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ORIGINAL ARTICLE

Survival of motor neuron protein downregulates miR-9 expression in patients with spinal muscular atrophy

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Abstract Spinal muscular atrophy (SMA) is a lethal hereditary disease caused by homozygous absence of the survival of the motor neuron (SMN) 1 gene (*SMN1*), and it is the leading genetic cause of infant mortality. The severity of SMA is directly correlated with SMN protein levels in affected patients; however, the cellular regulatory mechanisms for SMN protein expression are not completely understood. In this study, we investigated the regulatory effects between SMN expression and miR-9a, a downstream noncoding small RNA. Using an inducible SMN short hairpin RNA interference (shRNAi) system in NSC 34 and human skin fibroblast cells, cellular miR-9 levels and SMN protein repression were time-dependently upregulated. Conversely, cellular miR-9 levels decreased when HeLa cells were transfected with SMN protein fused with green fluorescent protein. In SMA-like mice spinal cords and human primary skin fibroblasts isolated from patients with different degrees of SMA, human SMN exhibited a disease severity-dependent decrease, whereas cellular miR-9 levels increased. These results clearly suggested that cellular SMN proteins regulated miR-9 expression and that miR-9 expression was related to SMA severity. Thus, miR-9 may be a marker for SMA prognosis.

Conflicts of interest: All authors declare no conflicts of interest.

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Introduction

Proximal spinal muscular atrophy (SMA) is an autosomal recessive disease that is characterized by degeneration of the anterior horn cells. SMA is subdivided into the following four clinical types on the basis of age at onset and the level of motor function: (1) severe type I; (2) intermediate type II; (3) mild type III; and (4) adult-onset type IV [1—3]. Two survival of motor neuron (*SMN*) genes, *SMN1* and *SMN2*, are located on chromosome 5q13. Loss-of-function mutations of both copies of the telomeric gene *SMN1* are correlated with the development of SMA [4].

The nearly identical centromeric gene *SMN2*, which is typically not mutated in SMA, appears to modify the disease severity based on the amount of full-length SMN protein that it produces. The SMN protein levels are correlated with disease severity in both patients with SMA and an SMA-like mouse model [4—7]. Major issues in SMA treatment are how to increase the level of the full-length SMN protein and improve disease severity that is regulated by a downstream effect in patients with SMA. No curative therapy for SMA is currently available.

MicroRNAs (miRNAs) are a newly identified group of small RNAs. These are short, noncoding sequences that control gene-expression profiles through translational regulation modifications [8]. Through their interactions with the 3′-untranslated regions of messenger RNAs (mRNAs), miRNAs can trigger translational repression and play a key role in several developmental timing pathways [8,9]. Furthermore, many miRNAs have been shown to regulate various processes during early embryonic development and in human diseases, such as neurogenesis, tumorigenesis, angiogenesis, and metastasis [10]. These links highlight the importance of miRNA research to understand human disease development and devise novel therapies. However, although there is increasing information on miRNA function in developmental systems and some disease entities, such as cancer [11,12], there is very little information on how miRNAs may function during the pathogenesis of neurodegenerative diseases, such as SMA [2].

Recently, many studies have proposed that miRNAs may also play important regulatory roles in neurodegenerative diseases. New discoveries of miRNAs have changed some of our understanding of disease mechanisms and have introduced an entirely novel level of regulatory control over gene expression [13]. In the nervous system, many miRNAs are essential for developmental timing, cell proliferation, cell death, and patterning as well as for the function and identity of neural cell populations, such as miR-9 and miR-124 [14—16]. The mature products of miR-9, which are highly expressed in the brain, are considered to have regulatory roles because of their complementarity to messenger RNA [17,18]. A number of specific miR-9 targets have been proposed, including the RE-1-silencing transcription factor (REST) and its partner corepressor for element-1-silencing transcription factor (CoREST) [19].

In this study, we aimed to identify downstream molecules in patients with SMA. We found that miR-9 was an important downstream molecule of SMN protein. Its SMA disease severity type-related expression pattern suggested that miR-9 may be a marker for determining SMA prognosis.

Materials and methods

Cloning of human SMN

The human SMN (hSMN) protein was cloned from a human placenta complementary DNA (cDNA) library as previously described [20]. Scrambled siRNA and siRNAs against SMN (siRNA1: 5′-UAAAGUCAAU GGACGUAAUAGUAGC-3′; siRNA2: 5′-UACUAUUAGCUACUUCACAGGUCGG-3′; siRNA3: 5′-AAAUGUCAGAAUCAUCACUCUGGCC-3′; siRNA4: 5′-UGGCUAAGUGGU GUCGUCAUCAGCA-3′) were purchased from Invitrogen. Cellular miR-9-expression levels were determined using the following primer pairs: primer 1 (TCTTTGGTTATCTAGCTGT ATGA) and primer 2 (TCATACAGCTAGAGATAACCAAAGA).

