ORIGINAL ARTICLE

Single-nucleotide substitution T to A in the polypyrimidine stretch at the splice acceptor site of intron 9 causes exon 10 skipping in the *ACAT1* gene

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Abstract

Background

β-ketothiolase (T2, gene symbol *ACAT1*) deficiency is an autosomal recessive disorder, affecting isoleucine and ketone body metabolism. We encountered a patient (GK03) with T2 deficiency whose T2 mRNA level was <10% of the control, but in whom a previous routine cDNA analysis had failed to find any mutations. Genomic PCR-direct sequencing showed homozygosity for c.941-9T>A in the polypyrimidine stretch at the splice acceptor site of intron 9 of *ACAT1*. Initially, we regarded this variant as not being disease-causing by a method of predicting the effect of splicing using in silico tools. However, based on other findings of exon 10 splicing, we eventually hypothesized that this mutation causes exon 10 skipping.

Methods

cDNA analysis was performed using GK03's fibroblasts treated with/without cycloheximide (CHX), since exon 10 skipping caused a frameshift and nonsense-mediated mRNA decay (NMD). Minigene splicing experiment was done to confirm aberrant splicing.

Results

cDNA analysis using fibroblasts cultured with cycloheximide indeed showed the occurrence of exon 10 skipping. A minigene splicing experiment clearly showed that the c.941-9T>A mutant resulted in transcripts with exon 10 skipping. There are few reports describing that single-nucleotide substitutions in polypyrimidine stretches of splice acceptor sites cause aberrant splicing.

Conclusion

We showed that c.941-9T>A induces aberrant splicing in the *ACAT1* gene. Our ability to predict the effects of mutations on splicing using in silico tools is still limited. cDNA analysis and minigene splicing experiments remain useful alternatives to reveal splice defects.

Introduction

β-Ketothiolase deficiency (OMIM #203750, *607809), a defect of mitochondrial acetoacetyl-CoA thiolase (T2, EC 2.3.1.9, gene symbol *ACAT1*, RefSeq NM_000019.3), is an autosomal recessive disorder, affecting isoleucine and ketone body metabolism (Fukao et al. 2014). Since 1971 (Daum et al. 1971), more than 100 T2-deficient patients

have been identified (Mitchell and Fukao 2001). 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 (Fukao et al. 1990). *ACAT1* spans approximately 27 kb and contains 12 exons (Kano et al. 1991). We have identified more than 70 gene mutations, 15% of which cause aberrant splicing (Fukao et al. 2010). Most are located at the highly conserved dinucleotide "ag" at the splice acceptor site and "gt" at the splice donor site. We also identified some exonic mutations that cause aberrant splicing by activating cryptic splice sites within their exons (Nakamura et al. 2001; Fukao et al. 2008) or by altering the consensus sequences of exonic splice enhancer sites (Fukao et al. 2010).

It is still a challenge to predict whether single-nucleotide substitutions in polypyrimidine stretches of splice acceptor sites cause disease. We herein describe that a c.941-9T>A mutation in the polypyrimidine stretch at the splice acceptor site of intron 9 of *ACAT1* induces exon 10 skipping and causes β-ketothiolase deficiency.

Materials and Methods

Case presentations

GK03

A case report on GK03 was previously published (Middleton et al. 1986). Briefly, he was a Laotian male infant. When he was 10 months old, he was admitted to a medical center because of vomiting, diarrhea, and acidosis. Three of the patient's siblings had died of unknown causes at <6 months of age. The results of a neurological examination were grossly normal. Laboratory findings showed severe ketoacidosis; the arterial blood pH was 7.16 and the serum concentration of bicarbonate was 6 mEq/L. The urine test for ketones was strongly positive. The patient was treated with intravenous fluids and bicarbonate, and the acidosis resolved in 24 h. Urinary organic acid analysis revealed increased excretion of 3-hydroxybutyrate and 2M3HB, but not tiglylglycine. Potassium ion-activated acetoacetyl-CoA thiolase activity was reduced in the fibroblasts of this patient; the +K$^+$/−K$^+$ ratio was reported to be 1.1. Northern blot analysis showed a decreased amount (<10% of that in the control) of T2 mRNA (Fukao et al. 1990) and routine cDNA analysis did not reveal any mutations at that time. The molecular basis of T2 deficiency in this patient was not resolved. Growth and development were normal.

