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ORIGINAL ARTICLE

Mutation-adapted U1 snRNA corrects a splicing error of the dopa decarboxylase gene

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Abstract

Aromatic L-amino acid decarboxylase (AADC) deficiency is an inborn error of monoamine neurotransmitter synthesis, which results in dopamine, serotonin, epinephrine and norepinephrine deficiencies. The *DDC* gene founder mutation IVS6 + 4A > T is highly prevalent in Chinese patients with AADC deficiency. In this study, we designed several U1 snRNA vectors to adapt U1 snRNA binding sequences of the mutated *DDC* gene. We found that only the modified U1 snRNA (IVS-AAA) that completely matched both the intronic and exonic U1 binding sequences of the mutated *DDC* gene could correct splicing errors of either the mutated human *DDC* minigene or the mouse artificial splicing construct *in vitro*. We further injected an adeno-associated viral (AAV) vector to express IVS-AAA in the brain of a knock-in mouse model. This treatment was well tolerated and improved both the survival and brain dopamine and serotonin levels of mice with AADC deficiency. Therefore, mutation-adapted U1 snRNA gene therapy can be a promising method to treat genetic diseases caused by splicing errors, but the efficiency of such a treatment still needs improvements.

Introduction

Splicing of pre-mRNAs involves the excision of introns and ligation of exons, which requires the recognition of splice sites located at the 5' and 3' end of introns (1). The splice sites are recognized by the U snRNPs U1, U2, U4, U5 and U6, and each U snRNP is formed by a complex of different proteins and a U-specific small nuclear RNA (snRNA) (2). At the initial steps of the splicing process, U1 is recruited to the splice donor site. U1 snRNA binds to the splice donor through Watson-Crick base pairing, involving the last three nucleotides of the exon (positions -3 to-1) and the first six nucleotides of the downstream intron (3).

Splice donor mutations lower the binding affinity of U1 and cause splicing defects in approximately 15% of patients with monogenetic diseases (4). In 2002, modified U1 snRNAs were shown to suppress the expression of a target transgene (5). However, only a handful of gene therapy studies employing U1 snRNA have been reported. In patient-derived cells, mutation-adapted U1 snRNAs were shown to correct a splice defect (6–9). Published animal studies include rescued dystrophin synthesis and muscle function in *mdx* mice (10,11) and increased plasma hFVII levels in a mouse model of haemophilia (12). U1 snRNP-dependent therapy in cancer are also possible (13).

Aromatic L-amino acid decarboxylase (AADC) deficiency (MIM #608643) is a rare neurotransmitter synthesis disorder that results in deficiencies in monoamine neurotransmitters dopamine and serotonin and downstream metabolites (14,15).

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Clinical manifestations of AADC deficiency include hypotonia, hypokinesia, oculogyric crises, autonomic dysfunction and developmental delay, and most patients die in childhood (16–19). AADC deficiency is more common in Taiwan than in other countries because of a founder splice site mutation (IVS6 + 4A > T) in the Taiwanese population (19). This mutation destroys the splice donor consensus sequence of intron 6, which results in aberrant splicing of the gene (20,21).

Treatments for AADC deficiency, including a combination of vitamin B6, dopamine agonists, monoamine oxidase inhibitors and anticholinergics, are generally ineffective (18). To develop new treatments for AADC deficiency, we knocked in the IVS6 + 4A > T mutation and created a mouse model of AADC deficiency (Ddc^{KI} mice). Young homozygous Ddc^{KI} mice exhibit biochemical and movement abnormalities similar to those in patients with AADC deficiencies (22). Previously, we have applied gene therapy by using the AAV9 vector via intracerebroventricular injection of neonatal mice (23) or intraperitoneal injection of young mice to express AADC under the control of a ubiquitous CMV promoter or a neuronal preferential synapsin I promoter (24).

In this study, we employed a different strategy. We tried to repair the splicing of the mutated gene so the restored AADC expression will be under its intrinsic control mechanisms. We demonstrated the efficacy and safety of such a method in both cell culture and a mouse model.

