SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 15: 3879-3884, 2017

A novel mutation (c.121-13T>A) in the polypyrimidine tract of the splice acceptor site of intron 2 causes exon 3 skipping in mitochondrial acetoacetyl-CoA thiolase gene

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Received August 2, 2016; Accepted March 10, 2017

DOI: 10.3892/mmr.2017.6434

Abstract. Mitochondrial acetoacetyl-CoA thiolase (T2) (gene symbol: *ACAT1*) deficiency is an autosomal recessive disorder affecting isoleucine catabolism and ketone body utilization. In this study, mutational analysis of an Indian T2-deficient patient revealed a homozygous mutation (c.121-13T>A) located at the polypyrimidine tract of the splice acceptor site of intron 2, and exon 3 skipping was identified by cDNA analysis using cycloheximide. We made three mutant constructs (c.121-13T>A, T>C, and T>G substitutions) followed by making a wild-type minigene construct that included an *ACAT1* segment from exon 2 to 4 for a splicing experiment. The minigene splicing experiment demonstrated that exon 3 skipping was induced not only by c.121-13T>A mutation, but also by the other two substitutions. It was difficult to predict the effect of these mutations on splicing using *in silico* tools, as predictions of different tools were inconsistent with each other. The minigene splicing experiment remains the most reliable method to unravel splicing abnormalities.

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Abbreviations: 2M3HB, 2-methyl-3-hydroxybutyrate; CHX, cycloheximide; NMD, nonsense-mediated mRNA decay; T2, mitochondrial acetoacetyl-CoA thiolase

Key words: mitochondrial acetoacetyl-CoA thiolase deficiency, T2 deficiency, splice acceptor site, polypyrimidine tract, aberrant splicing, exon skipping

Introduction

Mitochondrial acetoacetyl-CoA thiolase (T2) (EC 2.3.1.9, gene symbol *ACAT1*) deficiency (Online Mendelian Inheritance in Man [OMIM] #203750, *607809), well known as β-ketothiolase deficiency, is an autosomal recessive disorder affecting the isoleucine catabolic pathway and ketone body metabolism (1,2). T2 plays important role in ketone body utilization in extrahepatic tissues through thiolysis of acetoacetyl-CoA to acetyl-CoA. In hepatic ketogenesis, T2 mediates interconversion between acetoacetyl-CoA and acetyl-CoA. Another thiolase, mitochondrial 3-ketoacyl-CoA thiolase, can compensate for T2 deficiency in ketogenesis but to a lesser extent in ketolysis. Accordingly, T2 deficiency results in ketoacidosis (1,2). Since 1971 (3), more than 100 T2-deficient patients have been identified worldwide (1, unpublished observation). This disorder is clinically characterized by intermittent ketoacidotic episodes, often triggered by infections. Patients usually have no symptoms between episodes unless a prior episode caused a lasting neurological impairment (2). The distinctive laboratory feature is an increased urinary excretion of 2-methyl-3-hydroxybutyrate (2M3HB), 2-methylacetoacetate, and tiglylglycine, derived from intermediates of isoleucine catabolism. Blood acylcarnitine analysis commonly show elevated C5:1 carnitine derived from tiglyl-CoA and C5-OH carnitine from 2-methyl-3-hydroxybutyryl-CoA (2). However, some patients with atypical clinical and/or laboratory findings have been reported (4). The severity of the clinical features varies from patient to patient, but follow-up studies revealed that T2 deficiency, in general, has a favorable outcome (1,5).

The human *ACAT1* gene is located at chromosome 11q22.3-23.1. It spans approximately 27 kb and contains 12 exons and 11 introns (6). T2 cDNA is approximately 1.5 kb long and encodes a precursor protein of 427 amino acids (7). So far, more than 70 *ACAT1* mutations have been identified, 20% of which cause aberrant splicing (8).

Herein, we report the molecular basis of T2 deficiency in a patient, in whom a minigene experiment demonstrated that a single nucleotide substitution of T to A in the polypyrimidine stretch at the -13 position of the splice acceptor site of intron 2 (c.121-13T>A) causes exon 3 skipping in *ACAT1* gene. We also discuss why it was difficult to predict the effect of this mutation on splicing using *in silico* tools.

