研究成果の刊行に関する一覧表

書籍

| 著者氏名 | 論文タイトル名 | 書籍全体の編集者名 | 書　籍　名 | 出版社名 | 出版地 | 出版年 | ページ |
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| 浦澤林太郎, 久保徹夫, 深尾敏幸 | 急性胃腸炎を契機に急激な意識障害を起こした９ヶ月男児 | 遠藤文夫, 山口清次, 高柳正樹, 深尾敏幸 | 症例から学ぶ先天代謝異常症～日常診療からのアプローチ～ | 診断と治療社 | 東京 | 2009 | 100-102 |
| 深尾敏幸 | ケトン体 | 高柳正樹 | 小児科臨床ピクシス 見逃せない先天代謝異常 | 中山書店 | 東京 | 2010 | 印刷中 |
| 深尾敏幸 | ケトーシス | 高柳正樹 | 小児科臨床ピクシス 見逃せない先天代謝異常 | 中山書店 | 東京 | 2010 | 印刷中 |
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| **Fukao T**, Zhang G, Matsuo N, Kondo N | CpG islands around exon 1 in Succinyl-CoA:3-ketoacid CoA transferase (SCOT) gene were hypomethylated even in human and mouse hepatic tissues where SCOT gene expression was completely suppressed. | Molecular Medicine Reports | 3 | 335-359 | 2010 |
| **Fukao T**, Nguyen HT, Nguyen NT, Vu DC, Can NTB, Pham ATV, Nguyen KN, Kobayashi H, Hasegawa Y, Bui TH, Niezen-Koning KE, Wanders RJA, de Koning T, Nguyen LT, Yamaguchi S, Kondo N | A common mutation, R208X, identified in Vietnamese patients with mitochondrial acetoacetyl-CoA thiolase (T2) deficiency | Mol Genet Metab | In press | | 2010 |
| **Fukao T**, Horikawa R, Naiki Y, Tanaka T, Takayanagi M, Yamaguchi S, Kondo N | A Novel Mutation (c.951C>T) in an Exonic Splicing Enhancer Results in Exon 10 Skipping in the Human Mitochondrial Acetoacetyl-CoA Thiolase Gene | Mol Genet Metab | In press | | 2010 |
| 高柳正樹, 村山圭, 長坂博範, 真山義民、鶴岡智子、藤浪綾子 | 先天性有機酸代謝異常症全国調査（１９９０－１９９９） | 日本マス・スクリーニング学会誌 | １９ | 243-248 | 2009 |
| 山口清次 | 新生児突然死の予防：タンデムマスによる早期発見. | 日本周産期・新生児医学会雑誌 | ４５ | 973-976 | 2009 |

# Ⅳ. 研究成果の刊行物・別刷

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# Different Clinical Presentation in Siblings with Mitochondrial Acetoacetyl-CoA Thiolase Deficiency and Identification of Two Novel Mutations

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Mitochondrial acetoacetyl-CoA thiolase (T2) catalyzes 2-methylacetoacetyl-CoA cleavage into acetyl-CoA and propionyl-CoA in isoleucine catabolism and interconversion between acetyl-CoA and acetoacetyl-CoA in ketone body metabolism. T2 deficiency is a rare metabolic disease of autosomal recessive inheritance. The disorder is characterized by intermittent ketoacidotic episodes. The onset of clinical symptoms is in the infant or toddler period. The frequency of episodes declines with age, stopping before adolescence. Here we report two siblings with this disorder. The proband (GK65) is a French girl born from non-consanguineous parents. She presented several ketoacidotic episodes with 5 hospitalizations from age 2 to 4 years, the first of them complicated by ketoacidotic coma. Minor episodes, which are generally provoked by infections or high protein intake, still persist at age of 16 years. Molecular analysis of the *T2* gene has revealed the compound heterozygosity of c.578T>C (M193T) and IVS8+5g>t. The latter mutation results in skipping of exon 8. In contrast, the younger brother (GK65b) had a unique ketoacidotic crisis at the age of 6 years that is the oldest-age first crisis among T2-deficient patients reported thus far. Despite the mild phenotype, he carried the same T2 gene mutations as his sister (GK65). Furthermore, T2 catalytic activity and T2 protein were not detected in the fibroblasts derived from GK65 and GK65b. In conclusion, the siblings with the same T2 gene mutations present different clinical severity. Diagnostic testing for asymptomatic siblings is important in the management of T2-deficient families.

**Keywords:** T2 deficiency/mitochondrial acetoacetyl-CoA thiolase deficiency/β-ketothiolase deficiency/genotype/phenotype correlation/mutation

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Mitochondrial acetoacetyl-CoA thiolase (T2) deficiency (OMIM 203750) is an inborn error of metabolism that affects the catabolism of isoleucine and ketone bodies. It is a rare disease of autosomal recessive inheritance with less than 100 patients described in the literature. 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 (T2D) is easily diagnosed by urinary organic acid analysis, characterized by massive excretion of tiglylglycine, 2-methyl-3-hydroxybutyrate and 2-methylacetoacetate (Fukao et al. 2001, 2003). T2D usually has a favorable outcome (Fukao et al. 2001), but there is a risk of death and neurological sequelae from an acute ketoacidotic episode. Diagnosis is confirmed by measurement of T2 activity on cultured skin fibroblasts (Zhang et al. 2004). T2D is caused by mutations in the *ACAT1 (T2)* gene located on chromosome 11q22.3-q23.1 (Fukao et al. 1990; Kano et al. 1991). T2D is very heterogeneous at the genotype level with at least 50 different mutations described (Fukao et al. 1995, 2001).

We present here the cases of two siblings diagnosed with T2D who were quite different in terms of onset and frequency of ketoacidotic episodes.

## Clinical Report

The proband (GK65) is a French girl born from non-consanguineous parents in 1992. She was well until 25

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months of age when she presented her first ketoacidotic episode following rhinopharyngitis. After a 48-hour period of anorexia and vomiting she was admitted to hospital because of alterations of consciousness and dyspnea. Blood gas analysis revealed severe metabolic acidosis with a pH of 6.98 and $HCO_3$ of 3.5 mmol/l. Urine dipstick testing showed massive ketonuria. Additional standard blood tests were normal. Clinical improvement was obtained by intravenous glucose and bicarbonate infusion therapy within 36 hours. There have not been any neurological sequelae due to this severe ketoacidotic coma.

Metabolic analyses were performed during the acute episode. Urinary organic acid chromatography revealed massive excretion of tiglylglycine (330 $\mu$mol/mmol creatinine), 2-methyl-3-hydroxybutyrate (692 $\mu$mol/mmol creatinine) and 2-methylacetoacetate (non-quantifiable), characteristic of T2 deficiency. Diagnosis was then confirmed by enzymatic measurement of T2 activity on cultured skin fibroblasts by a coupled assay with tiglyl-CoA as the substrate, as previously reported (Gibson et al. 1992). T2 activity markedly decreased in the cells from our patient (3% of control cells). Under stable conditions after this first episode, the urinary organic acid profile still showed excretion of 2-methyl-3-hydroxybutyrate (148 $\mu$mol/l for a normal value < 14), but no significant excretion of tiglylglycine.

There were four further hospitalizations for less severe ketoacidotic episodes which occurred until the age of 4 years, three of them following infections. Mild episodes, mostly induced by infections, once or twice per year, were successfully managed by the patient and his parents at home and still persisted at age 16 years. Attacks were also provoked by high protein intake (such as an egg and a steak during the same meal). Episodes are now easily controlled by sweet liquid, such as coke. Bicarbonate mixed with coke was the treatment of choice before age 10.