Results

Modified U1 snRNA rescues the splicing of a mutated *DDC* minigene

The IVS6 + 4A > T mutation of the human *DDC* gene causes a 37-bp insertion after exon 6 because of the presence of the +37 bp cryptic splice donor site within intron 6. We constructed a *DDC* minigene in which a human *DDC* cDNA is interrupted between exons 6 and 7 by a shortened human intron 6 (Fig. 1A). When a minigene containing the IVS6 + 4A > T mutation was expressed in N2A cells, the 37-bp-inserted mRNA was the only spliced mRNA species (Fig. 1B). After we cotransfected the cells with a 3-fold molar excess of U1 vectors U1, U1m, or IVS, the results did not change (Fig. 1B). However, the cotransfection of IVS-AAA resulted in correct splicing of half the spliced mRNA (Fig. 1C).

Modified U1 snRNA improved the splicing of a mutated mouse artificial splicing construct

The mouse *Ddc* gene does not have the +37 bp cryptic splice donor found in human intron 6. We made an artificial splicing construct (In5-6) that contains a gene fragment spanning mouse *Ddc* gene from exon 5 to exon 7 except that the internal portion of intron 6 was removed to allow the construct to fit in an expression vector (Fig. 2A). Correct splicing of this construct connected exons 5, 6 and 7 (Fig. 2B). Splicing of a construct containing the IVS6 + 4A > T mutation resulted in the occurrence of shorter mRNA caused by skipping of exon 6 during splicing (Fig. 2B). When we cotransfected U1 vectors U1, IVS, or IVS-AAA, only IVS-AAA decreased the portion of exon 6 skipping (Fig. 2B). A 9-fold molar excess of IVS-AAA was required to effectively prevent exon 6 skipping (Fig. 2C and D).

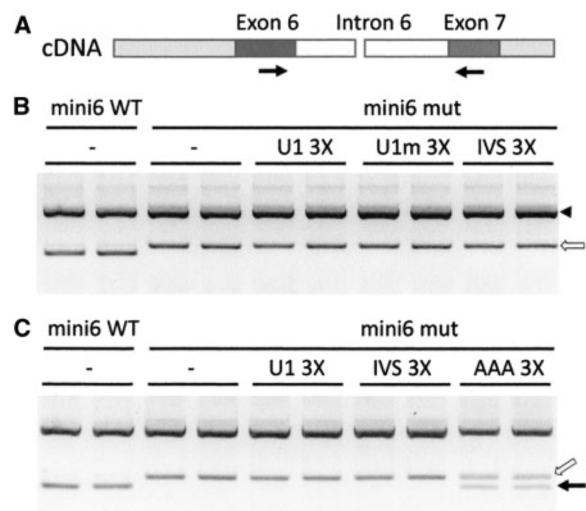

Figure 1. Results from the *DDC* minigene splicing assay. (**A**) A diagram of a region of human *DDC* cDNA in which an internally deleted human *DDC* intron 6 is inserted between exon 5 and 6. Locations of PCR primers (arrows) are indicted. (**B**) Results of RT-PCR after the expression of wild-type (WT) or IVS6 + 4A > T mutated (mut) minigenes in N2A and a 3-fold molar excess of U1 vectors (U1, U1m, or IVS) in N2A cells. The arrow indicates correctly spliced mRNA, the open arrow indicates 37-bp inserted mRNA, and the arrowhead indicates un-spliced mRNA. (**C**) A comparison between effects of U1, IVS, and IVS-AAA (AAA). Only IVS-AAA rescues the splicing error.

