mutations which cause aberrant splicing by activating cryptic splice sites within their exons [15, 24].

We herein report a novel exonic mutation—c.951C>T (the 11th nucleotide in exon 10). It was first regarded to be a silent mutation, D317D, but was associated with exon 10 skipping in cDNA analysis. The c.951C nucleotide is located in a possible exonic splicing enhancer sequence, SF2/ASF, and C>T substitution results in a deviation from its consensus sequence. We showed by a minigene splicing experiment that the substitutions in this exonic splicing enhancer caused exon 10 skipping.

MATERIALS AND METHODS

Case Report

The patient (GK64), a female, was born to non-consanguineous Japanese parents. She was well until 7 months of age when she presented with a one-week history of coughing and appetite loss. She developed convulsions and was admitted to a hospital. Laboratory findings showed blood pH 6.769, bicarbonate 2.8 mmol/L, base excess -28.2 mmol/L, ammonia 213 μmol/L, and blood glucose 0.45 mmol/L. She was referred to the National Center for Child Health and Development on a mechanical ventilation support. Urinary organic acid analysis at the time of crisis showed huge amounts of acetoacetate and 3-hydroxybutyrate with dicarboxylic acids; 2-methyl-3-hydroxybutyrate and tiglylglycine were not evident at that time. She was successfully treated by intravenous glucose infusion. Later, she had an upper respiratory infection and her urinary ketone was 2+ at the age of 8 months. At that time, urinary organic acid analysis showed the presence of 2-methyl-3-hydroxybutyrate. However, tiglylglycine, another characteristic urinary organic acid in T2 deficiency, was not detected. Skin biopsy and a fibroblast culture were performed and finally she was diagnosed as having T2 deficiency by enzyme assay.

Cell culture, enzyme assay and immunoblot analysis

The fibroblasts were cultured in Eagle's minimum essential medium containing 10% fetal calf serum. Acetoacetyl-CoA thiolase activity was assayed, as described in [26]. Immunoblot analysis was done, as described in [27].

Mutation detection

Genomic DNA was purified from the fibroblasts with Sepa Gene kits (Sanko Junyaku, Tokyo, Japan). Mutation screening was performed at the genomic level by PCR and direct sequencing using a primer set for 12 fragments including an exon and its intron boundaries [13]. RNA was prepared from the fibroblasts using an ISOGEN kit (Nippon Gene, Tokyo, Japan). RT-PCR and sequencing after subcloning into a pGEM-T Easy vector (Promega, Madison, USA) were performed as described previously [7], except for the following point. A full-coding sequence of human T2 cDNA was amplified as a single fragment using a sense primer (5'-[-40]AGTCTACGCCTGTGGAGCCGA[-20]-3') and an antisense primer (5'-[1326]TTCTGGTCACATAGGGTT[1309]-3').

Transient expression analyses

Transient expression analysis of T2 cDNAs was done using a pCAGGS eukaryote expression vector [28], as described in [19]. After transfection, the cells were further cultured at 37°C for 72 hr, and then they were harvested and kept at -80°C until use. The cells were freeze-thawed and sonicated in 50 mM sodium phosphate (pH 8.0), 0.1% Triton X-100. After centrifugation at 10,000 x g for 10 min, the supernatant was used in an enzyme assay for acetoacetyl-CoA thiolase activity and for immunoblot analysis.

Splicing experiment

A fragment (about 4 kb long) from the middle part of exon 9 to the middle part of exon 11 was amplified by Phusion DNA polymerase (New England BioLabs, Ipswich, USA) using control genomic DNA. The primers used in this amplification included the EcoR I linker sequence, as follows:

Ex 9 (EcoR I) primer (exon 9, sense) 5'-cagctgcgaatt^{842}CCAGTA CACTGAATGATGGAGCAGCT^{873}-3'

Ex 11 (EcoR I) primer (exon 11, antisense) 5'-cctccattggaatt^{1122}CACTTTT TGGGGATCAATCT^{1103}-3'.

The amplified fragment, after digestion with EcoR I, was subcloned into an EcoR I site of the pCAGGS expression vector from which the Hind III and Pst I sites were deleted. The subcloned PCR fragment did not contain PCR errors, at least in the sequence of exon 9, the 3' and 5' splice sites of intron 9, exon 10, the 3' and 5' splice sites of intron 10, and exon 11. We deleted about a 0.5-kb Hind III-Pst I inner fragment in intron 9 and a 1.1-kb Hind III-Pst I inner fragment in intron 10 to reduce the minigene construct length. In order to make an mutant construct, in vitro mutagenesis was done on the wild-type fragment in the pUC118 vector, and then the mutant fragment was subcloned into the pCAGGS expression vector as a cassette of an about 870-bp Pst I-Hind III fragment including exon 10. We made three mutant constructs which altered the SF2/ASF site (c.947C>T, c.951C>T, and c.952G>A). Moreover, we also made 3 further mutant constructs with additional substitution of c.941G for C at the first nucleotide of exon 10.

Two μg of these expression vectors were transfected into 5X10^5 cells of SV40-transformed fibroblasts using Lipofectamine 2000. At 48 hours after transfection, RNA was extracted from the cells. The first strand cDNA was transcribed with a rabbit β-globin-specific antisense primer (β-glo2) (5'-^{461}AGCCACCACCTTCTGATA -3') and then amplified with the Ex10 (EcoRI) primer on T2 exon 10, and another rabbit-specific antisense primer (β-glo3) (5'-^{443}GGCAGCCTGCACCTGAGGAGT -3') to amplify the chimera cDNA of human T2 and rabbit β-globin.

