Exon 10 skipping in *ACAT1* caused by a novel c.949G>A mutation located at an exonic splice enhancer site

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Abstract. Beta-ketothiolase deficiency, also known as mitochondrial acetoacetyl-CoA thiolase (T2) deficiency, is an autosomal recessive disease caused by mutations in the acetyl-CoA acetyltransferase 1 (*ACAT1*) gene. A German T2-deficient patient that developed a severe ketoacidotic episode at the age of 11 months, was revealed to be a compound heterozygote of a previously reported null mutation, c.472A>G (p.N158D) and a novel mutation, c.949G>A (p.D317N), in *ACAT1*. The c.949G>A mutation was suspected to cause aberrant splicing as it is located within an exonic splicing enhancer sequence (c. ^{947}CTGACGC) that is a potential binding site for serine/arginine-rich splicing factor 1. A mutation in this sequence, c.951C>T, results in exon 10 skipping. A minigene construct was synthesized that included exon 9-truncated intron 9-exon 10-truncated intron 10-exon 11, and the splicing of this minigene revealed that the c.949G>A mutant construct caused exon 10 skipping in a proportion of the transcripts. Furthermore, additional substitution of G for C at the first nucleotide of exon 10 (c.941G>C) abolished the effect of the c.949G>A mutation. Transient expression analysis of the c.949G>A mutant cDNA revealed no residual T2 activity in the mutated D317N enzyme. Therefore, c.949G>A (D317N) is a pathogenic missense mutation, and diminishes the effect of an exonic splicing enhancer and causes exon 10 skipping. The present study demonstrates that a missense mutation, or even a synonymous substitution, may disrupt enzyme function by interference with splicing.

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Abbreviations: 2M3HB, 2-methyl-3-hydroxybutyrate; 2M-AcAc, 2-methylacetoacetate; *ACAT1*, acetyl-CoA acetyltransferase 1; ESE, exonic splice enhancer; SR, serine- and arginine-rich; T2, mitochondrial acetoacetyl-CoA thiolase; TIG, tiglylglycine

Key words: acetyl-CoA acetyltransferase 1, thiolase deficiency, beta-ketothiolase deficiency, minigene, exonic splice enhancer, aberrant splicing

Introduction

Beta-ketothiolase deficiency, also termed mitochondrial acetoacetyl-CoA thiolase (T2) deficiency (Online Mendelian Inheritance in Man nos. 607809, 203750), is an inherited autosomal recessive disease caused by mutations in the acetyl-CoA acetyltransferase 1 (*ACAT1*) gene (1-4). T2 deficiency affects ketone body metabolism and isoleucine catabolism. The first description of T2 deficiency was in 1971 (2). Currently, >100 cases have been identified worldwide, with no ethnic predisposition (5).

T2 deficiency typically presents between 6 and 18 months of age with intermittent ketoacidotic episodes; patients are generally asymptomatic between episodes. Urinary organic acid analysis typically reveals increased excretion of 2-methyl-3-hydroxybutyrate (2M3HB), 2-methylacetoacetate (2M-AcAc), and tiglylglycine (TIG). However, certain cases with atypical clinical and/or biochemical presentations have been identified (6). This disorder often has a favorable outcome, when damaging ketoacidotic episodes are avoided (3).

The human *ACAT1* gene (that encodes T2) is located on chromosome 11q22.3-23.1, spans ~27 kb, and comprises 12 exons and 11 introns (7,8). The human T2 cDNA is ~1.5 kb long and encodes a precursor protein of 427 amino acids, including a 33-amino-acid leader polypeptide (9). Numerous different mutations (>50) have been identified in *ACAT1*, 20% of which cause aberrant splicing (10). Although the majority

of mutations that cause aberrant splicing are located at splice acceptor or donor sites (11-15), certain exonic mutations have been identified to activate cryptic splice sites within their exons or alter the consensus sequences of exonic splice enhancer (ESE) sites (16,17).

The present study reports a novel exonic mutation, c.949G>A (nucleotide 9 in exon 10). A minigene splicing experiment revealed that this mutation alters the sequence of an ESE, serine/arginine-rich splicing factor 1 (SF2/ASF) binding site, which results in exon 10 skipping.

