NOVA1 Promotes *SMN2* Exon 7 Splicing via Binding the UCAC Motif and Increases SMN Protein Expression

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Abstract

Spinal muscular atrophy (SMA) is a rare hereditary neuromuscular disease with high lethality rate in infants. Homologous genes *SMN1* and *SMN2* were reported to be SMA pathogenic factors. Studies showed that high inclusion of SMN2 exon 7 increased SMN expression which in turn ameliorated the severity of SMA. The inclusion rate of SMN2 exon 7 was higher in neural tissues than that in non-neural tissues. Expression of splicing factors that regulate inclusion of SMN2 exon 7 were significantly increased in neural tissues compared to non-neural ones. A positive correlation was checked between expression of neuro-oncological ventral antigen 1 (NOVA1) and SMN in central nervous system. In addition, reduced number of neurons in the spinal cord anterior horn was determined by Nissl staining in SMA mice from postnatal day 1 to 7 continuously. Meanwhile, NOVA1 was presented in motor neurons and gradually decreased as SMA ongoing. Moreover, SMN2 exon 7 inclusion and protein level were enhanced by overexpressing NOVA1, while the enhancement was reversed when NOVA1 knockdown in vitro. Finally, the "YCA Y" motif (Y is pyrimidine, U or C) was located in the exon 7 of SMN2 and was critical for NOVA1 binding and promoting the inclusion of exon 7. Mutagenesis experiments revealed that CA was essential for the exon 7 inclusion while less influence was detected by changing order of Y in the motif. Collectively, NOVA1 interacted with "YCA Y" motif in exon 7 of SMN2 and thus enhanced the inclusion of exon 7 in SMN2 which in turn increased the level of SMN protein. Our data may provide new insights into the treatment of SMA disease.

Introduction

Spinal muscular atrophy (SMA), a rare hereditary neuromuscular disease, is mainly characterized by degeneration of alpha motor neurons in the anterior horn of the spinal cord, which affects the innervation of skeletal muscles, leading to muscle weakness, muscle atrophy, paralysis or even death [1]. The survival of motor neuron 1 (*SMN1*) gene, one of the SMA pathogenic genes, was located at 5q13. Deletion of exon 7 and 8 or only exon 7 results in inability of functional full length SMN protein [2]. SMN is a protein known for its housekeeping role in the SMN-Gemin multiprotein complex [3]. It is involved in the assembly of small nuclear protein (SnRNP), regulating the splicing of precursor mRNA (pre-mRNA), and also participating in a variety of physiological processes, including stress response, axon transport, cytoskeletal dynamics, mitochondrial and bioenergy pathways, and ubiquitin pathways [4]. Please insert a sentence on the inadequacy of current study on SMN.

Two homologous genes, *SMN1* and *SMN2*, were reported to be presented in human genome. The 6th nucleotide was C in exon 7 of *SMN1* gene, while a T in the same location of *SMN2* gene. Although *SMN2* can produce stable SMN protein as *SMN1*, while only 10% of the transcripts was full length that encoded stable SMN protein, the rest was exon 7 truncated (*SMN Δ 7*) transcripts that produced truncated proteins without biological functions and extremely unstable [5]. Hence, the variation of the 6th nucleotide was the key factor that influenced inclusion of exon 7 [6]. Clinical studies have shown that the severity of SMA disease is negatively correlated with the amount of functional SMN protein and *SMN2* copy numbers [7, 8]. Amount of full-length SMN protein generated by *SMN2* is not sufficient to compensate for the defect

of *SMN1*, however, promoting the inclusion of *SMN2* exon 7 can effectively ameliorate the severity of SMA disease and improve survival of patients [9–11]. Hence, transcripts of *SMN2* gene that exon 7 included or excluded account more and more importance in treatment of SMA disease.

Alternative splicing is one of the important mechanisms that regulate protein diversity and the complexity of gene abundance, and is involved in the occurrence and development of many diseases. Two types of splicing factor (SF) are involved in regulating pre-mRNA splicing. A typical type I SR protein that promotes splicing of pre-mRNA contains an RNA recognition motif (RRM) and RS region with repetitive sequences of serine and arginine [12, 13]. While type II SFs inhibit splicing of pre-mRNA, for example the HNRNPs protein with KH domain [14]. Related studies have shown that the splicing factor NOVA, which is specifically expressed in neurons, exhibits splicing promotion or inhibition depending on which binding sites the protein located [15]. The SR and HNRNP protein families have been proved to play an important role in the regulation of *SMN2* splicing [16, 17]. For example, Hua Yimin et al. blocked the binding of HNRNP A1/A2 to the intron splicing silencer located in *SMN2* intron 7, and thus significantly increased the inclusion of *SMN2* exon 7 and up-regulated the expression of full-length SMN protein [18]. Moreover, the nerve-specific expressed splicing factor NOVA exerted key role in the development of neural system and neurological diseases [19], while the relationship between NOVA family and SMA disease remains unclear.