The youngest brother (GK65b), born in 2000, presented his first and up to now the only ketoacidotic episode at the age of 6 years following rotavirus gastroenteritis. He suffered from fever, anorexia and vomiting for about 3 days. At hospitalization he presented general alteration and dyspnea. Blood pH was 7.15 with $HCO_3$ of 9.8 mmol/l. He also presented massive ketonuria. T2 deficiency was suspected because of his sister's medical history and the characteristic urinary organic acid profile.

The parents had not taken him to metabolic counselling and no urinary organic acid analysis had been performed until this severe episode. The boy didn't present any ketoacidotic episode earlier in life despite infections like chickenpox and several operations with general anesthesia (hydrocella at the age of 3 years, adenoidectomy at the age of 5 years, transtympanic drainage at the age of 6 years). Another operation performed after the diagnosis of T2D at 7 years of age (transtympanic drainage), as well as high protein intake, didn't provoke any attack either. Diagnosis of T2D was confirmed by enzymatic analysis on cultured

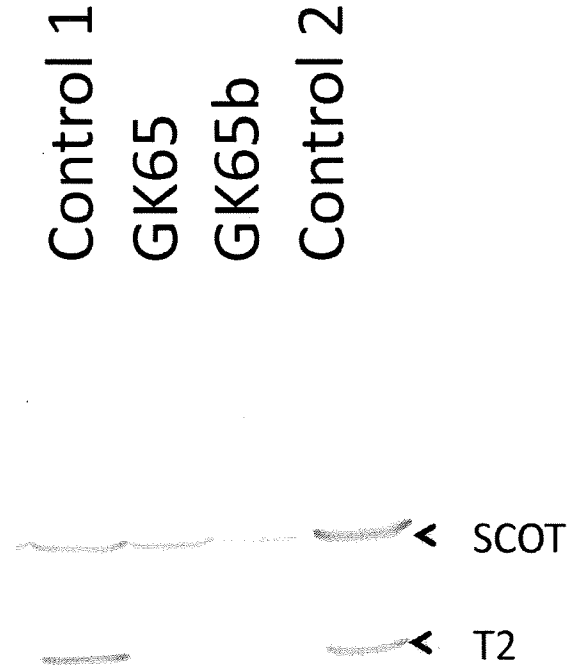


Fig. 1. Immunoblot analysis. Thirty $\mu$g of fibroblast protein extract were applied to each lane. The first antibody was a mixture of anti-T2 antibody and anti-SCOT antibody. The positions of the bands for T2 and SCOT are indicated with arrows. No T2 protein was detected in the fibroblasts of GK65 and GK65b.

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

| Fibroblasts | *acetoacetyl-CoA thiolase acitvity* | | |
|---|---|---|---|
| | $-K^+$ | $+K^+$ | $+K^+/-K^+$ |
| Present control | 3.6 | 6.5 | 1.8 |
| GK65 | 3.8 | 3.7 | 1.0 |
| GK65b | 4.3 | 4.1 | 1.0 |

Enzyme activity is expressed as nmol/min/mg protein. Potassium-ion-activated acetoacetyl-CoA thiolase activity was not detected in the fibroblasts of GK65 and GK65b since the ratio of $+K^+/-K^+$ was 1.0.

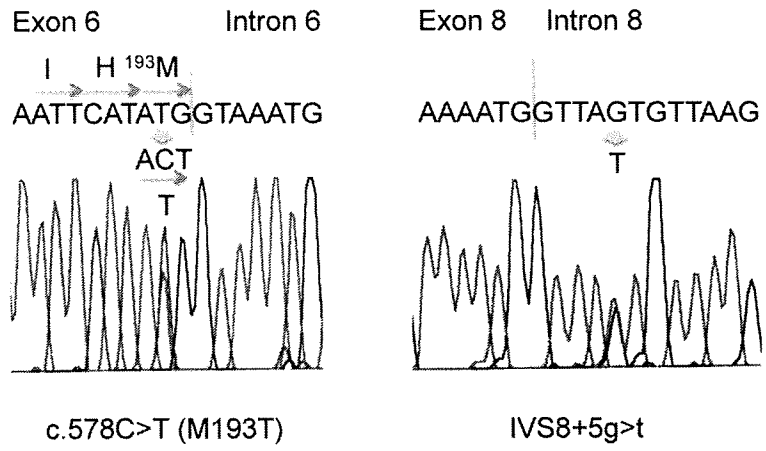


Fig. 2. Mutations identified in GK65 at the genomic level. GK65 was a compound heterozygote of c.578C > T (M193T) and IVS8+5g > t. The results of direct sequencing of genomic PCR fragments are shown. The same mutations were identified in GK65b.

skin fibroblasts by measuring T2 activity using acetoacetyl-CoA as the substrate with/without potassium ions (Robinson et al. 1979).

The fibroblasts from GK65 and GK65b were re-examined simultaneously at Gifu University in 2008. The result of a T2 enzyme assay using acetoacetyl-CoA as the substrate with/without potassium ions is shown in Table 1. No potassium ion-activated acetoacetyl-CoA thiolase activity was detected in GK65 or GK65b. Immunoblot analysis showed that T2 protein was not detectable in the samples from GK65 and GK65b, whereas succinyl-CoA:3-ketoacid CoA transferase (SCOT) protein was clearly detected in all samples (Fig. 1). Routine mutation screening at the genomic level (Fukao et al. 2007) revealed that both patients were compound heterozygotes of c.578T>C (M193T) from the mother and IVS8+5g>t from the father. cDNA analysis of the siblings revealed the presence of exon 8 skipping in about half of their cDNA clones. This result indicates that IVS8+5g>t caused the exon skipping.

## Discussion

We have described two sibling patients affected by T2 deficiency with quite different clinical presentations. The older sister (GK65) presented several episodes of decompensation with 5 hospitalizations from age 2 to 4 years, the first of them complicated by ketoacidotic coma. Minor episodes, which are generally provoked by infections or high protein intake, still persist at age 16 years. The younger boy (GK65b) did not have urinary organic acid analysis, although he was born after the confirmation of his sister's diagnosis, and presented only one ketoacidotic attack at the age of 6 years.

The case presentations of GK65 and GK65b include important messages. First, the most important management of T2D is the prevention of severe ketoacidotic crises. GK65b did not have any presymptomatic tests despite the diagnostic confirmation of T2D in GK65 before GK65b's birth. If GK65b had been analyzed by urinary organic acid analysis after his birth, he could have been diagnosed as having T2D and the severe ketoacidotic episode at the age of 6 might have been avoided. A presymptomatic test can accurately detect the existence of an underlying potential T2D condition. Presymptomatic diagnosis of T2D prevents severe ketoacidosis and its risk of sequelae or even death. Second, to our knowledge, the first ketoacidotic crisis at the age of 6 years is the oldest- age first crisis among T2 deficient patients to date. Fukao et al. (2001) described the median onset of symptoms at the age of 15 months (3 days to 48 months) in 26 cases of enzymatically confirmed T2D. GK65b had not experienced any ketoacidotic crisis in spite of infections like chickenpox and several operations with general anesthesia. However, at the age of 6 years, rotavirus gastroenteritis induced severe ketoacidotic crisis. Gastroenteritis is indeed the most frequent pre-existing condition for ketoacidotic crises in T2D subjects. The different clinical courses between these siblings might be based on the possibility that the parents' attention to the elder sister prevented a severe ketoacidotic crisis in the younger boy beforehand. Third, the elder sister, GK65, still experiences minor episodes at the age of 16 years. Those episodes occurred once or twice a year with urinary ketone-positive manifestations. They were easily managed at home by the patient and her parents. The frequency of attacks in T2D decreases with age, the last being reported at the age of 10 years in a previous follow-up study (Fukao et al. 2001). Nevertheless, we cannot rule out the possibility that some patients in the follow-up study also experienced such mild episodes without reporting them. Though GK65 experienced ketotic events even after the age of 10 years, the fact that such events could be managed at home is also informative.