GK124

GK124 was a girl born to American parents with no significant family history. She had previously been well with

normal development and health. At 5 years and 11 months of age, she had a 4-day history of fever, lethargy, and decreased appetite. She had also experienced one episode of emesis. The night prior to admission, her parents had noted that she was breathing quite heavily and had a reduced level of consciousness. She was also described as being delusional. When she was admitted to hospital, she had extreme acidosis. Capillary blood gas revealed pH of 7.058, bicarbonate 3.4 mmol/L, and base excess −26 mmol/L; her lactate was 2.4 mmol/L, glucose was 4.8 mmol/L, sodium 138 mmol/L, potassium 4.1 mmol/L, and chloride 110 mmol/L. The anion gap was 21 mmol/L. Initial urine had 3+ ketones. The acylcarnitine profile was normal and plasma carnitine levels were slightly low (these were collected 3 days after admission). Initial urine organic acids collected on the day of admission showed large amounts of lactic acid, 3-hydroxybutyrate, and acetoacetate. Urine organic acid analyses were repeated several times. On several occasions, 2M3HB was present along with 3-hydroxybutyrate and acetoacetate.

She was treated with intravenous fluid including glucose and bicarbonate. Her blood gas normalized in 48 h. Cranial CT scan was normal. She was discharged after 10 days. After discharge, it was noted that her speech was slightly slurred and her gait was abnormal. Cranial magnetic resonance imaging (MRI) revealed bilateral small ovoid areas with an increased T2 signal within the basal ganglia at the junction of the posterior putamen and globus pallidus.

Although metabolic investigations were not suggestive of any specific metabolic condition, a diagnosis of T2 deficiency was suspected because of severe metabolic acidosis with a sequela of a basal ganglia lesion. Acetoacetyl-CoA thiolase activity in fibroblasts of 12 nmol/min/mg protein was within the normal range (8.9–20.6 nmol/min/mg protein), but the enzyme was not stimulated by the addition of potassium to the reaction. Mutation analysis of *ACAT1* reported no disease-associated mutations in exons 1–12. The results of targeted array CGH with exon-level resolution were normal (both mutation analysis and array CGH were performed at GeneDx).

Although metabolic, enzymatic, and molecular studies did not confirm a diagnosis of deficiency, the suspicion of this diagnosis led to repeat molecular testing. This testing was performed at the Baylor College of Medicine, 3 years after the first molecular testing. The results revealed homozygosity for c.941-9T>A in intron 9.

The corresponding author was consulted about the pathological meaning of c.941-9T>A. At the time of writing, this patient is 11 years of age and is doing well academically. However, she continues to have an abnormal gait with dystonic posturing of the left leg. Her most

recent MRI performed at 11 years of age showed stable bilateral basal ganglion changes. She has not suffered any further episodes of acidosis.

Ethical consideration

This study was approved by the Ethics Committee of the Graduate School of Medicine, Gifu University, Gifu, Japan, and was carried out in accordance with the principles of the Declaration of Helsinki.

Fibroblasts and enzyme assay

Fibroblasts from GK03 and controls were cultured in Eagle's minimal essential medium containing 10% fetal calf serum. Acetoacetyl-CoA thiolase enzyme assay was performed as described previously (Williamson et al. 1971) with modifications (Fukao et al. 1996). We calculated the mean values and standard errors of acetoacetyl-CoA thiolase activity, in the absence and presence of potassium ions, of three independent experiments.

Immunoblot analysis

Immunoblot analysis was performed using fibroblasts' protein extract, as described previously (Fukao et al. 1997), with a mixture of an anti-human T2 and anti-SCOT (succinyl-CoA:3-ketoacid CoA transferase) antiserum used as the first antibody.

Mutation screening at the genomic level

Genomic DNA was purified using Sepa Gene kits (EIDIA, Tokyo, Japan) from GK03's fibroblasts. Mutation analysis was performed by PCR amplification of each exon and its boundaries (at least 18 bases from the exon/intron boundaries in both directions) with a pair of intronic primers described previously (Fukao et al. 1998), followed by direct sequencing. The genomic sequence was obtained from RefSeq (accession number NG_009888.1/NM_000019.3).

