รายงานวิจัยฉบับสมบูรณ์

โครงการ Evaluation of antisense oligomer as a molecular treatment for spinal muscular atrophy in mouse models of SMA

โดย

Dr. Chalermchai Mitrpant

July 2014

สัญญาเลขที่ MRG055

รายงานวิจัยฉบับสมบูรณ์

โครงการ Evaluation of antisense oligomer as a molecular treatment for spinal muscular atrophy in mouse models of SMA

Dr. Chalermchai Mitrpant

Department of Biochemistry, Faculty of Medicine,

Siriraj Hospital, Mahidol University

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

Abstract

Project Code: MRG055

Project Title: Evaluation of antisense oligomer as a molecular treatment for Spinal Muscular Atrophy in mouse models of SMA

Investigator: Dr. Chalermchai Mitrpant

E-mail Address: chalermchai.mit@mahidol.ac.th

Project Period: July 2012-June 2014

Spinal muscular atrophy (SMA) is caused by loss of the Survival Motor Neuron 1 (*SMN1*) gene, resulting in reduced SMN protein. Humans possess the additional *SMN2* gene (or genes) that does produce low level of full-length SMN, but cannot adequately compensate for loss of *SMN1* due to aberrant splicing. The majority of *SMN2* gene transcripts lack exon 7 and the resultant SMNΔ7 mRNA is translated into an unstable and nonfunctional protein. Splice intervention therapies to promote exon 7 retention and increase amounts of full-length *SMN2* transcript offer great potential as a treatment for SMA patients. Several splice silencing motifs in *SMN2* have been identified as potential targets for antisense oligonucleotide mediated splice modification. A strong splice silencer, (ISS-N1) is located downstream of exon 7 in *SMN2* intron 7. Antisense oligonucleotides targeting this motif (PMO-10-29) promoted *SMN2* exon 7 retention in the mature SMN2 transcripts, with increased SMN expression detected in SMA fibroblasts. We systematically optimised the coordinate of AO (-10-34) that promote exon 7 retention in SMA fibroblast cultures.

We wish to confirm efficiency of this phosphorodiamidate morpholino oligomer (PMO) in mice model of SMA. Single injection of PMO(-10-34) or control were intracerebroventricularly injected into SMA mice and SMA carrier mice. Single ICV injection of PMO (-10-34) can effectively enhanced expression of FL-SMN in SMA carrier mice. Furthermore, the PMO (-10-34) gives the longest survival reported to date after a single dosing by ICV.

Keywords: Spinal muscular atrophy, antisense oligomer, Neuromuscular disorders

บทคัดย่อ

รหัสโครงการ: MRG055

ชื่อโครงการ: **การทดลองใช้** antisense oligomer **เพื่อเพิ่มระดับ โปรทีน** SMN **ใน สัตว์ทดลอง**

ชื่อนักวิจัย: เฉลิมชัย มิตรพันธ์

อีเมล์: chalermchai.mit@mahidol.ac.th

ระยะเวลาดำเนินงาน: กรกฎาคม 2554 - มิถุนายน 2556

โรคกล้ามเนื้อฝ่อลีบ spinal muscular atrophy (SMA) เกิดจากการขาดหายไปของยีน survival motor neuron 1 (*SMN1*) มีผลให้ผู้ป่วยสร้างโปรตีน SMN ลดลง มนุษย์มียีน SMN สองชุดกล่าวคือ *SMN1* และ *SMN2* ทว่ายีน *SMN2* ผลิตโปรตีน SMN (FL-SMN) ได้ในระดับต่ำ จึงไม่สามารถชดเชยการขาดหายไปของยีน *SMN1* mRNA ที่ถูกสร้างจากยีน *SMN2* ส่วนใหญ่จะไม่มี exon 7 (SMNΔ7) เป็นผลให้โปรตีน SMN ที่ผลิตได้ไม่เสถียรถูกทำลาย ได้เร็วกว่าปกติ แนวทางการรักษาระดับโมเลกุลแบบหนึ่งของโรคนี้คือการใช้สาร antisense oligomer (AO) จับ pre-mRNA ของ *SMN2* บริเวณ intron7 (ISS-N1) เพื่อเปลี่ยน การแสดงออกของ *SMN2* mRNA ให้สร้างโปรตีน SMN (FL-SMN) สาร AO (PMO(-10-29)) ได้ถูกทดสอบสามารถเพิ่มปริมาณ FL-SMN ในเซลล์เนื้อเยื่อเกี่ยวพันจากผู้ป่วย SMA

