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Unverricht-Lundborg disease: development of splicing therapeutic approaches for a patient with an homozygous mutation in the cystatin B gene

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Introduction

Unverricht-Lundborg disease (ULD or EPM1) is the most common form of progressive myoclonic epilepsy (PME) worldwide. It is an autosomal recessive neurodegenerative disorder characterized by action myoclonic jerks, generalized tonic-clonic seizures, marked photosensitivity and juvenile ataxia with gradual worsening. Actually, there is no etiologic treatment for ULD, symptomatic pharmacologic and rehabilitative management are the mainstay of patient care (1).

ULD is caused by mutations in the cystatin B gene (*CSTB*) localized on chromosome 21q22.3 and encodes an inhibitor of several lysosomal cathepsins (2-4). Thirteen *CSTB* mutations have been described as causal of EPM1 (5). The most common one, responsible for about 90% of the abnormal alleles is an unstable expansion of a dodecamer repeat in the 5' UTR promoter region (6). The remaining twelve mutations are missense, nonsense, frameshift and splice-site mutations that may lead to abnormal RNA processing (5). Most frequently, splice-site mutations interfere with exon recognition in pre-mRNA transcripts and lead to exon skipping or intron retention. In general, more than 15% of disease-causing point mutations affect classical splice-sites (7,8). A mutation in a splice donor site (SDS) often interferes with proper complex formation of the splice factor U1 snRNP with pre-mRNAs (9). The part of the U1 snRNP that binds the SDS by Watson-Crick base pairing is called U1 snRNA (U1). After U1 has recognized a SDS, the complex recruits other components of the spliceosome to further promote splicing of the pre-mRNA (10).

The increasing knowledge of RNA biology is stimulating new approaches of RNA-based strategies to achieve therapy in alternative to conventional gene replacement therapies. The vast majority of RNA-based approaches have exploited, *in vitro* and *in vivo*, antisense sequences to either mask natural splice sites, to induce skipping of defective exons, or newly generated cryptic sites, to favour the use of the canonical ones (11). On the other hand, the use of U1snRNA complementary to the mutated site has been described as a potentially therapeutic strategy to correct 5'splice site defects dependent on U1 binding (12,13).

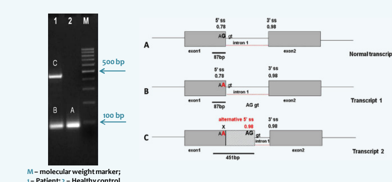
Recently, our group described a Portuguese ULD patient who is homozygous for a new synonymous mutation (c.66G>A; p.Q22Q) located at the last nucleotide of exon 1 which leads to missplicing of *CSTB* transcripts (14). Here, to overcome the pathogenic effect of the SDS mutation, we have exploited both antisense oligonucleotide and U1 snRNA mediated therapeutic strategies to correct the splice defect in the patient cell line.

Workflow & Results

Study of the impact of the *CSTB* gene mutation at cDNA level

→ RT-PCR analysis from healthy control and ULD patient

• Mutation c.66G>A on exon 1



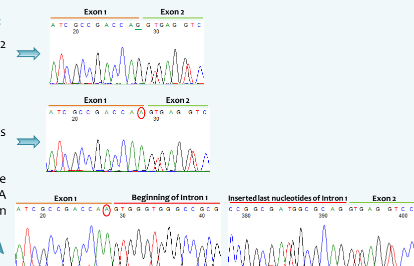
The RT-PCR analysis of cDNA with primers for exon 1 (Fw) and 2 (Rv) allowed to observe:

- In the healthy control the amplification of a fragment with 87 bp corresponding to exons 1 and 2 (lane 2) – normal transcript A;

- In the patient (lane 1):

- A fragment of normal size (87 bp) corresponding to exon 1 and 2 presenting the synonymous change G>A in the last nucleotide of exon 1 (transcript 1-B);

- A second fragment of abnormal size (451 bp) with partial inclusion of 364 bp of intronic sequence from intron 1 due to the activation of a cryptic splice-site inside the intron. The abnormal RNA (r.[66G>A, 65_66ins66+364bp]), predicts an abnormal peptide with a premature truncation (transcript 2-C).



