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Research paper

Use of modified U1 small nuclear RNA for rescue from exon 7 skipping caused by 5′-splice site mutation of human cathepsin A gene☆

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ABSTRACT

Cathepsin A (CTSA) is a multifunctional lysosomal enzyme, and its hereditary defect causes an autosomal recessive disorder called galactosialidosis. In a certain number of galactosialidosis patients, a base substitution from adenine to guanine is observed at the +3 position of the 7th intron (IVS7 +3a > g) of the *CTSA gene*. With this mutation, a splicing error occurs; and consequently mRNA lacking the 7th exon is produced. This skipping of exon 7 causes a frame shift of the transcripts, resulting in a non-functional CTSA protein and hence galactosialidosis. This mutation seems to make the interaction between the 5′-splice site of intron 7 of pre-mRNA and U1 small nuclear RNA (U1 snRNA) much weaker. In the present study, to produce properly spliced mRNA from the *CTSA gene* harboring this IVS7 +3a > g mutation, we examined the possible usefulness of modified U1 snRNA that could interact with the mutated 5′-splice site. Toward this goal, we first prepared a model system using a mutant *CTSA mini gene* plasmid for delivery into HeLa cells. Then, we examined the effectiveness of modified U1 snRNA on the formation of properly spliced mRNA from this mutant *CTSA mini gene*. As a result, we succeeded in obtaining improved formation of properly spliced CTSA mRNA. Our results suggest the usefulness of modified U1 snRNA for rescue from exon 7 skipping caused by the IVS7 +3a > g mutation of the *CTSA gene*.

1. Introduction

In the various processes involved in eukaryotic gene expression, the splicing of nuclear mRNA precursor (pre-mRNA) is one of the critical steps of RNA processing. During this splicing process, introns are precisely excised at the exon/intron boundaries, and adjacent exons are joined together to form the mature mRNA. This splicing reaction is initiated by the association of U1 small nuclear RNA (U1 snRNA) with the 5′-splice site (ss) of pre-mRNA (Krämer et al., 1984; Zhuang and Weiner, 1986; Roca et al., 2013). In general, the third to eleventh bases of U1 snRNA (5′-ACUUACCUG) bind to the 5′-ss of pre-mRNA by RNA-RNA base paring. The consensus sequence of the 5′-ss was reported to be 5′-MAG/GURAGU, with "/" indicating the boundary between exon and intron, and "M" and "R" representing A or C and A or G, respectively (Mount, 1982). In many cases, however, the nucleotide sequences of the 5′-ss do not completely match with the above consensus sequence. Even though not completely conserved, such a 5′-ss can be recognized by U1 snRNA, and the splicing reaction is initiated. However, if the degree of conservation of the 5′-ss is too low, the association of U1 snRNA with pre-mRNA does not properly occur, and a splice defect can thus be induced (Faustino and Cooper, 2003; Wang and Cooper, 2007; Havens et al., 2013).

Cathepsin A (EC 3.4.16.5), hereafter abbreviated as CTSA, is a multifunctional lysosomal enzyme showing the catalytic activities of deamidase, esterase, and carboxypeptidase. In addition, this enzyme is essential for the stabilization of β-galactosidase and the activation of

Abbreviations: bp, base pair(s); cDNA, complementary DNA; CTSA, cathepsin A; EGFP, enhanced green fluorescent protein; kbp, kilobase pairs; kDa, kilodaltons; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; pre-mRNA, mRNA precursor; rAAV, recombinant adeno-associated virus; RT-PCR, reverse transcription-PCR; U1 snRNA, U1 small nuclear RNA; SD, standard deviation; SDS, sodium dodecyl sulfate; ss, splice site; ss-cDNA, single-stranded complementary DNA

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neuraminidase. Therefore, a genetic defect of CTSA causes a significant decrease in the activities of β-galactosidase and neuraminidase, resulting in an autosomal recessive lysosomal storage disorder called galactosialidosis (Shimmoto et al., 1993; Hiraiwa, 1999; Ketterer et al., 2017). About 80 patients with galactosialidosis have been reported globally. Based on the age of onset and severity of their symptoms, patients are classified as early infantile type, late infantile type, or juvenile/adult type. More than 60% of these patients have been reported in Japan, and most of them are of the juvenile/adult type (Hossain et al., 2016).