โครงการนี้ทำการทดสอบประสิทธิภาพของสาร AO (PMO(-10-34)) โดยการฉีดเข้าทางโพรงสมอง (intracerebroventricular injection) ของหนูที่ป่วยเป็น SMA (SMA mice) และหนูพาหะ SMA (SMA carrier mice) ผลที่ได้คือสาร PMO(-10-34) สามารถเพิ่มปริมาณ FL-SMN ใน SMA carrier และสามารถยืดอายุเฉลี่ยของหนู SMA mice

คำหลัก: โรคกล้ามเนื้อฝ่อลีบ, spinal muscular atrophy

1. Background

Spinal muscular atrophy (SMA) is the leading genetic cause of death in children under the age of 2 years. Patients with SMA will loss motor neuron in the ventral horn cells, the most striking pathological finding leading to muscle degeneration and atrophy. Depending upon the onset and severity of disease, patients with SMA are classified as SMA types I, II or III. SMA type I is the most severe form, resulting in death in infancy, whilst the milder forms, SMA types II and III are less severe phenotype. SMA is caused most commonly by genomic deletions of the Survival Motor Neuron 1 (*SMN1*) gene on chromosome 5, resulting in the absence of functional SMN protein. Although humans have extra centromeric copies of the SMN gene, the *SMN2* (or genes) cannot adequately compensate for *SMN1* loss, since the majority of *SMN2* gene transcripts lack exon 7. The C>T polymorphism near the beginning of *SMN2* exon 7 leads to the loss of the exon 7 from 90% of *SMN2* gene transcripts (SMN2□7) and substantially reduced full-length SMN (FL-SMN) transcript and protein. Since the milder forms of SMA, SMA type II and type III, are associated with extra copies of the *SMN2*, an approach aimed at enhancing *SMN2* expression has been pursued as a genetic treatment for SMA patients. Alternative strategy to induced FL-SMN is that the use of antisense oligomer (AO) to alter the splicing pattern of *SMN2* toward full-length transcript

Antisense oligomers (AOs) have been used to modify pre-mRNA splicing, most commonly by masking splicing motifs essential for exon recognition so that the targeted exon is excised from the mature transcript. In addition to exon skipping, AO can also be used to mask splice silencing motif and therefore promoting exon inclusion. From our previous study, we identified a set of AOs which effectively induces *SMN2* exon 7 inclusion in type I SMA fibroblasts and the selected AO can efficiently induces FL-SMN transcript with substantial increased level of full-length protein.

We propose in this application to determine that, to what extent, this AO can induce FL-SMN transcript and protein in heterozygous SMA mice, and whether the level of induced SMN could prolong the survival of the severe mouse model of SMA. This

project will be the proof-of-concept experiment to assure whether this approach could be the molecular genetic therapy for patients with SMA.

2. Objectives

- To confirm the effect of PMO induced *SMN2* exon 7 inclusion *in vitro*.

- To determine the effect of PMO induced exon 7 inclusion on induction of FL-SMN transcript and protein in heterozygous SMA mice (SMN carrier).

- To determine whether the level of induced *SMN* could prolong the survival of the severe mouse model of SMA.

