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Original Research Article

Usefulness of functional splicing analysis to confirm precise disease pathogenesis in Diamond-Blackfan anemia caused by intronic variants in *RPS19*

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Abstract

Diamond-Blackfan anemia (DBA) is mainly caused by pathogenic variants in ribosomal proteins and **22** responsible genes have been identified to date. The most common causative gene of DBA is *RPS19* [NM_001022.4]. Nearly 180 *RPS19* variants have been reported, including three deep intronic variants outside the splicing consensus sequence (c.72-92A>G, c.356+18G>C, and c.411+6G>C). We also identified one case with a c.412-3C>G intronic variant. Without conducting transcript analysis, the pathogenicity of these variants is unknown. However, it is difficult to assess transcripts because of their fragility. In such cases, in *vitro* functional splicing assays can be used to assess pathogenicity. Here, we report functional splicing analysis results of four *RPS19* deep intronic variants identified in our case and in previously reported cases. One splicing consensus variant (c.411+1G>A) was also examined as a positive control. Aberrant splicing with a 2-bp insertion between exons 5 and 6 was identified in the patient samples and minigene assay results also identified exon 6 skipping in our case. The exon 6 skipping transcript was confirmed by further evaluation using quantitative RT-PCR. Additionally, minigene assay analysis of three reported deep intronic variants revealed that none of them showed aberrant splicing and that these variants were not

considered to be pathogenic. In conclusion, the minigene assay is a useful method for functional splicing analysis of inherited disease.

Key words: minigene assay, Diamond-Blackfan anemia, intronic variant, functional splicing analysis

Introduction

Diamond–Blackfan anemia (DBA) is a rare inherited autosomal dominant bone marrow failure disorder, characterized by normocellular bone marrow with erythroid hypoplasia, congenital anomalies, and a predisposition for malignancies.[1,2] Transcriptional abnormalities related to ribosomal dysfunction are the central mechanisms of DBA, and result in *TP53* pathway activation.[3] DBA is mainly caused by pathogenic variants in ribosomal proteins, and mutations in 20 ribosomal protein genes (*RPS19*, *RPS24*, *RPS17*, *RPL35A*, *RPL5*, *RPL11*, *RPS7*, *RPL26, RPS10*, *RPS26*, *RPL15*, *RPL31*, *RPS29*, *RPS28*, *RPL27*, *RPS27*, *RPS15A*, *RPL35*, *RPL18,* and *RPL9*) have been identified as responsible for DBA.[4-18] Additionally, extremely rare variants in non-ribosomal proteins like TSR2 (a ribosomal maturation factor) and GATA1 (an erythroid transcription factor), have been identified as responsible for DBA.[12,19] The most common causative gene of DBA is *RPS19*. *RPS19* reads were mapped to the human reference sequence NM_001022.4. Haploinsufficiency-mediated reduced *RPS19* expression causes selective activation of the p53 pathway in erythroid progenitor cells but not in cells from other hematopoietic lineages. Activation of the p53 pathway results in erythroid-specific cell cycle arrest and apoptosis in patients with DBA.[20,21]

According to The Human Gene Mutation Database (HGMD) (https://portal.biobase-

international.com/hgmd/pro/start.php), nearly 180 *RPS19* variants have been reported, 29 of which are splicing variants.[22] Most of these splicing variants are located in the splicing consensus sequence within two bases immediately before and after exons (AG and GT), and only three variants, c.72-92A>G, c.356+18G>C, and c.411+6G>C, are located outside these regions.[23,24] Although cases with these variants might exhibit splicing abnormalities, this has not been proven and pathogenicity of these variants is recorded as uncertain in the HGMD.

In general, transcriptional analysis is required to determine the effects of variants located outside of the splicing consensus sequence. However, mRNA is often difficult to analyze because it is unstable, leading to insufficient quality, or because of decreased expression levels due to nonsense-mediated mRNA decay pathway activation. In recent years, *in vitro* functional splicing analyses using minigene constructs have been used as an alternative approach to assess the pathogenicity of splicing variants in various inherited diseases.[25-31] To date, few reports of splicing analysis using a minigene assay have been published in the field of inherited hematological disease.[32-41] The only report that describes using the minigene assay in cases of congenital bone marrow failure is a functional splicing analysis of familial DBA caused by the *RPS7* variant.[42] No reports have described using minigene assays for DBA patients with variants in the *RPS19* gene,

the most common causative gene of DBA.