Molecular analysis revealed that the affected siblings

are compound heterozygotes of M193T from the mother, and IVS8+5g>t from the father. Most reported mutations in T2D are single-based substitutions resulting in nonsense, missense, or splice mutations of the T2 gene (Fukao et al. 1995, 2001). T2 gene deletion and tandem duplication also have been reported (Zhang et al. 2006; Fukao et al. 2007).

The IVS8+5g>t mutation resulted in a drastically reduced Shapiro and Senapathy score (Shapiro and Senapathy 1987) at the authentic splice donor site of intron 8 from 82 (TG/gttagt) to 68 (TG/gttatt). Exon 8 skipping was identified in almost half of the cDNA clones from GK65 and GK65b and no other nucleotide substitutions in exon 8 or surrounding introns (~100bp) were identified. The importance of G at position +5 was previously well documented. Buratti et al. (2007) summarized 346 aberrant splice donor sites that were activated by mutations in 166 human diseases. Point mutations leading to cryptic splice donor site activation were most common in the first intron nucleotide, followed by the fifth nucleotide. Substitutions at position +5 were exclusively g>a transitions. In our case, no cryptic splice donor site was apparently activated and the substitution was g>t at position 5. We previously identified several mutations, which resulted in exon 8 skipping in the T2 gene. Among them, IVS8+1g>t caused exon 8 skipping in almost all transcripts (Fukao et al. 1992) and c.816C>T (Q272X) caused exon 8 skipping in some transcripts (Fukao et al. 1994). In both cases, no aberrant splicing variants using any other cryptic splice sites were identified. This may indicate no available cryptic splice donor site in intron 8. We concluded that IVS8+5g>t is responsible for exon 8 skipping in GK65 and GK65b.

T2D is very heterogeneous at the genotype level with at least 50 different mutations described (Fukao et al. 1995, 2001). We identified two novel T2 gene mutations in this family. No genotype-phenotype correlation could be identified and mutant siblings can thus present different clinical severity (Fukao et al. 1995, 2001), as these siblings did. T2D usually has a favorable outcome, but there is a risk of death and neurological sequelae due to an acute ketoacidotic episode (Ozand et al. 1994; Fukao et al. 2001). Hence diagnostic testing for asymptomatic siblings is important in the management of T2D families.

## Acknowledgments

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# A common mutation, R208X, identified in Vietnamese patients with mitochondrial acetoacetyl-CoA thiolase (T2) deficiency

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ABSTRACT

Mitochondrial acetoacetyl-CoA thiolase (T2) deficiency is an inborn error of metabolism affecting isoleucine catabolism and ketone body utilization. This disorder is clinically characterized by intermittent ketoacidotic episodes with no clinical symptoms between episodes. In general, T2 gene mutations are heterogenous. No common mutations have been identified and more than 70 mutations have been identified in 70 patients with T2 deficiency (including unpublished data). We herein identified a common mutation, R208X, in Vietnamese patients. We identified R208X homozygously in six patients and heterozygously in two patients among eight Vietnamese patients. This R208X mutation was also identified heterozygously in two Dutch patients, however, R208X mutant alleles in the Vietnamese have a different haplotype from that in the Dutch, when analyzed using Msp I and Taq I polymorphisms in the T2 gene. The R208X mutant allele was not so frequent in the Vietnamese since we could not find that mutant allele in 400 healthy Vietnamese controls using the Nla III restriction enzyme assay. DNA diagnosis of T2 deficiency may be applicable to the Vietnamese population.



## Introduction

Mitochondrial acetoacetyl-CoA thiolase (T2) (EC 2.3.1.9, gene symbol *ACAT1*) deficiency (OMIM 203750, 607809) is an autosomal recessive disorder, commonly known as β-ketothiolase deficiency. Since 1971 [1], more than 90 patients with it have been identified (including personal communications) [2]. This disorder is clinically characterized by intermittent ketoacidotic episodes with no clinical symptoms between episodes. T2 plays a role in ketolysis in extrahepatic tissues. T2 also catalyzes thiolysis of 2-methylacetoacetyl-CoA in isoleucine catabolism. Hence, T2-deficient patients usually have urinary excretion of 2-methyl-3-hydroxybutyrate, 2-methylacetoacetate and tiglylglycine, which are hallmarks derived from intermediates in isoleucine catabolism. The severity of the clinical features varies from patient to patient but follow-up studies reveal that, in general, T2 deficiency has a favorable outcome [3].

Human T2 cDNA is about 1.5 kb long and encodes a precursor protein of 427 amino acids, including a 33-amino-acid leader polypeptide [4]. The T2 (*ACAT1*) gene spans approximately 27 kb, and contains 12 exons [5]. We have identified more than 70 gene mutations ([6–23] and unpublished data). In general, T2 gene mutations are heterogenous and many patients have unique mutations. Several mutations have been identified in more than two independent families, but as far as we know, no common mutations have yet been identified in T2 deficiency.

We herein report identification of a common mutation, R208X, in Vietnamese patients.

*Abbreviation:* T2, mitochondrial acetoacetyl-CoA thiolase.

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## Materials and methods

### *Vietnamese patients*

Ten Vietnamese patients (who were not related to each other) from the northern half of Vietnam (around Hanoi) were suspected of having T2 deficiency from urinary organic acid analysis in Shimane University from 2005 to 2009. All patients belonged to the major Vietnamese ethnic group, the Kinh. 2-Methyl-3-hydroxybutyrate and tiglylglycine were detected in their urinary organic acid profiles. A typical acylcarnitine profile of elevated levels of C5:1 and C5OH was also detected in the nine patients analyzed. Among them, samples from eight patients were available for this study. All eight had developed severe ketoacidotic crises and were referred to the National Hospital of Pediatrics in Hanoi for intensive care or evaluation. Their clinical presentations are summarized in Table 1. Among the eight patients, GK74 died at 25 months due to a severe second ketoacidotic crisis after confirmation of chemical diagnosis at 18 months. GK70 experienced only one ketoacidotic crisis at 30 months which was severely complicated by delayed mental development with convulsions, hypotonia and DQ 60. The other cases have achieved normal development thus far.

### *Dutch patients*

GK36 is a Dutch girl born from non-consanguineous parents in 1994. The parents are Dutch Caucasians. She was consulted for evaluation of motor skills and suspected of having T2 deficiency at the age of 1 y 10 m by urinary organic acid analysis. 2-Methyl-3-hydroxybutyrate, 2-methylacetoacetate, and tiglylglycine were detected in her urine in asymptomatic condition. She did not experience severe ketoacidotic crisis until the age of 13 y 7 m. Her development is now normal.

GK35 was evaluated at the age of 19 months because of recurrent episodes of hypoglycemia with metabolic acidosis. He is the third child of healthy non-consanguineous Dutch parents. Urinary organic acid analysis prompted the diagnosis of T2 deficiency. He was treated with a mild protein restriction in infancy, as well as L-carnitine. Psychomotor development has been uneventful and episodes of hypoglycemia and acidosis have not reccured. At present the patient is nearly 14 years old.

### *Urinary organic acid analysis*

Urinary organic acid analyses for these Vietnamese patients were done in Shimane university using dried filter paper, as described in Ref. [24].

### *Mutation detection*

This study was approved by The Ethical Committee of The Graduate School of Medicine, Gifu University. Genomic DNA in the Vietnamese patients and their families was purified from blood with QIAamp DNA blood mini kits (Qiagen Inc., Valencia, CA, USA). Genomic DNA from the Dutch patients was extracted from fibroblasts using SepaGene kit (Sanko Junyaku, Tokyo, Japan). Mutation screening was performed at the genomic level by PCR and direct sequencing using a primer set for fragments, including an exon and its intron boundaries [11].

