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Functional analysis of suspected splicing variants in CLCN5 gene in Dent disease 1

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Abstract

Background In recent years, the elucidation of splicing abnormalities as a cause of hereditary diseases has progressed. However, there are no comprehensive reports of suspected splicing variants in the CLCN5 gene in Dent disease cases. We reproduced gene mutations by mutagenesis, inserted the mutated genes into minigene vectors, and investigated the pathogenicity and onset mechanisms of these variants.

Methods We conducted functional splicing assays using a hybrid minigene for six suspected splicing variants (c.105G>A, c.105+5G>C, c.106−17T>G, c.393+4A>G, c.517−8A>G, c.517−3C>A) in CLCN5. We extracted information on these variants from the Human Gene Mutation Database. We reproduced minigene vectors with the insertion of relevant exons with suspected splicing variants. We then transfected these minigene vectors into cultured cells and extracted and analyzed the mRNA. In addition, we conducted in silico analysis to confirm our minigene assay results.

Results We successfully determined that five of these six variants are pathogenic via the production of splicing abnormalities. One showed only normal transcript production and was thus suspected of not being pathogenic (c.106−17T>G).

Conclusion We found that five CLCN5 variants disrupted the original splice site, resulting in aberrant splicing. It is sometimes difficult to obtain mRNA from patient samples because of the fragility of mRNA or its low expression level in peripheral leukocytes. Our in vitro system can be used as an alternative to in vivo assays to determine the pathogenicity of suspected splicing variants.

Keywords CLCN5 · Splicing · Minigene · Variant · In silico

Introduction

Dent disease is an X-linked genetic disease of tubulopathy characterized by low-molecular-weight proteinuria, hypercalciuria, and renal calcification [1]. Approximately 60% of Dent disease cases are caused by mutations in the CLCN5 gene, which are referred to as Dent disease 1 (OMIM#300,009). In the kidney, the CLCN5 gene is involved in the synthesis of CIC-5 at proximal tubules, ascending limb of Henle’s loop, and the intercalated cells of the collecting duct [1]. CIC-5 is a type of voltage-gated chloride channel involved in regulating cell volume, membrane potential, and transepithelial transport. A mouse model of CIC-5 knockout demonstrated that impairment of the endocytic traffic is the major cause not only of tubular proteinuria, but also of hypercalciuria and consequently kidney stones [2].
In the Human Gene Mutation Database (HGMD) Professional (https://portal.biobase-international.com/hgmd/pro/start.php), most reported mutations of the CLCN5 gene are missense or nonsense mutations, followed by deletions and splicing variants located at obvious splice sites within two bases immediately before and after exons (AG-GT). However, some previously reported suspected splicing variants were outside these regions [1, 3–6]. Although cases with such mutations might exhibit splicing abnormality, this has not been proven.

RNA splicing is a process involving the removal of the intervening, noncoding sequences of genes (introns) from pre-mRNA and the joining of the protein-coding sequences (exons) together, to enable translation of mRNA into a protein. Recent research has underlined the abundance and importance of splicing variants in the etiology of inherited diseases [7–11]. The most effective method to determine whether the selected variants affect splicing is to analyze the mRNA extracted from the relevant tissue of patients. However, the extraction of RNA from the affected organs is sometimes difficult. In addition, the analysis of mRNA is usually difficult because of its low expression levels in peripheral leukocytes or its fragility. In silico algorithms were developed to predict splicing abnormalities and are available as tools, but they do not always predict the exact splicing abnormalities. If the appropriate material for RNA analysis is not available, an alternative method is a minigene splicing assay—an in vitro hybrid system that allows exon trapping.

Against this background, in this study, the pathogenicity of six variants in cases of Dent disease one, in which splicing abnormalities were suspected, was analyzed using in vitro (minigene assay) and in silico assays.

**Materials and methods**

Among the variants in the CLCN5 gene reported as the causative agents of Dent disease one, we selected six variants suspected of being associated with splicing abnormalities from HGMD Professional, which were outside the obvious splice sites (not within two bases immediately before and after exons: AG-GT). In general, variants in the acceptor and donor sites (AG-GT sites) always alter the interactions between pre-mRNA and proteins involved in intron removal. We excluded these AG-GT consensus site variants from this study. Each variant is shown in Table 1.