Nonsense-mediated mRNA decay (NMD) inhibition

To determine whether any observed reduction of the transcript was due to NMD (Maquat 2005), we inhibited NMD in fibroblasts using CHX (Sigma, St. Louis, MO, USA), a general protein translation inhibitor. Fibroblasts were left untreated or cultured in the presence of 200 μg/mL CHX for 7 h before RNA extraction. This analysis was performed as described previously (Hernan et al. 2011).

cDNA analysis

Total RNA was isolated from fibroblasts using ISOGEN kits (Nippon Gene, Tokyo, Japan). Total RNA (5 μg) was reverse-transcribed in 20 μL of 50 mM Tris–HCl pH 7.5, 75 mM KCl, 3 mM MgCl$_2$, 10 mM dithiothreitol, 0.5 mM dNTPs, and 200 U M-MLV reverse transcriptase (Life Technologies, Rockville, MD, USA) with a primer mixture including 5 pmol of each of T2-specific antisense primer (T2 135 5'-$^{c.1360}$TGACCCACAGTAGTCACAC-3'), *GAPDH*-specific antisense primer (*GAPDH*3 5'-$^{c.1040}$GTG CTCTTGCTGGGGCTG-3'), and oligo dT primers. The above preparation was incubated at 37°C for 1 h. One microliter of this cDNA solution served as a PCR template. Sequencing was performed after the full coding sequence of T2 cDNA had been amplified (c. 1–1284).

For cDNA analysis with/without CHX treatment, the cDNA fragment c.732–c.1142 was amplified using the following primer set:

Primer T2 Ex8 (sense) 5'-$^{c.732}$TCAACCAGATGTAGTG GT-3'.

Primer T2 114 (antisense) 5'-$^{c.1142}$ACAGCTCCTCCAT TGATATTC-3'.

To evaluate the total amount of mRNA products, *GAPDH* cDNA was also amplified with the following primer set:

*GAPDH*1 (sense) 5'-$^{c.4}$GGGAAGGTGAAGGTC-3'.

*GAPDH*2 (antisense) 5'-$^{c.1015}$AGGGGTCTTACTCCTTG GAG-3'.

These cDNA sequences were obtained from RefSeq (accession number NM_002046.4 for *GAPDH*, and NM_000019.3 for T2). After 30 PCR cycles, amplified fragments were separated by electrophoresis on a 5% (w/v) polyacrylamide gel and a 1% (w/v) agarose gel, and extracted using a Geneclean II kit (BIO 101, Vista, CA, USA). Sequences of the amplified fragments were confirmed by direct sequencing.

Quantitative real-time PCR

Quantitative real-time PCR was performed using a Light-Cycler FastStart DNA Master CYBR Green I Kit (Roche, Mannheim, Germany). The amplification reaction was performed using a LightCycler 1.5 (Roche). Specific primers were designed for the target gene using the Primer 3 software program (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). To amplify wild-type T2 cDNA, primers for exon 9 and exon 10 were used. *GAPDH* cDNA was amplified as an internal control.

ACAT1-9L (sense) 5'-$^{c.814}$CAGAAAGAAAATGGCACA GTAACAG-3'.

ACAT1-10R (antisense) 5'-$^{c.976}$GAAAATCAATAGGTTC TACAGCAGC-3'.

GAPDH L (sense) 5'-$^{c.930}$CATTTCCTGGTATGACAACG-3'.

GAPDH R (antisense) 5′-^{c.1047}TCCTCTTGTGCTCTTG
CTGG-3′.

The amplification reaction for the SYBR Green I assays
contained 1× SYBR Green I PCR Mastermix and 250 nM
of each primer. The PCR conditions were 95°C for
10 min, followed by 50 cycles of 95°C for 10 sec, 63°C
for 15 sec, and 72°C for 1 min. Melting curves were gen-
erated after amplification. Ct values of T2 and *GAPDH*
cDNAs for each sample were determined by the second
derivative maximum method. Then, the amount of T2
cDNA of GK03 fibroblasts relative to that of control
fibroblasts was calculated with adjustment using the rela-
tive *GAPDH* cDNA amount.

Minigene splicing experiment

A wild-type exon 10 minigene splicing construct was pre-
viously established using a pCAGGS eukaryote expression
vector (Fukao et al. 2013). To make a mutant construct,
in vitro mutagenesis was performed using the Quik-
Change Site-Directed Mutagenesis Kit (Stratagene, La
Jolla, CA, USA). Two micrograms of these expression vec-
tors were transfected into 5×10^5 SV40-transformed
fibroblasts using Lipofectamine 2000 (Invitrogen, Carls-
bad, CA, USA). At 48 h after transfection, RNA was
extracted from the cells. The first-strand cDNA was tran-
scribed with a rabbit β-globin-specific antisense primer
(β-glo2) (5′-^{c.461}AGCCACCACCTTCTGATA-3′) and then
amplified with the Ex10 (*Eco*RI) primer on T2 exon 10,
and another rabbit-specific antisense primer (β-glo3)
(5′-^{c.443}GGCAGCCTGCACCTGAGGAGT-3′) to amplify
the chimeric cDNA of human T2 and rabbit β-globin. A
study of normal and aberrant splicing constructs was per-
formed by sequencing and 5% polyacrylamide gel elec-
trophoresis of the PCR-amplified cDNA fragments.