Allele-specific RT-PCR

We performed allele specific RT-PCR using mismatched primers:
c. 556G allele (D186)-specific sense primer, 5'-^{530}TTTGATTGTAAAA GACGGGCTAT̲CTG^{556}-3'
c. 556T allele (Y186)-specific sense primer, 5'-^{530}TTTGATTGTAAAA GACGGGCTAT̲CTT^{556}-3'
The bold G or T represents the D186Y mutation site of c.556G > A. The underlined T indicates a mismatch introduced to the 4th nucleotide to assist

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allele-specific-RT-PCR.

Antisense primer 5'-^{1065}GGCTTCTTTACTTCCCACATTGCA^{1041}-3'

cDNA with exon 10 gave a 535-bp fragment and cDNA with exon 10 skipping gave a 470-bp fragment.

RESULTS and DISCUSSION

Enzyme assay and immunoblot analysis

Potassium-ion-activated acetoacetyl-CoA thiolase activity was absent in GK64's fibroblasts (-K$^+$ 3.8, +K$^+$ 3.9 nmol/min/mg of protein; Control fibroblasts -K$^+$ 4.7, +K$^+$ 7.8 nmol/min/mg of protein), confirming the diagnosis of T2 deficiency. Succinyl-CoA:3-ketoacid CoA transferase activity was 6.3 nmol/min/mg of protein (control fibroblasts 5.6 nmol/min/mg of protein). In immunoblot analysis, GK64's fibroblasts had a reduced but significant amout of T2 protein (Fig.1). We then performed immunoblot analysis using twofold serially diluted samples of two controls and GK64's fibroblasts from 30 to 3.75 μg. The relative amount of T2 protein in GK64 fibroblasts were estimated to be 25 % of controls (data not shown).

Mutation screening at the genomic level and cDNA level.

Routine genomic PCR and sequencing of exons 1~12 identified two nucleotide substitutions, c.556G>T(D186Y) in exon 6 and c.951C>T(D317D) in exon 10. Both c.556G>T and c.951C>T were noverl nucleotide substitutions in the T2 gene. No further mutations were identified by genomic mutation screening. Since the latter substit. on does not alter amino acid, we performed RT-PCR analysis. A full-coding region was amplified using a pair of primers on a 5'-noncoding region and a 3'-non-coding region, allowing one to show the segregation of these two substitutions. After subcloning, 8 clones had c.556G>T(D186Y) but not c.951C>T(D317D). Two clones had exon 10 skipping without c.556G>T(D186Y) . The exon 10 skipping causes a frame shift and premature termination at c.1011TAA. We re-sequenced the genomic region around exon 10 (IVS8-88~IVS9+44) again, but only c.951C>T(D317D) was detected. We regarded c.951C>T(D317D) , the 11th nucleotide of exon 10, as the cause of exon 10 skipping which was detected in GK64's cDNA. Since the splice acceptor site of intron 9 might be weak because of the first nucleotide of exon 10 being C, we hypothesized that exonic splicing enhancer (ESE) sequences would be necessary for accurate exon recognition of exon 10 and that c.951C>T might disrupt the exonic splicing enhancer and result in exon 10 skipping.

Transient expression analysis of D186Y mutant cDNA

Transfection of wild-type T2 cDNA gave a high acetoacetyl-CoA thiolase activity in the presence of potassium ion. Transfection of D186Y mutant cDNA gave no significant thiolase activity compared with mock cDNA transfection (Fig. 2A) Immunoblot analysis showed that mutant D186Y protein was detected with 1/3 fold amount of wild-type protein (Fig.2B). These results indicate that the D186Y mutant protein is a stable protein but retains no residual activity. Even when incubation was done at a lower temperature (30 °C) after transfection, no residual T2 activity was detected (data not shown). This result confirmed that the D186Y mutation is a causative mutation in one allele, and is consistent with the fact that GK64's fibroblasts had T2

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protein with about a 1/4 fold amount of controls'.

Searches for exonic splicing enhancer

We searched the possible ESE sequences which can be affected by c.951C>T, using ESE finder 3.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process =home) [30-31] and found that this mutated site, c.951 C>T, was located in a possible SF2/ASF site, c.947CTGA951CGC (7th~13th nucleotides in exon 10). The substitution made a deviation from the consensus sequence of SF2/ASF, as shown in Fig. 3A.

Mini gene splicing constructs

We previously successfully performed minigene splicing experiments using a pCAGGS expression vector [8, 24, 29]. Since our minigene construct produces human T2-rabbit β-globin fusion mRNA, we could amplify this specific mRNA by RT-PCR using a combination of a human T2 sense primer and a rabbit β-globin antisense primer. We made a minigene construct including exon 9-truncated intron 9-exon 10-truncated intron 10-exon 11 for a splicing experiment, as shown in Fig. 3B. We made the c.951C>T mutant constructs and two additional mutant constructs (c.947C>T or c.952G>A) which also altered the SF2/ASF site, as shown in Fig. 3A 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. Hence, we made three constructs with an additional substitution of 941G for C at the first nucleotide of exon 10 to strengthen the splice acceptor site of intron 9.

Splicing experiment

We performed a minigene splicing experiment. As shown in Fig. 3C, exon 10 skipping was induced in all three mutant constructs. Normally spliced transcripts with the inclusion of exon 10 were also produced in these mutant transcripts. The ratio of signal intensity of transcripts with exon 10 skipping to that of normally spliced transcripts in three independent experiments was highest in c.951C>T, followed by c.952G>A among these three mutants.