In this study, we identified a novel intronic variant in *RPS19* intron 5 (c.412-3C>G) in a sporadic case with DBA. We confirmed pathogenicity of this variant using *in vitro* minigene functional splicing analysis and by analyzing the patient's sample. We then applied this *in vitro* approach to reported intronic variants located outside the apparent splicing consensus sequence in cases with DBA.

Materials and Methods

We performed the minigene assay for four *RPS19* variants (c.412-3C>G, identified in a case we diagnosed as DBA, and c.72-92A>G, c.356+18G>C, and c.411+6G>C, reported in HGMD as of uncertain significance). We initiated *RPS19* cloning using wild-type genomic DNA, and used the PrimeSTAR mutagenesis basal kit (Takara Bio, Kusatsu, Japan) to introduce these four variants by site-directed mutagenesis.

Detailed case description: A 1-month-old boy referred to our hospital with anemia. He had no family history of anemia and anomaly. Blood examination showed macrocytic hyperchromic anemia (hemoglobin: 4.7 g/dl [normal range: 10.7–17.1]; mean corpuscular volume: 119 fL [normal range: 91–111]; mean corpuscular hemoglobin concentration: 32.4% [normal range: 28.1% to 35.5%]) and elevated erythrocyte adenosine deaminase levels 1.74 IU/gm of hemoglobin [normal range: below 1.59]. He

was diagnosed with DBA from normocellular bone marrow with erythroid hypoplasia (below 1% of erythroblasts). Anemia was steroid-dependent and often worsened during infections. He received allogenic bone marrow transplantation from an unrelated HLA identical donor. One year after transplantation he is maintaining a good clinical course.

After obtaining written informed consent from the patient's parents, we conducted genetic testing with targeted sequencing using next generation sequencing (NGS) and Sanger sequencing of the genes responsible for DBA (Table 1).

NGS samples were prepared using a Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA) to capture 16 genes (Table 1) responsible for DBA, following the manufacturer's instructions. Amplified target libraries were sequenced using MiSeq (Illumina, San Diego, CA, USA) and analyzed with CLC Genomics Workbench (CLC bio, Aarhus, Denmark).

For transcriptional analysis, total RNA was extracted from the patient's peripheral blood mononuclear cells using Lymohoprep (Veritas, Tokyo, Japan) and TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reverse-transcribed into cDNA using the RNA to cDNA Ecodry Kit (Takara Bio, Kusatsu, Japan). Reverse-transcriptional polymerase chain reaction (RT-PCR) amplification was performed (described in Supplementary Data). PCR products were separated on a 1.5% agarose gel. After

purification, cDNA was subcloned into the pT7Blue T-vector (Merck, Darmstadt, Germany) using *E. coli* HST-08 competent cells (Takara Bio, Kusatsu, Japan) and Sanger sequencing was performed using a 3130 genetic analyzer (Thermo Fisher Scientific, Tokyo, Japan).

The hybrid minigene construct was created in the pcDNA 3.0 mammalian expression vector (Inivitrogen, Carlsbad, CA) by inserting a test sequence fragment, and its flanking introns, into the multi-cloning site with an intervening intron between two exons (exon A and B) of the minigene construct (H492) (Figure 1). We cloned DNA fragments containing a couple of exons and introns around the target *RPS19* variant using in-fusion cloning methods.[43] We analyzed the variant detected in our case and three other variants obtained from HGMD and suspected of being associated with *RPS19* splicing abnormalities. The additional variants were located outside the obvious splice consensus site (two bases immediately before and after exons) (Table 2).

For the c.412-3C>G variant identified in this study, genomic DNA from the patient was cloned. For other variants (c.72-92A>G, c.356+18G>C, and c.411+6G>C), we initiated cloning with wild-type genomic DNA and then used the PrimeSTAR mutagenesis basal kit to introduce each variant using site-directed mutagenesis. The primer sequences are shown in Supplementary Table 1. The c.411+1G>A variant, which causes exon 5 skipping

in *RPS19*, was selected as a positive control.[44] To analyze whether variants in the last intron influence splicing, we made an artificial splice donor site in the non-coding region of exon 6 (Supplementary Figure 1).