Materials and methods

Clinical summary of patient. The female patient was born to second-degree consanguineous Indian parents. She experienced age-appropriate development with no significant illnesses until 10 months of age, when she was admitted to a hospital with vomiting, loose stools, poor feeding, fast breathing, and lethargy for one day. Her laboratory investigations showed normal hemogram levels, in addition to levels of blood glucose of 5.4 mmol/l, serum sodium of 138 mEq/l, serum potassium of 4.5 mEq/l, lactate of 0.6 mmol/l, marginally elevated ammonia of 146 μmol/l, and urinary ketone of 3+. An arterial blood gas analysis showed a severe metabolic acidosis with pH 6.88, pO_2 127 mmHg, pCO_2 10 mmHg, and HCO_3^- 4.5 mmol/l. Blood cultures and C-reactive protein were negative.

The patient was successfully treated with intravenous fluids (10% dextrose with electrolytes), bicarbonate infusion, and intravenous carnitine supplementation (100 mg/kg/day); blood glucose level was kept at a high normal range to induce insulin secretion which suppressed ketogenesis. The acidosis gradually resolved, and the patient's condition improved. Urinary gas chromatography-mass spectrometry revealed an increased excretion of lactic acid, 3-hydroxybutyrate, and 2M3HB. Plasma acylcarnitine profiling by tandem mass spectrometry showed an elevated C5-OH carnitine and C5-OH/C2 ratio. She is now 3-years old, and has attained age-appropriate development without any further ketoacidotic episodes thus far.

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 contained within the Declaration of Helsinki. The participant provided informed consent to participate in the study.

Enzyme assay and immunoblot analysis. The patient's and controls' fibroblasts were cultured in an Eagle's minimal essential medium containing 10% fetal calf serum. Protein concentration was determined by the method of Lowry using bovine serum albumin as a standard. Enzyme assay for T2 activity was performed as described (9). In brief, using supernatants from cell extracts, we spectrophotometrically monitored the decrease of acetoacetyl-CoA absorbance at 303 nm, which is the result of thiolysis of acetoacetyl-CoA to acetyl-CoA. We measured the difference of thiolase activity in the absence and the presence of potassium ions, which specifically stimulate T2; such a difference represents T2 activity (9). Immunoblot analysis was performed using rabbit polyclonal antibodies and Proto Blot® Western Blot AP system (Promega Corp., Madison, WI, USA) as described by Fukao *et al* (10); a mixture of an anti-human T2 antibody and anti-human succinyl-CoA: 3-oxoacid CoA transferase antibody was used as the first antibody. We applied 30 μg proteins extracted from each of patient's and two controls' fibroblasts. Kaleidoscope Prestained Standards (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used as molecular size markers.

Mutation analysis at the genomic DNA level. Genomic DNA was purified from the patient's fibroblasts using a Sepa Gene® (EIDIA Co., Ltd., Tokyo, Japan). A mutation screening was performed at the genomic level by PCR and direct sequencing using a set of primer pairs that amplify exons with their intron boundaries as previously described (11). The genomic *ACAT1* sequence was obtained from NCBI Reference Sequence no. NG_009888.1.

cDNA analysis. The patient's and controls' fibroblasts were cultured in two flasks, one of which was treated with 200 μg/ml of cycloheximide (CHX) (Sigma, St. Louis, MO, USA) for 5 h before RNA extraction, as previously described (12). Total RNA was isolated from fibroblasts using ISOGEN kit (Nippon Gene Co., Ltd., Tokyo, Japan). Thereafter, the isolated 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 dithiothretiol, 0.5 mM dNTPs, and 200 U of M-MLV reverse transcriptase (Life Technologies, Rockville, MD, USA) with a primer mixture including 5 pmol of both a *ACAT1*-specific antisense primer, T2-135 (5'-$^{c.*76}$TGACCCACAGTAGTCACAC-3'), and oligo dT primers.

The above preparation was incubated at 37˚C for 1 h. A total of 1 μl of this cDNA solution served as a PCR template. The positions of the PCR primers were numbered in relation to the adenine of the initiation methionine codon, which was assigned position +1. The *ACAT1* cDNA fragment was amplified (c.-40-646) using Primer T2-4 (sense) (5'-$^{c.-40}$AGTCTACGC CTGTGGAGC-3') and Primer T2-64 (antisense) (5'-$^{c.646}$TAG CATAAGCGTCCTGTTCA-3') (13). The *ACAT1* cDNA sequence was obtained from Gen Bank accession number NM_000019.3. After 30 PCR cycles, amplified fragments were separated by electrophoresis on a 5% (w/v) polyacrylamide gel. The amplified fragments were also separated in 1% (w/v) agarose gel, extracted using a Geneclean® II kit (BIO 101, Vista, CA, USA), and analyzed by direct sequencing.