Results and Discussion

We investigated the molecular basis of T2 deficiency in a
patient, GK03, whose fibroblasts had a reduced T2 mRNA
level of <10% that of controls by Northern blot analysis
(Fukao et al. 1990). Previous routine cDNA analysis had
failed to find any mutations. In immunoblot analysis
(Fig. 1), no T2 protein was detected in GK03's fibroblasts
when 30 μg of protein extract was applied. In our system,
the detection limit of T2 protein was at 3.75 μg of pro-
tein extract in twofold serial dilution samples of control
fibroblasts. Hence, the level of T2 protein in GK03 fibrob-
lasts was estimated to be less than one-eighth the control
level. Genomic PCR and direct sequencing identified a
homozygous substitution of c.941-9T>A with no other
mutations. This intronic variant resulted in a slight
decrease in the Senapathy and Shapiro score (S&S score)

from 90.48 to 85.17. Therefore, we initially regarded it as
a non-disease-causing substitution. We hypothesized that
another mutation in GK03 produced a reduced amount
of wild-type T2 mRNA. However, we failed to discover
any mutations in the promoter region or the 3′ noncod-
ing regions including the polyadenylation site that could
affect mRNA stability.

We recently identified another T2-deficient patient
(GK124) who had the same c.941-9T>A substitution
with no other mutations. We also recently showed, using
minigene splicing experiments, that mutations (c.949G>A,
c.951C>T) within the exonic splicing enhancer sequence
(ESE, SF2/ASF) within exon 10 resulted in exon 10 skip-
ping (Fukao et al. 2010; Otsuka et al. 2016). Moreover,
such effects of ESE mutations were canceled by a substi-
tution from C to G at the first nucleotide of exon 10.
This implies that the intron 9 splice acceptor site is weak
and the ESE within exon 10 is necessary for accurate
splicing, although the S&S score of this acceptor site is
high. Consequently, even if the c.941-9T>A variant results
in a subtle change in the S&S score, we hypothesized that
this variant also caused exon 10 skipping.

First, we performed cDNA analysis again using GK03's
fibroblasts treated with/without cycloheximide (CHX),
since exon 10 skipping caused a frameshift and nonsense-
mediated mRNA decay (NMD). As shown in Fig. 2,
without CHX, cDNA with the normal length was pre-
dominant. A faint band with a shorter length was also
visible in polyacrylamide gel electrophoresis, but it was
hardly detectable in agarose gel electrophoresis. This
explains why we only detected the wild-type cDNA
sequence in the previous study. Real-time PCR showed
that the wild-type T2 mRNA level decreased to 8%
(±1%) of that in the control (data not shown); this value
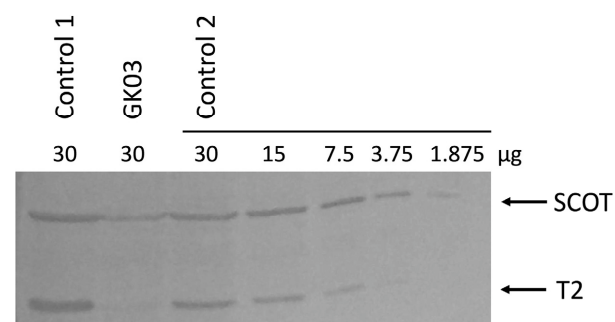was compatible with the findings of a previous Northern

Figure 1. Immunoblot analysis. The amount of fibroblast protein
extract applied is indicated in each lane. T2 protein was obtained
from GK03 fibroblasts and two healthy control fibroblasts. The first
antibody was a mixture of an anti-T2 antibody and an anti-SCOT
(succinyl-CoA: 3-ketoacid CoA transferase) antibody. The positions of
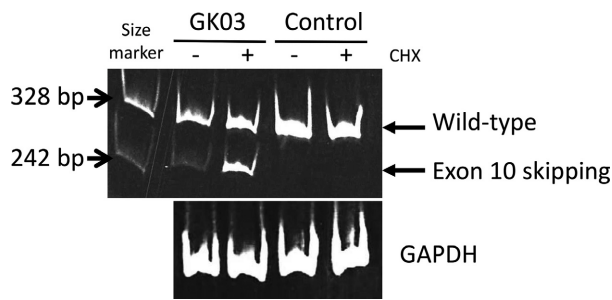the bands for T2 and SCOT are indicated by arrows.