Hybrid minigenes were transfected into HEK293T and HeLa cells for splicing assays as described elsewhere.[45,46] Both HEK293T and HeLa cells were obtained from the Cell Bank, Riken Bio Resource Center (Tsukuba, Japan). Total RNA was reverse-transcribed into cDNA and PCR was performed using specific primers (Supplementary Data). PCR products were analyzed by electrophoresis and Sanger sequencing.

RPS19 quantitative mRNA analysis was conducted using SYBR® green real-time RT-PCR amplification in a 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA). The analytical method is described in the Supplementary Data.

Additionally, we predicted the strength of the splicing domain in each variant using the Human Splicing Finder (https://www.umd.be/HSF3/).[47] Scores obtained for potential splice sites using MaxEnt Scan matrix are shown in Table 2.

All procedures were reviewed and approved by the Institutional Review Board of Kobe University School of Medicine. Written informed consent was obtained from the parents.

Results

Analysis of the c. 412-3C>G variant identified in our case

Using NGS and Sanger sequencing, we detected a novel intronic variant, c.412-3C>G, near the splice acceptor site of the last exon (exon 6) of *RPS19* (Figure 2a). mRNA analysis revealed two kinds of transcripts in our patient, one transcript contained normal, unmutated sequence and the other had a two base pair insertion between exons 5 and 6 (Figure 2b, c). This indicates that the novel splice acceptor site preferentially works as an exon 6 splice acceptor site (Supplementary Figure 2).

An RT-PCR product containing the expected exon 4 to 6 sequence between the cassette exons A and B was obtained from the minigene encoding the wild-type sequence. In contrast, RT-PCR from the hybrid minigene, containing the patient sequence with the intronic variant (c.412-3 C>G), showed two products: (1) small amount of transcript with a two bp (AG) insertion between exons 5 and 6; and (2) an exon 6 skipping transcript (Figure 2d).

Quantitative mRNA analysis was performed to assess *RPS19* and *RPL5* expression levels in our patient and a normal control. Comparison of mRNA levels in the patient and the normal control revealed that *RPL5* expression levels were almost the same and that *RPS19* expression in the patient was almost half that of the control (Figure 2e).

c.72-92A>G, c.356+18G>C, and c.411+6G>C variants minigene assays

In vitro analysis revealed that no obvious aberrant splicing RT-PCR products were obtained from minigenes encoding the previously reported c.72-92A>G, c.356+18G>C, and c.411+6G>C *RPS19* variants. The RT-PCR product from the minigene construct that included the c.72-92A>G variant, which encoded four exons (exons 2 and 3 between cassette exons A and B), contained all exons (Figure 3a). RT-PCR products from the minigene constructs including the 356+18G>C variant, and the c.411+6G>C variant, each encoding five exons (exons 4–6 between cassette exons A and B), also contained all exons in their entirety (Figure 3b, c). In contrast, the RT-PCR product from the minigene encoding the c.411+1G>A variant, which was reported to cause exon 5 skipping,[44] produced a single band 55 bases smaller than that from the minigene encoding the wild-type sequence. Sanger sequencing confirmed that this was an exon 5 skipping transcript (Figure 3d).

Discussion

In this study, we used targeted exome sequencing to detect a novel *RPS19* variant in a patient with DBA. However, because this is a novel variant located outside of the obvious splice consensus sequence, within two bases immediately before and after exons (AG and GT), its pathogenicity is unknown. To confirm the pathogenicity of the c.412-3

C>G variant, we conducted *in vivo* mRNA analysis and *in vitro* functional splicing analysis using a hybrid minigene construct. Using the patient's sample, transcriptional analysis revealed a single aberrant splicing product with a 2-bp insertion between exons 5 and 6. Minigene assay analysis detected an exon 6 skipping transcript in addition to the 2-bp insertion. To confirm the minigene assay results, we conducted quantitative RT-PCR using the patient's sample. Using this approach, we confirmed exon 6 skipping by the detection of decreased *RPS19* mRNA expression. Additionally, we investigated three reported intronic variants in *RPS19* located outside of the obvious splicing consensus sequence using the minigene assay. These variants had unknown pathogenicity and none of them exhibited aberrant splicing.