Here, according to the tissue specificity of *SMN2* exon 7 inclusion and splicing factor expression in Taiwanese SMA mouse model, we screened out NOVA1 that closely related to *SMN2* exon 7 inclusion from a great number of splicing factors. At the same time, the dynamic expression of NOVA1 in tissues from SMA mice at different age was correlated with the progress of SMA disease. In vitro, the regulatory mechanism of NOVA1 on *SMN2* exon 7 inclusion was revealed by the minigene system and RNA pull down experiment. This study will provide new ideas for correcting *SMN2* gene splicing and SMA disease treatment.

Materials And Methods

Animals

Total 50 mice of SMA mice (genotype *smn*^{-/-}*SMN2*^{tg/0}) and littermate control ones (genotype *smn*^{+/-}*SMN2*^{tg/0}) at P1, P4 and P7 were used in this study. The background strains are FVB inbred mice. The parental mice were donated from Hua-Lab of the Institute of Neuroscience in Soochow University, and were cultured in the Specific pathogen free (SPF) barrier system in Experimental Animal Center of Nantong University, where the animal experiment was carried out. All mice were free to water and food, and the light cycle was 12h light and 12h dark. The use of experimental animals adhered to the 3R principle, and the animal experiment had been approved by the Experimental Animal Ethics Management Committee of Nantong University (IACUC No. 20181008-088). The raise condition and the ethic number should be supplied.

RT-PCR

Total RNA was extracted from tissues or cell line by TRIzol reagent following the protocol of the manufacturer (Vazyme R401-01). In brief, 1µg of each RNA sample was used per 20µL reaction for first-strand cDNA synthesis with oligo (dT)₁₈ and M-MLV reverse transcriptase. The RT-PCR reaction system used to detect the inclusion level of *SMN2* exon 7 was 12.5µL 2× Taq master mix (Vazyme P112), 1µL Forward primer, 1µL Reverse primer, 1µL cDNA and 9.5µL ddH₂O. The above mixture were amplified using 28 PCR cycles, including 95°C for 15s, 60°C for 30s and 72°C for 45s, which was using to detect *SMN2* exon 7 inclusion level in SMA mice. Primers was as follows: E6-F: Cy5-5'-ATAATTCCCCCACCACCTCCC-3', E8-467R: 5'-TTGCCACATACGCCTCACATAC-3'. RT-PCR reaction procedure used for detecting the inclusion level of *SMN2* exon 7 at cell level was 26 cycles 95°C for 15s, 60°C for 30s and 72°C for 25s. Primers used for RT-PCR were as follows: *SMNT7F2*: 5'-TACTTAATACGACTCACTATAGGCTAGCCTCG-3', Ex8-29to52-R: Cy5-5'-TCTGATCGTTTCTTTAGTGGTGTC-3'.

PCR products were separated on 2% agarose gels, followed signals were quantitated by Image J software, and exon 7 inclusion was calculated by a formula: Exon 7 inclusion % = [FL(exon 7 inclusion) / (FL + Δ7(exon 7 exclusion))] × 100%

qPCR

The optional parameters for 10 µL qPCR reaction system was 5µL 2× ChamQ Universal SYBR qPCR Master Mix (Vazyme Q711), 0.5µL Forward primer, 0.5µL Reverse primer, 1µL cDNA and 3µL ddH₂O. PCR reaction procedure was 45 cycles 95°C for 10s, 60°C for 20s, and 72°C for 20s.

The primer sequence used for qPCR were as Supplementary (Table 1) and the mRNA levels showed by heat maps.

Western blot

Protein samples were separated by 10% SDS-PAGE and electroblotted onto PVDF membranes (Millipore FFP39), followed by 5% skim milk as blocking solution which can block non-specific bands. Then they were incubated with primary antibody overnight at 4 °C and secondary antibody for 2h at room temperature. Among them, primary antibodies were respectively anti-SMN antibody (BD Biosciences, 610647), anti-NOVA1 antibody (Abcam ab183024) and anti-β-actin antibody (Santa Cruz sc-47778). Secondary rabbit anti-mouse (D110087) and goat anti-rabbit antibodies (D110058) were purchased from Sangong. Protein signals were detected with the Tanon-5200Multi Gel Imaging System (Tanon Science & Technology).