Materials and methods

Patient clinical summary. The female patient (GK63) was born to non-consanguineous German parents. She was in good health until 11 months of age when she was admitted to a hospital with vomiting, acidotic breathing and somnolence. No hepatomegaly or cardiomegaly was presented. Laboratory investigations revealed the following: Blood pH 7.09; base excess, -22 mmol/l; blood glucose, 1.5 mmol/l; lactate, 1.98 mmol/l; pyruvate, 0.1 mmol/l; and ketone bodies in the urine. Urinary organic acid analysis at the time of a ketoacidotic episode revealed excess excretion of 2M3HB, 2M-AcAc and TIG. The patient was treated with intravenous glucose, bicarbonate, fluids and electrolytes, and the condition improved within 48 h. Repeated urinary organic acid analysis revealed excess excretion of the metabolites listed above. The management plan included avoiding prolonged fasting, protein restriction to 1.2-1.4 g/kg/day and 200 mg/kg/day L-carnitine supplementation. The patient is currently 8 years old and has not experienced further episodes. She has developed normally and has average intelligence (IQ=99).

Mutation analysis. After parental consent was obtained, mutation analysis was performed as part of the diagnostic work-up of the patient using genomic DNA isolated from cultivated fibroblasts and the SePaGene DNA extraction kit® (Sanko Junyaku Co., Ltd., Tokyo, Japan). Amplification of the 12 *ACAT1* exons, with their intron boundaries, was performed by polymerase chain reaction (PCR) using primer pairs and conditions as previously described (18). The 12 fragments were sequenced using a BigDye® Terminator version 1.1 Cycle Sequencing kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and an ABI PRISM® 3130xl genetic analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.).

ESE identification. ESE finder version 3.0 (rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process) was used to search for ESE sequences.

Minigene splicing experiment. A minigene construct, including a segment of *ACAT1* extending from the middle of exon 9 to the middle of exon 11 (amplified by primers containing an *Eco*RI linker sequence), was engineered using a pCAGGS eukaryote expression vector as previously described (16,17,19,20). This minigene construct produces a human T2-rabbit β-globin fusion mRNA; therefore, reverse transcription-PCR amplification of this specific mRNA was performed using a combination of a human T2 sense primer and a rabbit β-globin antisense primer, as previously described (16,17,19). A KOD-Plus-Mutagenesis kit® (Toyobo Co., Ltd., Osaka, Japan) was used to synthesize mutant constructs: c.949G>A with/without c.941C>G (substitution of G for C at the first nucleotide of exon 10); c.951C>T with/without c.941C>G; and c. 941C>G (Fig. 1) (17).

Wild-type and mutant constructs were transfected using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) into $5x10^5$ SV40-transformed fibroblasts derived from a T2-deficient patient (GK03), which were established by our group previously (9). A previous study demonstrated that, T2 mRNA was markedly decreased and T2 protein expression was virtually undetectable in GK03 fibroblasts (9). RNA was extracted at 48 h post-transfection using an ISOGEN kit® (Nippon Gene Co., Ltd., Tokyo, Japan), according to the manufacturer's instructions. Transcription of the first-strand cDNA was performed using a rabbit β-globin-specific antisense primer (β-glo2) (5'-^{461}AGCCACCACCTTCTGATA-3'), as described previously (12). Amplification was performed with the Ex9 (*Eco*RI) primer on T2 exon 9 (5'-cagctgcgaatt^{842}CCA GTACACTGAATGATGGAGCAGCT^{873}-3', lower case characters indicate linker sequence), and another rabbit-specific antisense primer (β-glo3) (5'-^{443}GGCAGCCTGCACCTG AGGAGT-3'), as described previously (12). Amplified fragments were electrophoresed on a 5% polyacrylamide gel with pUC13/*Hpa*II DNA ladder marker, which was generated in our lab using HpaII (Nippon Gene Co., Ltd.).

Transient expression analysis. Transient expression analysis of the D317N mutant cDNA was performed using the pCAGGS eukaryotic expression vector (Institute for Medical Genetics, Kumamoto University Medical School, Kuhonji, Japan) (21) as described previously (21,22). Following transfection, cells were cultured at 37˚C for 72 h, and then harvested and stored at -80˚C prior to use. The cells were freeze-thawed and sonicated in 50 mM sodium phosphate (pH 8.0) containing 0.1% Triton X-100. Following centrifugation at 10,000 x *g* for 10 min at 4˚C, the supernatant was used in an enzyme assay for acetoacetyl-CoA thiolase activity, as previously described (22). The mean values and standard errors of acetoacetyl-CoA thiolase activity, in the absence or presence of potassium ions, of three independent experiments were calculated.