### *Restriction enzyme assay to detect R208X*

The R208X substitution (CACG to CATG) creates a new Nla II site.

**Table 1**
Clinical and molecular summary of T2-deficient patients.

| | Consanguinity | Sex | Death of elder siblings | The first crisis | | | | | | | | Prognosis | | | Mutations | | Polymorphisms | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Onset of 1st crisis | Preceding illness | pH | HCO3 | BE | Glucose | NH3 | Unconsciousness | Poly-pnea | Mechanical ventilation | Number of crises | Present age | Condition | Paternal allele | Maternal allele | Msp I P | Taq I P |
| *Vietnamese* | | | | | | | | | | | | | | | | | | | | |
| GK70 | – | M | | 34 m | Pneumonia | | | | 5.5 | 196 | Coma | + | + | 1 | 4.5 | Delayed | R208X | 163_167del 5ins2 | Mm | Tt |
| GK72 | – | M | Sis 24 m | 12 m | Pneumonia | 6.93 | 4 | −26 | 2 | | Coma | + | | 2 | 4 | Good | R208X | R208X | MM | TT |
| GK73 | – | M | Bro 11 m | 13 m | | 7.08 | 2.4 | −27 | 5.8 | 86 | Coma | + | | 1 | 4 | Good | R208X | R208X | MM | TT |
| GK74 | – | M | | 18 m | Pneumonia | 6.9 | 2 | −20 | 5.9 | 96 | Coma | + | + | 2 | Died at 2 y | | IVS10-1g>c | R208X | Mm | Tt |
| GK75 | – | M | Bro 27 m | 11 m | Acute diarrhea | 6.88 | 1.5 | | 5.3 | 39.4 | Lethargy | + | | 1 | 2 | Good | R208X | R208X | MM | TT |
| GK76 | – | M | | 13 m | | 7.11 | 3.3 | −26 | 19.4 | | Coma | + | | 2 | 4 | Good | R208X | R208X | MM | TT |
| GK79 | – | F | | 12 m | Acute diarrhea | 7.1 | 4 | −20 | 6 | | Lethargy | + | | 2 | 3.5 | Good | R208X | R208X | MM | TT |
| GK80 | – | F | | 9 m | Acute diarrhea | 6.89 | 2.6 | −28 | 5.5 | 130 | Coma | + | + | 1 | 1 | Good | R208X | R208X | MM | TT |
| *Dutch* | | | | | | | | | | | | | | | | | | | | |
| GK35 | – | M | | 12 m | Pneumonia | 7.13 | 8 | −20.5 | 3 | | Lethargy | + | | 2 | 14 | Good | R208X | IVS11+2t>c | mm | Tt |
| GK36 | – | F | | No acute episodes | | | | | | | | | | 0 | 13 | Good | R208X | IVS10-2a>c | mm | Tt |

GK79 was evaluated in stable condition after two acute acidotic episodes and diagnosed as having T2 deficiency.
GK36 was suspected of having T2 deficiency by organic acid analysis performed for evaluation of motor skills at the age of 1 y 9 m.
Deaths of elder siblings due to metabolic decompensation were noted in 3 patients: Sis 24 m means a sister died at 24 months of age.
In Dutch patients, familial segregation of these mutations was not determined.

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A fragment (264 bp), including exon 7 and its surrounding introns, was amplified using the following primers:

In6s (in intron 6, −79 ~ −60) 5′-CACTATAAGTTAGGCAAAGT-3′

In7as (in intron 7, +39 ~ +20) 5′-TGAAAAGTCTATTCATCCTT-3′

After PCR amplification, an aliquot of an amplicon was digested with Nla III, then subjected to a 5% polyacrylamide gel.

*R208X mutant allele in Vietnamese population*

We used the above restriction enzyme assay to detect the R208X mutant allele in 400 healthy Vietnamese controls. The fragment which included exon 7 was amplified from a blood filter 1.25 mm in diameter using Amplidirect Plus (Shimadzu Biotech, Tsukuba, Japan).

*Msp I and Taq I polymorphisms*

There are two well-known polymorphisms in the T2 gene. One is c.13G/C in exon 1, which can be detected by the absence/presence of the Msp I site [7]. The other is IVS9+84C>T, which can be detected by the presence/absence of the Taq I site [25]. These polymorphic sequences were determined by direct sequencing.

## Results and discussion

*Identification of gene mutations in Vietnamese patients*

From 2005 to 2009, 10 Vietnamese patients (who were not related to each other) from the northern half of Vietnam (around Hanoi) were suspected of having T2 deficiency from typical profiles of urinary organic acids (Fig. 1). In this report, eight of the 10 patients were investigated at the DNA level. Their clinical presentations are summarized in Table 1. The National Hospital of Pediatrics in Hanoi covers an area of about 40 million people in the northern part and some middle parts of Vietnam. Since most of the very sick children are referred to this hospital, most T2-deficient patients who develop severe ketoacidotic crisis are expected to be examined in this hospital. This area has approximately 555,000 newborns per year. The birth years of the 10 T2-deficient patients were from 2003 to 2008. Hence, if all the patients with T2 deficiency in this area were identified in this hospital, the incidence of T2 deficiency is calculated to be about 1 in 333,000 newborns in this area (10 T2-deficient patients/555 × 6 years). Some patients may die before referral to this hospital, so the incidence of T2 deficiency may be more than this value.

We first confirmed T2 deficiency in GK70's fibroblasts. Acetoacetyl-CoA thiolase activities with and without potassium ions were 3.5 and 3.7 nmol/min/mg protein (4.8 and 10.1 in control fibroblasts), respectively, showing no potassium ion-activated acetoacetyl-CoA thiolase activity. This indicated that GK70 had T2 deficiency. Immunoblot analysis showed that the T2 protein was not detectable in GK70's fibroblasts (data not shown). Mutation analysis at the genomic level showed that GK70 was a compound heterozygote of c.622C>T (R208X) from the father and c.163_167delTTTTTinsAA from the mother. The latter mutation resulted in F55del and L56K. This mutation was not identified in 50 control Vietnamese subjects.

The other 7 Vietnamese patients who were suspected of having T2 deficiency from urinary organic acid analysis were analyzed using DNA samples. In the cases of GK72, GK73, GK74, and GK75, we sequenced all exons and their surrounding introns (~100 bp) and identified the mutations shown in Table 1. Fig. 2A shows the result of direct sequencing of the fragment, including exon 7, in GK73 and his parents. GK73 was a homozygote of R208X. Since the R208X mutation was identified in eight of 10 mutant alleles in these 5 Vietnamese patients (GK70, GK72–75), in cases of GK76, GK79, and GK80, we screened the presence of R208X first and revealed that the three patients were homozygotes of R208X.

*Allele frequency of R208X in Vietnamese population*

Since these 8 families with T2 deficiency were not related to each other, we expected that the R208X mutant allele would be prevalent in the Vietnamese population. We screened the R208X mutation in 400 Vietnamese healthy subjects, using the restriction enzyme assay (Fig. 2b). We could not identify R208X in 800 Vietnamese alleles. This may indicate that the frequency of R208X homozygous T2-deficient patients in a Vietnamese population is less than 1/640,000. As discussed above, we first expected to detect some heterozygotes of the R208X mutation when we examined 800 alleles, but this time we could detect no heterozygote of R208X.