3. Methodology

Animal model and intracerebroventricularly injection (ICV): The process of breeding mice, intracerebroventricular (ICV) injection of PMO, monitoring survival and weight and tissue collection will be undertaken at The Ohio State University. Collected tissue of the mice will be transferred to Faculty of Medicine, Siriraj Hospital for RT-PCR, quantitative RT-PCR Western blotting and immunofluorescence analysis. The □7 carrier breeding mice (*SMN2+/+; Smn+/-; SMNΔ7+/+*) will be crossed to generate three types of offspring varying in the mouse Smn genotype: *Smn +/+*, *Smn +/-*, *Smn -/-* as previously described. All breeding and subsequent use of animals in this study were approved by the IACUC of The Ohio State University, Columbus, Ohio. The ICV injections will be performed on the cryo-anesthetized P0 pup (day-of-birth) using pulled glass capillary needle. The pup will be hand-mounted over a back-light to visualize the intersection of the cranial sutures (*bregma*). A pulled capillary needle with attached injection assembly will be inserted 1 mm lateral and 1 mm posterior to *bregma*, and then tunneled 1mm deep to the skin edge. An opaque tracer (Evans blue, 0.04%) will be used to the reagent to visualize the borders of the lateral ventricle after injection of 2 μl of PMO. Brain and spinal cord tissues will be collected and sent to Thailand.

Morpholino AO Preparation: Morpholino oligomer will be supplied by Gene Tools (Philomath, USA) and based upon the sequence previously described our previous study. The PMO(-10-34) as well as a sham control PMO were resuspended in sterile 0.9% sodium chloride, aliquoted, and mixed with Evans Blue (final concentration

0.04%); three different molar concentrations will be prepared (High: 6 mM=40.5 μg/μL; Middle: 4 mM=27 μg/μL; Low: 2 mM=13.5 μg/μL). Stock solutions were stored at -20°C, working solutions at 4°C.

RT-PCR analysis: RNA will be isolated from tissues homogenized using Trizol (Invitrogen) and purified using the RNeasy kit (Qiagen) according to the manufacturer's instructions. RT-PCR will be performed as previously described. The FL-hSMN specific primers will be used to distinguish the *SMN2* transcript from the SMNΔ7 transgene transcripts as the terminal portion of exon 8 is lacking in the SMNΔ7 transgene and therefore the amplification only occurs from *SMN2* transcripts which contain this terminal region of exon 8.

Western blotting: Western blot analysis of adult and neonatal tissue will be modified from previously described. Briefly, 150 μL of blending buffer (62.5 mM Tris-HCl, pH 6.8, 5 mM EDTA, 10% SDS) was added to the tissue sample and the sample homogenized and sonicated. **Equal** volume of loading buffer was added (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 200 mM DTT, 0.2% bromophenol blue) and protein extracts were loaded onto a 12.5% SDS polyacrylamide gel. The separated proteins will be transferred to a 0.2 μm pore size PVDF membrane (BR 128950261, Bio-Rad) using the sandwich western transfer system. The membrane will be developed using Westernbreeze protein detection kit with primary antibody (either hSMN-KH, 1:20, a generous gift of Dr. Hua and Krainer or hSMN Ab (Santacruz)) overnight. Quantification of the protein band will be determined using G Box Chemi gel documentation with GeneTools software (Syngene). After detection of SMN protein, the membrane was stripped and re-incubated with mAb mouse-anti ß-actin (A5441, Sigma, 1:50,000) for 1 hr. Mouse ß-tubulin antibody (1:300) was used as an internal loading control for human SMN protein expression.

Immunohistochemistry: Spinal cords will be harvested, fixed, frozen and sectioned at P7 as previously described. Tissue sections were stained with rabbit anti-SMN Rab MAb (Epitomics, clone EPR4429) (1:1000) and Alexa Fluor® 488 goat anti-rabbit IgG (Molecular probes, A11008) (1:1000). Images will be captured using an Axio

Imager M2, epifluorescent microscope, Axio camera with Axiovision imaging software (Carl Zeiss) and further processed with Adobe Photoshop CS5 software.