Moreover, additional substitution of G for C at the first nucleotide of exon 10 resulted in normal splicing in these three mutants. Hence, the SF2/ASF exonic splicing enhancer was only necessary in the case of C at the first nucleotide of exon 10 in the experiment. This clearly showed that c.941C, the first nucleotide of exon 10, makes the recognition of exon 10 or the splice acceptor site of intron 9 and requires an exonic splicing enhancer for the accurate splicing of exon 10. These results confirmed that c.951C>T diminished the effect of the exonic splicing enhancer and caused exon 10 skipping.

Effects of c.951C>T mutation on splicing

In the minigene splicing, normally spliced transcripts were detected in the construct with c.951C>T. This may mean that not only exon-10-skipped transcripts but also normally splicied transcripts can be produced in the c. 951C>T mutant allele. However, when we analyzed 10 clones of full-length cDNA, 8 clones were from the allele with c.556G>T(D186Y). Two clones had exon 10 skipping but no cDNA clones with c.951C>T were found. In direct sequencing of full-length cDNA fragments, we

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found a possible faint signal for c. 951T in the major signal for c.951C (Fig.4B). Hence, the presence of normally spliced transcripts from c.951C>T was further confirmed by allele-specific RT-PCR. As shown in Fig. 4A, both c.556T(Y186) allele- and c.556G(D186) allele-specific RT-PCR gave a fragment with the expected size in the case of GK64, and only the latter gave a fragment in the case of a control. In direct sequencing of GK64's fragment of the c.556G(D186) allele, c.951 was T (normally spliced transcripts in the c.951C>T mutant allele) (Fig.4B). An additional faint fragment with exon 10 skipping was also seen in GK64's c.556G(D186) allele-specific PCR. Exon 10 skipping causes frame shift and should result in nonsense-mediated mRNA decay; hence, the amount of cDNA with exon 10 skipping in the D186 allele was smaller than that of normally spliced cDNA. Based on cDNA analysis, a small amount of normally spliced mRNA with c.951C>T(D317D) was also produced and hence GK64 retained some residual T2 activity from this mutant allele. This finding is in accord with GK64's urinary organic acid profiles. We previously showed that urinary organic acid analysis shows no elevated tiglylglycine and relatively small amount of 2-methyl-3-hydroxybutyrate even during ketoacidotic crisis and subtle elevation of 2-methyl-3-hydroxybutyrate under stable conditions in patients with mutations which retain some residual T2 activity [3, 18, 19].

The importance of the exonic splicing enhancer

The accurate removal of introns from pre-mRNA is essential for correct gene expression. However, the information contained in splice sites, including the splice donor site, branch site and splice acceptor site, is insufficient for a precise definition of exons [32-35]. Recently, it was established that exon sequence has elements which contribute to exonic recognition. Additional regulatory elements exist in the form of exonic splicing enhancers[32, 33]. Exonic variants may inactivate an ESE, resulting in insufficient exon inclusion.

Exonic splicing enhancers are known to play a particularly important role in exons with weak splice sites. Although the splice acceptor site of intron 10 has a relatively high Shapiro and Senapathy Score [35] of 90.5, the site deviates from the consensus sequence at position +1, by the replacement of the G nucleotide with C. In computer analysis using ESE finder, the mutation c.951C>T was located on an exonic splicing enhancer, the SF2/ASF site. SF2/ASF is a prototypical serine- and arginine-rich protein (SR family) with important roles in splicing and other aspects of mRNA metabolism. One classical function of SR proteins bound to exonic sequences is to stimulate recognition of the flanking splice sites[36]. Using the minigene approach, we have demonstrated that not only the c.951C>T substitution but also c.947C>T and c.952G>A, all of which affected the SF2/ASF site, resulted in insufficient exon 10 inclusion. This phenomenon was completely corrected by a substitution of G for C at the first nucleotide of exon 10. We therefore suggest that the weak splice acceptor site of intron 10 is normally compensated for by an ESE (SF2/ASF).

There are several precedent reports on exonic splicing enhancer mutations in other genes [37-39]. For example, two synonymous mutations in exon 5 identified in pyruvate dehydrogenase-deficient patients (the c.483C>T and c.498C>T variants) disrupt a putative exonic splicing enhancer, the SRp55 binding site[37]. These synonymous mutations result in the incomplete inclusion of PDHA1 exon 5 in the minigene splicing

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experiment and this effect is corrected following the restoration of a perfect consensus sequence for the 5' splice site by site-directed mutagenesis. The mutation in the SRp55 binding site is affected in the case of the weak 5' splice site selection in this case and the mutation in SF2/ASF site was affected in the case of the weak 3' splice site selection in our case. c.1918C>G (pR640G) in exon 14 in the APC gene, which was found in a familial adenomatous polyposis (FAP) patient, was revealed to be sufficient to cause exon 14 skipping [38]. Minigene splicing experiments showed a mechanism involving disruption of an ASF/SF2 exonic splicing enhancer element. Systemic analysis of 24 mutations in PAH exon 9 showed that three of them affected exonic splicing enhancer motifs and resulted in exon 9 skipping [39]. These facts indicate that we should consider that any mutations in an exon may affect splicing of the exon.

Importance of cDNA analysis

If mutation analysis were done only at the genomic level, this c.951C>T(D317D) mutation would be regarded as a silent mutation. However, the main character of this mutation was an ESE mutation which causes exon 10 skipping. Any nucleotide substitutions have the possibility to affect splicing efficiency. This indicates the importance of cDNA analysis to understand the character of mutations properly.