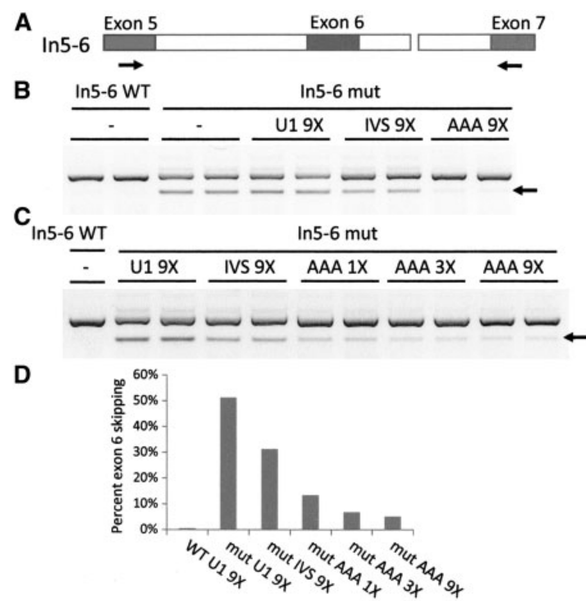

Figure 2. Results from the mouse *Ddc* artificial splicing assay. (**A**) A diagram of the mouse artificial splicing construct (In5-6) in which intron 6 is internally deleted. Locations of PCR primers (arrows) are indicated. (**B**) Results of RT-PCR after the expression of wild-type (WT) or IVS6 + 4A > T mutated (mut) splicing constructs and a 9-fold molar excess of U1 vectors (U1, U1m, and IVS-AAA) in N2A cells. The arrow indicates the exon 6-skipped mRNA. Only IVS-AAA improves splicing of the mutated mRNA. (**C**) A dose-responsive experiment for IVS-AAA. (**D**) Results from quantitative PCR shown as percent exon 6 skipping. The Y-axis indicates the percent exon 6 skipping.

Gene therapy with modified U1 snRNA improved survival and increased brain neurotransmitter levels of AADC deficiency mice

To demonstrate the therapeutic effect of modified U1 snRNA, we produced an AAV9 vector that expresses IVS-AAA. Newborn

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Ddc^{KI} mice were injected into bilateral cerebral ventricles 2 µl of phosphate-buffered saline (PBS) or $1x10^{10}$ vg/µl (low dose) or $2x10^{10}$ vg/µl (high dose) of AAV9-IVS-AAA vector. Because Ddc^{KI} mice were fragile after intraventricular injection, we sacrificed the mice at two weeks of age. The results revealed that all high-dose IVS-AAA-injected Ddc^{KI} mice survive to two weeks, while only half of the PBS-injected Ddc^{KI} mice did so ($p = 0.0229$, Logrank test) (Fig. 3A). However, IVS-AAA injection did not improve the body weight of the Ddc^{KI} mice, and very few mice in either groups survive to 4 weeks that rotarod test can be performed (Fig. 3B). We further measured brain neurotransmitter levels in these mice. High-dose IVS-AAA injection significantly elevate brain dopamine and serotonin levels ($p = 0.006$ in both dopamine and serotonin) to around 10% of the wide-type mouse levels (Fig. 4). The high-dose IVS-AAA-injected Ddc^{KI} mice had higher levels of correctly spliced mRNA in the striatum than the PBS-injected Ddc^{KI} mice (Supplementary Material, Fig. S1).

Discussion

Design of a therapeutic U1 snRNA

The sequences of a specific splice donor can differ from the consensus sequences. The three nucleotides of the *DDC* gene splice donor (TTTgtaagt) are quite different from the U1 consensus sequence (GUCcauuca). Our data revealed that the U1 snRNA vector sequences needed to be completely complementary to the sequences of the Ddc splice donor in order to see a therapeutic effect. Our results are similar to a previous report that full complementarity of U1 increased the recognition of the mutant splice donor site (9). In the current study, we demonstrated in a cell model that a modified U1 snRNA improved the splicing of an IVS6 + 4A > T mutated donor site in human *DDC*. We then showed a similar effect on the mouse sequences in cells and finally proved a therapeutic effect by gene therapy in a mouse model of AADC deficiency.

Benefit of a therapeutic U1 snRNA over gene therapy

We are now conducting a gene therapy clinical study using adeno-associated virus (AAV) type 2 vector to express an AADC cDNA in bilateral putamens of patients with AADC deficiency, and our preliminary results revealed that gene therapy only partially improves the motor function of patients (16). However, when we employed a wider spreading AAV vector (AAV9) in the gene therapy of mice with AADC deficiency, we observed hyperactivity of the mice, most likely due to the overexpression or ectopic expression of the transgene. One solution to control expression in gene therapy is to use a specific or authentic promoter. Unfortunately, the AADC gene promoter, as with many other genes, is not well defined. In contrast, if we improve the splicing of the mutated gene, the gene products are still under the control of its endogenous promoter.