Cell culture for mouse spinal cord and human SMA skin fibroblasts and knockdown assay

An inducible SMN knockdown system in NSC 34 and human skin fibroblast cells was provided by Dr Ting (Academia Sinica, Taiwan) [21,22]. The hSMN protein was cloned from a human placenta cDNA library as previously described [20]. The NSC 34 cells and human skin fibroblasts from patients with SMA were cultured in high-glucose Dulbecco's modified Eagle's medium with L-glutamine and sodium pyruvate (Gibco), penicillin and streptomycin (Gibco), and 10% fetal bovine serum (HyClone) at 37°C in a 5% CO_2 incubator. For the knockdown assay, 1×10^5 cells were cultured in a six-well plate. After culturing overnight, scrambled siRNA and siRNA against SMN were transfected using Lipofectamine transfection kit (GIBCO/BRL). Cells were knocked down for48 hours and then treated by doxycycline (1 μg/mL) to decrease SMN expression [23].

Western blot analysis and immunohistochemical staining

Western blot analysis and immunohistochemical staining were performed as previously described [23—25]. The primary antibodies used were against β-actin (1:2000; Santa Cruz, I-19), SMN (1:1000; Santa Cruz, SC-32313), and hemagglutinin tag (1:2000; Sigma).

Quantitative real-time reverse transcription polymerase chain reaction

Total RNA was extracted from cells with TRIzol (Invitrogen) and cDNA was generated using 1 mg of DNase-treated RNA with oligo-dT primers and TermoScript (Invitrogen) following the manufacturer's instruction. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed using premade primers/probes [glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Mm99999915 and miR-9 MIMAT0000441]. GAPDH was used as the internal standard. Gene expression was normalized and analyzed using the ΔC_t method with 7500 System Software (Applied Biosystems). Results were presented as fold difference of the mean compared with the control ($\Delta\Delta C_t$). All reactions were performed at least in triplicate.

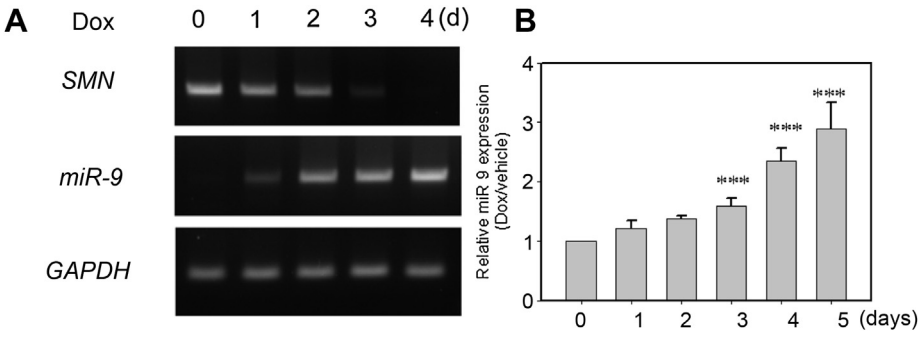

Figure 1. Downregulation of survival of motor neuron (SMN) protein expression upregulates miR-9 noncoding RNA expression. (A) Cellular SMN protein expression was knocked down with an inducible shRNAi system in NSC 34 cells [doxycycline (Dox), 1 μg/mL]. (B) miR-9 levels increased in SMN-shRNAi cells (mean ± standard deviation; $n = 3$; $***p < 0.001$). GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

Northern blot analysis

Total RNA was extracted from cells and mouse spinal cord with TRIzol reagent (Life Technologies). The total RNA (20 μg/sample) was loaded and separated on a 4% agarose/formaldehyde gel and then transferred to nylon membranes (Amersham Pharmacia Biotech). Blots were hybridized to a radiolabeled miR-9 cDNA probe (1—178 bp) and subsequently hybridized with a GAPDH control probe to ensure integrity and equal loading of RNA samples.

Mice

SMA-like mice in this study were obtained from Dr. Jong (Graduate Institute of Medicine, KMU) and the related generation and housing methods were followed as described previously [7].

Patients

This study assessed a total of 16 patients with different types of SMA from Kaohsiung Medical University Hospital (KMUH-IRB-980125). Clinical information about the patients with different types of SMA was obtained as described previously [26].

Statistical analyses

All experiments were performed at least three times. Results for RT-PCR are given as means ± standard deviations. Means were compared by the two-tailed Student test. A p value < 0.05 was considered statistically significant.

