is very important to diagnose T2 deficiency with mild mutations properly. Our results raised the possibility that T2-deficient patients with mild mutations had been misinterpreted as normal by the coupled assay with tiglyl-CoA.

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