ACKNOWLEDGEMENTS

ABBREVIATIONS

T2, mitochondrial acetoacetyl-CoA thiolase

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LEGENDS TO FIGURES

Figure 1. Immunoblot analysis. The amount of fibroblast protein extract applied was indicated in each lane. 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. Cont 1 and Cont 2 were healthy controls and GK01 was a disease control being cross reactive material-negative.

Figure 2. Transient expression analysis of D186Y mutant cDNA

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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 the potassium ion are shown together with the standard deviation of three independent experiments.

B) Immunoblot analysis. The protein amounts applied are shown above the lanes. The first antibody was a mixture of an anti-T2 antibody and an anti-SCOT antibody.

Figure 3. Minigene splicing experiment

A) Minigene splicing constructs.

Sequence differences among 8 minigene splicing constructs. Mutations introduced are underlined.

B) Schematic presentation of minigene splicing construct.

The minigene construct has a T2 gene fragment from c.842 of exon 9 and intron 9 (from +1 to a Hind III site, 475-bp open box) and intron 10 (from a Pst I site to -1, 680-bp open box) and exon 11 (to c. 1122). In the cases of mutant constructs, the region around exon 10, highlighted in gray, was replaced as a cassette. Thick lines and black boxes indicate pCAGGS vector sequences.

C) Detection of chimeric cDNAs derived from transfected minigenes. First-strand cDNA was reverse-transcribed using the glo2 primer. cDNA amplification was done using Ex9(EcoRI) and glo3 primers. Normal splicing and aberrant splicing produced 309-bp and 244-bp PCR fragments, respectively. The PCR fragments were electrophoresed on 5% polyacrylamide gel. Fragments with exon 10 skipping are shown by arrows.

Figure 4. Allele-specific cDNA amplification.

A) Allele specifc PCR fragments were electrophoresed on 5% polyacrylamide gel. C, control cDNA; 64, GK64's cDNA; N, negative control. An arrow indicates cDNA with exon 10 skipping. B) Direct sequencing of the antisense strand at the c.951c>T (D186Y) site. Y186 allele, Y186 allele-specific PCR fragment; D186 allele, D186 allele-specific PCR fragment.

12

Fig.1

St Cont Cont GK GK
1   2   64 01
15 30 30 30 30   μg

← SCOT

← T2

Fig.2

A)

D186Y

■ plus K

☐ minus K

Mock

Wild-type

0   50   100 150 200 250 300   nmol/min/mg protein

B)

Wild-type	D186Y
10 5 2.5 1.25	10 5 2.5 1.25 μg

← SCOT
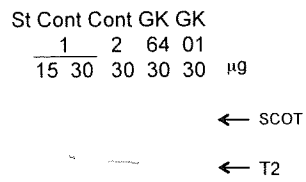
← T2
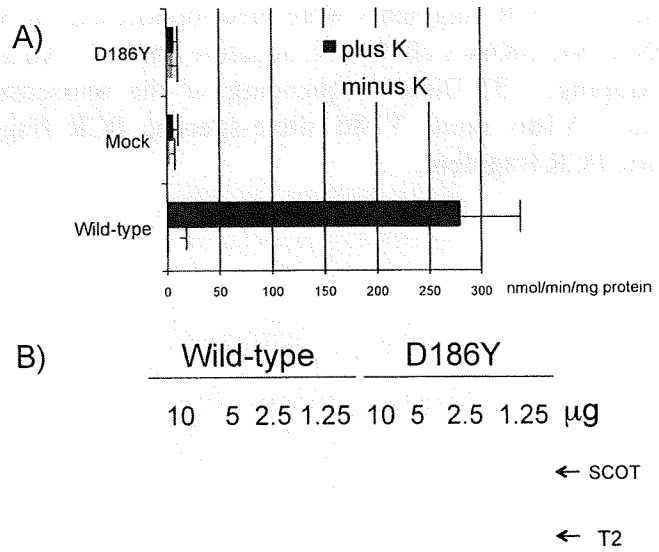
Fig.3

A)

SF2/ASF

Intron 9   Exon 10   [CACACGA / GTGCGTAG logo]

Wild-type ——acag| CATTTGCTGACGCTGCT.....

951C>T   CATTTGCTGA_T_GCTGCT
952G>A   CATTTGCTGAC_A_CTGCT
947C>T   CATTTG_T_TGACGCTGCT
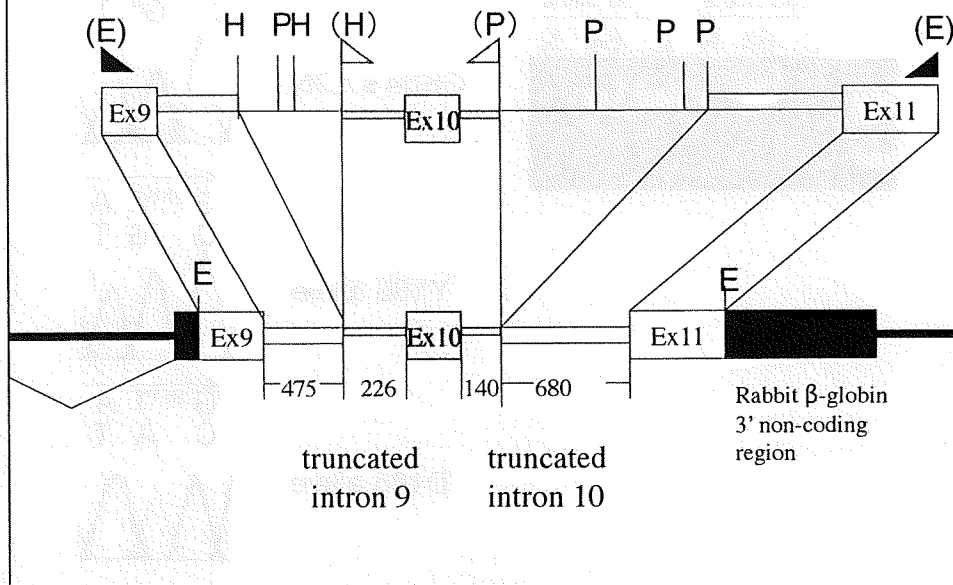941C>G   _G_ATTTGCTGACGCTGCT
941C>G, 951C>T   _G_ATTTGCTGA_T_GCTGCT
941C>G, 952G>A   _G_ATTTGCTGAC_A_CTGCT
941C>G, 947C>T   _G_ATTTG_T_TGACGCTGCT