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Figure 2. cDNA analysis using CHX-treated fibroblasts. Five percent polyacrylamide gel electrophoresis of amplified T2 cDNA fragments using RNAs extracted from cycloheximide (CHX)-treated and -untreated fibroblasts from the patient and a control. Bands corresponding to normal transcripts and those with exon 10 skipping are depicted by arrows.

blot analysis. In contrast, upon treatment with CHX, cDNA with a shorter length due to exon 10 skipping was clearly identified in GK03's fibroblasts. This indicates that transcripts with exon 10 skipping undergo NMD. The band with exon 10 skipping was not identified in control

fibroblasts, even with CHX treatment. Accordingly, we confirmed that exon 10 skipping had occurred in GK03's fibroblasts.

Next, we examined whether c.941-9T>A can induce exon 10 skipping using a minigene splicing experiment. For this splicing experiment, we used a previously established wild-type minigene construct including the following components: exon 9–truncated intron 9–exon 10–truncated intron 10–exon 11 (Fukao et al. 2010) (Fig. 3A). We created the c.941-9T>A mutant construct using in vitro mutagenesis. Since our minigene construct produces human T2-rabbit β-globin fusion mRNA, we could amplify the minigene-specific mRNA by RT-PCR using a combination of a human T2 sense primer and a rabbit β-globin antisense primer. As shown in Fig. 3B, exon 10 skipping was induced in the case of the c.941-9T>A mutant construct; normally spliced transcripts with the inclusion of exon 10 were also produced. In contrast, the wild-type construct had no band corresponding to exon 10 skipping.

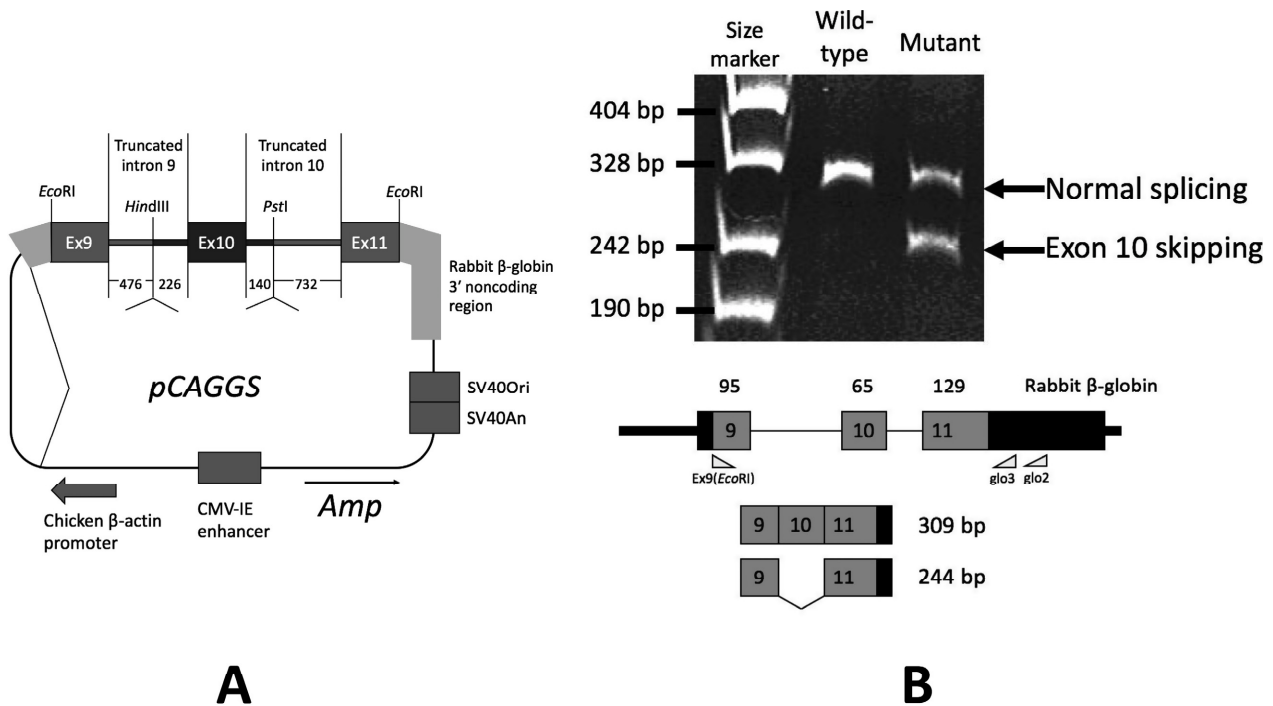The above findings explain the molecular basis by which c.941-9T>A mutation causes exon 10 skipping.