*Identification of gene mutations in Dutch patients*

We previously identified the c.622C>T(R208X) mutations heterozygously in two Dutch patients (GK35 and GK36). As shown in Table 1, GK35 and GK36 were compound heterozygotes of R208X and IVS11+2t>c, and R208X and IVS10-2a>c. We previously identified mutations in the other 3 Dutch patients as follows: GK04 was a compound heterozygote of G183R and IVS10-2a>c; GK04's father, GK05, was a compound heterozygote of G183R and IVS8+1g>t [6]; GK17 was a compound heterozygote of IVS7-46_c.752del68bp and IVS11+2t>c [9]. Hence among 9 mutant

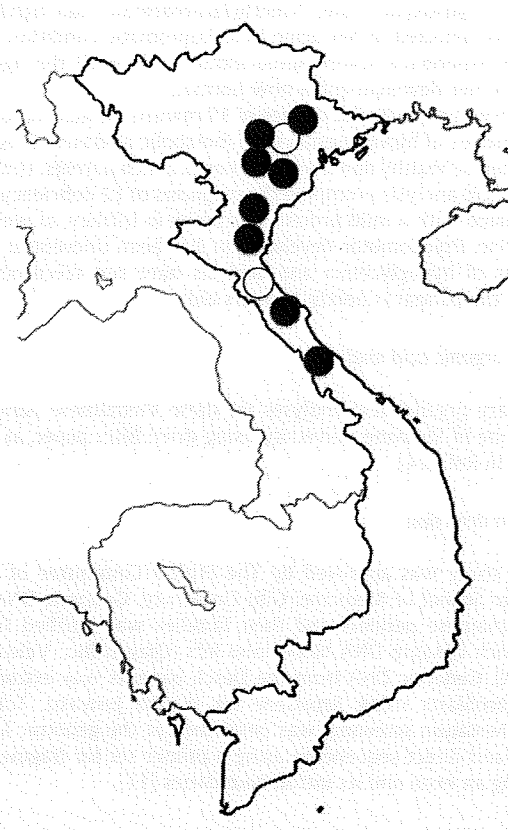

**Fig. 1.** T2-deficient patients identified in Vietnam. A closed circle indicates a T2-deficient patient whose mutations were confirmed. One open circle indicates a probable T2-deficient patient whose sample was not available for mutation analysis.

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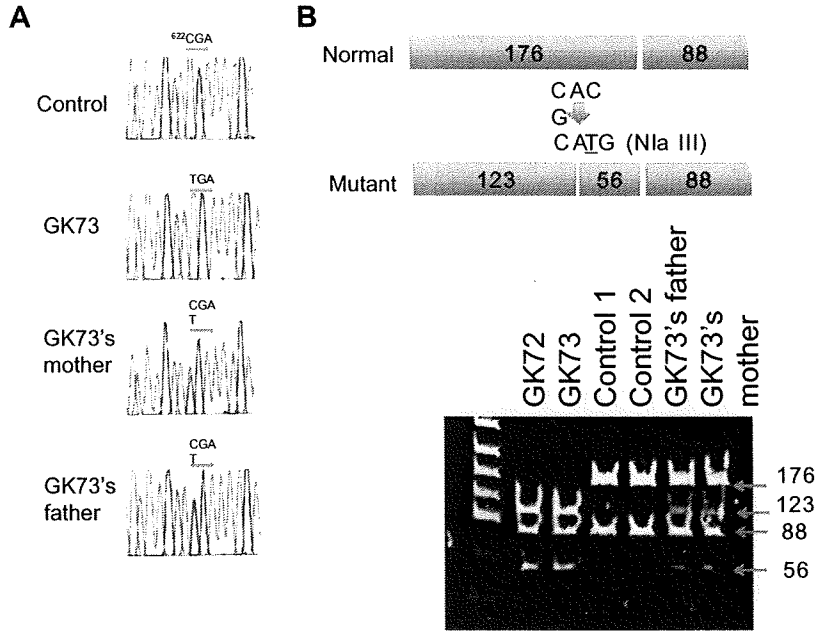


**Fig. 2.** Identification of R208X mutation. (A) Genomic direct sequencing of exon 7. GK73 had a homozygous c.622C>T (R208X) mutation and the parents were carriers of this mutation. (B) Restriction enzyme assay to detect R208X by NIa III. PCR fragments digested with NIa III were separated on a 5% polyacrylamide gel. Samples from two homozygotes (GK72 and GK73), two controls, and two heterozygotes (GK73's father and mother) are shown as representative data.

alleles in a Dutch population, R208X, IVS11+2t>c, and IVS10-2a>c were identified in two mutant alleles. IVS11+2t>c was also identified in other Caucasian patients (GK09, GK28). This IVS11+2t>c caused aberrant splicing using a cryptic splice site just 4 bp downstream of the authentic site, resulting in a 4-bp insertion to T2 mRNA [7].

*Haplotyping of R208X mutant allele in Vietnamese and Dutch populations*

Two T2 gene polymorphisms were reported [7,25]. One is c.13G/C in exon 1, which can be detected by the absence/presence of the Msp I site [7]. The heterozygosity of the Msp I polymorphism was reported to be 0.34 on NCBI SNP (http://www.ncbi.nlm.nih.gov/SNP/). The other is IVS9+84C>T, which can be detected by the absence/presence of Taq I site [25]. The heterozygosity of the Taq I polymorphism in Japanese population was reported to be 0.5. As shown in Table 1, the R208X allele in Vietnamese patients had an MT haplotype, which was confirmed by familial analysis. However, since the R208X mutant allele in Dutch patients had an m instead of an M, the haplotype of the R208X mutant allele in Dutch patients was different from that in Vietnamese patients.

These data suggested that the R208X mutation is a founder mutation in the Vietnamese population but independently occurred from R208X in the Dutch population.

*Common mutations in T2 deficiency*

To date, more than 70 different mutations have been identified in more than 70 T2-deficient patients (including unpublished data). Among the mutations, only a few were identified in more than two independent families: for example c.149delC in the Japanese population [12], c.455G>T(G152A) [18], c.890C>T(Q272X) [8], IVS8+1g>t [6], c. 890C>T(T297M)[10] and IVS11+2t>c [6] in the Caucasian population. As far as we know, no common mutations have been identified in T2 deficiency. However, as described above, the R208X mutation has been identified in 87.5% of mutant alleles in the Vietnamese population. The remarkably high incidence of the R208X mutation among Vietnamese T2-deficient patients is similar to the high incidence of c.727G>T in the G6PC gene among Japanese Glycogen storage disease-type Ia patients, and of the K329E mutation in the ACADM gene among Caucasian medium-chain acyl-CoA dehydrogenase-deficient patients [26–28]. DNA diagnosis of T2 deficiency may be applicable to the Vietnamese population. We have not analyzed mutations in Asian populations other than Vietnam and Japan, hence we do not know whether the R208X mutation is prevalent in Southeast Asia or not. We are planning to examine mutations in other Southeast Asian countries.

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# CpG islands around exon 1 in the succinyl-CoA:3-ketoacid CoA transferase (SCOT) gene are hypomethylated even in human and mouse hepatic tissues where SCOT gene expression is completely suppressed

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**Abstract.** In ketone body metabolism, hepatocyte-specific silencing of the succinyl-CoA:3-ketoacid CoA transferase (SCOT) gene appears to be physiologically important to avoid a futile cycle in the liver, whereas the SCOT gene is expressed in extrahepatic tissues. It is not possible to explain hepatocyte-specific silencing by cis-elements in the 2.2-kb 5' flanking region. The molecular basis of this gene silencing is unknown thus far. In the present study, the methylation status of CpG islands around exon 1 in the SCOT gene was analyzed by sodium bisulfite treatment and by sequencing of genomic DNA from the HepG2, Chang liver and HeLa human cell lines, and also from mouse liver, heart and kidney cells. Most CpG dinucleotides in the CpG island of the human SCOT promoter region were not methylated in the DNA of HeLa and Chang cells, while HepG2 DNA was hypomethylated in this CpG island. CpG dinucleotides in the mouse SCOT CpG island were almost completely unmethylated in the liver DNA as well as in the heart and kidney DNA. CpG islands around the promoter region of the SCOT gene were hypomethylated in the DNA from both human HepG2 cells and mouse liver. Hence, methylation status does not contribute to hepatocyte-specific SCOT gene silencing.