Fig.3

B)

truncated intron 9

truncated intron 10

Rabbit β-globin 3' non-coding region

Fig.3

C)

W-T 941C>G

947	951	952	941C>G plus
			947	951	952
C>T	C>T	G>A	C>T	C>T	G>A

← Normal splicing
← Exon 10 skipping

```
   95        65        129
  ┌─┐      ┌──┐      ┌──┐
──┤9├──────┤10├──────┤11├────────
  └─┘      └──┘      └──┘
  Ex9(EcoRI)              ◁◁
                         glo3 glo2
```

```
┌─┬──┬──┬──┐
│9│10│11│██│  309 bp
└─┴──┴──┴──┘
┌─┬──┬──┐
│9│  │11│██│  244 bp
└─┴╲╱┴──┘
```

Fig.4

A)

| Y186 allele | | | D186 allele | | |
| C | 64 | N | C | 64 | N |

B)

GK64's cDNA

G ⁹⁵¹Cᵀ A
C Gᴬ T

Y186 allele

G ⁹⁵¹C A
C G T

D186 allele

G ⁹⁵¹T A
C A T

先天性有機酸代謝異常症全国調査（1990-1999）

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要　旨

　先天性有機酸代謝異常症の発生頻度，生命予後および生活の質についての情報は，この疾患に対する診断法や治療法を考える基礎となるものである．今回本邦における有機酸代謝異常症の実態を把握する目的で，1990年から1999年までの10年間における調査を全国規模で行った．

　メチルマロン酸血症3.88名／100万人，プロピオン酸血症1.35名／100万人，高乳酸血症8.41名／100万人の疾患発生頻度と考えられた．全疾患の発生率を合計すると16.09名／100万人であった．今回の調査では有機酸代謝異常症の全症例が補足されてはいないので，実際の発生率はこれより遙かに高いものと推察された．

　さらに死亡率はきわめて高く，死亡にいたらないまでも生活の質という面からもみても疾患の負担はきわめて大きい事が確認された．

　今後有機酸代謝異常症を対象にした新生児マススクリーニングの有効性を検証していくことは重要なことであり，今回の調査はその基礎的資料となると思われた．

キーワード

　先天性有機酸代謝異常，疾患発生率，死亡率，生活の質，新生児マススクリーニング

A．研究目的

　1980年代後半ころよりガスクロマトグラフィー質量分析期などの分析機器の進歩によって，従来診断の難しかった先天性有機酸代謝異常症が数多く診断されるようになってきている．[1] 先天性有機酸代謝異常症の発生頻度，生命予後および生活の質についての情報は，この疾患に対する診断体制や治療方法を考える基礎となるものである．[2] わが国における先天性有機酸代謝異常症についての情報は少なく，多田らの行った1976年から1984年の間の調査報告が最後である．[3] 今回本邦における有機酸代謝異常症の実態を把握する目的で，これら疾患の診断が日本全国で比較的容易になった，1990年から1999年までの10年間における調査を全国規模で行なった．

B．研究方法

　全国の医療機関に，一次調査として調査依頼表を送付した．送付先は全国の大学病院などの医育機関121施設，小児専門医療機関29施設および300床以上で小児科を標榜している病院489施設の合計639施設である．調査内容は1990年1月よりより1999年11月までに経験した先天性有機酸代謝異常症の，疾患名，性別，生年，イニシャル，生命予後である．今回の調査ではアミノ酸代謝異常症にともなうものや糖原病に見られるような二次的なものは対象から除外した．

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二次調査として回答症例数の多かった5疾患すなわちメチルマロン酸血症，プロピオン酸血症，ホロカルボキシラーゼ欠損症，イソ吉草酸尿症，グルタル酸尿症Ⅱ型合計121症例について生命予後，死亡年齢，ビタミン反応性，生活の質，最近一年間の入院回数を調査した．生活の質の評価は評点尺度法により行い[4]，評価者は各主治医にお願いした．

C. 研究結果

一次調査は依頼した639施設のうち400施設より回答があった．全体の回答率は62.6％であった．その内訳は医育機関66施設，小児専門医療機関14施設および300床以上で小児科を標榜している病院320施設であり，それぞれの回答率は54％，48％，65％であった．回答のあった400

施設のうち，何らかの有機酸代謝異常症の症例を経験した施設は103施設であり回答のあった施設の約25.8％であった．報告された症例数は合計299症例であり，医育機関よりは145症例，小児専門医療機関よりは58症例，300床以上で小児科を標榜している病院から96症例報告された．二次調査に回答いただいたのは121症例中87例であり，回答率は71.9％であった．

疾患別患者数：

今回の調査と多田らの調査の疾患別の集計表を表1に示した．メチルマロン酸血症は68症例（男33，女35），プロピオン酸血症は32症例（男10，女22），ホロカルボキシラーゼ欠損症は11症例（男4，女7）であった．このほかイソ吉草酸血症5症例，グルタル酸尿症Ⅱ型5症例などの報告数が多かった．高乳酸血症は合計150症例の報告があった．これを表2に多田らの報告とあわせて示した．診断名については原則的に報告された病名をそのまま使用したので，分類に不十分なところがあるが，いわゆるミトコンドリア脳筋症といわれている疾患群に含まれる

表1. 疾患別集計表
今回の調査と多田らの調査を対比して表した.