4. Results

Optimisation of RT-PCR analysis, immunofluorescence and western blot analysis was undertaken. RT-PCR was optimized using RNA from untreated SMA fibroblasts. Two transcripts i.e. FL-SMN and SMNΔ7 were detected as shown in figure 1. For Immunofluorescence and Western blot analysis, frozen pig tissue was used for optimisation. Immunoblots of extract from pig muscle sections were incubated with either SMN or β-tubulin antibody (Figure 2). Double antibody incubation on immunoblot was tested to enable direct comparison between tested samples with internal protein expression control. (Figure 3) Frozen pig muscle was sectioned using cryotome machine and placed on silane coated slide. Anti-SMN antibody (MANSMA7) was used to detect SMN protein expressed in nuclei. (Figure 4)

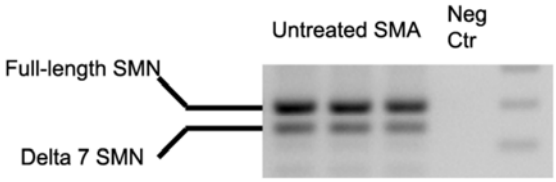

Figure 1: Splicing pattern of SMN expression in fibroblast culture from a type I SMA patient.

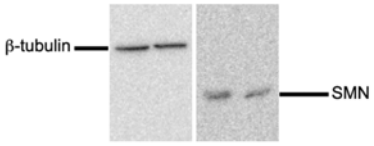

Figure 2: Immunoblots of protein extract from pig muscle sections were incubated with single antibody, as indicated.

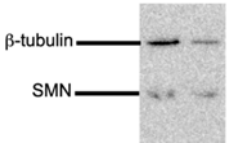

Figure 3: Immunoblot of protein extract from pig muscle sections was incubated with a mixture of two antibodies.

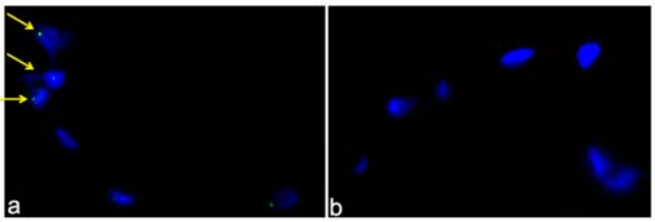

Figure 4: Immunofluorescence on pig muscle sections detected SMN protein localised in nuclei. Blue fluorescence of Hoeschst indicated nuclei. a; accumulated nuclear SMNs (GEMS) are present on the pig muscle section incubated with anti-SMN antibody and b; no antibody control.

We next determined efficiency of PMO(-10-34) in cultured fibroblasts from a type I SMA patient. RT-PCR analysis on the fibroblast cultures treated with PMO(-10-34) was conducted and shown in Figure 5. Western blot analysis on cultured SMA fibroblast treated with PMO(-10-34) confirmed its efficiency in inducing FL-SMN protein *in vitro*.

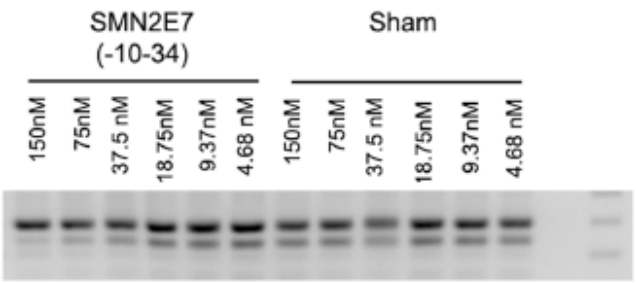

Figure 5: RT-PCR analysis of RNA from cultured fibroblast treated with PMO(-10-34) or sham PMO control at different concentrations.