Toxicity of U1 snRNA therapy

The injection of plasmids or AAV vectors encoding a modified U1snRNA increased plasma hFVII levels in a mouse model of haemophilia, but hepatocellular toxicity was observed (12). Off-target effects of U1 snRNA therapy have been considered, because the targeted sequences are composed of only 9 nucleotides. Towards the goal of overcoming the off-target effects, Fernandez Alanis et al. have proposed Exon Specific U1snRNA (ExSpeU1) to target non-conserved intronic sequences downstream of the splice donor site (25), but the sequences of mature mRNA will be modified. In the current study, we did not see toxic effects in the injected AADC deficiency mice within the study period.

Efficacy of the treatment

In the current study, bilateral intraventricular injection of 2×10^{10} vg/µl of IVS-AAA vector exhibited a therapeutic effect on DDC^{KI} mice, and a lower dose (1×10^{10} vg/µl) was not as effective. However, the injected mice survive did not gain weight at 2 weeks, and only few of them survive 4 weeks that rotarod test can be performed. This efficacy is lower than these from gene therapy with AAV9-CMV-hAADC (2×10^{10} vg/µl) that the injected mice gained weight at 2 weeks, the increase in brain dopamine levels to 30% of wild-type mice, and survival beyond 4 weeks (23). This may be explained by a low efficiency of the U1snRNA, for example, a 9-fold molar excess of the IVS-AAA plasmid over the splicing construct was required to obtain a maximal effect

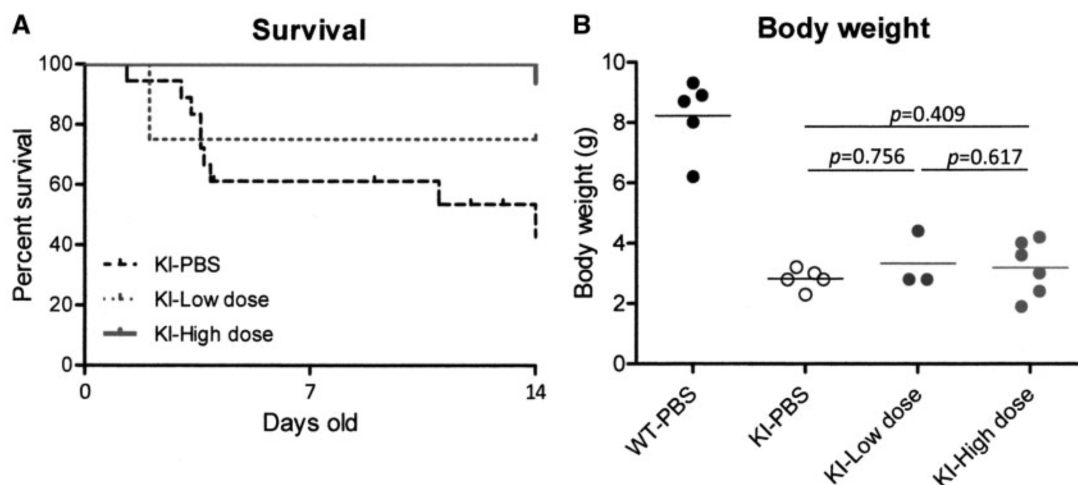

Figure 3. Survival and body weight after gene therapy with modified U1 snRNA in mice with AADC deficiency. Wild-type (WT) were injected with PBS, and Ddc^{KI} (KI) mice were injected with PBS, low-dose, or high-dose IVS-AAA vector. (**A**) Kaplan-Meier survival curve demonstrates that the high-dose IVS-AAA injected KI mice had a better survival than injected with PBS. (**B**) Body weights of the PBS or IVS-AAA injected mice were similar.