Transcript analysis is necessary to assess the biological effect of intronic variants located outside of splicing consensus sequences. However, there are several reasons that may cause this to be unsuccessful. First, abnormal transcripts can be difficult to amplify by RT-PCR because of the influence of nonsense-mediated mRNA decay for products of truncating variants. Second, in many cases, it is difficult to extract RNA from organs that express a large amount of target transcript. In addition, abnormal transcripts cannot be identified using transcript analysis in cases with variants causing splicing abnormalities including exon skipping of the first or last exon because the mRNA of the target gene is

not expressed[31].

To resolve these issues, in addition to traditional mRNA analysis we conducted an *in vitro* splicing assay using hybrid minigene construction using a sample of the patient's peripheral blood. The minigene assay revealed the presence of a 2-bp insertion between exons 5 and 6, and that *RPS19* exon 6 (the last exon) was not translated in the minigene derived from the patient sequence. This indicates that the *RPS19* variant allele mRNA was extremely reduced, as only the normal *RPS19* transcript and the 2-bp insertion transcript were detected by standard RT-PCR analysis of the patient's mRNA. To confirm this result, we conducted quantitative mRNA analysis of the *RPS19* gene and successfully proved its pathogenicity. Therefore, in this study, the minigene assay was successfully applied to accurately elucidate that the disease pathogenesis in this patient occurred through aberrant splicing.

Additionally, to the best of our knowledge, this is the first report showing the pathogenicity of a last exon splicing acceptor site variant using a hybrid minigene construct. Our group previously reported the usefulness of the combination of minigene assay and quantitative mRNA analysis for the elucidation of pathogenicity of a novel intronic variant located in the intron 1 splicing donor site, which caused a reduction in target mRNA expression thorough exon 1 skipping. In this study, we also show reduction

of *RPS19* mRNA expression through last exon (exon 6) skipping using the combination of minigene assay and quantitative mRNA analysis. This result shows that the minigene assay can be used to analyze intronic variants that fall around the last exon of the target gene.

In this study, for the first time, we also conducted a comprehensive exploration of *RPS19* gene splicing patterns in splicing variants located outside obvious splicing consensus sequence and reported as having uncertain pathogenicity. Although these cases were clinically diagnosed with DBA, the detected variants were registered as of uncertain pathogenicity in HGMD because they were located outside of the splicing consensus sequence and no transcript analyses were conducted. Our *in vitro* analysis revealed that minigene constructs encoding these three variants (c.72-92A>G, c.356+18G>C, and c.411+6G>C) only produced normal transcripts. *In silico* analysis, using Human Splicing Finder (https://www.umd.be/HSF3/) also showed that splice site scores are not changed or only mildly changed in these three cases in contrast to those of other pathogenic intronic variants (Table 2). These results show that these reported variants are unlikely to cause splicing abnormalities and suggest the presence of unidentified *RPS19* variants or variants in other DBA causative genes in these patients.

Recently, we examined the pathogenicity of reported intronic variants located

outside obvious splicing consensus sequences, within two bases immediately before and after exons (AG and GT), in some inherited kidney diseases including in Alport syndrome and Dent disease.[25,26] In these reports, hybrid minigene analysis was useful for assessing whether the variant in question causes aberrant splicing. The results of the present study suggest that *in vitro* functional analysis using hybrid mini gene constructs may be useful for estimating the pathogenicity of novel intronic variants, especially those located outside splice consensus sequences, in the field of inherited hematological disorders.

Recently, splicing abnormalities have gained attention as a potential target of treatment. Splice alteration, through the use of antisense oligonucleotides, has emerged as an effective approach for the treatment of inherited diseases caused by splicing abnormalities. Eteplirsen and Nusinersen have already been approved by the FDA as splicing modulation drugs for Duchenne muscular dystrophy and spinal muscular atrophy, respectively.[48,49] Additionally, our group developed exon skipping therapy using antisense oligonucleotides for Alport syndrome, one of the most common inherited kidney diseases.[50] In the future, splicing targeted therapy may be applied to the treatment of other inherited diseases. For this reason, it is critically important to determine the pathogenicity of intronic variants with unknown significance.