The gene for human CTSA is located on 20q13.1 (Wiegant et al., 1991). It consists of 15 exons (one 5′-non-coding exon and 14 coding exons) spanning approx. 7.5 kilobase pairs (kbp) and encodes a 480 amino acid-inactive precursor protein (Shimmoto et al., 1996). In the human *CTSA gene*, more than 10 missense mutations have been reported to cause galactosialidosis (OMIM®, 2009). In addition, an adenine to guanine base substitution at the +3 position of the 7th intron (NG_008291.1:g.7363A > G) is frequently found in juvenile/adult-type Japanese galactosialidosis patients (homozygous for this mutation or heterozygous with the other allele being a missense mutation) (Shimmoto et al., 1990, 1993). In this manuscript, this mutation is referred to as IVS7 +3a > g. With such a mutation, mature mRNA lacking the 7th exon (SpDEx7) is formed. Skipping of the 7th exon causes a frame shift in the transcripts, and thus functional mature CTSA protein is not produced. As a result, the IVS7 +3a > g mutation of the *CTSA gene* causes galactosialidosis. As treatment for galactosialidosis, transplantation of cells overexpressing human CTSA (Zhou et al., 1995; Hahn et al., 1998) and enzyme replacement therapy (Itoh et al., 2016) are currently being investigated. In addition, the injection of a recombinant adeno-associated virus (rAAV) vector expressing human CTSA under the control of a liver-specific promoter was reported to be effective for galactosialidosis treatment of CTSA-deficient mouse (Hu et al., 2012); and a low-molecular-weight chemical chaperone was reported to increase the endogenous β-galactosidase activity in CTSA-deficient cells (Hossain et al., 2016). However, for the development of new therapies for human galactosialidosis, a novel approach for treatment of this disorder is necessary.

In the IVS7 +3a > g mutation of the *CTSA gene*, it is predicted that U1 snRNA cannot interact with the 5′-ss of the 7th intron of the pre-mRNA; and, hence, skipping of the 7th exon occurs. To negate the splice defect caused by the 5′-ss mutation, the application of a U1 snRNA of which its 5′-end was modified to enable association with the mutated 5′-ss was reported (Havens et al., 2013). In the present study, we constructed a model experimental system using a *CTSA mini gene* harboring the IVS7 +3a > g mutation and examined possible modified U1 snRNA-mediated rescue from the splice defect of the human *CTSA gene* caused by this mutation.

2. Materials and methods

2.1. Construction of expression plasmids encoding U1 snRNAs

All recombinant DNA experiments were performed according to the guidelines of Tokushima University.

A genomic DNA fragment encoding the human *U1 snRNA gene* (accession number V00591), from its own promoter to the transcription termination signal, i.e., nucleotide sequence from −393 to +199 (nucleotide sequence numbered taking the 5′-end of mature U1 snRNA as +1) (Murphy et al., 1982), was prepared from human genomic DNA (Clontech, Palo Alto, CA, USA) by use of the polymerase chain reaction (PCR). The obtained DNA fragment was subcloned into pUC19. In this paper, the expression plasmid encoding wild-type U1 snRNA is referred to as the wild-type U1 plasmid. The expression plasmids of modified U1 snRNA were prepared by PCR using mutated oligonucleotide primers and the wild-type U1 plasmid as a template. Details of the plasmid construction are summarized in Supplemental methods.