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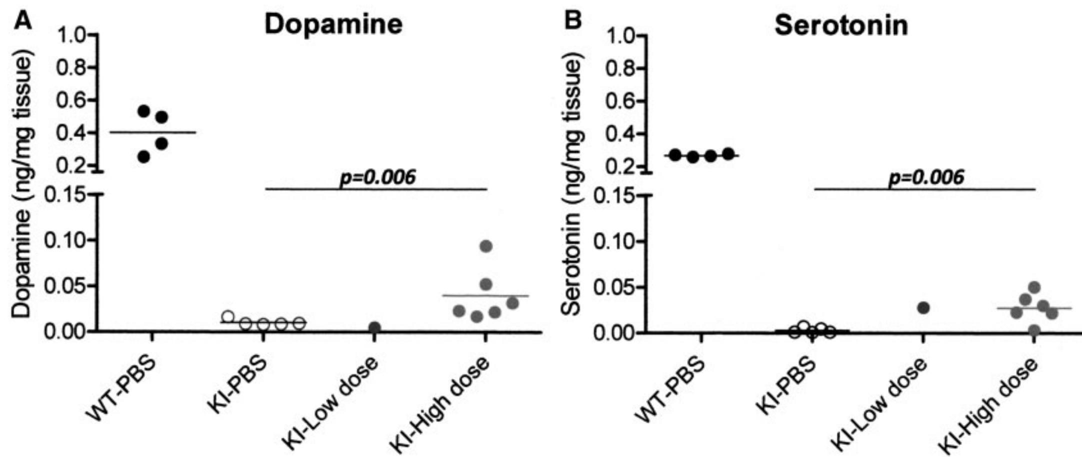

Figure 4. Brain neurotransmitter levels after gene therapy with modified U1 snRNA in mice with AADC deficiency. (**A**) Brain dopamine levels were higher in the high-dose IVS-AAA injected Ddc^{KI} (KI) mice than in PBS injected KI mice ($p = 0.006$). (**B**) Brain serotonin levels were higher in the high-dose IVS-AAA injected Ddc^{KI} (KI) mice than in PBS injected KI mice ($p = 0.006$).

in the cell models. Further developments of U1snRNA therapy will be necessary to be able to translate this treatment to human trials.

Materials and Methods

U1 snRNA expression vectors

The mouse U1 snRNA gene was cloned into pcDNA3.1 vector by polymerase chain reaction (PCR) with the upper primer 5'-GAGA GGCAGACGTCACTTCC and lower primer 5'-TGATGCTCGCTT TTTCTCCT. Splicing donor consensus sequences include the last three nucleotides in the exon and the first six nucleotides in the intron. Modified U1 genes were created by the PCR-mediated mutagenesis method that IVS-AAA matches both the exonic and intronic sequences of the *DDC* gene intron 6 donor, IVS only matches the intronic sequences, and U1m partially matches the exonic sequences (Fig. 5). To construct the adeno-associated virus (AAV) type 9 vector, the expression cassette consisted of a cytomegalovirus immediate-early promoter followed by the first intron of the human growth hormone gene, U1 gene and simian virus 40 polyadenylation signal sequence (16). The AAV9 vector was packaged and titred at the University of Florida Powell Gene Therapy Center Vector Core Laboratory using previously published methods (26). The titre of the viral preparations used in this study was 1.96×10^{13} viral genomes (vg) per ml.

Human DDC minigene construct

The *DDC* minigene was constructed by inserting an internally truncated intron 6 into plasmid pAAV-hAADC. The shortened human intron 6 was composed of a piece of 5' intron sequences containing the splice donor and the +37 cryptic splice donor, and 3' intron sequences containing the splice acceptor. To detect aberrant splicing, a PCR crossing the exon 6 and 7 border gives a product size of 202 bp for correctly spliced mRNA and 165 bp for aberrantly spliced mRNA.

Mouse artificial splicing construct

The intron 6 splice donor sequences are completely identical between the human *DDC* gene and mouse *Ddc* gene. The mouse artificial splicing construct was composed of mouse *Ddc* gene partial exon 5 (3' sequences), intron 5, exon 6, a shortened intron 6 (5' sequences containing the splice donor and 3' sequences containing the splice acceptor), and partial exon 7 (5' sequences). These sequences were combined and cloned into the pcDNA3.1 vector (Plasmid In5-6). A PCR crossing exon 6 gives a product size of 285 bp for correctly spliced mRNA and 141 bp for exon 6-skipped mRNA.