For hybrid minigene functional splicing analysis for the initial case, we created

an artificial splicing donor site in the non-coding region of *RPS19* exon 6. In general, the last intron is not correctly spliced in minigene assays because it does not contain a splicing consensus sequence beginning with a GT. Indeed, the minigene construct containing the patient's sequence without any alterations did not produce any *RPS19* exon 6 mRNA in preliminary experiments (Supplementary Figure 1a). For this reason, we created a new splice donor site within the non-coding region of exon 6 using mutagenesis. This artificial splice donor site worked correctly (Supplementary Figure 1b). There are two limitations of this study. Firstly, there is a potential that this artificial manipulation can affect the splicing mechanism of the introns in which the target variants are located. However, it is unlikely that alteration of the intron immediately after exon 6 will affect intron 5 splicing because splicing usually proceeds in an intron-by-intron manner. Secondly, the general drawback of using a minigene assay to analyze the pathogenicity of mutations identified by NGS is that it requires a great deal of effort to apply. However, the minigene assay can take as little as two weeks and multiple mutations can be analyzed simultaneously.

In conclusion, our study demonstrates that the minigene assay is a useful, non-invasive method for functional splicing analysis of inherited hematological disorders, especially when standard transcriptional analysis fails to correctly detect abnormal mRNA or when a sample is not available from the patient. This assay could be adapted for all other

inherited diseases.

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Declaration of interest statement

The authors have no conflicts of interest to disclose.

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Table 1. List of genes analyzed by target sequencing in this study

No.	Gene	Location
1	*RPL11*	Chromosome 1
2	*RPL5*	Chromosome 1
3	*RPS27*	Chromosome 1
4	*RPS7*	Chromosome 2
5	*RPL35A*	Chromosome 3
6	*RPS14*	Chromosome 5
7	*RPS10*	Chromosome 6
8	*RPS24*	Chromosome 10
9	*RPS26*	Chromosome 12
10	*RPS17*	Chromosome 15
11	*RPS17L*	Chromosome 15
12	*RPL26*	Chromosome 17
13	*RPL27*	Chromosome 17
14	*TP53*	Chromosome 17
15	*RPS19*	Chromosome 19
16	*GATA1*	Chromosome X

Table 2. List of variants cloned into the minigene construct

	Variant	Location of variant	mRNA	*In vitro* (minigene assay)		*In silico* (MaxEnt score)			Reference
				Genetic region cloned	Result	Original Splice site score	Variant score	Novel splice site score	
No. 1	c.72-92A>G	Intron 2	N/A	Introns 1–3	Normal transcript	10.14	10.14		[19]
No. 2	c.356+18G>C	Intron 4	N/A	Intron 3–exon 6	Normal transcript	8.84	8.84		[20]
No. 3	c.411+1G>A	Intron 5	Exon 5 skipping	Intron 3–exon 6	Normal transcript	11.08	2.90		[40]
No. 4	c.411+6G>C	Intron 5	N/A	Intron 3–exon 6	Normal transcript	11.08	9.88		[20]
No. 5	c.412-3C>G	Intron 5	Normal transcript and insertion of AG between exons 5 and 6	Intron 3–exon 6	Exon 6 skipping or insertion of AG between exons 5 and 6	13.52	1.50	8.93	Our case