2.2. Construction of CTSA mini gene plasmids

Genomic DNA fragments encoding the human *CTSA gene* (accession number NG_008291.1) were isolated by PCR using human genomic DNA (Clontech) as a template. Two regions, from exon 6 to exon 8, and from exon 3 to exon 11, were amplified with the primer pairs of primer 6 (sense) and primer 8 (antisense), and primer 3 (sense) and primer 11 (antisense), respectively. The obtained DNA fragment was subcloned into pcDNA3.1/Hygro(−) mammalian expression plasmid (Invitrogen, Carlsbad, CA, USA), in which DNA fragment encoding CTSA was sandwiched between those encoding enhanced green fluorescent protein (EGFP, Clontech) and c-Myc tag. These mini gene plasmids enabling transcription of the pre-mRNA of parts of the *CTSA gene* are referred to as wild-type *CTSA mini gene* plasmids (“exons 6–8” or “exons 3–11”). Details of the plasmid construction are summarized in Supplemental methods and Supplemental results Fig. S1A.

For construction of the *CTSA mini gene* plasmid harboring the IVS7 +3a > g mutation, the overlap extension PCR method was carried out with mutated oligonucleotide primers (Ho et al., 1989) using a wild-type *CTSA mini gene* plasmid as a template. The plasmids including the mutation were referred to as mutant *CTSA mini gene* plasmids (“exons 6–8” or “exons 3–11”). The detailed nucleotide sequence of *CTSA mini gene* plasmid (exons 3–11) is shown in Supplemental results Fig. S1B and C.

2.3. Cell culture and transfection

HeLa cells were cultured in Minimum Essential Medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (MP Biomedicals, Santa Ana, CA, USA) and 1% MEM Non-essential Amino Acids (Sigma-Aldrich). COS7 cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) containing 10% fetal bovine serum. Eighteen to 24 h before transfection, 3.5×10^5 cells were suspended in 2 mL of culture medium and plated in each well of a 6-well plate.

For RNA analysis (HeLa cells), basically, a 1-μg aliquot of the *CTSA mini gene* plasmid and a 2-μg aliquot of U1 plasmid were mixed with 3 μL of X-treme GENE HP (Roche Diagnostics, Indianapolis, IN, USA) in 100 μL/well Opti-MEM (Invitrogen) and added to each culture well. For protein analysis (COS7 cells), a 2-μg aliquot of the *CTSA mini gene* plasmid, a 1-μg aliquot of U1 plasmid, and a 0.3-μg aliquot of EGFP expression plasmid (only EGFP-coding region was inserted into pcDNA3.1/Hygro(−) plasmid) were mixed with 5 μL of Lipofectamine 2000 (Invitrogen) in 500 μL/well Opti-MEM and added to each well.

After incubation for 24 h, total RNA was prepared from HeLa cells by use of an RNeasy Plus Kit (QIAGEN, Hilden, Germany) or ISOGEN II (Nippon Gene, Tokyo, Japan) according to the methods recommended by the suppliers. For protein analysis, COS7 cells were dissolved in RIPA buffer (Nacalai Tesque, Kyoto, Japan) supplemented with 0.1% sodium dodecyl sulfate (SDS), and their lysates were prepared.

2.4. Reverse transcription-PCR (RT-PCR)

To obtain single-stranded complementary DNA (ss-cDNA), total RNA from HeLa cells was reversely transcribed with oligo(dT) primer. For specific amplification of the transcripts derived from the *CTSA mini gene* plasmid, ss-cDNA was amplified by 2 rounds of PCR. Briefly, ss-cDNA corresponding to the region sandwiched between the T7 promoter and BGH polyadenylation signal region of the pcDNA3.1/Hygro (−) plasmid was first amplified by using the primer pair of T7 primer (sense) and BGH pA primer (antisense). Then, the reaction mixture was diluted with distilled water. To obtain the complementary DNA (cDNA) fragment corresponding to the region from exon 6 to exon 8 of the human *CTSA gene*, we amplified a diluted reaction mixture by using the primer pair of primer 6 and primer 8. The reaction mixtures of second PCR were subjected to polyacrylamide gel electrophoresis (PAGE) with DNA size marker (pUC19 digested with *Msp*I). The gel was stained with