Minigene splicing experiment. An *ACAT1* segment, from exon 2 to 4 (exon 2-intron 2-exon 3-intron 3-exon 4; approximately 2.4 kb long), was amplified from control genomic DNA using an Ex taq® (Takara Bio, Inc., Otsu, Japan). The used primers included the *Eco*RI linker sequence: a sense primer (Exon2-*Eco*RI) (5'-cgttcgaattc$^{c.77}$TAAGAT ATGTGGAACGGAGTTATG-3') and an antisense primer (Exon4-*Eco*RI) (5'-cgttcgaattc$^{c.321}$TGCCTGCCTTGTAGG AGC-3'). After sequence confirmation in a pGEM®-T Easy Vector (Promega Corp.), the *Eco*RI fragment was subcloned into a eukaryote expression vector pCAGGS (14). This was defined as a wild-type minigene splicing construct. We used a KOD-Plus-Mutagenesis Kit® (Toyobo Co., Ltd., Osaka, Japan) to make three mutant constructs: c.121-13T>A, T>C, and T>G. A total of 2 μg of each minigene splicing vector was transfected into $5x10^5$ cells of SV40-transformed T2-deficient fibroblasts using Lipofectamine® 2000 (Invitrogen, San Diego, CA, USA). At 48 h after transfection, RNA was extracted from

the cells. Our minigene constructs should produce human T2-rabbit β-globin fusion mRNAs. The first strand cDNA was transcribed with the rabbit β-globin-specific antisense primer (β-glo2) (5'-^{461}AGCCACCACCTTCTGATA-3') and then amplified with the Exon2-*Eco*RI primer on *ACAT1* exon 2, and another rabbit-specific antisense primer (β-glo3) (5'-^{443}GGCAGCCTGCACCTGAGGAGT-3') to amplify the chimeric cDNA of human *ACAT1* and rabbit β-globin. A study of normal and aberrant splicing in the four constructs was performed by sequencing and 5% polyacrylamide gel electrophoresis of the PCR-amplified cDNA fragments.

Results

Enzyme assay and immunoblot analysis. The potassium ion-activated acetoacetyl-CoA thiolase activity was markedly reduced in the patient's fibroblasts; in the absence of potassium (-K^+) it was 11.5, and in the presence of potassium (+K^+) it was 12.6 nmol/min/mg of protein, leading to a +K^+/-K^+ ratio of 1.1 (the control fibroblasts -K^+ 5.1, +K^+ 8.0 nmol/min/mg of protein, +K^+/-K^+ ratio of 1.6). In immunoblot analysis, no T2 protein was detected in the patient's fibroblasts (Fig. 1). These data confirm the diagnosis of T2 deficiency in the patient.

Mutation analysis at the genomic DNA level. As shown in Fig. 2, a homozygous substitution, c.121-13T>A, was revealed in the DNA fragment around exon 3 of *ACAT1*. No other mutations were identified.

cDNA analysis. The amplification of the *ACAT1* cDNA (c.-40-646) from control's fibroblasts produced a single fragment with the expected size of 686 bp. However, two faint fragments were amplified from CHX-untreated patient's fibroblasts; one had the same mobility as that of control's fibroblasts and the other was smaller (568 bp). The smaller fragment was predominantly amplified from CHX-treated patient's fibroblasts (Fig. 3A). The sequencing of this smaller fragment revealed an exon 3 skipping (Fig. 3B). Since exon 3 skipping causes frame-shift, *ACAT1* mRNAs with exon 3 skipping was subjected to nonsense-mediated mRNA decay (NMD).

Minigene splicing experiment. We hypothesized that the patient's mutation, c.121-13T>A, in intron 2 caused exon 3 skipping, hence minigene splicing experiment was performed. In case of wild-type minigene splicing construct, normal splicing was predominantly observed although small amount of transcript with exon 3 skipping was also detected. In case of c.121-13T>A construct, almost all transcripts skipped exon 3, confirming this mutation identified in the patient was responsible for the exon skipping. Furthermore, c.121-13T>G and unexpectedly c.121-13T>C also resulted in predominate exon 3 skipping (Fig.4).