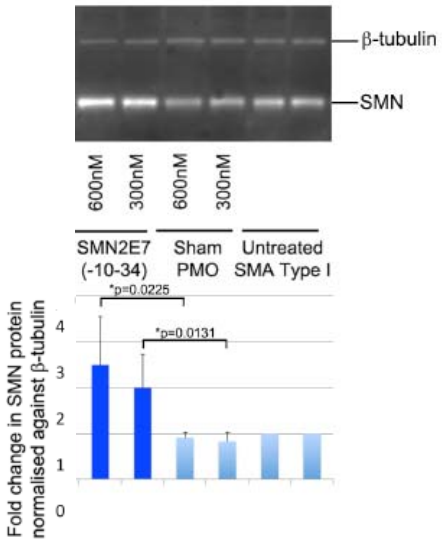

Figure 6: Western blot analysis of SMN and β-tubulin in type I SMA patient cells transfected with PMOs or unrelated negative control and lower panel shows the fold change in SMN, normalised against β-tubulin, as determined by densitrometric analysis. P-values indicates statistical significance in SMN expression between PMO(-10-34) and sham PMO.

We subsequently selected frozen brain and spinal of SMA carrier mice, which were injected with 6 mM of PMO(-10-34). Those mice were sacrificed at 7, 21 or 65 days after single ICV injection. Human SMN protein expression was enhanced at all three time points in both brain and spinal cord tissue. (Figure 6) While single ICV injection of PMO(-10-34) in SMA mice led to prolong mean survival of 135 days, sham treated SMA mice lived only for 14 days.

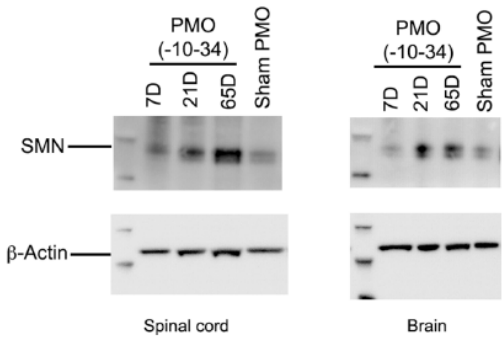

Figure 7: Western blot analysis of SMN and β-actin in a; spinal cord and b; brain of SMA carrier mice intracerebroventricularly injected with 6 mM of PMO(-10-34) or scrambled PMO control. Tissues were collected at 7, 21, 65 days after injection.

As this AO exerts its effect through prevention of the binding of hnRNPA1 on silencing motif in intron 7, we further explore the role of hnRNPA1 in SMN2 splicing. We initially design primer to evaluate mRNA transcript of hnRNPA1 in fibroblast cultures. RT-PCR analysis of hnRNPA1 using four different primers at 55c and 60c of annealing temperatures was undertaken. Two isoforms i.e. hnRNPA1 and hnRNPA1b are previously reported and hnRNPA1 is a major isoform expressing FL-hnRNPA1 protein (320 amino acid). hnRNPA1b transcript has an inclusion of exon 7b and hnRNPA1b protein contains extra 52 amino acid inserting in glycine rich domain of hnRNPA1 protein. We further determine expression level and splicing pattern of hnRNPA1 in type I SMA fibroblast (most severe phenotype), type II SMA fibroblast and normal fibroblast using comparable amount of RNA template. The result suggested that hnRNPA1 expression itself could be a determining factor for SMA phenotype. Moreover hnRNPA1b isoform was reported to have a role on splice site selection.