Results

Mutation screening reveals a novel mutation in the ACAT1 gene. Analysis demonstrated that patient GK63 was a compound heterozygote, with a previously reported null mutation (23), c.472A>G (N158D) and a novel mutation, c.949G>A (D317N) in the *ACAT1* gene, located in exons 6 and 10, respectively. The latter mutation is located at a potential SF2/ASF target ESE sequence, as is the previously identified c.951C>T mutation (18). Genomic mutation screening identified no further mutations.

c.949G>A results in exon 10 skipping. As presented in Fig. 2, in fibroblasts transfected with the minigenes, exon 10 skipping occurred in c.949G>A and c.951C>T mutant transcripts. Normally spliced transcripts with the inclusion of exon 10 were also produced from these mutant constructs. The aberrant splicing was induced to a greater extent in c.949G>A compared with c.951C>T mutant transcripts. The addition

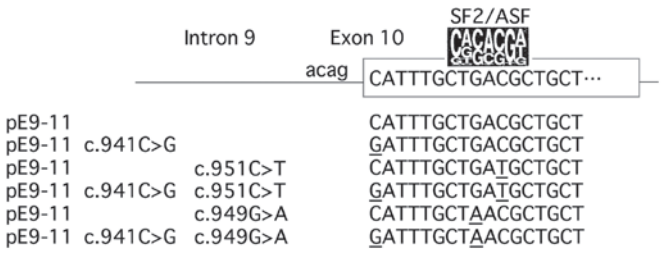

Figure 1. Minigene constructs. Mutants were produced from an *ACAT1* fragment containing exon 10. Five mutant constructs were created: c.949G>A with/without c.941C>G (substitution of G for C at the first nucleotide of exon 10); c.951C>T with/without c.941C>G; and c.941C>G. SV40-transformed fibroblasts were transfected with these constructs. *ACAT1*, acetyl-CoA acetyltransferase 1.

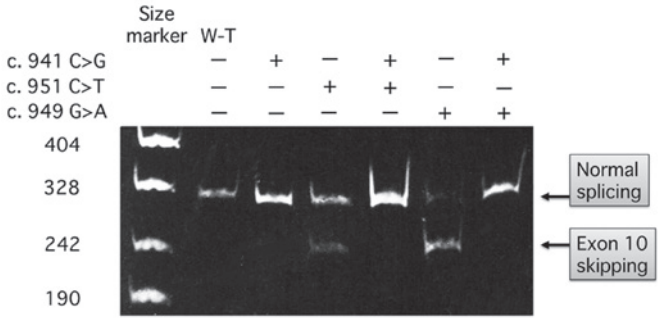

Figure 2. Exon skipping in minigene construct transcripts. Minigene construct transcripts were analyzed by reverse transcription-polymerase chain reaction. The upper bands are 309 bp, which reflect normal splicing (inclusion of exon 10), whereas the lower bands are 244 bp, which reflect aberrant splicing (exon 10 skipping). Exon 10 skipping was induced in c.949G>A and c.951C>T mutant transcripts (to a greater extent in the former compared with the latter). Normally spliced transcripts (including exon 10) were also produced from the mutant constructs. The additional c.941C>G mutation resulted in normal splicing in c.949G>A and c.951C>T constructs. The W-T and c.941C>G mutant constructs produced normal transcripts. W-T, wild-type.

of the c.941C>G mutation resulted in normal splicing from c.949G>A and c.951C>T mutant constructs.

D317N mutant protein does not have acetoacetyl-CoA thiolase activity. c.949G>A resulted in exon 10 skipping in the majority of transcripts, however, normally spliced transcripts were also detected in the minigene splicing experiment. Therefore, it was investigated whether the D371N mutant protein retains residual T2 activity via transient expression analysis of wild-type and mutant cDNAs. Wild-type T2 protein produced high acetoacetyl-CoA thiolase activity in the presence of potassium ions, which represents T2 activity. D317N mutant protein did not retain any potassium-ion-dependent acetoacetyl-CoA thiolase activity (Fig. 3). Based on the minigene splicing experiment and transient expression analysis, c.949G>A was determined to be a null mutation.