Discussion

Aberrant splicing-causing mutations are increasingly recognized in several human diseases. These mutations result in the complete skipping of an exon, holding of an intron, or an introduction of a new splice site within an exon or intron (15). Most of these mutations, including that of *ACAT1*, are located at the highly conserved sequences at splice sites: the -1/-2 position (ag) at the splice acceptor site (13,16) and the +1/+2/+5 position at the splice donor site (13,17,18). However, some exonic mutations have been also demonstrated to result in aberrant splicing by activating cryptic splice sites within their exons (19,20) or by altering the consensus sequences of exonic splice enhancer sites (8).

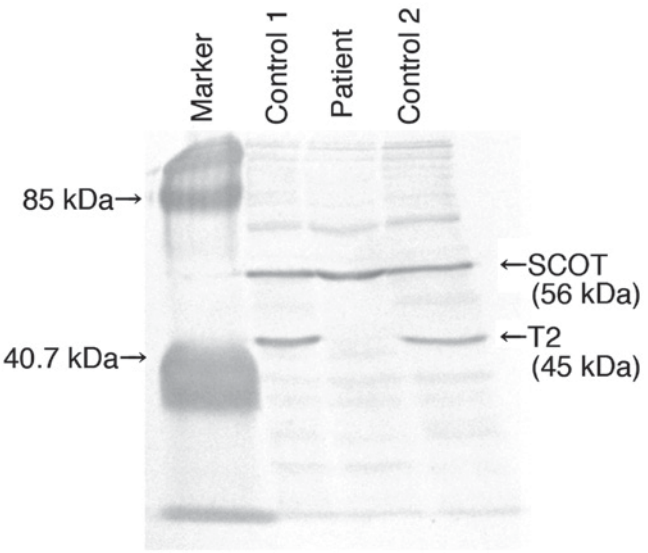

Figure 1. Immunoblot analysis. We applied 30 μg proteins extracted from each of patient's and two controls' fibroblasts. The first antibody was a mixture of an anti-T2 antibody and an anti-succinyl-CoA: 3-oxoacid CoA transferase (SCOT) antibody. The molecular weight of T2 protein was confirmed by standard marker. The positions of the bands for T2 and SCOT are indicated by arrows.

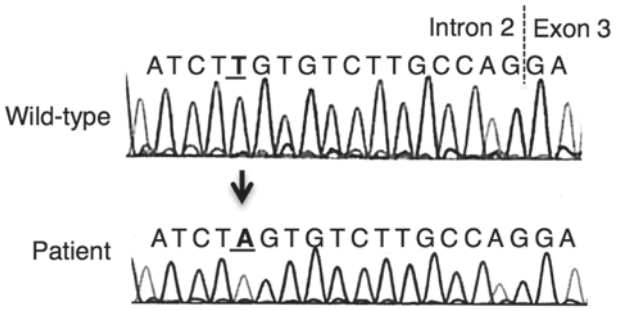

Figure 2. Genomic DNA analysis. A homozygous substitution (c.121-13T>A), shown by arrow, is demonstrated in the DNA fragment around exon 3 of *ACAT1* gene.

We identified herein a novel homozygous *ACAT1* mutation, c.121-13T>A, that caused aberrant splicing. Using Human Gene Mutation Database and Single Nucleotide Polymorphism Database, this mutation has not yet been reported in *ACAT1* gene. This c.121-13T>A mutation is located at the polypyrimidine tract of intron 2. Splice acceptor site mutations in the polypyrimidine tract have not been so commonly reported. However, we previously reported a homozygous *SLC25A20* mutation, c.199-10T>G, in two patients with carnitine-acylcarnitine translocase deficiency (21). The c.199-10T>G mutation has been shown to reside at a consensus branch point sequence, resulting in skipping of exons 3 and 4 or exon 3 alone, which leads to a truncated protein (22).

Table I. Analysis of *in silico* tools for splicing defect prediction.