疾患名	1990-1999 今回報告 症例数	1976-1984 多田ら報告 症例数
高乳酸血症	150	57
メチルマロン酸血症	68	42
プロピオン酸血症	32	17
イソ吉草酸尿症	5	6
ホロカルボキシラーゼ欠損症	11	2
グルタル酸尿症Ⅰ型	3	0
グルタル酸尿症Ⅱ型	5	0
βケトチオラーゼ欠損症	2	1
ジカルボン酸尿症	2	2
メープルシロップ尿症	1	0
グリセロールキナーゼ欠損症	3	0
メトロクロトニルCoAカルボキラーゼ欠損症	1	0
3-ヒドロキシ3-メチルグルタコン酸尿症	3	0
アルカプトン尿症	2	0
フルクトース2フォスファターゼ欠損症	1	0
高シュウ酸尿症	1	0
3-ヒドロキシ3-メチルグルタリルCoA リアーゼ欠損症	1	0
全身性カルニチン欠損症	2	0
極長鎖アシルCoA脱水素酵素欠損症	1	0
トランスロケース欠損症	1	0
その他	4	1
合計	299	128

表2. 高乳酸血症診断名別集計表

疾患名	1990-1999 今回報告 症例数	1976-1984 多田ら報告 症例数
高乳酸血症	22	32
ミトコンドリア脳筋症	26	5
KSS	3	
MELAS	37	
MARRF	1	
リー脳症	18	
ピアソン症候群	3	
ピルビン酸脱水素酵素複合体欠損症	26	16
ピルビン酸カルボキシラーゼ欠損症	1	2
電子伝達系comlexⅠ欠損症	1	
チトクロームCオキシダーゼ欠損症	11	2
NADH チトクロームC リダクターゼ欠損症	1	
合計	150	57

略語
KSS : Kearns-Sayer Syndrome.
MELAS : Mitochondrial myopathy, Encephalopathy, Lactic Acidosis, Stroke-like episodes
MARRF : Myoclonic Epilepsy associated with Ragged Red Fibers

ものが多く報告された．そのほかピルビン酸脱水素酵素複合体の異常症に含まれる疾患も多く報告されている．今回の報告で特記すべきは脂肪酸代謝異常症の症例が数は少ないものの報告されていることである．

　多田らの調査の方法は206施設に調査を行い回答率が70％であった．多田らの報告と較べると，全体の症例数は146症例から299症例と2倍以上の増多を示している．ことに高乳酸血症が66症例から150症例と大きく増多している．絶対数は増加しているが疾患の発生比率には大きな変化は認められなかった．

生命予後：

　表3に一次調査における生死についての調査結果を示した．メチルマロン酸血症全体における生存率は多田らの報告では30％であったが，今回は66％と改善が認められている．症例のビタミン反応性を加えた二次調査において，ビタミンB12不応型の生存率は56％（20例／36例）

と極めて低く，ビタミンB12反応型は全例が生存している（12例／12例）事が判明した．プロピオン酸血症の生存率は多田らの報告では67％であり，今回は47％と生存率の低下が認められた．高乳酸血症の生命予後は多田らの報告では生存率は64％であり，今回の調査では症例全体の生存率は63％と生存率の改善は認められなかった．今回の調査におけるホロカルボキシラーゼ欠損症，イソ吉草酸尿症は生命予後良好で，その生存率はそれぞれ91％，100％であった．

　二次調査の結果を用いてメチルマロン酸血症，プロピオン酸血症において，カプランマイヤー法により生存曲線を作成した．（図1）この検討の対象症例としたのは1990年以後に生まれたそれぞれ21症例（ビタミンB12不応型17症例，反応型4例），プロピオン酸血症10症例である．メチルマロン酸血症ビタミンB12不応型，プロピオン酸血症の5年生存率はそれぞれ50％，70％であった．メチルマロン酸血症ビタミンB12

表3. 各疾患における生存率

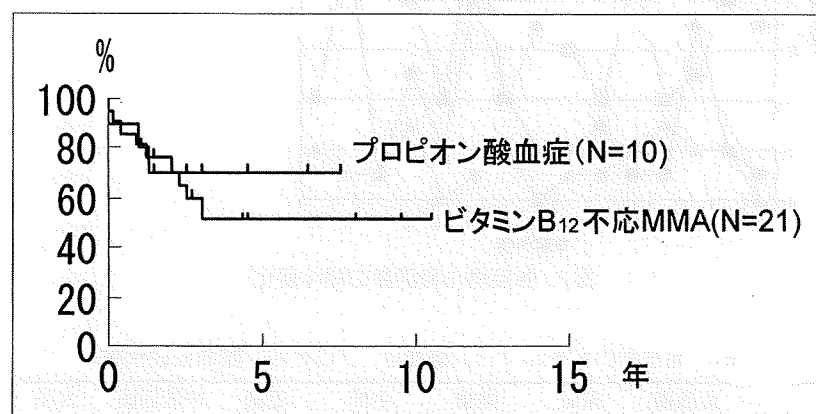|	症例数	生存者数	死亡者数	不明	生存率（%）
メチルマロン酸血症全体	68	43	20	2	63
メチルマロン酸血症ビタミンB12反応型	12	12	0	0	100
メチルマロン酸血症ビタミンB12不応型	36	20	14	1	56
プロピオン酸血症	32	16	14	2	50
ホロカルボキシラーゼ欠損症	11	10	1	0	91
イソ吉草酸尿症	5	5	0	0	100
グルタル酸尿症Ⅱ型	4	2	2	0	50

