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Securinine enhances *SMN2* exon 7 inclusion in spinal muscular atrophy cells

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ABSTRACT

Spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by the degeneration of motor neurons in the spinal cord, leading to muscular atrophy. SMA is caused by deletions or mutations in the *survival motor neuron* gene (*SMN1*) on chromosome 5q13. A second copy of the *SMN* gene (*SMN2*) also exists on chromosome 5, and both genes can produce functional protein. However, due to alternative splicing of the exon 7, the majority of SMN protein produced by *SMN2* is truncated and unable to compensate for the loss of *SMN1*. Increasing full-length SMN protein production by promoting the exon 7 inclusion in *SMN2* mRNA or increasing *SMN2* gene transcription could be a therapeutic approach for SMA. In this study, we screened for the compounds that enhance *SMN2* exon 7 inclusion by using *SMN2* minigene-luciferase reporter system. We found that securinine can increase luciferase activity, indicating that securinine promoted *SMN2* exon 7 inclusion. In addition, securinine increased full-length *SMN2* mRNA and SMN protein expression in SMA patient-derived lymphoid cell lines. To investigate the mechanism of securinine effect on *SMN2* splicing, we compared the protein levels of relevant splicing factors between securinine-treated and untreated cells. We found that securinine downregulated hnRNP A1 and Sam68 and upregulated Tra2-β1 expression. However, securinine, unlike HDAC inhibitors, did not enhance *tra2-β1* gene transcription, indicating a post-transcriptional mechanism for Tra2-β1 upregulation. Furthermore, we treated SMA-like mice with securinine by i.p. injection and found that securinine treatment increased *SMN2* exon 7 inclusion and SMN protein expression in the brain and spinal cord. According to our results, securinine might have the potential to become a therapeutic drug for SMA disease.

1. Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disease caused by motor neuron degeneration in the spinal cord [1]. SMA is one of the most common inherited diseases which cause infant death. SMA occurs in approximately 1 in 10,000 newborns and has a carrier frequency of approximately 1 in 50 [2]. The disease was classified into three major types and one minor type according to disease severity and onset age. SMA type I is the most severe type, leading to death within the first two years of age. SMA type II is the intermediate severe type with a childhood onset. Patients with SMA type III have onset of symptoms after 18 months and are able to achieve adult stage. SMA type IV is the mildest type with an adult onset [3,4].

SMA is caused by *SMN1* (*survival of motor neuron 1*) gene deletion or mutation, resulting in SMN protein deficiency [5]. A small amount of SMN protein is produced from a gene similar to *SMN1* called *SMN2* (*survival of motor neuron 2*). The major

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difference between *SMN1* and *SMN2* is the 6th nucleotide of exon 7 (a C to T transition). The transition causes exon 7 exclusion in most *SMN2* mRNA and produces unstable SMN protein [6]. Deficient SMN protein causes motor neuron death, which leads to SMA disease symptoms. However, the specific role of the ubiquitously expressed SMN protein in motor neuron cells remains unclear.

The mechanism of *SMN1* and *SMN2* mRNA alternative splicing has been known as two hypotheses: 1. *SMN1* gene exon 7 contains an exonic splicing enhancer (ESE) sequence, which promotes ASF/SF2 binding and causes *SMN1* mRNA exon 7 inclusion [7]. 2. The C-to-T transition of *SMN2* exon 7 loses the ESE sequence and produces an exonic splicing silencer (ESS) sequence, which promotes hnRNP A1 binding and leads to *SMN2* mRNA exon 7 skipping [8,9]. The known splicing factors involved in *SMN2* exon 7 splicing include SRp30c [10], Tra2-β1 [11], TDP-43 [12], TIA1 [13], Sam68 [14], hnRNP A1 [15], hnRNP Q [16], hnRNP G [17], and other factors [18]. According to previous studies, SRp30c, Tra2-β1, TDP-43, TIA1, hnRNP Q and hnRNP G promote *SMN2* exon 7 inclusion. In contrast, hnRNP A1 and Sam68 inhibit *SMN2* exon 7 inclusion.

The functional SMN complex, containing SMN protein, Gemin2-8 and other proteins, is involved in snRNP complex assembly [19], mRNA translocation [20], signal recognition particle (SRP) biogenesis [21], and neuron-specific apoptosis [22]. The SMN protein also associates with telomerase and is likely to function in human telomerase biogenesis [23]. The SMN complex concentrates as dot structures in the nucleus named gems. The number of gems is known as an indicator of SMN protein expression levels and SMA severity [24].