Nissl staining

Nissl staining are carried out according to the kit (Beyotime C0117). Mouse is anesthetized on ice, then the spinal cord tissue is taken out, fixed with 4% formaldehyde at 4 °C overnight, washed in phosphate

buffered saline (PBS) and embedded in paraffin blocks. 4 μ m paraffin sections were cut for Nissl Staining. In brief, the paraffin sections were dewaxed, rehydrated and washed twice with PBS for 2 minutes each time. Subsequently, put the tissue section in Nissl staining solution and stain for 3-10min at room temperature. Wash the stained sections with pure water twice, each for a few seconds. The tissue sections were dehydrated twice with 95% ethanol, each dehydration time was 2 min. Xylene was transparent twice, each time for 5 minutes. Finally, seal the sheet with neutral gum.

Immunofluorescence

The spinal cord tissues were fixed with 4% formaldehyde at 4 °C overnight, dehydrated with 30% sugar solution and embedded in Tissue Freezing Medium (O.C.T), 6 μ m frozen sections by freezing microtome were rehydrated before immunofluorescence staining. In brief, the frozen sections were placed in an oven at 60°C, dried for 30 minutes, washed twice with PBS for 2 minutes each, and were incubated with 0.3% Triton X-100 permeabilization solution at room temperature for 30 minutes. After washing with 0.01M PBS, add 5% BSA blocking solution and block for 30 minutes at room temperature. Dilute the primary antibody (anti-NOVA1 Abcam ab183024; anti-ChAT antibody: Abcam ab34419) with 0.01M PBS in proportion, add it dropwise to the sliced tissue, and place it in a humid box in a refrigerator at 4°C overnight. Wash 3 times with 0.01M PBS, 5min each time. Dilute the secondary antibody (Cy3-Goat anti-Rabbit IgG Sangong D110062 and FITC-Donkey Anti-Chicken IgY Sangong D110201) and DAPI (Sangong E607303) with 0.01M PBS proportionally, and incubate for 2h in a humid box at room temperature in dark. Wash 3 times with 0.01M PBS, 15min each time. Mount the slide with a fluorescent mounting tablet and take pictures in the dark.

Plasmid construction

The specific construction method of *SMN2* minigene was similar to those previously described by Hua-Lab [16]. The vector used for NOVA1 overexpression plasmid was pCGT7. The multiple cloning sites are XbaI and KpnI. NheI and XbaI are homologous enzymes. This feature can be used to construct the NOVA1 overexpression plasmid. Primer sequences used for NOVA1 overexpression plasmid were as follow: hNOVA1 Nhe1-F: 5'- ATGCTAGCATGATGGCGGCAGCTCCC-3', hNOVA1 Kpn1-R: 5'- ACGGTACCTCAACCCACTTTCTGAGG-3'. PCR reaction system was 4 μ L 5 $\times$ Phusion HF buffer, 0.4 μ L 10mM dNTPs, 1.2MI, 1.2 μ L, 1~10ng cDNA, 1.2 μ L DMSO, 0.3 μ L Phusion DNA polymerase, and ddH₂O which added to make up to 20 μ L. Reaction procedure was 45 cycles, including 95°C for 15s, 60°C for 20s and 72°C for 20s/kb. The PCR product was purified with PCR clean-up kit (Axygen AP-PCR-250). Through restriction enzyme digestion, ligation, transformation, amplification, plasmid extraction, and sequencing to verify the amplification results, finally NOVA1 overexpression plasmid was obtained.

Cell culture and transfection

The human glioma cell line U87MG and the human embryonic kidney (HEK293T) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone SH30243.01) supplemented with 10% (v/v) FBS (LONSERA S711-001S), 100U/mL penicillin and 100 μ g/mL streptomycin (HyClone SV30010), which were

cultured at 37°C in a humidified 5% CO₂ atmosphere. 1 µg of the *SMN2* minigene plasmid and *NOVA1* overexpression plasmid or empty vector plasmid were all transfected into cells that had been planted in a 6-well plate one day in advance. After 48h of transfection, cells were collected to extract RNA or protein. In the mutation experiment, *SMN2* mutation minigene was also transfected with 1 µg. Transfection reagent of petroleum equipment institute (PEI) (Sigma 408727) was used to transfect plasmid. When knocking down *NOVA1*, the *NOVA1* siRNA sequences were as follow: *NOVA1*-sense: 5'-AGACAAUUGUUCAGUUGCA-3', antisense: 5'-UGCAACUGAACAAUUGUCU-3'. The negative control sequences were *NOVA1* siNC-sense: 5'-UUCUCCGAACGUGUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGGAGAATT-3'. siRNAs were designed by online software (<http://biodev.extra.cea.fr/DSIR/DSIR.html>) and synthesized by Shanghai Genepharm company were transfected using Lipofectamine 2000 (Life Technologies).