## Introduction

Ketone bodies are important vectors of energy transfer from the liver to extrahepatic tissues, especially when glucose is in short supply (1). Succinyl-CoA:3-ketoacid CoA transferase (SCOT; EC2.8.3.5; locus symbol OXCT) catalyzes the rate-determining step of ketone body utilization (ketolysis) in extrahepatic tissues. SCOT protein is abundant in the heart, brain and kidney, and has been detected in all extrahepatic tissues tested (2). In extrahepatic tissues, SCOT-activated acetoacetyl-CoA is cleaved to acetyl-CoA by mitochondrial acetoacetyl-CoA thiolase (T2). Acetyl-CoA is then converted to fuel via the Krebs cycle.

SCOT mRNA expression is almost completely suppressed in the human liver, the site of most ketone body synthesis (2). This can be viewed as a mechanism to avoid futile cycling. On the other hand, T2 is abundant in the liver, where it is also involved in ketogenesis and isoleucine catabolism. Notably, rat hepatoma cell lines exhibit various degrees of SCOT protein expression, while expression was scarcely detected in rat hepatocytes (3). Presumably, this may allow hepatoma cells to use ketone bodies as an energy source.

Hereditary SCOT deficiency is one cause of ketoacidosis and, typically, elevated serum levels of ketone bodies are present even when a patient is well nourished and not acutely ill. We previously cloned human SCOT cDNA (4) and the human SCOT gene and reported its structural organization (5). We also previously investigated the basis of SCOT deficiency at the molecular level (5-9).

In previous studies, we investigated the control of SCOT gene expression, especially the mechanism of SCOT gene silencing in hepatic tissue. We recently demonstrated that two GC boxes in the SCOT promoter region are essential for promoter activity, but failed to identify cis-elements as responsible for the complete hepatocyte-specific suppression of the SCOT gene in the 2.2-kb 5' flanking region (10).

Genomic analysis also showed high GC contents in the promoter, in which there are many CpG sites, both in human and mouse SCOT genes. It is well known that gene expression is affected by epigenetic control. A DNA region with a high level of CpG methylation in association with a low level of chromatin histone acetylation is inactive for transcription, whereas a DNA region with a low level of CpG methylation in association with a high level of chromatin histone acetylation is active for transcription. In the present study, we investigated the methylation status of CpG islands around exon 1 of the

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*Key words:* succinyl-CoA:3-ketoacid-CoA transferase, OXCT, gene silencing, CpG island, methylation

SCOT gene in both human cell lines and mouse tissues (including the liver) and found that these SCOT CpG islands were, in general, hypomethylated in both human and mouse hepatic DNA.

## Materials and methods

*Samples.* Genomic DNA was extracted from HeLa, HepG2 and Chang liver cells. Adult mouse genomic DNA was obtained from the heart, kidney and liver. Animal handling and experimentation were carried out in accordance with the guidelines set by the Institutional Animal Care and Use Committee of Gifu University.

*Sodium bisulfite treatment.* The bisulfite conversion of genomic DNA was performed using a previously described protocol with minor modifications (11-13). Approximately 500 ng of genomic DNA was digested overnight with *Hin*dIII, boiled for 1 min, denatured by adding freshly prepared 3 M NaOH for a final concentration of 0.3 M, and incubated at 42°C for 30 min. A fresh solution of 3.8 M sodium bisulfite was prepared and adjusted to pH 5.0 with NaOH and 20 mM hydroquinone by gentle mixing at 37°C. Final concentrations of 3.4 M sodium bisulfite and 1 mM hydroquinone were added to the denatured DNA for a final volume of 100 µl. The DNA was gently mixed in this sodium bisulfite/hydroquinone solution, overlaid with mineral oil and incubated at 55°C for 6 h. After recovering the aqueous phase from under the oil, the unbound bisulfite was removed from the DNA using microspin S-200HR columns (Pharmacia Biotech). The purified DNA sample was subsequently mixed and incubated with freshly prepared NaOH (0.3 M final concentration) at 37°C for 20 min. The NaOH was removed using microspin S-200HR columns, and the flow-through (<100 ml) contained the converted DNA ready for amplification.

*PCR conditions.* PCR amplifications were carried out in 50-µl reaction mixtures containing 2-8 µl of bisulfite-treated genomic DNA. PCR amplification was performed under the following general conditions: 94°C for 5 min, followed by 40 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min and 1 cycle at 72°C for 7 min.

For human SCOT CpG islands around exon 1, including the basic promoter region, the following two sets of primers were used: fragment 1, hMETH1 (sense) 5'-GGGTTTTGAATTTTAGGTTAAGATTTATTT-3'; hMETH11 (antisense) 5'-ACTTTACCTTATACCAAATTA CCCAAATC-3'; and fragment 2, hMETH2 (sense) 5'-GAT TTGGGGTAATTTGGTATAAGGTAAAGT-3'; hMETH22 (antisense) 5'-CCATAACTAACCCAACCTCAATTCTA AAC-3'.

For mouse SCOT CpG islands around exon 1, including the basic promoter region, the following two sets of primers were used: fragment 1, mMETH1 (sense) 5'-TTAGTA AGAGATTTTTTAGGTTTTTGGTAA-3'; mMETH11 (antisense) 5'-CCCTACACCTTCAATTTACCTTATACAAA-3'; and fragment 2, mMETH2 (sense) 5'-TTGAAGGTGTAGGGGG GTAAGAGGAAGGTT-3'; mMETH22 (antisense) 5'-CCTT CCCAAAAAC(G/A)TC(G/A)ACCTAAAACC-3'.

The primer positions are shown in Figs. 1 and 3.

*Cloning sequencing and analysis of PCR products.* Amplified fragments were separated following electrophoresis on a 1% (w/v) agarose gel and extracted using a Geneclean II kit (Bio 101, Vista, CA). Isolated PCR fragments were ligated into a pGEM-T Easy vector (Promega) for subcloning. Sequencing was carried out using ABI PRISM™ Cycle Sequencing kits (Perkin-Elmer Corp., Foster City, CA) and analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). We sequenced ten randomly picked clones after subcloning them into a pGEM-T Easy vector.

## Results

*Methylation status in CpG islands around exon 1 in the human SCOT gene.* As shown in Fig. 1, more than 80 CpG dinucleotides exist within 1,000 bases. The 5' flanking region of the SCOT gene includes two GC boxes and transcription starting sites, and its GC content is 68.3%. Exon 1 consists of 79 nucleotides and its GC content is 63.3%. The 5' region of intron 1 (~500 bp) also has a high GC content of 64.4%. Genomic DNA was extracted from HeLa, Chang liver and HepG2 cells. This region was divided into two fragments, which were analyzed by bisulfite sequencing.

Fig. 2 shows the percentage of CpG methylation at individual CpG dinucleotides (the numbering of CpG dinucleotides is as shown in Fig. 1) in these cell lines. In general, this region was almost entirely composed of non-methylated DNA in the HeLa and Chang liver cells. CpG dinucleotides no. 63-83 were rather more methylated than the other dinucleotides in HepG2 DNA. DNA from HepG2 cells was the most methylated among these three cell lines, and was regarded as hypomethylated in this region.