N/A: not available

Figure 1. Schema for the hybrid minigene

Figure 1

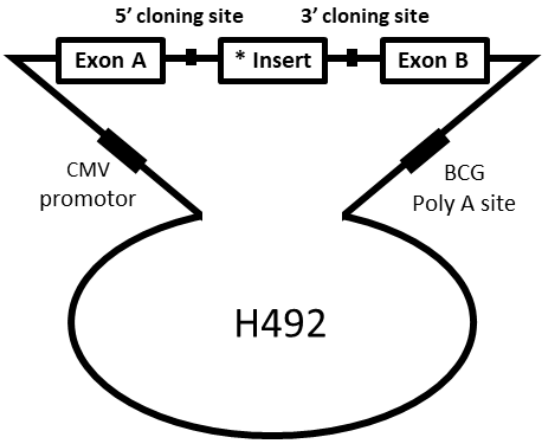

Figure 2. Transcript analysis in our patient and his family

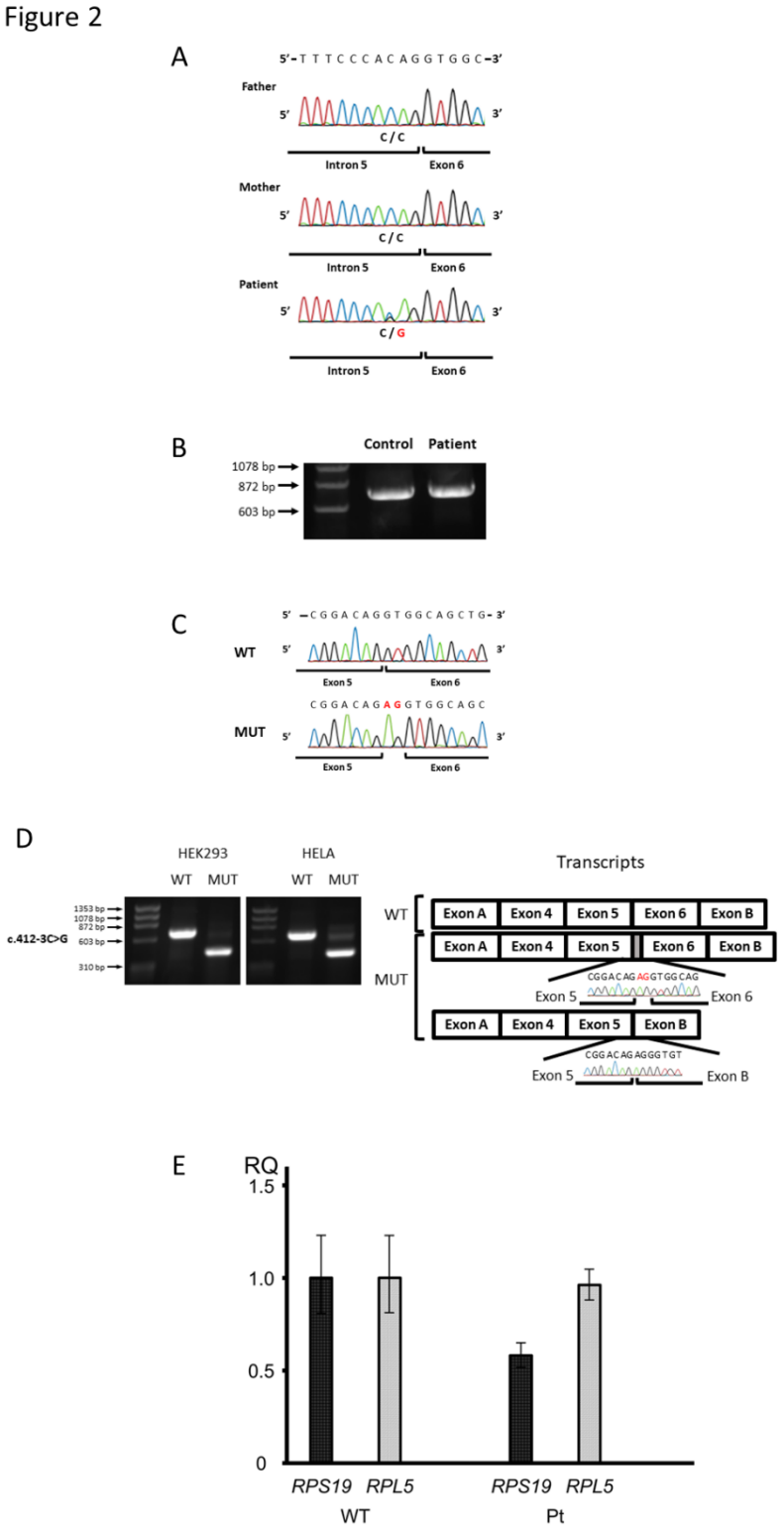

Figure 3. *In vitro* splicing analysis for c.72-92A>G, c.356+18G>C, and c.411+6G>C variants