Point mutation

The PCR reaction system for constructing *SMN2* mutation minigene was similar to that of *NOVA1* overexpression plasmid. reaction condition was 94°C for 10s, T_m°C for 20s and 72°C for 15~30s/kb, which had 30 cycles in total. The annealing temperature was adjusted according to the primer T_m value, or the annealing temperature was determined by gradient PCR. Mutation primers were listed in Supplementary data (Table 2). Agarose electrophoresis was used to detect the products after PCR amplification (usually 25 µL reaction system). 1 µL DpnI enzyme was added to the remaining PCR product, digested in a 37°C water bath for 4 hours. 8 µL of the product was taken for transformation after digestion, and a single colony was selected for amplification. The germs were collected and plasmids were extracted and sequenced to verify whether the mutation was successful.

RNA pull down

The biotin-labeled RNA probes, WT (5'-AGGUGCUCACAUUCCU-3') and Mut (5'- AGGUGCUCACAUUCCU-3'), were purchased from Shanghai Genepharm company, which were used for RNA pull down experiment. After washing the streptavidin agarose beads (Sigma S1638-1ML) with streptavidin washing buffer three times, biotin labeled RNA was added to the precleared beads, and the mixture was incubated at 4 °C for 1-4 h on the 100 RPM rotator. Then, the beads were precipitated and cleaned by RNA-streptavidin interaction buffer. 100 µL of nucleoprotein extracted with a protein concentration of 3-5 µg/µL from U87MG cells was mixed with the beads. The tubes were kept at 4 °C overnight on the 100 RPM rotator. After centrifugation, the supernatant was discarded. An appropriate volume of protein loading buffer was added then the mixture was placed in a water bath at 90 °C for 10 minutes. After cooling on ice, samples were centrifuged at 12000 rpm 4 °C 10 minutes and the supernatant was take for western blot analysis.

Statistical analysis

The software SPSS 16.0 was used for statistically analyzing. The data were expressed as mean ± standard deviation, and statistical significance of difference between groups was analyzed by Student's t test and one-way ANOVA. *P* < 0.05 indicates that the difference was statistically significant. *P* < 0.01 indicates that the difference was extremely significant.

Results

Inclusion of *SMN2* exon7 was high in neural tissues

Manipulation protein expression of SMN via *SMN2* splicing regulation became an effective way for the treatment of SMA diseases. However, fewer reports were focused on the differences of inclusion of *SMN2* exon 7 in the tissues involving in SMA disease individuals. This study offered certain evidences for treating SMA disease.

Severe type SMA mice harbored two copies of *SMN2* (*smn*^{-/-}, *SMN2*^{2tg/0}) [20], and appeared symptoms similar to type I SMA patients at day 4 after birth.

To study the differences on the inclusion of *SMN2* exon 7 in different tissues, we conducted research on 8 tissues: heart, liver, spleen, lung, kidney, brain, spinal cord, and muscle in SMA and control mice at postnatal day 4. Interestingly, we found that the *SMN2* splicing, the inclusion of *SMN2* exon 7, was different between tissues, and high rate of exon 7 inclusion were observed in nerve tissues in both SMA mice and control ones (*smn*^{+/-}, *SMN2*^{2tg/0}) (Figure 1).

Expression of Splicing Factors in control and SMA mice

Exon inclusion or not was determined by splicing events under regulation of splicing factors. Classic splicing factors, such as HNRNP, SR and NOVA family members, have been confirmed to regulate a variety of splicing events. In order to clarify the relationship between the expression of splicing factors in SMA mice and the differential splicing *SMN2* among tissues, we studied their expression in neural and non-neural tissues of SMA or control mice at postnatal day 4 by qPCR and showed as heat maps. Results showed that the expressions of 20 members of HNRNP, SR and NOVA family were different among tissues, while NOVA was highly expressed in nerve tissues in both SMA mice (Figure 2B) and control mice (Figure 2A).

NOVA1 was Down-regulated with the Development of the Disease

NOVA1 and NOVA2 were members of NOVA family. NOVA1 was predominantly expressed in the ventral spinal cord and the midbrain [21], NOVA2 was localized in the neocortex and hippocampus [22]. Meanwhile, the *NOVA1*-deficient mice produced by gene editing had abnormal motor neurons in the brainstem and spinal cord, then exhibited motor dysfunction, and died within 10 days after birth, similar to the symptoms of SMA mice [23]. To explore the relationship between the neuro-specific expression of the *NOVA* family members and progression of SMA disease, mRNA and protein levels of *SMN* and *NOVA1* were detected in spinal cord of SMA mice at postnatal day 1(P1), 4(P4) and 7(P7).