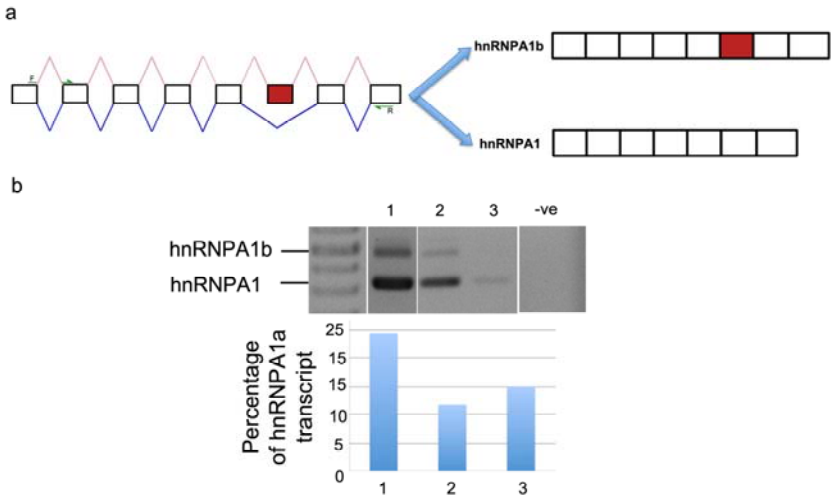

Figure 8: Splicing pattern and level of expression of hnRNPA1 transcript: a) schematic representation of splicing pattern of hnRNPA1 which encodes two transcripts i.e. hnRNPA1 and hnRNPA1b. hnRNPA1b containing casette exon 8 have 156 nucleotides larger than hnRNPA1 transcript. The green arrows indicate the priming location of PCR primers; b) RT-PCR analysis of RNA from three samples including 1. a type I SMA individual, 2. an SMA carrier and 3. a normal individual is shown with densitometric analysis.

5. Critical appraisal and suggestion

This proposal was to evaluate the efficiency of phosphorothioate morpholino oligomer (PMO) compound to increase survival motor neuron (SMN) expression in a mouse model of spinal muscular atrophy (SMA). We demonstrated that single intracerebroventricular administration of the newly developed AO (PMO-10-34) can prolong median survival time of the severe mouse model of SMA. The expression of induced SMN protein in PMO treated mice compared to mock PMO treated mice at three time points, i.e. 7 days, 21 days and 65 days post single ICV injection to determine how long the induced SMN protein last. This PMO also was able to efficiently alter splicing pattern of *SMN2* gene product and enhance substantial amount of SMN protein in heterozygous SMA carrier mice. We observed increased expression of SMN protein in both brain and spinal cord from treated heterozygous mice in all time point. In the mean time, we also observed that heterozygous mice injected with PMO gained more weight compared to control heterozygous mice. In SMA, loss of survival motor neuron protein (SMN) protein leads to motor neuron death and clinical phenotypes. We evaluated the effect of antisense compound (PMO) in a mouse model of SMA. Without any treatment, SMA mice will succumb to the disease within 14 days postnatal. We observed that life span of severe SMA mice intracerebroventricularly injected with PMO at postnatal day 1 (P0) is significantly extended. We proposed to assess the efficiency of PMO in increasing expressed SMN protein in heterozygous SMA mice. Heterozygous SMA mice carry heterozygous endogenous mouse Smn expression with transgenic human *SMN2*, and therefore have a normal life expectancy. These mice then are good model to determine induced SMN protein from PMO.

เอกสารแนบหมายเลข 3

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

Improved Antisense Oligonucleotide Design to Suppress Aberrant *SMN2* Gene Transcript Processing: Towards a Treatment for Spinal Muscular Atrophy. PLoS One. 2013 Apr 22;8(4): e62114. doi: 10.1371/journal.pone.0062114. Print 2013.

2. การเสนอผลงานในที่ประชุมวิชาการ

Mitrpant C., Porensky P.N., Zhou H., Price L., Muntoni F., Fletcher S., Wilton S.D., Burghes A.H.M. P.6.5 Improved antisense oligonucleotide design to suppress aberrant SMN2 gene transcript processing. The 16th International Congress of The World Muscle Society, Asilomar, USA. Neuromuscular Disorders, Volume 23, Issues 9–10, October 2013, Page 771.