Results

Reduced SMN protein expression induces miR-9 expression in NSC 34 and human skin fibroblast cells

To examine a possible regulatory role for miRNAs in patients with SMA, associations between SMN protein and miR-9 expression were first evaluated using NSC 34 neuroblastoma cells [21]. Cellular SMN protein levels showed a time-dependent decrease after treatment with doxycycline (1 μg/mL) using an inducible SMN shRNAi system. Of interest, cellular miR-9 expression increased over time (Fig. 1A and B). This was also found in normal human skin fibroblast cells. As shown in Fig. 2, cellular miR-9 levels showed a time-dependent increase when cellular SMN protein levels were knocked down using gene-specific shRNAi. These

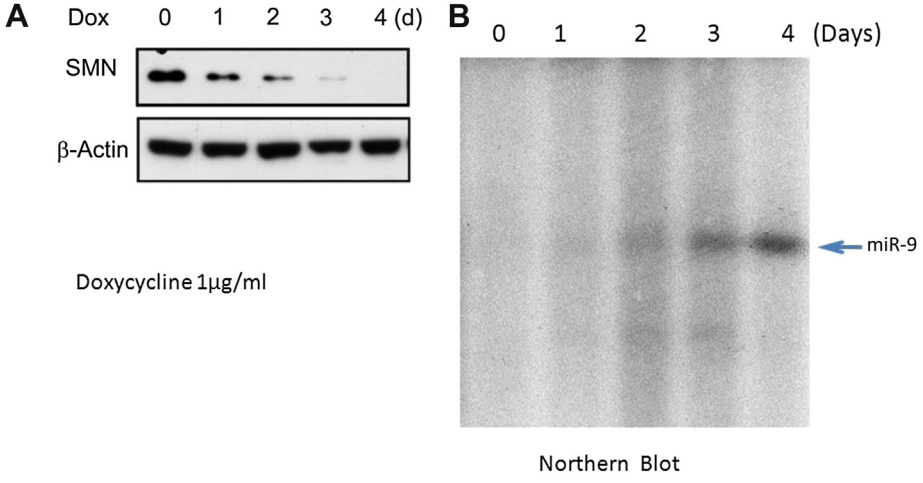

Figure 2. Downregulation of survival of motor neuron (SMN) protein expression upregulates miR-9 noncoding RNA expression in human skin fibroblast cells. (A) Cellular SMN protein expression was knocked down with an inducible shRNAi system in human skin fibroblast cells [doxycycline (Dox), 1 μg/mL]. (B) miR-9 levels increased in SMN-shRNAi skin fibroblast cells. miR-9 was detected by Northern blotting.

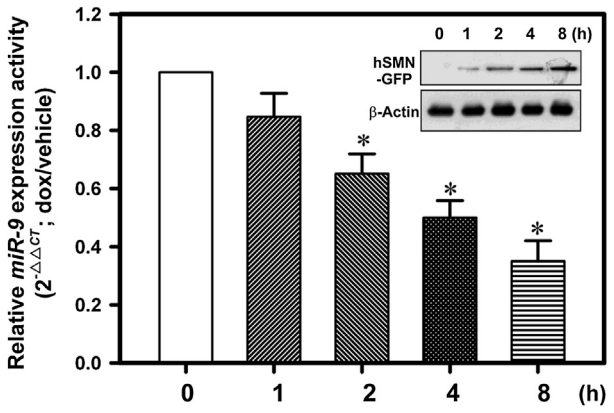

Figure 3. Forced survival of motor neuron (SMN) protein expression downregulates cellular miR-9 expression (mean ± standard deviation; $n = 3$; * $p < 0.001$). Dox = doxycycline; GFP = green fluorescent protein; hSMN = human SMN.

results showed that cellular miR-9 expression was regulated by SMN protein in NSC 34 and human skin fibroblast cells.

hSMN protein overexpression reduces miR-9 expression in HeLa cells

The regulatory effect of SMN protein on cellular miR-9 was further characterized by forcing the hSMN expression fused with green fluorescent protein (GFP) in HeLa cells, which had a high endogenous miR-9 expression. As shown in Fig. 3, using an anti-GFP antibody, hSMN protein fused with GFP was detected in HeLa cells in a time-dependent manner. Concomitantly, cellular miR-9 levels decreased over time. This showed that SMN protein indeed regulated cellular miR-9 expression.

Cellular miR-9 levels exhibit disease severity-dependent upregulation in SMA-like mice and patients with SMA

The miR-9 expression has also been determined in SMA-like mice [7]. Total proteins and total RNA from the spinal cords of SMA-like mice with different levels of disease progression were prepared to determine hSMN and miR-9 expressions. The hSMN-expression level exhibited a disease severity-dependent decrease in the total proteins that were isolated from SMA-like mice spinal cords. However, cellular miR-9 levels increased with disease severity (Fig. 4A). This severity-dependent phenomenon was also investigated in primary skin fibroblast cells established from patients with SMA. As shown in Fig. 4B, cellular miR-9 expression, as determined by semiquantitative RT-PCR, also showed a significant disease severity-dependent increase in patients with SMA.