In control mice, results showed that full length *SMN* (*SMN*FL) mRNA and protein levels were significantly up-regulated from P1, P4 to P7 (Figure 3A, 3B, 3C). Meanwhile, the expression levels of *NOVA1* were significantly increased at P4 and P7 compared to P1, and NOVA1 protein levels at P7 significantly enhanced compared with P4 (Figure 3D, 3E, 3F).

In SMA mice, results showed that *SMN*FL mRNA and protein levels were significantly decreased from P1 to P4 and P7 (Figure 3G, 3H, 3I). Meanwhile, the expression levels of *NOVA1* were significantly decreased at P4 and P7 compared to P1, and NOVA1 protein levels at P7 significantly decreased compared with P4 (Figure 3J, 3K, 3L).

NOVA1 expressed in motor neurons and decreased with the development of SMA mice

Results above showed that the expression of NOVA1 was correlated with that of SMA pathogenic protein SMN. Nissl staining showed that the number of the neurons in spinal anterior horn decreased continuously from P1, P4 to P7 when compared to control mice (Figure 4A). Bundles of hollow Nissl positive plaque were found in the SMA anterior horn regions compared with that of control ones. At the meantime, we found that motor neuron marker ChAT and NOVA1 were partially co-localized in spinal anterior horn at the time points (Figure 4B). Taken together, NOVA1 expression was correlated with splicing of SMA related gene *SMN2*, SMN protein expression and number of motor neurons in spinal cord anterior horn in SMA mice, which indicated that NOVA1 may be involved in the development of SMA disease.

NOVA1 Regulated the Inclusion of *SMN2* Exon 7 in vitro

NOVA1 may participate in the development of SMA disease. However, as a RNA binding protein, whether NOVA1 can regulate the splicing of *SMN2* and affect the severity of SMA disease was unknown. Therefore, to study the mechanism of NOVA1 on *SMN2* splicing, that is, the inclusion of *SMN2* exon 7, we transfected NOVA1 small interfering RNA (siRNA) and plasmid into U87MG cells.

The expression of NOVA1 mRNA (Figure 5A) and protein (Figure 5B, 5C) was decreased after knocking down by siRNA. The inclusion of *SMN2* exon 7 was significantly reduced as the expression NOVA1 was obviously knocked down (Figure 5D, 5E). Meanwhile, SMN protein level was also decreased (Figure 5F, 5G). In contrast, NOVA1 mRNA (Figure 5H) and protein (Figure 5I, 5J) expression was up-regulated after plasmid transfection. Expression of NOVA1 was correlated with the inclusion rate of *SMN2* exon 7 (Figure 5K, 5L), and the protein expression of SMN was increased (Figure 5M, 5N). NOVA1 expression did not change after SMN knockdown (Supplementary Figure 1). In short, NOVA1 significantly promoted the inclusion of *SMN2* exon 7 and increased the expression of SMN protein.

NOVA1 Bound to the UCAC Motif on Exon 7 of *SMN2*

As a very important neuro-specific splicing factor, NOVA1 binds to the YCAY motif in the mRNA precursor of target gene through its KH domain to regulate alternative splicing. In this motif, "CA" is the key factor to splicing regulation. Interestingly, one "UCAC" motif was found in exon 7 of *SMN2* (34 to 37 bp) by sequence analysis, which was consent to YCAY motif (Figure 6A). To confirm that NOVA1 targeted the UCAC sequence to regulate the inclusion of *SMN2* exon 7, mutation technology was used to delete the sequence UCAC on *SMN2* exon 7 and change the "UCAC" to "UAAC" in *SMN2* minigene to perform *SMN2* splicing research (Supplementary Figure 2).

SMN2 wild type and mutated minigenes were transferred into HEK293T cells for 48 hours respectively. Results showed that deletion of the sequence UCAC significantly reduced the inclusion level of *SMN2* exon 7. In addition, after UCAC was mutated to UAAC, the inclusion level of *SMN2* exon 7 decreased more significantly (Figure 6B, 6C).

In order to confirm that NOVA1 targeted the UCAC sequence to regulate *SMN2* splicing, RNA pull down experiments were performed with biotin-labeled RNA probes of WT (UCAC) and Mut (UAAC) respectively. The nucleoprotein extract group was used as a positive control, and the RNA probe (no RNA) group was used as a negative control. Results showed that in the *SMN2* WT (UCAC) group, NOVA1 was detected in the pull down protein. On the contrary, NOVA1 was not detected in the *SMN2* Mut (UAAC) group (Figure 6D), indicating that NOVA1 protein bound to *SMN2 mRNA*, and the binding site was the UCAC sequence (34 to 37 bp) in exon 7 of *SMN2*.