A

B

Figure 3. Minigene splicing experiment. (A) Schematic presentation of minigene splicing construct. pCAGGS expression vector was used for this construct. The minigene construct had a T2 gene fragment from c.842 of exon 9 and intron 9 (from +1 to a *Hin*dIII site, 476 bp) and intron 10 (from a *Pst*I site to −1, 732 bp), and exon 11 (to c. 1122). In the cases of mutant constructs, the region around exon 10, highlighted in black, was replaced as a cassette. (B) Minigene splicing experiment. Detection of chimeric cDNAs derived from transfected minigenes. First-strand cDNA was reverse-transcribed using the glo2 primer. cDNA amplification was performed using Ex9 (*Eco*RI) and glo3 primers. Those fragments were electrophoresed on 5% polyacrylamide gel. Normal splicing and exon 10 skipping produced 309-bp and 244-bp PCR fragments, respectively.

Table 1. Prediction of splicing effects by in silico tools.

Gene	OMIM*	RefSeq	Mutation	Intron	Reference sequence	Mutant sequence	3′ Senapathy and Shapiro score Reference	3′ Senapathy and Shapiro score Mutant	Prediction of Human Splicing Finder (HSF) HSF matrices (acceptor site)	Prediction of Human Splicing Finder (HSF) MaxEnt (3′ motif)	Reported effects	Ref. No.
ACAT1	**607809**	**NM_000019.3**	**c.941-9T>A**	**9**	cttttttaa acag/C	ctttt**a**ttaa acag/C	**90.48**	**85.17**	**Not significant**	**Probably no impact on splicing**	**Exon 10 skipping**	**Present case**
SLC25A20	613698	NM_000387.5	c.199-10T>G	2	gtgattcct tgcag/G	gtga**g**tcct tgcag/G	92.50	86.88	New acceptor site	Broken WT acceptor site	Exon 3 skipping or exon 3 and 4 skipping	Ogawa et al. (2000)
LMNA	150330	NM_170707.3	c.937-11C>G	5	ccacccccc ttcag/C	cca**g**ccccc ttcag/C	83.29	82.67	New acceptor site	New acceptor site or broken WT acceptor site	40-bp insertion	Carboni et al. (2011)
AGL	610860	NM_000642.2	c.2682-8A>G	20	ctctgaatt ttcag/G	ctctga**g**ttt tcag/G	88.59	88.59	New acceptor site	Broken WT acceptor site	7-bp insertion	Ben Rhouma et al. (2013)
FANCA	607139	NM_000135.2	c.710-5T>C	7	ttatggttt ttcag/A	ttatggttt **c**tcag/A	89.29	88.35	Not significant	Probably no impact on splicing	Exon 8 skipping	Mattioli et al. (2014)
HBB	141900	NM_000518.4	c.316-12T>C	2	tcttcctcc cacag/C	tc**t**tcctcc cacag/C	89.07	86.57	Not significant	Probably no impact on splicing	Could reduce splicing efficiency	Waye et al. (2013)
HBB	141900	NM_000518.4	c.316-7C>A	2	tcttcctcc cacag/C	tcttcct**a**c cacag/C	89.07	88.14	Not significant	Probably no impact on splicing	Could reduce splicing efficiency	Waye et al. (2013)
F9(FIX)	300746	NM_000133.3	c.392-8T>G	4	tttgcttctt ttag/A	tttgct**g**ct tttag/A	85.96	84.86	Not significant	Probably no impact on splicing	Exon 5 skipping	Montejo et al. (1999)
F9(FIX)	300746	NM_000133.3	c.392-9T>G	4	tttgcttct tttag/A	tttgc**g**tc ttttag/A	85.96	83.46	Not significant	Probably no impact on splicing	Exon 5 skipping	Lewandowska (2013)

In the mutant sequence column, mutated nucleotides are shown in bold type and underlined. WT, wild type.

c.941-9T>A mutation at the polypyrimidine stretch of the splice acceptor site of intron 9 results in insufficient exon 10 recognition. This mutation causes exon 10 skipping in most, but not all, transcripts. The c.941-9T>A mutation effect on splice site selection seems to be incomplete. Transcripts with exon 10 skipping are rapidly degraded by NMD. Hence, under steady-state conditions, only normal T2 mRNA can be detected at a level <10% of that in controls. Therefore, individuals homozygous for this mutation, GK03 and GK124, had <10% activity from such residual normal T2 mRNA.