図1. メチルマロン酸血症，プロピオン酸血症における生存曲線
略語　MAA:Methylmalonic acidemia

反応型は全例生存している．今回の調査ではいずれの疾患においても死亡症例は3才までに発生しており，それ以後には一名の死亡症例もなかった．メチルマロン酸血症において年長児の腎不全による死亡例も知られている．今回調査期間を10年としたことより，これら年長児死亡例は生存曲線作成の対象患者にならなかったものと思われる．

経年的発生頻度の推移：

年次別発生頻度を代表的な疾患である，メチルマロン酸血症，プロピオン酸血症，高乳酸血症について図2に示す．最近5年間でいずれの疾患も報告数が増多している傾向が見られた．

年間発生数：

有機酸血症の報告数は調査期間後半で増多しているので，より正確な年間の発生率を検討するため，最近5年間（1995-1999）の症例の集計を行った．メチルマロン酸血症4.6名／年，プロピオン酸血症1.6名／年，高乳酸血症10.0名／年の発生率であった．これを全国の出生数で補正するとメチルマロン酸血症3.88名／100万人，プロピオン酸血症1.35名／100万人，　高乳酸血症8.41名／100万人　の疾患発生頻度　と考えられた．全疾患の発生率を合計すると16.09名／

100万人である．今回の調査では全有機酸代謝異常症例が補足されているとは考えられないので，実際の発生率はこれより遙かに高いものと推察される．

地域別患者数：

表4にメチルマロン酸血症68症例，プロピオン酸血症32例の各地方別の発生数を示した．メチルマロン酸血症は西日本で，プロピオン酸血症は東日本に多く発生している．このほかの疾患では地域的な症例の偏在は認められなかった．なお地域別の回答率には大きな差は認められなかった．

生活の質：

評点尺度法[7]を用いた生活の質についての評価のまとめを表5に示した．これにおいてもメチルマロン酸血症ビタミンB_{12}不応型，プロピオン酸血症においてその得点はそれぞれ60点台であり，他疾患に比してその評価は低く報告された．グルタル酸尿症II型の症例の評価は80点であり比較的低値であった．
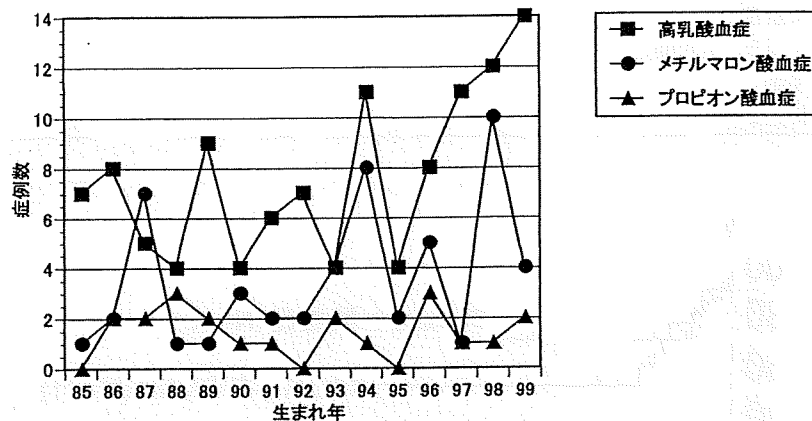
最近一年の入院回数の分析をおこなったが特徴ある結果を得られなかった．

図2．各疾患の発症数の経年変化

表4．地方別のメチルマロン酸血症，プロピオン酸血症症例数

	北海道	東北	関東	中部	近畿	中国四国	九州	計
メチルマロン酸血症	0	8	15	7	12	9	17	68
プロピオン酸血症	0	9	10	5	2	0	6	32

表5. 各疾患における評点尺度法による生活の質の評価

	症例数	平均得点	標準偏差
メチルマロン酸血症ビタミンB_{12}不応型	17	69.1	23.7
メチルマロン酸血症ビタミンB_{12}反応型	11	92.3	8.7
プロピオン酸血症	13	68.8	23.2
ホロカルボキシラーゼ欠損症	8	87.5	10.7
イソ吉草酸尿症	3	88.3	
グルタル酸尿症Ⅱ型	2	80	

D. 考　案

　先天性有機酸代謝異常症の発生頻度は，各疾患をすべて合せると少なく見積もっても16.09名／100万人以上と考えられた．これまでに学会などに報告されている症例数の合計などと比べ，明らかに報告数の少ない疾患もあるので，今回の調査によっても多くの症例が集計もれになっていると思われる．さらに診断がなされずにいる症例はさらに多いものと考えられることより，おそらく本当の発生頻度はこれよりも高いものと考えられた．