Development of Splicing Therapeutic Approaches for an ULD patient with the synonymous mutation c.66G>A

⌘ U1 snRNA-mediated Gene Therapeutic Approach

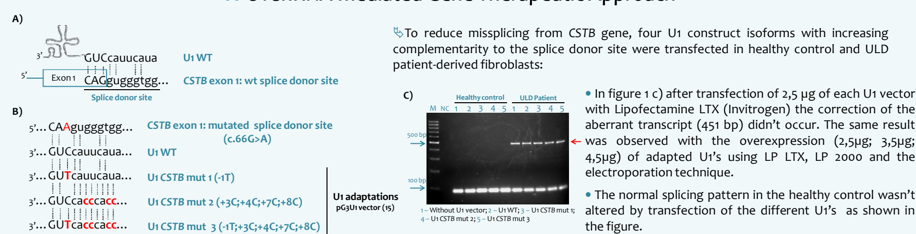


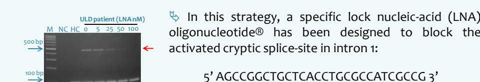
Figure 1: A) U1 small nuclear RNA annealing to the splice donor site of *CSTB* exon 1. B) Strategy to increase complementarity of the U1 with the mutated splice donor site of *CSTB*. Sequence description of the three U1 snRNA-adapted vectors constructed. C) RT-PCR analysis after the U1 snRNA-mediated gene therapeutic approach with the U1WT and U1 mutation-adapted vectors in healthy control and patient fibroblast cells.

To reduce missplicing from *CSTB* gene, four U1 construct isoforms with increasing complementarity to the splice donor site were transfected in healthy control and ULD patient-derived fibroblasts:

- In figure 1 c) after transfection of 2,5 µg of each U1 vector with Lipofectamine LTX (Invitrogen) the correction of the aberrant transcript (451 bp) didn't occur. The same result was observed with the overexpression (2,5µg; 3,5µg; 4,5µg) of adapted U1's using LP LTX, LP 2000 and the electroporation technique.
- The normal splicing pattern in the healthy control wasn't altered by transfection of the different U1's as shown in the figure.

⌘ Antisense Oligonucleotide (LNA) Therapeutic Approach

→ RT-PCR analysis after LNA transfection in patient fibroblasts



In this strategy, a specific lock nucleic-acid (LNA) oligonucleotide® has been designed to block the activated cryptic splice-site in intron 1:

5' AGCCGGCTGCTCACCTGCGCCATCGCCG 3'

- Normal splicing pattern of a single transcript with the synonymous change G>A was successfully rescued after LNA transfection (Lipofectamine LTX) in ULD patient cells. The therapeutic effect showed to be dose-dependent, being the correction achieved with 100 nM of LNA.

Conclusion

Here we have developed antisense oligonucleotide and U1snRNA-mediated therapeutic strategies to correct the splice defect caused by the presence of the mutation c.66G>A in the cystatin B gene.

1. In a first approach, to reduce missplicing from *CSTB* gene, we generated four U1 construct isoforms with increasing complementarity to the SDS. Transfection of patient-derived fibroblasts with different concentrations of the adapted U1 vectors did not allowed the correction of the aberrant transcript.

2. In a second strategy, a specific lock nucleic-acid (LNA) oligonucleotide was designed to block the activated cryptic splice-site in intron 1. Normal splicing pattern of a single transcript with the synonymous change G>A was successfully rescued after LNA transfection in the ULD patient cells. The therapeutic effect showed to be dose-dependent.

Actually, there is no etiologic treatment for ULD, therefore these results suggest that antisense therapy might be a potential alternative or adjunct treatment strategy for patients holding splicing changes in *CSTB* gene. As far as we know this is the first report of a patient tailored therapy in cells of an ULD patient.

References

1 – Kälviäinen R, *et al.* Epilepsia - Review 2008; 49(4):549-556.
2 – Lehesjoki AE, *et al.* Proc Natl Acad Sci USA. 1991; 88:3696-3699.
3 – Lehesjoki AE, *et al.* Hum Mol Genet. 1993; 2:1229-1234.
4 – Pennachio LA, *et al.* Science; 1996; 271:1731-1734.
5 – Human Gene Mutation Database Professional Release 2013 (http://www.biobase-international.com/product/human-gene-mutation-database)
6 - Joensuu T, *et al.* Epilepsia – Review 2008; 49:557-563.
7 – Faustino NA and Cooper TA. Genes Dev. 2003; 17:419-437.
8 - López-Bigas N, *et al.* FEBS Lett. 2005; 579(9):1900-1903.
9 – Zhuang Y and Weiner AM. Cell. 1986; 46:827-835.
10 – Wahl MC, *et al.* Cell. 2009; 136:701-718.
11 – Wood M, *et al.* PloS Genet. 2007; 3:e109.
12– Tanner G, *et al*. Hum Mutat. 2009; 30(2):255-63.
13 – Sánchez-Alcudia R, *et al.* Mol Genet Metab. 2011; 102(2):134-138.
14 – Pinto E, *et al.* Epilepsy Res. 2012; 99(1-2):187-190.
15 – Susani L, *et al*. Hum Mutat. 2004; 24(3):225-35.

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