Dealing with the YCAY motif, "CA" and the pyrimidines at both ends of the sequence are particularly important. Based on the principals of avoiding pseudo-binding site and not to form new "YCAY" binding site in *SMN2* exon 7, the order of Y in UCAC motif was shifted as UCAU, CCAU, and CCAC. Meanwhile, mutation in the core "CA" sequence was designed as UGAC, UUAC, UAGC, UGGC, UUGC and UCGC (Figure 6E). We found that when the UCAC sequence was mutated to UCAU, CCAU, UGAC, UUAC, UAGC, UGGC, and UUGC, the inclusion of *SMN2* exon 7 was reduced (Figure 6F, 6G, 6H). Among them, the "CA" mutation had a more significant impact on the inclusion. However, CCAC and UCGC mutations significantly up-regulated the inclusion of exon 7 (Figure 6F, 6G, 6H). In short, the UCAC sequence was the key sequence for NOVA1 to specifically bind to *SMN2* and regulate splicing.

Discussion

Spinal muscular atrophy, which is caused by the failure in synthesizing of functional SMN protein for the *SMN1* mutation, has become one of the rare diseases that seriously affect the quality of human life. Many scholars have studied the splicing of the *SMN2* gene that is parallel to *SMN1* and found that promoting the inclusion of *SMN2* exon 7 can effectively improve the severity of SMA disease [11].

Research on the treatment of SMA disease has never stopped, including sodium butyrate [24] and induced pluripotent stem cells [25]. Although these methods have certain prospects, their efficacy is minimal or concerning with safety issues. In recent years, gene therapy and antisense oligonucleotides become popular in the treatment of SMA. Spinraza is the first antisense oligonucleotide drug developed by Hua Yimin to treat SMA in children and adults [26]. In addition, Zolgensma, the first gene therapy drug used to treat spinal muscular atrophy in children under 2 years, was also approved by the FDA for clinical treatment in May 2019 [27]. However, multiple systems were affected in SMA disease [28], these drugs have their advantages in one or more systems while their limitation and side effects cannot be ignored. At the same time, they cannot completely and effectively cure SMA, which brings greater economic and life pressure to patients. The development of more effective SMA treatment drugs is urgently demanded.

Alternative splicing refers to the process in which a pre-mRNA generates different splicing isoforms of mRNA through different splicing methods or selection of different splicing sites. There are five basic forms of alternative splicing, namely exon skipping, intron retention, alternative 5' splice site, alternative 3' splice site and mutually exclusive exon [29]. Among them, exon skipping is the most common one. In humans, approximately 95% of multi-exon genes are alternatively spliced [30]. One-third of all hereditary diseases may have splicing components [31], especially in the development of the nervous system, which affects the differentiation and migration of neurons, the growth and guidance of axons and the formation and function of synapses. It has an important impact on the growth and development of nervous tissue and the occurrence of neurological diseases, such as tau protein and Alzheimer's disease [32], myelin and demyelinating diseases [33], and AMPA receptors and epilepsy [34]. As a neurological disease caused by *SMN2* alternative splicing, treatment of SMA disease from the perspective of splicing is currently the most prospective research direction.

There are many limitations to treatment of SMA diseases by directly targeting human beings. Mouse is a common animal model with highly homologous genome sequences and similar physiological processes to humans, and plays an important role in the research and treatment of SMA disease. SMA mouse was generated by Hung Li laboratory with symptoms similar to human SMA. they carried two copies of *SMN2* gene (genotype *smn*^{-/-}, *SMN2*^{2tg/0}), and developed typical symptoms of SMA four days after birth, which indicated that gene polymorphism might exert significant role in regulating the pathological change during this period [20]. At the same time, the pathological changes in different tissues and organs of SMA mice were different. Tissue specificity of *SMN2* splicing was revealed by previous studies on SMA type III mice [35], which hinted importance of splicing changes of *SMN2* in SMA mice.

In this study, we also detected the inclusion of *SMN2* exon 7 in 8 different tissues of SMA mice at postnatal day 4. Interestingly, we found that the inclusion of *SMN2* exon 7 was different among tissues and to a highflyer degree in nerve tissues in SMA and control mice. Different expression of certain splicing factors in neural and non-neural tissues was speculated to be possible for various inclusion rates. Based on this, we selected three types of classic splicing factors HNRNP, SR and NOVA family and detected their expressions. Members of three types of classic splicing factors family, such as HNRNP, SR and NOVA, were picked out and checked to determine their expression in different tissues. Enhanced expression of NOVA1 was detected in neural tissues in both control and model mice [21]. In addition, clinical studies have shown that SMA patients onset the disease at 6 months and died of respiratory diseases before the age of 2. Researches by Meilin Qin and others showed that the development of lung cancer and other respiratory diseases was closely related to NOVA1 [36, 37]. NOVA1 and NOVA2 were highly expressed in lung tissues besides the brain and spinal cord in SMA mice that indicated the role of NOVA in the respiratory system.