Discussion

Proximal SMA is an autosomal recessive neurodegenerative disease that is characterized by degeneration of anterior horn cells. No effective therapy is currently available for this disease. In this study, we aimed to determine a possible regulatory effect between SMN proteins and miR-9, a newly proposed small noncoding RNA molecule, in patients with SMA. Our results showed that SMN protein downregulated miR-9 expression under normal conditions. However, cellular miR-9 levels showed an SMA disease severity-dependent increase that was concomitant with decreased cellular SMN protein levels in the cells of patients with SMA. This suggested that miR-9 was an important regulator related to SMA severity and that it could be a target for improving SMA prognosis.

In this study, we first showed that miR-9 expression negatively correlated with SMN protein expression in skin

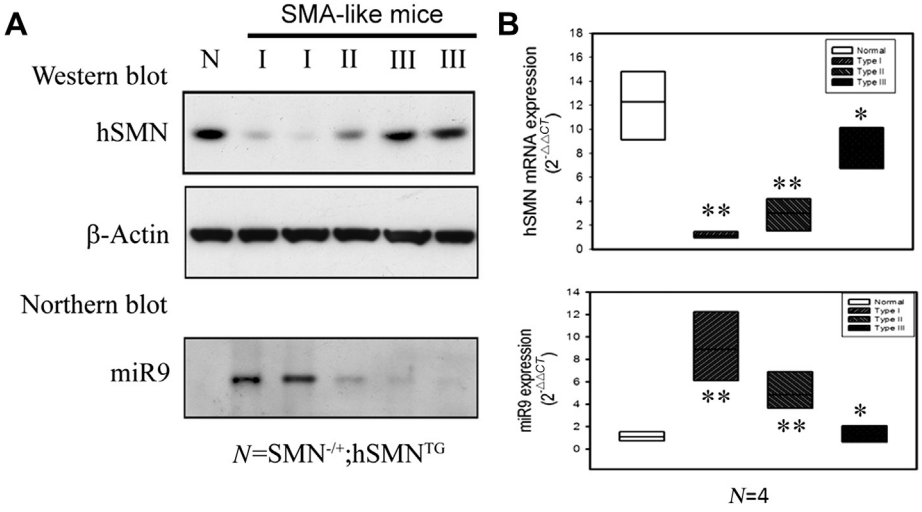

Figure 4. Cellular miR-9 expression exhibits disease severity-dependent increases in spinal muscular atrophy (SMA)-like mice and patients with SMA. (A) miR-9 expression exhibits disease severity-related increases in SMA-like mice. (B) miR-9 expression exhibits disease severity-related increases in skin fibroblast cells of patients with SMA (mean ± SD; $N = 4$; * $p < 0.05$ and ** $p < 0.001$). hSMN = human SMN; mRNA = messenger RNA.

fibroblast cells of patients with SMA. miR-9, a brain-enriched noncoding small RNA, was first cloned in 2001 [17]. Most research on miR-9 has focused on cancer-related studies, and its possibility as a tumor suppressor miRNA was suggested [27—29]. Packer et al. first reported that the miR-9 levels with upstream RE1 sites significantly decreased in the cortices of patients with Huntington's disease (HD) compared with those of healthy controls [19]. The miR-9 expression decreased in patients with early HD onset, and it was found that it was processed from the same primary transcript from the following three genomic loci: *miR-9-1*, *miR-9-2*, and *miR-9-3*. Both *miR-9-1* and *miR-9-3* have upstream RE1 sequences that can be occupied by REST.

miR-9 targets two components of the REST complex, namely, REST and CoREST. These data provided evidence for a double-negative feedback loop between REST complexes and *miR-9* [19]. REST factor, also known as neuron-restrictive silencer factor, contains eight Cys_2His_2 zinc fingers and acts as a silencer to repress neural genes in non-neuronal cells [30—32]. In undifferentiated neuronal progenitor cells, REST acts as a master negative regulator of neurogenesis [31,33]. The pathological function of REST in human disease remains controversial despite the repression effects of REST that were found in many tumorigenesis studies.

In this study, we found that miR-9 expression negatively correlated with SMN protein expression in the primary fibroblast and exhibited disease severity-dependent expression in patients with SMA. By detecting SMN protein expression in primary fibroblasts with SMN1 homologous mutants, the severity of SMA may be predictable in the future and provide for a more suitable therapeutic approach. These results suggest that miR-9 may be a marker for SMA prognosis and, thus, it warrants further investigation.

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