Cells, transfection and mRNA quantitation

N2A cells were grown in DMEM medium supplemented with 10% FCS. Cells were seeded in 6-well culture plates one night before transfection. Transfection was conduction using Lipofectamine 2000 (Life). Cells were harvested 2 days after transfection. RNA was extracted using the Trizol reagent, and reverse transcription (RT) was performed using Superscript II reverse transcriptase and oligo dT primer. Quantitative PCR (qPCR), on a StepOnePlus real-time PCR system (Thermo-Fischer Scientific), was used to determine the amount of mRNA. In the mouse artificial splicing study, qPCR reactions, employing the KAPA SYBR FAST qPCR Kits (Kapabiosystems), specific to either the correctly spliced or exon 6-skipped products were designed and percentage correct splicing was calculated as the amount of correctly spliced mRNA divided by the total *Ddc* mRNA (correctly splicing + aberrantly splicing). Mouse brain mRNA quantitation employed a TaqMan Real-Time PCR assay (Thermo-Fischer Scientific). Expression of GAPDH was used as an internal control and mRNA expression levels relative to the PBS-injected wild-type mice was calculated.

Animals

All experimental procedures were approved and performed in accordance with the guidelines of the National Taiwan University College of Medicine and College of Public Health Institutional Animal Care and Use Committee (IACUC No. 20110134). The mouse model of AADC deficiency (Ddc^{KI} mice) was generated by knocking-in a splicing mutation into the *Ddc* gene (22). Age-matched wild-type (WT) and heterozygous (Het) littermates and untreated Ddc^{KI} mice were used as controls.

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Symbol	Sequence	Notes
U1	GUCcauucaua-5′	U1 consensus RNA sequence
U1m	GAGcauucaua-5′	Modified U1 RNA sequence
IVS	GUCcatacaua-5	Modified U1 RNA sequence
IVS-AAA	AAAcatacaua-5′	Modified U1 RNA sequence
DDC	TTTgtatgtcc-3′	DDC mutated gene sequence

Figure 5. The mouse U1 snRNA consensus (U1), modified U1 (U1m, IVS, and IVS-AAA) and mutated human *DDC* gene sequences. Exonic sequences are in upper case and intronic sequences are in lower case.

Intracerebroventricular (ICV) injection

Neonatal Ddc^{KI} mice within 24 h of birth (P0) received bilateral injection into the lateral ventricle 2 μl of 1×10^{10} vg/μl (low dose), 2×10^{10} vg/μl (high dose) AAV9-IVS-AAA vector or phosphate-buffered saline (PBS) using a 10-μl Hamilton syringe (Hamilton, 84853, 801RN26S/2"2, Reno, NV) with a 31-gauge needle (Hamilton, 31GA 0.5" PT2 RN, Reno, NV) as previously described (27). Mice were subjected to cryoanaesthesia until their activity slowed. The needle was inserted perpendicularly to the skull surface at two-fifths of the distance along the line between the eye and lambda with a depth of 2 mm. After bilateral injection of the ventricles, the pups were placed on a warming pad until they were warm and active. The injected pups were then returned to their mothers. Survivals and body weight of each injected pups were recorded and analysed.

Mouse brain neurotransmitter measurements

Immediately following euthanasia at 2 weeks of age, mouse brain halves were flash-frozen in liquid nitrogen and stored at $-80\,^{\circ}$C for neurotransmitter measurements. To determine neurotransmitter levels, brain tissues were deproteinized with 0.2 M perchloric acid and centrifuged three times at 14,000*g* for 5 min. Dopamine and serotonin levels were analysed using high-performance liquid chromatography (HPLC) and a previously reported protocol consisting of electrochemical detection after separation with a Thermo Scientific Hypersil GOLD HPLC column (Part Number: 25005-154630; 5 μm particle size) (28).

Statistical analysis

The data are presented as the mean in each figure. Statistical analyses were performed using the SPSS (Statistical Package for the Social Sciences) statistical software package, version 11.5. The Mann-Whitney rank test was applied for statistical analyses between groups. The percentage of survival was analysed using Log-rank test. A p value <0.05 was considered significant.

Supplementary Material

Supplementary Material is available at *HMG* online.

Conflict of Interest statement. None declared.

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