A splice acceptor site consists of two parts. One is the proximal region from −3 to +1 with the consensus sequence cag/G. The other distal region is the polypyrimidine stretch from −14 to −5. As it is usually possible for some purine residues to be present in this region, it is difficult to determine whether a nucleotide substitution in this region is a disease-causing mutation or functionally neutral. This is one of the reasons why mutations located in polypyrimidine stretches have rarely been reported (Lewandowska 2013). In carnitine acylcarnitine translocase deficiency, c.199-10T>G in *SLC25A20* is a common mutation, resulting in the skipping of exons 3 and 4 or exon 3 alone (Ogawa et al. 2000; Vatanavicharn et al. 2015). This mutation changed the S&S score from 92.50 (gtgattccttgcag/G) to 86.88 (gtgagtccttgcag/G). It has been reported that ag dinucleotides in polypyrimidine stretches are very rare. There are also few reports describing that single-nucleotide substitutions in polypyrimidine stretches of splice acceptor sites were proved to cause aberrant splicing (Montejo et al. 1999; Ogawa et al. 2000; Carboni et al. 2011; Ben Rhouma et al. 2013; Lewandowska 2013; Waye et al. 2013; Mattioli et al. 2014). We thus examined whether in silico tools can predict splice aberration caused by these polypyrimidine stretch mutations (Table 1). We used Human Splicing Finder (HSF) (http://www.umd.be/HSF3/HSF.html), which combines 12 different algorithms to identify and predict the effects of mutations on splicing signals (Desmet et al. 2009). Among them, in some cases, the prediction of splicing effects was successful, but it was not in others. HSF failed to predict exon 10 skipping in the present case.

We showed that c.941-9T>A induces aberrant splicing in the *ACAT1* gene. In silico tools still have a limited ability to predict splicing abnormalities. cDNA analysis and minigene splicing experiments remain useful alternatives to reveal splice defects.

Acknowledgments

Conflict of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

References

Ben Rhouma, F., H. Azzouz, F. M. Petit, M. B. Khelifa, A. B. Chehida, F. Nasrallah, et al. 2013. Molecular and biochemical characterization of a novel intronic single point mutation in a Tunisian family with glycogen storage disease type III. Mol. Biol. Rep. 40:4197–4202.

Carboni, N., M. Floris, A. Mateddu, M. Porcu, G. Marrosu, E. Solla, et al. 2011. Aberrant splicing in the LMNA gene caused by a novel mutation on the polypyrimidine tract of intron 5. Muscle Nerve 43:688–693.

Daum, R. S., P. H. Lamm, O. A. Mamer, and C. R. Scriver. 1971. A "new" disorder of isoleucine catabolism. Lancet 2:1289–1290.

Desmet, F. O., D. Hamroun, M. Lalande, G. Collod-Beroud, M. Claustres, and C. Beroud. 2009. Human Splicing Finder: an online bioinformatics tool to predict splicing signals. Nucleic Acids Res. 37:e67.

Fukao, T., S. Yamaguchi, M. Kano, T. Orii, Y. Fujiki, T. Osumi, et al. 1990. Molecular cloning and sequence of the complementary DNA encoding human mitochondrial acetoacetyl-coenzyme A thiolase and study of the variant enzymes in cultured fibroblasts from patients with 3-ketothiolase deficiency. J. Clin. Invest. 86:2086–2092.

Fukao, T., X. Q. Song, S. Yamaguchi, T. Hashimoto, T. Orii, and N. Kondo. 1996. Immunotitration analysis of cytosolic acetoacetyl-coenzyme A thiolase activity in human fibroblasts. Pediatr. Res. 39:1055–1058.

Fukao, T., X. Q. Song, G. A. Mitchell, S. Yamaguchi, K. Sukegawa, T. Orii, et al. 1997. Enzymes of ketone body utilization in human tissues: protein and messenger RNA levels of succinyl-coenzyme A (CoA):3-ketoacid CoA transferase and mitochondrial and cytosolic acetoacetyl-CoA thiolases. Pediatr. Res. 42:498–502.

Fukao, T., H. Nakamura, X. Q. Song, K. Nakamura, K. E. Orii, Y. Kohno, et al. 1998. Characterization of N93S, I312T, and A333P missense mutations in two Japanese families with mitochondrial acetoacetyl-CoA thiolase deficiency. Hum. Mutat. 12:245–254.

Fukao, T., A. Boneh, Y. Aoki, and N. Kondo. 2008. A novel single-base substitution (c.1124A>G) that activates a 5-base upstream cryptic splice donor site within exon 11 in the human mitochondrial acetoacetyl-CoA thiolase gene. Mol. Genet. Metab. 94:417–421.