NOVA, as splicing factor specifically expressed in nerves, can regulate the inclusion of GABAA receptor $\gamma 2$ [38], Dcc intron 16 [39] and Sept8 exon 10b [40], thereby affecting development of the motor system, function and survival of motor neurons. In addition, NOVA is also involved in other neurological diseases, such as the occurrence and development of familial autonomic dysfunction (FD) diseases [41]. In this

experiment, we found that the expression of NOVA1 was correlated with the inclusion of *SMN2* exon 7, and was significantly down-regulated as the SMA disease progressed in SMA mice. Immunofluorescence experiments also confirmed that NOVA1 was expressed along with the anterior horn motor neurons of the spinal cord in SMA mice that might rely on its promotion on the inclusion of exon 7 in *SMN2*. The NOVA-deficient mice created by Kirk B Jensen and others through gene editing technology have motor dysfunction and died due to motor neuron apoptosis within 10 days after birth [23, 42]. The symptoms were similar to SMA mice studied in this experiment, suggesting an association between the expression of NOVA1 and the severity of SMA. Subsequently, we first revealed that NOVA1 was highly expressed in neural tissues and enhanced translation of SMN protein via promoting the inclusion of *SMN2* exon 7.

Previous study showed that NOVA1 regulates alternative splicing by combining its KH domain with the YCAY motif in the target gene mRNA precursor [15, 43]. Mutation of YCAY to YAAAY can eliminate the regulatory effect of NOVA1 on alternative splicing [37]. Fortunately, the UCAC sequence was found in exon 7 of *SMN2*. Interestingly, when we mutated the pyrimidine combination at both ends of the UCAC sequence and the core sequence "CA" in it separately, the inclusion of *SMN2* exon 7 in most mutation groups were significantly reduced, and the core sequence "CA" had more significant impact on it. Therefore, the "CA" sequence in YCAY motif is critical in NOVA1 interaction. Besides, few mutations of UCAC to CCAC or UCGC significantly promoted the inclusion of exon 7, which was speculated that these mutations might form new RNA-binding sites, and further verification was needed to determine the new splicing regulation mechanism.

Decreased synthesis and loss of function of *survival motor neuron (SMN)* dominated the development of SMA disease. As a ubiquitously expressed protein, SMN protein is involved in small nuclear ribonucleoprotein (snRNP) biosynthesis, pre-mRNA splicing, apoptosis, axon transport, cytoskeleton dynamics, mitochondria and bioenergy pathways and other activities [44–46]. Current study showed that with the aggravation of SMA disease, SMN protein was significantly down-regulated, which is consistent with the results of Ewout J.N. Groen et al. [47], and all the evidences confirmed temporal and spatial expression of SMN affected the severity of SMA disease. However, SMN protein was translated with the template of full length *SMN2* mRNA with *SMN2* exon 7, and its expression level was significantly down-regulated when exon 7 inclusion reduced, which may not be able to synthesize snRNP normally. Therefore, a positive correlation between *SMN2* exon 7 inclusion and SMN protein expression was established. In this study, both NOVA1 and SMN proteins were simultaneously down-regulated in SMA mice. Similarly, NOVA1 up-regulated both *SMN2* FL and SMN protein in vitro. Related studies have shown that SMN protein can interact with splicing factors, such as SMN and HNRNPR in the axons bodies, axon terminals and cytoplasm of mouse motor neurons [48]. So, it was speculated that there may be a protein-protein interaction between NOVA1 and SMN protein, in other words, NOVA1 may also directly bind to SMN protein to regulate protein expression and affect the severity of SMA disease, and the regulation mechanism need further study[49, 50].

In short, with the continuous development of SMA disease, the expression of NOVA1, which had the same inter-tissue differences with *SMN2* splicing, was significantly down-regulated, and it can target binding to

the 34 to 37 UCAC sequence in *SMN2* exon 7 to regulate *SMN2* splicing, which is of great significance for the treatment of SMA disease.

Conclusion

The inclusion rate of *SMN2* exon 7 was higher in neural tissues, and the splicing factor of *NOVA1* was higher in neural tissues too from many splicing factors. *NOVA1* was presented in motor neurons and decreased as SMA ongoing. *SMN2* exon 7 inclusion and protein level were enhanced by overexpressing *NOVA1*, while reversed when *NOVA1* knockdown. *NOVA1* could bind the “YCA Y” motif in exon 7 thus enhanced mRNA levels of *SMN2 FL* and SMN protein. The data may provide new target gene of *NOVA1* for treatment of SMA disease.