*Methylation status in CpG islands around exon 1 in the mouse SCOT gene.* As shown in Fig. 3, more than 80 CpG dinucleotides exist within 960 bases around exon 1 as well as the human SCOT gene. Genomic DNA was extracted from mouse kidney, heart and liver tissues. This region was divided into two fragments, which were analyzed by bisulfite sequencing. Unexpectedly, the DNA from mouse liver was completely unmethylated in these 81 CpG dinucleotides, as was the DNA from mouse kidney and heart.

## Discussion

Liver-specific SCOT gene silencing appears to be physiologically important. This silencing is observed in humans and mice. Previously, we studied the molecular basis of this liver-specific SCOT gene silencing. In the present study, we compared the methylation status of the CpG islands around exon 1 of the human SCOT gene in two hepatoma cell lines (HepG2 and Chang liver cells) and in the HeLa cervical cancer cell line. We showed that SCOT mRNA and protein were detectable in Chang liver cells and the HeLa cervical cancer cell line, but not in the HepG2 cell line, indicating that the latter maintains the characteristics of liver cells in ketone body metabolism (10). In general, the CpG islands around exon 1 were non-methylated in HeLa and Chang liver cells and were hypomethylated (up to 60%) in the HepG2 cell line. Since SCOT gene silencing was almost complete, the difference among these cell lines

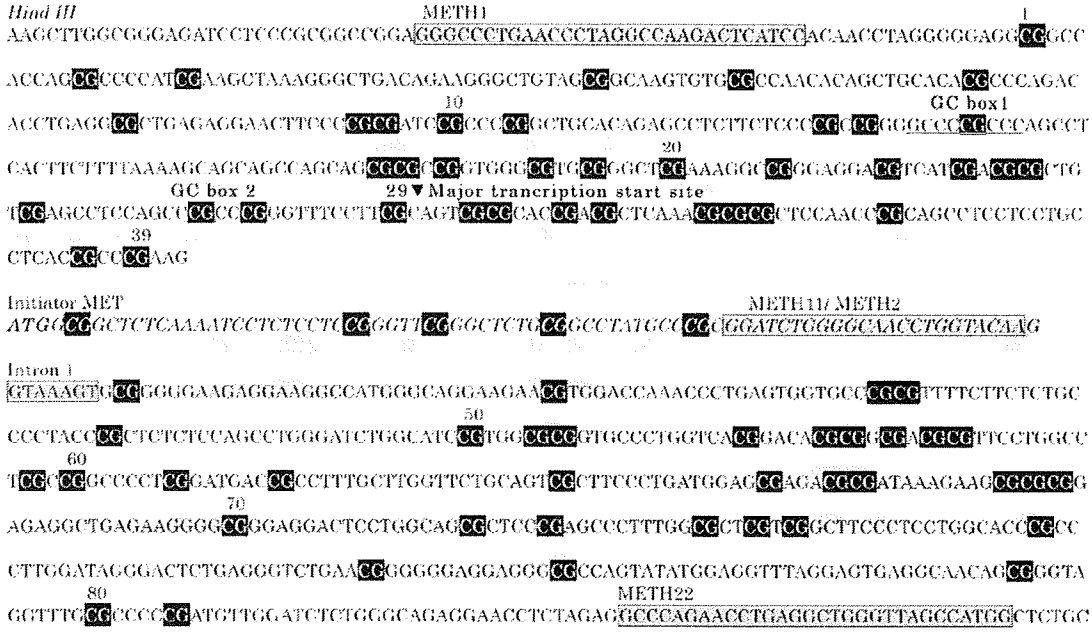


Figure 1. CpG islands around exon 1 of the human SCOT gene. CpGs are indicated by a black background. CpG dinucleotides are numbered from a 5' to 3' orientation. Two GC boxes and major transcription starting points are indicated. The nucleotides in exon 1 are indicated by italicized characters. The positions of the primers used in the PCR after bisulfite treatment are indicated by a grey background.

| | 5' | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CpG number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| HepG2 | 10 | 0 | 10 | 10 | 30 | 0 | 10 | 0 | 10 | 0 | 0 | 10 | 10 | 10 |
| Chang | 10 | 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HeLa | 0 | 0 | 10 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 10 | 0 | 0 |

| CpG number | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HepG2 | 30 | 10 | 30 | 10 | 10 | 10 | 10 | 10 | 10 | 0 | 0 | 10 | 10 | 10 |
| Chang | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HeLa | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 |

| CpG number | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HepG2 | 60 | 0 | 10 | 40 | 30 | 30 | 10 | 30 | 30 | 60 | 10 | 40 | 0 | 0 |
| Chang | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HeLa | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| CpG number | 43 | 44 | 45 | 46 | 47 | 48 | 49 | 50 | 51 | 52 | 53 | 54 | 55 | 56 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HepG2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 40 | 10 | 0 | 0 | 30 | 10 | 20 |
| Chang | 0 | 0 | 0 | 0 | 10 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HeLa | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 0 | 0 |

| CpG number | 57 | 58 | 59 | 60 | 61 | 62 | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HepG2 | 0 | 20 | 10 | 10 | 0 | 30 | 30 | 40 | 40 | 30 | 40 | 40 | 40 | 50 |
| Chang | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HeLa | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| CpG number | 71 | 72 | 73 | 74 | 75 | 76 | 77 | 78 | 79 | 80 | 81 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| HepG2 | 40 | 40 | 30 | 20 | 40 | 30 | 40 | 30 | 40 | 40 | 40 |
| Chang | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HeLa | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Figure 2. Methylation status in the CpG island around exon 1 of the human SCOT gene. Ten clones of each PCR fragment after bisulfite treatment were sequenced. Percentages of methylated CpG dinucletides are shown. The number of CpG dinuceltides is shown in Fig. 1.

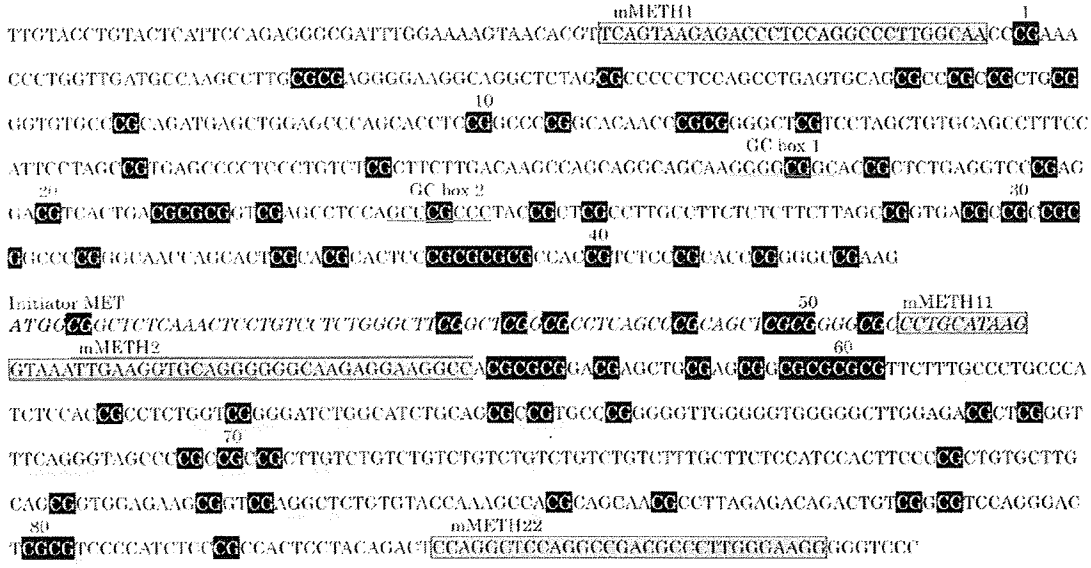


Figure 3. CpG islands around exon 1 of the mouse SCOT gene. CpGs are indicated by a black background. CpG dinucleotides are numbered from a 5' to 3' orientation. Two GC boxes and major transcription starting points are indicated. The nucleotides in exon 1 are indicated by italicized characters. The positions of the primers used in PCR after bisulfite treatment are indicated by a grey background.

was likely due to the mechanism of gene silencing. Since hepatocyte-specific silencing is observed not only in humans, but also in mice, we analyzed the methylation status around exon 1 of the mouse SCOT gene. Most CpG dinucleotides in the CpG islands around exon 1 of the mouse SCOT gene were not methylated in the hepatic DNA or in the DNA from the heart and kidney. Hence, the methylation status around exon 1 of the human and mouse SCOT genes does not contribute to hepatocyte-specific SCOT gene silencing.