Fukao, T., R. Horikawa, Y. Naiki, T. Tanaka, M. Takayanagi, S. Yamaguchi, et al. 2010. 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. 100:339–344.

Fukao, T., Y. Aoyama, K. Murase, T. Hori, R. K. Harijan, R. K. Wierenga, et al. 2013. Development of MLPA for human ACAT1 gene and identification of a heterozygous Alu-mediated deletion of exons 3 and 4 in a patient with mitochondrial acetoacetyl-CoA thiolase (T2) deficiency. Mol. Genet. Metab. 110:184–187.

Fukao, T., G. Mitchell, J. O. Sass, T. Hori, K. Orii, and Y. Aoyama. 2014. Ketone body metabolism and its defects. J. Inherit. Metab. Dis. 37:541–551.

Hernan, I., M. J. Gamundi, E. Planas, E. Borras, M. Maseras, and M. Carballo. 2011. Cellular expression and siRNA-mediated interference of rhodopsin cis-acting splicing mutants associated with autosomal dominant retinitis pigmentosa. Invest. Ophthalmol. Vis. Sci. 52:3723–3729.

Kano, M., T. Fukao, S. Yamaguchi, T. Orii, T. Osumi, and T. Hashimoto. 1991. Structure and expression of the human mitochondrial acetoacetyl-CoA thiolase-encoding gene. Gene 109:285–290.

Lewandowska, M. A. 2013. The missing puzzle piece: splicing mutations. Int. J. Clin. Exp. Pathol. 6:2675–2682.

Maquat, L. E. 2005. Nonsense-mediated mRNA decay in mammals. J. Cell Sci. 118:1773–1776.

Mattioli, C., G. Pianigiani, D. De Rocco, A. M. Bianco, E. Cappelli, A. Savoia, et al. 2014. Unusual splice site mutations disrupt FANCA exon 8 definition. Biochim. Biophys. Acta 1842:1052–1058.

Middleton, B., K. Bartlett, A. Romanos, J. Gomez Vazquez, C. Conde, R. A. Cannon, et al. 1986. 3-Ketothiolase deficiency. Eur. J. Pediatr. 144:586–589.

Mitchell, G. A., and T. Fukao. 2001. Chapter 102. Inborn errors of ketone body catabolism. Pp. 2327–2356 in C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle, eds. Metabolic and molecular bases of inherited disease. 8th ed. McGraw–Hill, New York.

Montejo, J. M., M. Magallon, E. Tizzano, and J. Solera. 1999. Identification of twenty-one new mutations in the factor IX gene by SSCP analysis. Hum. Mutat. 13:160–165.

Nakamura, K., T. Fukao, C. Perez-Cerda, C. Luque, X. Q. Song, Y. Naiki, et al. 2001. A novel single-base substitution (380C>T) that activates a 5-base downstream cryptic splice-acceptor site within exon 5 in almost all transcripts in the human mitochondrial acetoacetyl-CoA thiolase gene. Mol. Genet. Metab. 72:115–121.

Ogawa, A., S. Yamamoto, M. Kanazawa, M. Takayanagi, S. Hasegawa, and Y. Kohno. 2000. Identification of two novel mutations of the carnitine/acylcarnitine translocase (CACT) gene in a patient with CACT deficiency. J. Hum. Genet. 45:52–55.

Otsuka, H., H. Sasai, Y. Aoyama, A. Elsayed, K. Vassiliki, L. Martin, et al. 2016. Exon 10 skipping caused by a novel mutation (c.949G>A) located in an exonic splice enhancer site in ACAT1 gene. Mol. Med. Rep. 14:4906–4910.

Vatanavicharn, N., K. Yamada, Y. Aoyama, T. Fukao, N. Densupsoontorn, P. Jirapinyo, et al. 2015. Carnitine-acylcarnitine translocase deficiency: two neonatal cases with common splicing mutation and in vitro bezafibrate response. Br. Dev. 37:698–703.

Waye, J. S., B. Eng, L. Hellens, B. A. Hohenadel, L. M. Nakamura, and L. Walker. 2013. Mild beta(+)-thalassemia associated with two linked sequence variants: IVS-II-839 (T>C) and IVS-II-844 (C>A). Hemoglobin 37:378–386.

Williamson, D. H., M. W. Bates, M. A. Page, and H. A. Krebs. 1971. Activities of enzymes involved in acetoacetate utilization in adult mammalian tissues. Biochem. J. 121:41–47.