**In vitro assay**

To create hybrid minigene constructs, we used the H492 vector based on the pcDNA 3.0 mammalian expression vector (Invitrogen, Carlsbad, CA, USA) (Fig. 1) that we developed previously [11–15]. We cloned DNA fragments containing a couple of exons and introns around the target variant in the CLCN5 gene using In-Fusion cloning methods with In-Fusion®HD

| No.   | c. Genetic region cloned | Result                                      | Original score | Variant score | New splicing site score | Reference |
|-------|--------------------------|---------------------------------------------|----------------|---------------|-------------------------|-----------|
| 1     | c. 105 G > A            | N/A                                         | Intron 1–2     | 23 bp inclusion of intron 2 | 10.06     | 6.42                  | 10.47     | [1]        |
| 2     | c. 105 + 5 G > C        | 23 bp inclusion of intron 2                 | Intron 1–2     | 23 bp inclusion of intron 2 | 10.06     | 8.1                   | 10.47     | [4]        |
| 3     | c. 106–17 T > G        | Normal                                      | Intron 2–3     | Normal        | 11.08     | 10.24                 |           | [3]        |
| 4     | c. 393 + 4 A > G       | N/A                                         | Intron 3–4     | Exon 4 skipping | 8.1       | 4.15                  |           | [5]        |
| 5     | c. 517–8 A > G         | N/A                                         | Intron 5–6     | 7 bp inclusion of intron 5 partial deletion (32 bp) of exon 6 partial deletion (88 bp) of exon 6 skipping | 11.44     | 4.78                  | 7.42      | 5.30 3.95  | [1]        |
| 6     | c. 517–3 C > A         | Exon 6 skipping                             | Intron 5–6     | Partial deletion (32 bp) of exon 6 partial deletion (88 bp) of exon 6 skipping | 11.44     | 4.78                  | 5.30 3.95 |           | [6]        |

**Table 1** In vivo (Minigene) and in silico assays

N/A not available
Cloning Kit (Takara Bio Inc., Kusatsu, Japan), in accordance with the manufacturer’s instructions.

Because patients’ gDNA was not available, we initiated cloning from wild-type gDNA and then introduced mutations by site-directed mutagenesis using PrimeSTAR® Mutagenesis Basal Kit (Takara Bio Inc.), in accordance with the manufacturer’s instructions. The primers used are shown in Supplementary Table 1.

The hybrid minigenes were confirmed by sequencing and transfected into HEK293T and HeLa cells using Lipofectamine® 3000 Transfection Kit (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was extracted from cells after 36 h using the RNeasy® Plus Mini Kit (QIAGEN, Hilden, Germany).

Total RNA (1 μg) was reverse-transcribed using RNA to cDNA EcoDry™ Premix (Double Primed) (Takara Bio Inc.). PCR was performed using a forward primer corresponding to a segment upstream of exon A and reverse primer complementary to a segment downstream of exon B (Fig. 1). PCR products were analyzed by electrophoresis on a 1.5% agarose gel using a 50 bp DNA ladder and direct sequencing.

**In silico assay**

We predicted the splicing domain strength in each variant, using Human Splicing Finder (https://www.umd.be/HSF3/). As for potential splice sites, scores obtained using MaxEnt Scan matrix are shown in Table 1.

**Results**

Analysis of each cDNA revealed splicing abnormalities associated with five of the six variants (Table 1, Fig. 2, 3). We observed one transcript with a 23 bp inclusion of intron two at the end of exon two in two variants (c.105G>A and c.105+5G>C) (No. 1 and 2, Fig. 2a). In both cases, normal nucleotide sequences were not detected. We observed no change in one variant (No. 3, c.106-17T>G) (Fig. 2b). The variant c.393+4A>G led to skipping of the whole of exon 4 (No. 4, Fig. 2c). For the variant c.517–8A>G, we observed several transcripts: (1) inclusion of an intron five fragment (7 bp), (2) partial deletion (32 bp) of exon 6, (3) partial deletion (88 bp) of exon 6, and (4) exon 6 skipping (No. 5, Fig. 2d). For the variant c.517–3C>A, we observed the same transcripts as with No. 5 (No. 6, Fig. 2d), except for the inclusion of 7 bp from the intron 5 fragment (Fig. 2d).

**Discussion**

This is the first report describing a comprehensive exploration of CLCN5 gene splicing patterns in variants reported to be pathogenic. Most of the variants showed disruption of the original splice site and the creation of new splice sites, which were shown by in vitro minigene splicing assay and confirmed by in silico analysis.

Each result can be interpreted as follows. No. 1 (c.105G>A) and No. 2 (c.105+5G>C) result in the generation of an aberrant transcript because the original donor site is disrupted, and the existing candidate new donor site in intron 2 is activated. Both of them lead to the inclusion of a 23 bp intron 2 fragment (Fig. 3a). It was also expected that No. 4 (c.393+4A>G) leads to the skipping of exon 4 due to the disruption of the original donor site of intron 4. No. 5 (c.517–8A>G) and No. 6 (c.517–3C>A) also lead to exon skipping as a result of disruption of the original
of candidate splicing sites having a large score for splicing site (Table 1, score = 10.47 in No. 1 and 2). Variant No. 3 (c.106–17T>G) was suspected of not being pathogenic, a conclusion that was derived from both in vitro and in silico splicing assays. Actually, even in the original study [3], the authors detected only normal transcript from the patient’s mRNA extracted from the peripheral leukocytes. In addition, this patient had no proteinuria but showed nephrolithiasis and hypercalciuria. This clinical information suggested that the case was unlikely to involve Dent disease.