In normal hepatocytes, SCOT gene expression is almost completely suppressed, whereas all extrahepatic tissues tested exhibited SCOT expression to various degrees, as follows: myocardium > brain kidney adrenal glands > other tissues (2). The activation of acetoacetate to acetoacetyl-CoA by SCOT is essential for the use of ketone bodies as an energy source in extrahepatic tissues (1). The absence of SCOT in hepatocytes is an important element in energy metabolism, suppressing ketolysis in the liver that might otherwise create a futile cycle and interfere with the efficiency of ketogenesis. Another important aspect of SCOT gene regulation is that some hepatoma cell lines have detectable SCOT expression (3). Such dysregulation benefits tumor cells since there is a relationship between SCOT expression and their growth rate. Hence, this hepatocyte-specific suppression should be programmed and conserved in mammalians.

We recently demonstrated that two GC boxes in the SCOT promotor region are essential for promotor activity, but failed to identify the specific cis-elements responsible for the complete silencing of the SCOT gene in hepatic tissues in the 2.2-kb 5' flanking region (10). One possibility is that liver-specific elements, such as strong suppressors or silencers in the SCOT gene, may lie outside of the 2.2-kb 5' flanking region. Alternatively, other mechanisms of gene silencing, such as methylation and siRNA, may be involved in hepatocyte-specific SCOT gene silencing.

DNA methylation is an evolutionally conserved mechanism for the regulation of gene expression in mammals. Tissue- and disease-specific *de novo* methylation events are observed during somatic cell development/differentiation. Once established, DNA methylation patterns are thought to be stable. Generally, cytosine residues in CpG are methylated in the genome, especially within non-coding DNA, introns and repetitive sequences. Most CpG clusters, called CpG islands, which are frequently found in the proximal promoter regions of many genes, are unmethylated during normal cell development. However, there are exceptions, such as imprinted genes, genes on the inactive X chromosome and tissue-specific differentially methylated genes. DNA methylation plays a role in the regulation of tissue-specific gene expression (14,15). Comparative analysis between mice and humans suggests that some, but not all, tissue-specific differentially methylated regions are conserved (16).

At least 5% of 15,500 CpG islands in the mouse are differentially methylated (17), and 50 tissue-specific differentially methylated regions have been identified. The majority of the tissue-specific differentially methylated regions are associated with 5' promoter CpG islands, and may play important roles in establishing or maintaining gene silencing during or after tissue differentiation.

Here, we investigated whether the methylation status of CpG islands around exon 1, including the SCOT gene promoter in the liver, is different from other tissues which express the SCOT gene, such as heart and kidney tissue. We clearly demonstrated that most CpG dinucleotides in CpG islands around exon 1 of the SCOT gene were hypomethylated or non-methylated in DNA from human and mouse hepatic tissues, as well as DNA from SCOT-expressed cells and tissues. The tissue-specific-expressed human β-globin (18) and α2(1).collagen (19) genes were found to have CpG islands that remain unmethylated in tested tissues regardless of

expression. Since many CpG islands are located at genes that have a tissue-restricted expression pattern, it follows that CpG islands remain methylation-free, even when their associated gene is silent (20). In other words, a cluster of hypomethylated CpG dinucleotides may be a common characteristic of CpG islands, while some tissue-specific differentially methylated CpG islands have also been identified. Hence, the SCOT gene has a typical CpG island that remains un- or hypomethylated. Further analysis is needed to understand liver-specific silencing of the SCOT gene.

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# A Novel Mutation (c.951C>T) in an Exonic Splicing Enhancer Results in Exon 10 Skipping in the Human Mitochondrial Acetoacetyl-CoA Thiolase Gene

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Running title: exonic enhancer mutation causing exon 10 skipping

## ABSTRACT

Mitochondrial acetoacetyl-CoA thiolase (T2) deficiency is an inherited disorder affecting isoleucine catabolism and ketone body metabolism. A Japanese female developed a severe ketoacidotic attack at the age of 7 months. Urinary organic acid analysis showed elevated excretion of 2-methyl-3-hydroxybutyrate but not tiglylglycine. She was diagnosed as having T2 deficiency by enzyme assay using fibroblasts. Mutation analysis revealed a compound heterozygote of c556G>T(D186Y) and c.951C>T(D317D). Since c.951C>T does not cause amino acid change, we performed cDNA analysis and found that exon 10 skipping had occurred in the c.951C>T allele. A computer search using an ESE finder showed that an exonic splicing enhancer sequence, SF2/ASF, was located in CTGA$^{951}$CGC. We hypothesized that the exonic splicing enhancer is necessary for accurate splicing since the first nucleotide of exon 10 is C, which weakens the splice acceptor site of intron 9. We made a mini gene construct including exon 9-truncated intron 9-exon 10-truncated intron 10-exon 11 for a splicing experiment. We also made three mutant constructs which alter the SF2/ASF site (947C>T, 951C>T, 952G>A). An min-gene splicing experiment clearly showed that exon 10 skipping was induced in all three mutant constructs. Moreover, additional substitution of G for C at the first nucleotide of exon 10 resulted in normal splicing in these three mutants. These results confirmed that c.951C>T diminished the effect of the exonic splicing enhancer and caused exon 10 skipping.

Key Words: aberrant splicing, exonic mutation, splice site selection, mitochondrial acetoacetyl-CoA thiolase, inborn error of metabolism, SF2/ASF

## INTRODUCTION

Mitochondrial acetoacetyl-CoA thiolase (T2) (EC 2.3.1.9, gene symbol *ACAT1*) deficiency (OMIM 203750, 607809) is an autosomal recessive disorder, commonly known as β-ketothiolase deficiency. Since 1971 [1], more than 70 patients with it have been identified (including personal communications) [2]. This disorder affects the catabolism of isoleucine and the metabolism of ketone bodies, and is clinically characterized by intermittent ketoacidotic episodes with no clinical symptoms between episodes. T2-deficient patients usually have urinary excretion of 2-methyl-3-hydroxybutyrate, 2-methylacetoacetate and tiglylglycine, derived from intermediates in isoleucine catabolism. The severity of the clinical features varies from patient to patient but follow-up studies reveal that, in general, T2 deficiency has a favorable outcome [3].

The T2 gene (gene symbol: ACAT1) spans approximately 27 kb, contains 12 exons [4], and is located at 11q22.3-q23.1 [5]. Human T2 cDNA is about 1.5 kb long and encodes a precursor protein of 427 amino acids, including a 33-amino-acid leader polypeptide [6]. We have identified more than 70 gene mutations [7-25], 15 % of which cause aberrant splicing. Most were located at the highly conserved ag at the splice acceptor site and gt at the splice donor site. We also identified some exonic