Discussion

The present study revealed that patient GK63 was a compound heterozygote for a previously reported c.472A>G (N158D) and a novel c.949G>A (D317N) mutation in the *ACAT1* gene. The latter mutation is a missense mutation and an ESE mutation,

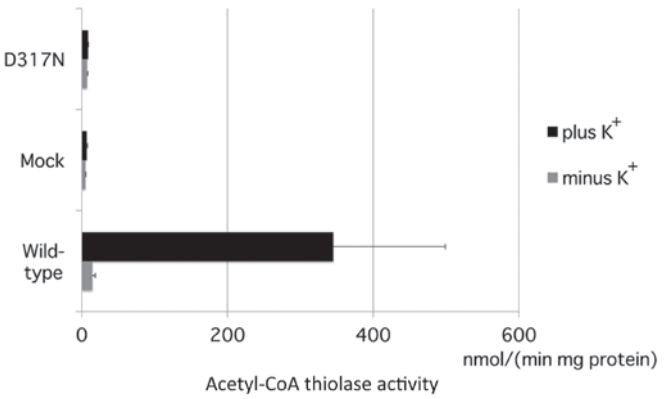

Figure 3. Transient expression analysis of the D317N mutant cDNA by the K^+-ion-activated acetoacetyl-CoA thiolase assay. Data are presented as the mean ± standard error acetoacetyl-CoA thiolase activities, in the absence and presence of K^+ ions, of three independent experiments. Wild-type T2 cDNA produced high acetoacetyl-CoA thiolase activity in the presences of potassium ions. D317N mutant cDNA-transfected cells produced no significant thiolase activity compared with mock control.

which induces exon 10 skipping. This mutation is located at the same codon as the previously reported c.951C>T mutation (D317D) (17).

The accurate removal of introns from pre-mRNAs is essential for functional gene expression. Splice sites, which include the splice donor site, branch site and splice acceptor site, do not contain all the information required for the precise definition of exons (23-26); exonic sequences also contribute. Regulatory elements in exons exist in the form of ESEs (23,24). Exonic variants may inactivate an ESE, resulting in incorrect exon inclusion. SF2/ASF is a prototypical serine- and arginine-rich (SR) family protein and is an important protein for splicing and mRNA metabolism. When bound to exonic sequences SR proteins mediate recognition of the neighboring splice site (27).

Our previous study identified that the c.951C>T mutation in *ACAT1* caused exon 10 skipping (18). It was demonstrated that c.951C>T is located within an ESE sequence for SF2/ASF (c. ^{947}CTGACGC; from the nucleotide 7-13 of exon 10). A minigene splicing experiment demonstrated that c.951C>T results in aberrant splicing. Thus, c.951C>T, despite being a synonymous substitution (D317D), was revealed to be a pathogenic mutation. In addition, it was demonstrated that two additional nucleotide substitutions located within the same ESE sequence, c.952G>A and c.947C>T, caused exon 10 skipping in some transcripts (18). The novel mutation (c.949G>A) is located within the same ESE sequence for SF2/ASF (c. ^{947}CTGACGC); therefore, c.949G>A may affect splicing in a similar manner to c.951C>T. In the absence of this information, molecular analysis of DNA from patient GK63 may only consider c.949G>A to be a missense mutation (D317N).

ESEs are more common in exons with weak splice sites (28). G is the preferred first nucleotide of an exon; however, *ACAT1* exon 10 starts with C (c.941C). Although the Shapiro and Senapathy score (26) of the splice acceptor site of intron 9 has a high score of 90.5, changing the first nucleotide of exon 10 from C to G (c.941C>G) further increases the score to 96.3 (26). It should be stressed that the additional c.941C>G substitution abolished the aberrant splicing and exon 10 skipping induced by all the ESE mutations in the present study (c.949G>A and

c.951C>T) and in our previous study (c.951C>T, c.952G>A and c.947C>T) (18). This may indicate that the effect of the ESE on splicing is weaker than the effect of G at position 941, the preferable first nucleotide of exon 10.

A substitution in the ESE sequence for SF2/ASF in exon 7 of the gene, survival of motor neuron 2 (*SMN2*) has been well characterized to cause exon 7 skipping in about 90% of transcripts (29). Although the Shapiro and Senapathy score of the splice acceptor site of intron 6 is very high (99.7) and the first nucleotide of exon 7 is G, substitution of only one nucleotide in the ESE causes exon 7 skipping in *SMN2* (29,30). This is in contrast with the findings of the present study. Splicing is a complex process and numerous factors, including ESEs/silencer and intronic splicing enhancer/silencer factors, influence splicing efficiency together with splice acceptor/donor sites (31).

In conclusion, the results of the present study demonstrate that *ACAT1* exonic mutations that affect ESE sequences may result in aberrant splicing. This may affect the activity of mitochondrial acetoacetyl-CoA thiolase. Ultimately, minigene splicing experiments remain the most useful method to detect the potential adverse effects of nucleotide substitution on gene splicing.

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