Recently, gene targeted therapy has been developed. Splice modulation through the use of antisense oligonucleotides has emerged as an effective technique for the treatment of splicing abnormalities. The US Food and Drug Administration (FDA) approved eteplirsen (Exondys 51) as the first exon-skipping drug for the treatment of Duchenne muscular dystrophy and nusinersen (Spinraza) for spinal muscular atrophy [16]. We can also pursue the development of gene targeted therapy for cases with aberrant splicing to correct splicing patterns. The CRISPR/Cas system is also a promising tool for correcting many genetic defects [17]. For this purpose, it is critically important to determine the pathogenicity of variants of unknown significance. We believe that the results of these splicing assays are important for studying the pathogenesis and treatment of diseases.

Lourdel et al. asserted that functional studies of CLCN5 mutations have made it possible to distinguish among the three different classes of mutation [18]. Class I mutations comprise nonsense, frameshift, and aberrant mRNA splicing mutations that lead to impairment of protein synthesis. As a consequence, Class I mutations impair processing and folding and the CIC-5 mutants of this type are retained within the endoplasmic reticulum and targeted for degradation by quality control mechanisms. We speculate that the five variants examined in this study lead to the impaired function of CIC-5.

Among three of the six variants, the original paper [3, 4, 6] showed the results of mRNA sequencing. In No. 2 (c.105+5G>C) and No. 3 (c.106–17T>G), these results were identical to our minigene assay results [3, 4]. Conversely, in No. 6 (c.517–3C>A), the mRNA sequencing results identified only exon 6 skipping [4], so this result is not completely concordant with ours. This discrepancy may be derived from the difference between in vivo and in vitro minigene assays using cultured cells. We also speculate that defense mechanisms such as nonsense-mediated decay, which eliminates abnormal mRNA in vivo, may occur in the living host [19]. Because the variants examined in this study were reported in a previous study, mRNA analysis using the patients’ samples was difficult. However, in the minigene assay, variants of interest can be inserted and their effects can be examined even when patients’ samples are not available. In addition, extracting mRNA from affected
organs is often difficult. Alternatively, peripheral leukocytes are usually used for in vivo mRNA analysis; however, their mRNA expression level is usually low and the mRNA is fragile. These conditions make mRNA analysis in vivo very difficult. In such cases, the minigene assay is a good tool for assessing splicing abnormalities.

Approximately 9% of all mutations reported in the HGMD are splicing mutations (18,761/208,368) (HGMD database, accessed on October 10, 2017), although this number may well be an underestimate. Here, we assessed only variants suspected of causing splicing abnormalities. However, even variants in exons can cause splicing abnormalities. Studies for the detection of splicing abnormalities have been conducted for a long time, but it is still difficult to accurately predict abnormal splicing caused by genetic variants [20]. Recently, the number of genes identified as being responsible for inherited kidney diseases has increased rapidly. Genetic disorders are present in any part of the renal and urinary tract. Recently, using minigene assay, we have detected splicing abnormalities in the COL4A5 gene as a cause of glomerular disease [11, 21]. In this report, we summarize splicing abnormalities associated with Dent disease 1, one of the most common renal tubulopathies. Next, we plan to analyze splicing abnormalities in the OCRL gene causing Lowe syndrome and in the GLA gene causing Fabry disease, using the same approach. Other inherited kidney diseases are also targets for this approach. Cases suspected of having inherited kidney diseases are less likely to undergo
renal biopsy than other diseases. As a result, the affected organ, the kidney itself, cannot be used for splicing analysis. However, analysis using minigene assay that does not require biological samples and may provide a comprehensive understanding of splicing abnormalities in inherited kidney diseases. Further analysis is thus required to further elucidate the mechanism of splicing.

In conclusion, this study provides proof of splicing abnormalities associated with some suspected variants in the CLCN5 gene. The incidence of genetic disorders due to splicing abnormalities has been underestimated, and further comprehensive studies should be conducted to clarify the contribution of splicing abnormalities to the onset of disease. In this study, we have focused on Dent disease, but we have also conducted a comprehensive approach for Alport syndrome [11, 21]. In future work, we should apply minigene assay to other common inherited kidney diseases such as Lowe syndrome and Fabry disease.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Review Board of Kobe University Graduate School of Medicine (IRB approval number 301) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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Correction to: Functional analysis of suspected splicing variants in \textit{CLCN5} gene in Dent disease 1

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