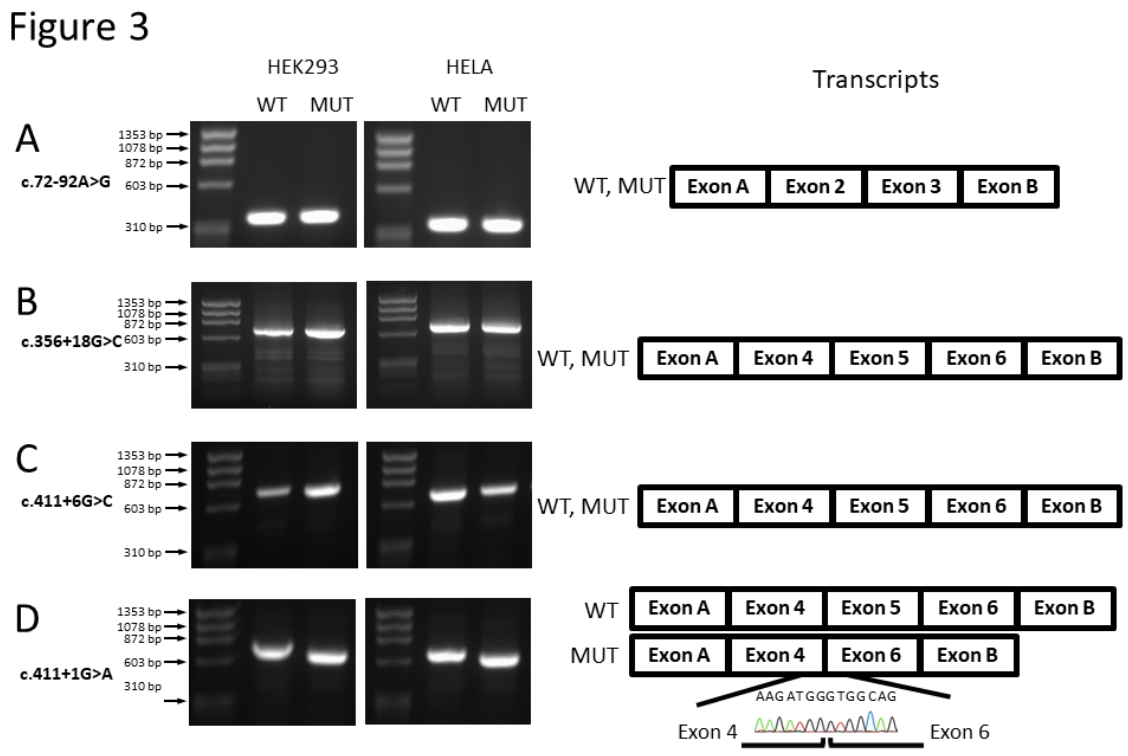

Figure legends

Figure 1. Schema for the hybrid minigene

The H492 vector has two cassette exons, A and B, between which is a multicloning site. The H492 vector also has cytomegalovirus (CMV) enhancer-promotor and bovine growth hormone (BGH) gene polyadenylation site.

Figure 2. Transcript analysis in our patient and his family

(A) The *RPS19* genomic DNA sequences of the patient and his parents. A heterozygous single-base substitution of C to G was detected in the patient. (B) Electrophoretic gel of the RT-PCR products obtained from the control and the patient. A single band was observed for each PCR product. The products were almost identical sizes. (C) PCR products were subcloned and sequenced. Wild-type and an otherwise wild-type *RPS19* sequence with two bases inserted between exons 5 and 6 were identified. (D) RT-PCR amplified products of minigene construct transcripts. As shown in the gel images, a 295-bp smaller band and 2-bp larger band are produced by the minigene construct with the c.412-3C>G variant in both in HEK 293T and Hela cells. Transcripts from the minigene construct with the c.412-3C>G variant show skipping of exon 6 and that the larger transcript has two bases inserted between exons 5 and 6. (E) Quantitative RT-PCR using mRNA from the patient and a normal control was performed to compare *RPS19* and

RPL5 expression. Relative quantification (RQ) of each gene was calculated, which is a fold change compared with the calibrator, *HPRT-1*. *RPS19* expression level in the patient, (RQ = 0.581) was approximately half that measured in the control (RQ = 1.000). The *RPL5* (RQ = 0.961) expression levels in the patient were almost equal to those in the control (RQ = 1.000).

Figure 3. *In vitro* splicing analysis for c.72-92A>G, c.356+18G>C, and c.411+6G>C variants

RT-PCR amplified products of minigene construct transcripts. (**a**, **b**, and **c**) Gel images and sequences of transcripts from the minigenes with c.72-92A>G (**a**), c.356+18G>C (**b**), and c.411+6G>C (**c**). Electrophoresis results reveal a band the same size as that produced by the wild-type construct. Transcript analysis shows that each transcript is the same as that of the wild-type construct. (**d**) As shown in the gel images, the transcript from the wild-type construct was larger than that from the minigene with the c.411+1G>A variant in both HEK 293T and Hela cells. The transcripts from the c.411+1G>A variant skipped exon 5.