　さらに死亡率はきわめて高く，死亡にいたらないまでも生活の質という面からみても疾患の負担は大きい．経済的負担も大きいことが報告されており[5]，先天性有機酸代謝異常症の患者・家族に対する負担は非常に重大であると考えられた．

　今回の調査においては15年前の多田らの報告に比べ，診断される先天性有機酸代謝異常症の症例数は増多しているものと考えられた．これは測定機器の進歩により，全国的にこれら疾患の診断が容易に出来るようになってきたことによる部分が大きいと考えられた．なかでも高乳酸血症の際立った増多は，ミトコンドリア病にたいする理解が急速に進んだことも大きいものと思われた．

　メチルマロン酸血症とプロピオン酸血症には発生率に明らかな地域差がある事が判明した．日本人の中にも遺伝子変異の頻度が異なる集団が存在することは，これによっても明らかであると思われた．

　Dionisi-Viciらの報告[6]によると，新生児発症のメチルマロン酸血症，プロピオン酸血症，イソ吉草酸尿症，合計15例の5年生存率は約45％である．今回の我々の検討では発症時期による検討は出来ていないが，ほぼ同程度の生存率であり，本邦，欧州を問わずこれら疾患の生命予後は不良である．

　近年有機酸代謝異常症を対象とした拡大マススクリーニングが，地域的に行われて来ている[7]スクリーニングのほとんど行われていない時期に，有機酸代謝異常症が与えている負担を正確に評価しておくことは，有機酸代謝異常症の新生児マススクリーニングの効果を科学的に評価する際に重要な必須の資料となりうると考えられた．

E. 結　論

　先天性有機酸代謝異常症の発生率は少なく見積もっても16.09名／100万人である．その生命予後は不良であり，生存し得ても後遺症などで生活の質も低い事が判明した．以前の調査に比して生存率に改善が認められている疾患もあるが，全体的には有機酸代謝障害の予後は不良であることは，最近になっても不変であると考えられた．

　今後有機酸代謝異常症を対象にした新生児マススクリーニングの有効性を検証していくことは重要なことであり，今回の調査はその基礎的資料となると思われた．

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National survey for congenital organic acidemias

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教育講演 4

新生児突然死の予防：タンデムマスによる早期発見

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> Key words
> 　新生児突然死
> 　SIDS
> 　脂肪酸代謝異常
> 　タンデムマス
> 　新生児マススクリーニング

1. はじめに

乳児突然死症候群（SIDS）は生後 2 ～ 4 カ月にピークがいわれるが，新生児突然死に遭遇することもある．また SIDS 乳幼児症例でも新生児期の病歴をみると，一過性多呼吸，一過性低血糖などの病歴を持っていることが少なくない．このような症例では，新生児期に SIDS の前兆を示したと考えられる．

新生児マススクリーニングの新技術としてタンデムマス法が注目されているが，その対象疾患の中には，SIDS や急性脳症，ライ症候群様の発症形態をとる先天代謝異常が含まれている [1] ~ [3]．今後タンデムマスを応用すれば，乳幼児突然死の予防あるいは病態解明に役立つ可能性がある．そこで，先天代謝異常の面から新生児突然死の予防，あるいは遭遇した時の対応について述べたい．

2. 新生児突然死の要因

乳幼児突然死症候群（SIDS）あるいは新生児突然死の原因として，表 1 のような仮説がある．すなわち 1）生物学的因子，2）生理学的要因，3）生化学的因子（一部の先天代謝異常），4）体質因子（単一遺伝子のみならず多因子遺伝），および 5）社会的環境因子（うつぶせ寝，母親の喫煙）などである．

3. 乳幼児突然死（SIDS）または 新生児突然死を起こす代表的な先天代謝異常

SIDS 様症状で発症する先天代謝異常の代表的な疾患として，表 2 のように脂肪酸代謝異常，有機酸代謝異常，アミノ酸代謝異常，糖新生系異常症などがある [4] [5]．このうち脂肪酸代謝異常，有機酸代謝異常は，微量の血液をタンデムマスデ分析することによって，比較的簡単に診断でき，注目されている．

1）脂肪酸代謝異常症

ミトコンドリア脂肪酸 β 酸化系の代謝障害である．この代謝系に異常があると，長時間空腹状態が続いたり，感染症，下痢などで急激にエネルギー需要の高まった時，急激に代謝不全状態におちいり，急性脳症，突然死などの経過をとる．

2）有機酸代謝異常症

アミノ酸の中間代謝過程にはたらく酵素の異常によって，中間代謝体である有機酸が体内に上昇して，ケトアシドーシス，高アンモニア，低血糖などをきたす．

3）アミノ酸代謝異常

尿素回路異常症，メープルシロップ尿症などで急性発作が起こることがある．

4）糖新生系異常症：

食後一定時間後に食事由来の炭水化物からのエネルギーが枯渇すると，肝臓や筋肉に蓄えていたグリコーゲンや，乳酸，アミノ酸からブドウ糖を産生して血糖を維持する代謝経路が働く．この経路の代表的な代謝疾患として，糖原病，糖新生異常症がある．

5）その他

内分泌疾患で急性の電解質異常や，低血糖をきたす高インスリン血症などがある．

表 1　新生児突然死を起こす要因として考えられること

分類	例
1）生物学的因子	未熟性 潜在的感染症，エンドトキシなど
2）電気生理学的因子	不整脈（QT 延長症候群など） 脳幹自律神経機能異常
3）生化学的因子	先天代謝異常（脂肪酸代謝異常など）
4）体質的素因	同胞，家族歴に SIDS など
5）環境社会的因子	うつぶせ 母親の喫煙など

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