Minigene construct	Shapiro and senapathy score	Analyzer splice tool	Human splicing finder (HSF)		Exon 3 skipping identified by minigene splicing experiment
			HSF matrices (acceptor site)	MaxEnt (3' Motif)	
Wild-type	92.50 (TTGTGTCTTGCCAG/G)	89.93			A small amount of transcripts
c.121-13T>A	86.88 (TAGTGTCTTGCCAG/G)	84.22	Activates a cryptic intronic acceptor site	A new acceptor site or broken wild-type acceptor site	Almost all transcripts
c.121-13T>G	86.88 (TGGTGTCTTGCCAG/G)	84.38	No impact on splicing	No impact on splicing	Almost all transcripts
c.121-13T>C	90.63 (TCGTGTCTTGCCAG/G)	86.72	No impact on splicing	No impact on splicing	Most transcripts

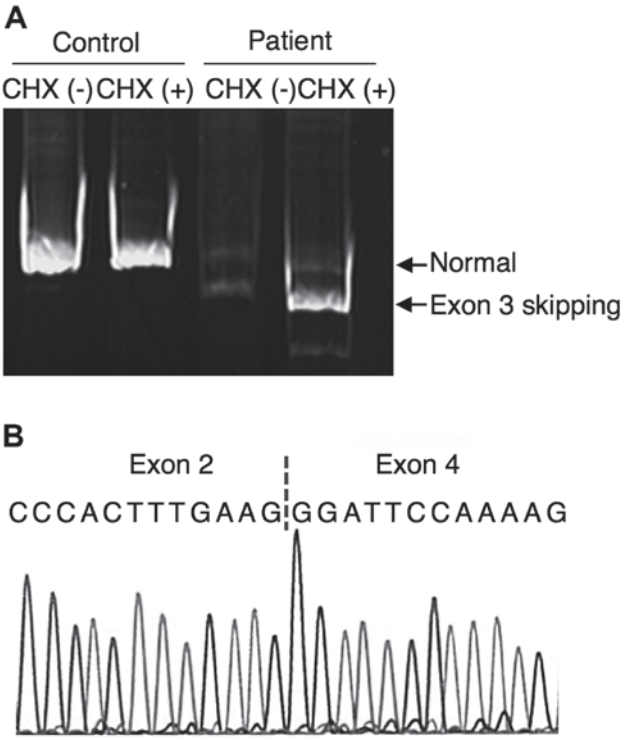

Figure 3. cDNA analysis. (A) A 5% polyacrylamide gel electrophoresis of amplified *ACAT1* cDNA fragments using RNAs extracted from cycloheximide-treated and cycloheximide-untreated patient's and control's fibroblasts. Bands corresponding to normal transcripts and those with exon 3 skipping are depicted by arrows. (B) Exon 3 skipping identified by sequencing in patient's *ACAT1* cDNA.

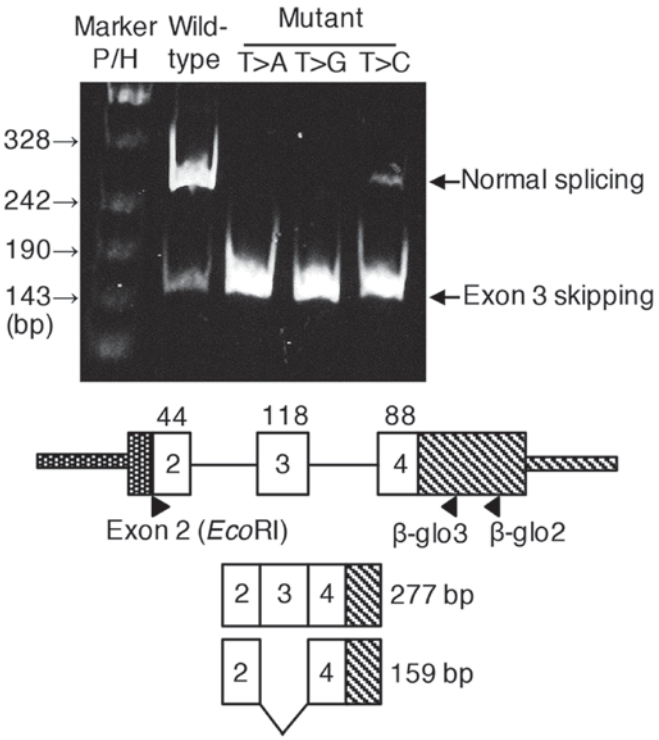

Figure 4. Minigene splicing experiment. For detection of chimeric *ACAT1* cDNA derived from transfected wild-type and mutant minigenes, the first strand cDNA was reverse transcribed using the β-glo2 primer. cDNA then was amplified using Exon2-*Eco*RI and β-glo3 primers. The amplified PCR fragments were electrophoresed on a 5% polyacrylamide gel. Bands corresponding to normal splicing (277 bp) and aberrant splicing with exon 3 skipping (159 bp) are demonstrated by arrows.