Abbreviations

SMA spinal muscular atrophy;

SMN survival of motor neuron;

FL Full-length;

NOVA1 neuro-oncological ventral antigen 1;

Pre-mRNA Precursor messenger RNA;

AS Alternative splicing;

SF Splicing factor;

RRM RNA recognition motif;

HNRNP Heterogenous nuclear ribnucleoprotein;

snRNP Small nuclear ribonucleoprotein;

SR Serine-arginine protein;

SPF Specific pathogen free;

Declarations

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Author contribution Liucheng Wu and Junjie Sun designed the experiments and supervised the project. Lili Du, Junjie Sun, Liucheng Wu and Zhiheng Chen performed the experiments. Liucheng Wu and Junjie

Sun analyzed the results. Yixiang Shao gave suggestions for the experiments and analysis. Lili Du and Liucheng Wu wrote the manuscript. Liucheng Wu and Junjie Sun revised the manuscript.

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Availability of Data Data and material used to support the study are included in this article.

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Figures

Figure 1

Expression pattern of full-length SMN2 in neural and non-neural tissues. (A) Inclusion ratio of SMN2 exon 7 in neural and non-neural tissues in control and SMA mice. (B) Relative gray density in different tissues in control mice (left panel) and average gray density in neural and non-neural tissues (right panel in B). (C) Relative gray density in different tissues in SMA mice (left panel) and average gray density in neural and non-neural tissues (right panel in C). $n=3$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Figure 2

Expression of splicing factors in control and SMA mice. Heat map of splicing factors mRNA level in control (A) and SMA mice (B). The original data to form heat maps derived from qPCR with primers in supplementary table 1.

Figure 3

SMN demonstrated similar expression pattern with NOVA1 in control and SMA mice. (A, D, G, J) results of mRNA expression of control mice (A. SMN, D. NOVA1) and SMA mice (G. SMN, J. NOVA1). (B, E, H, K) results of protein expression in control mice (B. SMN, E. NOVA1) and SMA mice (H. SMN, K. NOVA1). (C, F, I, L) gray density analysis for B, E, H, K. P1, P4, and P7 indicated sample from mice at age of postnatal day 1, 4 and 7. n=3. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 4

NOVA1 expressed in motor neurons and decreased with the development of SMA mice. (A) Nissl staining of spinal cord sections of control and SMA mice. The boxes indicated the location of anterior horn of spinal cord. Regions in box 1 and 2 was enlarged in the right panels. Arrows pointed out the neurons in site. n=3. (B) Immunofluorescence detection for ChAT (motor neuron, Green), NOVA1 (Red) and DAPI (Nuclear, Blue) expression in the spinal cord in control and SMA mice. The white box showed the region

of anterior horn of spinal cord. Enlarged views of region in the boxes were demonstrated in the right panels. White arrows indicated location of motor neurons. P1, P4, and P7 indicated sample from mice at age of postnatal day 1, 4 and 7. n=3. Scale bar=200um & 50um.

Figure 5

NOVA1 regulated SMN2 exon 7 inclusion in U87MG cells. Knockdown efficiency of NOVA1 was checked by qRT-PCR (A) and western blot (B) in U87MG cells. (C) Gray density analysis for (B). (D) Inclusion rate

of exon 7 of SMN2 after knocking down NOVA1 and (E) statistic result of (D). (F) Protein level of SMN after NOVA1 inhibition and gray density analysis (G). Overexpression efficiency of NOVA1 was checked by qRT-PCR (H) and western blot (I) in U87MG cells. (J) Gray density analysis for (I). (K) Inclusion rate of exon 7 of SMN2 after overexpression of NOVA1 and (L) statistic result of (K). (M) Protein level of SMN after NOVA1 overexpression and gray density analysis (N). n=3. *P<0.05, ***P<0.01. ***P<0.001.

Figure 6

The UCAC motif was the binding site of NOVA1 and played an important role in SMN2 splicing. (A) Schematic diagram of SMN2 minigene with NOVA1 binding site on exon7. (B) Inclusion of SMN2 exon7 under condition of UCAC deletion and UAAC mutation. (C) Statistic analysis for (B). (D) Western blot detection of NOVA1 protein after RNA pull down. (E) Schematic diagram of UCAC mutation cassette. (F) Detection of SMN2 splicing after mutation of pyrimidine in UCAC sequence. (G) Determination of SMN2 splicing after mutation of CA in UCAC sequence. (H) Statistical graph based on (F) and (G). Compared to the UCAC, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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