Because all SMA patients carry at least one copy of *SMN2* gene, increasing full-length SMN protein production by promoting the exon 7 inclusion in *SMN2* mRNA and/or increasing *SMN2* gene transcription could be a therapeutic approach for SMA. Recent therapeutic strategies have been classified into several types: small molecule treatment, protein-based therapy, gene therapy, stem cell based therapy, antisense oligonucleotide (ASO)-based therapy, and other therapies [25]. The small molecules used to treat SMA include histone deacetylase (HDAC) inhibitors, translation read-through compounds, quinazolines, hydroxyurea, antibiotics, signal transducing molecules, and neuroprotective compounds [25]. Some of these drugs were already in clinical trials. However, there is still no cure for SMA and the therapeutic drugs for SMA need further exploration. In this study, we screened for small compounds that promote *SMN2* exon 7 inclusion using *SMN2* minigene-luciferase reporter system [26] in NSC34 motor neuron cell line.

2. Materials and methods

2.1. Cell culture and transfection

NSC34 cell line and SMA type 1 fibroblast cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2. SMA type 1 fibroblast cells were primary cells established from SMA patient skin biopsies [27]. SMA type 1 patient-derived lymphoid cell lines we used were transformed with Epstein–Barr virus and became immortal cell lines [28]. SMA type 1 lymphoid cell lines were grown in RPMI 1640 containing 10% fetal bovine serum. NSC34 cells were transfected with pIRES-*SMN2* minigene-luciferase plasmid, which was provided by Dr. Zhang et al. [26], by Lipofectamine® 2000 Transfection Reagent (Invitrogen) according to manufacturer's instructions. After 24 h of transfection, the cells were selected by G418 (600 μg/mL) until the colony formation. Then, the cells were expanded from one single colony and used for compound screening.

2.2. Luciferase activity assay

The NSC34 cells that expressed *SMN2* minigene-luc or *Tra2-β1* promoter-luc were seeded 2×10^4 in 96-well plate and incubated for 48 h. The cells were treated with different compounds or different doses for 16 h. Then, the cells were lysed and assayed for luciferase activity by using Steady-Glo® Luciferase Assay System (Promega) according to manufacturer's instructions.

2.3. RNA extraction and RT-PCR

Total RNA was extracted from tissues or cells by TRIzol® Reagent (Invitrogen). One microgram RNA was reverse-transcribed into cDNA by SuperScript® III Reverse Transcriptase kit (Invitrogen). The PCR primers were as follows: *SMN2* forward, 5′-CCC ACC ACC TCC CAT ATG TCC-3′, and *SMN2* reverse, 5′-AAC TGC CTC ACC ACC GTG CTG-3′. The *SMN2* PCR reaction was carried out as following programs: 94 °C for 5 min, then 35 cycles of 94 °C for 30 s, 60 °C for 45 s, 72 °C for 1 min, and final extension at 72 °C for 7 min. The PCR products separated by 3% agarose gel electrophoresis included exon 7-containing *SMN2* mRNA product (421 bp) and exon 7-lacking *SMN2* mRNA product (367 bp).

2.4. Protein extraction and Western blotting

The proteins were isolated from cells by using RIPA lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP40, 1% sodium deoxycholate, 0.1% SDS) and analyzed in SDS-PAGE as the following description: the proteins were transferred onto nitrocellulose membrane (GE Healthcare) after electrophoresis. The nitrocellulose membranes were blocked with 5% skim milk solution in TBST for 60 min. The nitrocellulose membranes were incubated with the primary antibody in 1% skim milk solution for 60 min, and then incubated with HRP-conjugated secondary antibody for 60 min. After washing three times with TBST, the nitrocellulose membranes were exposed in the ECL plus substrate (Amersham) for five minutes. The chemiluminescence signals were detected by X-ray films and the expression levels were analyzed by LabWorks Image Analysis Software. The primary antibodies used were anti-SMN (BD), anti-TRA2B (Abcam), anti-SF2/ASF (Zymed Laboratories Inc.), anti-hnRNP A1 (9H10, Sigma-Aldrich), anti-SR protein (1H4, Zymed Laboratories Inc.), anti-Sam68 (Santa Cruz Biotechnology, Inc.), anti-TDP-43 (Abcam), anti-TIA1 (Abcam), and anti-Actin (Santa Cruz Biotechnology, Inc.). The secondary antibodies used were horse anti-mouse IgG-HRP (Cell Signaling), Goat anti-rabbit IgG-HRP (Life Technologies), and donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Inc.).

2.5. Immunofluorescence

SMA type 1 lymphoid cells were incubated on poly-lysine-coated coverslips for 2 h after being treated with 10 μM securinine for 72 h. Then, the cells were fixed by 4% paraformaldehyde for 30 min and permeabilized by 0.1% Triton X-100 for 10 min. The cells were blocked with 1% BSA for one hour and incubated with anti-SMN antibody (1:150) in 1% BSA for one hour. Then, the cells were incubated with rhodamine-conjugated goat anti-mouse IgG for one hour in dark. The cell nuclei were stained with DAPI (0.1 μg/mL) for 5 min in dark. The cells were examined under a confocal laser scanning microscope. The slides were washed with PBS for 3 times between each step. All solutions were prepared in PBS.

2.6. Cell viability

SMA type 1 lymphoid cells were seeded 1×10^4 in 96-well plate and treated with different doses of securinine for 60 h. The cell