T2 deficiency was confirmed in our patient by demonstrating the lack of T2 activity and protein in the patient's fibroblasts. cDNA analysis using RNA extracted from patient's fibroblasts revealed two faint fragments on electrophoresis, compared to a clearly-visible single fragment of control's fibroblasts. To investigate whether the observed decline of transcripts was due to NMD (23), we treated fibroblasts with CHX, a general protein translation inhibitor, before RNA extraction. cDNA analysis using CHX-treated patient's fibroblasts showed a predominance of the smaller-than-normal fragment, in which exon 3 skipping was confirmed by sequencing (r.121_238del); unless treated by CHX, such fragment was mostly subjected to NMD.

We hypothesized that the patient's mutation, c.121-13T>A, weakens the recognition of the splice acceptor site in intron 2. For elucidating the causal relationship between this mutation and exon 3 skipping by a splicing experiment, we made a wild-type minigene construct, including an *ACAT1* segment from exon 2 to 4, and the mutant constructs, c.121-13T>A, and another two constructs, c.121-13T>C, and c.121-13T>G substitutions for comparison. Minigene splicing experiment clearly showed that not only c.121-13T>A but also c.121-13T>C, and c.121-13T>G substitutions resulted in exon 3 skipping.

We examined whether *in silico* tools can predict exon 3 skipping caused by these polypyrimidine-tract mutations (Table I). It is noteworthy that the c.121-13T>A, T>C, and T>G mutations resulted in only a modest decrease in the score calculated using both the Shapiro and Senapathy method (24) and Analyzer Splice Tool (http://ibis.tau.ac.il/ssat/SpliceSiteFrame.htm). The 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 (25), also failed to predict the effects of c.121-13T>C and T>G on splicing. Based on these results, it was difficult to predict exon 3 skipping caused by c.121-13T>A, T>C, and T>G mutations that was clearly demonstrated by our minigene experiment (26).

In fact, bioinformatics predictions with *in silico* tools have been widely used to evaluate the potentially pathogenic effects of genetic mutations on splicing. However, the predictions of these algorithms mostly are not consistent with each other, or with the results of minigene splice experiments (27-29). Most *in silico* tools generate a numerical score as a measure of the strength of splicing motifs, but the score itself is meaningless without a clear threshold. Several criteria were developed to set a cutoff value to define aberrant-splicing causing mutations; however, these values are commonly arbitrary across various tools and studies, which may explain the inconsistency among different tools. Although most prediction tools perform successfully for mutations at the highly conserved splice sites (ag/gt), mutations at more distant sites, like c.121-13T>A in our study, are still representing a challenge. It seems that our knowledge about the splicing process has yet to be optimized (26,30).

We demonstrated by a minigene splicing experiment that a novel mutation (c.121-13T>A) results in aberrant splicing with exon 3 skipping in *ACAT1* gene. The c.121-13 position of *ACAT1* gene appears to be an originally low-recognized site. In the routine diagnostic practice, *in silico* tools can predict the potential consequences of mutations on splicing, but their results are not so reliable. The minigene splicing experiment remains the most reliable method to unravel splicing abnormalities.

The present study has some limitations. We only identified this mutation (c.121-13T>A) in one patient. However, in other disorders, similar splice site variants, especially in the polypyrimidine tract of the splice acceptor site should be also considered as disease-causing mutations. In addition, the technique of minigene splicing experiment, which is the most reliable method to detect aberrant splicing, is not widely available. Accordingly, bioinformatics predictions with *in silico* tools remains useful, but we should consider their current restrictions.

Acknowledgements

The authors thank Ms. Naomi Sakaguchi for her technical assistance. The present study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (nos. 26114708, 24591505, 16K09962 and 15K01693), Health and Labour Science Research Grants for Research on Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan, and the Practical Research Project for Rare/Intractable Diseases from Japan Agency for Medical Research and Development (AMED).

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