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Study on the role of Hsa-miR-382-5p in epidural fibrosis

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Abstract. – OBJECTIVE: To investigate the role and potential mechanism of human serum albumin (hsa)-micro ribonucleic acid (miR)-382-5p in epidural fibrosis formation after laminectomy.

MATERIALS AND METHODS: Cells were transfected with miR-382-5p mimic or miR-382-5p inhibitor. Then, 3-(4,5)-dimethylthiahiazol (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) assay was employed to detect the effect of miR-382-5p on proliferation, and Real-time reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting were used to examine the expressions of miR-382-5p and fibrosis-related proteins after treatment with transforming growth factor beta (TGF-β). Luciferase assay and immunofluorescent staining were done to confirm whether collagen I A1 is a target of miR-382-5p.

RESULTS: MTT assay demonstrated that miR-382-5p had no significant effect on fibroblast proliferation. Expressions of miR-382-5p and fibrosis-related proteins were remarkably increased after TGF-β treatment. Collagen I A1 was acknowledged as a target of miR-382-5p. MiR-382-5p mimic statistically enhanced the level of collagen I A1, and miR-382-5p enhanced the expressions of collagen I A1.

CONCLUSIONS: Increased miR-382-5p promotes epidural fibrosis by increasing collagen I A1 expression, and miR-382-5p may be a potential novel molecular target for the treatment of epidural fibrosis.

Key Words

Epidural fibrosis, MiR-382-5p, Collagen I, Laminectomy.

Introduction

Laminectomy is one of the most frequently-used surgical methods in spinal surgery. However, the adhesion of epidural scar after laminectomy severely affects the decompression effect of the operation, leading to failed back surgery syndrome (FBSS)[1-3]. Hence, it becomes a problem perplexing spinal surgeons, which is not resolved well. As a result, how to effectively prevent or reduce the adhesion of epidural scar after laminectomy is very important for the assurance of postoperative efficacy. FBSS is caused by many complex factors, but many researchers believe that the adhesion of epidural fibrous scar tissue after lumbar spinal canal decompression and laminectomy is one of the important causes[4,5]. The mechanism is as follows: surgical trauma after laminectomy leads to local inflammatory response and hematoma formation, and the mediation of inflammatory factors results in proliferation of fibroblasts, increased synthesis of collagen proteins and formation of a fibrous scar in the epidural surgery area, thus resulting in epidural fibrosis. Fibrous scar tissue stretches and squeezes the spinal dura mater and nerve root and affects blood supply, causing clinical symptoms such as pain in waist and lower extremities[6,7]. Previous studies[8,9] have proved that epidural scar formation after laminectomy is mainly caused by abnormal proliferation and excessive activation of local fibroblasts and massive accumulation of collagens. Therefore, how to suppress abnormal proliferation and over-activation of fibroblasts is a key for the research in this field.

Micro ribonucleic acids (miRNAs) are endogenous and highly conserved single-stranded non-coding RNAs with 18-22 nt in length, produced by the transcription of deoxyribonucleic acid (DNA). However, they have no open reading frames, so that they cannot be translated into proteins. As early in 1933, miRNAs were discovered for the first time in the embryonic development of nematodes, and the two miRNAs discovered were named as lin-4 and let-7[10-12]. Subsequent studies have suggested that miRNAs are widely expressed in plants and animals and are involved in many pathophysiological processes such as growth, proliferation, development, wound healing, and tumorigenesis. Most miRNA-encoded genomes are located in the intergenic regions or two-intron regions as a single gene or clustered

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family domain. MiRNAs are transcribed and synthesized in the nucleus and then translocated into the cytoplasm to play their roles. Transcribed precursor miRNAs are processed into mature double-stranded miRNAs with Drosha (a member of RNase II family) and Dicer (a member of RNase III family). Mature miRNA chains are selected according to the thermodynamic properties to exert their biological effects, and the complementary sequences are degraded. However, recent studies have revealed that the complementary strand also has biological effects. Previous studies have clarified that miR-382-5p plays a key role in fibroblast activation. Therefore, this work was conducted to explore its potential mechanism.

Materials and Methods

Cell Culture

Primary human fibroblasts were isolated from epidural scars. Fibroblasts were then used for the experiments at passages 3-4.

Drug Treatment and Cell Transfection

Fibroblasts were treated with transforming growth factor beta 1 (TGF-β1) (5 ng/mL) (Cell Signaling Technology, Danvers, MA, USA) for 24 h. Cells were transfected with miR-382-5p mimic or miR-382-5p inhibitor to simulate overexpression or knockdown of miR-382-5p.

Assay of Cell Proliferation

After 24 h of transfection, cells were collected from each group and inoculated into a 96-well plate at a density of 1×10^4 cells/well (100 μL/well). Each group covered 4-time points, namely, 6, 12, 24, and 48 h in triplicate. After cells adhered to the surface of each well, 50 μL [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) was added to each well for 4 h of culture at 37°C. Then, the supernatant was aspirated, and dimethyl sulfoxide (DMSO) (150 μL/well) was added for shaking for 10 min. After that, a microplate reader was used to determine the absorbance (A) of each well at a wavelength of 570 nM. The proliferation of cells in each group was calculated, and corresponding proliferation curve was drawn.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from fibroblasts using TRIzol (Invitrogen, Carlsbad, CA, USA). The expression levels of miR-382-5p, collagen I A1, collagen I A2, alpha-smooth muscle actin (α-SMA), and connective tissue growth factor (CTGF) were measured by quantitative RT-PCR (qRT-PCR) with aSABI SYBR Green Master MIX kit (Invitrogen, Carlsbad, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or U6 was used as a control.

Western Blotting

Total protein was extracted from treated fibroblasts using radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China), and the concentration of protein was determined by bicinchoninic acid (BCA) assay. Then, 30 μg protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and blocked with 5% non-fat dry milk. After that, the membrane was incubated with collagen I A1, collagen I A2, α-SMA, CTGF, and GAPDH primary antibodies (Abcam, Cambridge, MA, USA). Thereafter, the membrane was subjected to chemiluminescence assay using horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and enhanced chemiluminescence (ECL) detection reagents (Millipore, Billerica, MA, USA), and luminescence bands were quantified *via* densitometry. The results were normalized to GAPDH. All experiments were performed for 3 times.

Validation of Collagen I A1 as a Direct Target of miR-382-5p

To gain further insight into the molecular mechanism of miR-382-5p in regulating collagen I A1, miR-382-5p gene targets were determined by miRanda, TargetScan, PicTar and PITA.

Immunofluorescent Staining

Firstly, a 24-well plate was moistened using Dulbecco's modified Eagle medium (DMEM). Cells were passaged, re-suspended and inoculated on a coverslip at an appropriate density. Cells grew adherently. Then, the 24-well plate was taken out to remove the medium, and cells were rinsed with 1×phosphate-buffered saline (PBS) twice and fixed in 4% paraformaldehyde for 30-60 min, followed by 4 times of washing with 1×PBS (5 min/time). Next, cells were punched *via* 0.4% Triton-100 (diluted with 1×PBS, 4 μL Triton + 1 mL PBS) for 5 min and washed with 1×PBS for 3 times (5 min/time). After that, cells were blocked with 5% bovine serum albumin (BSA) (5% BSA

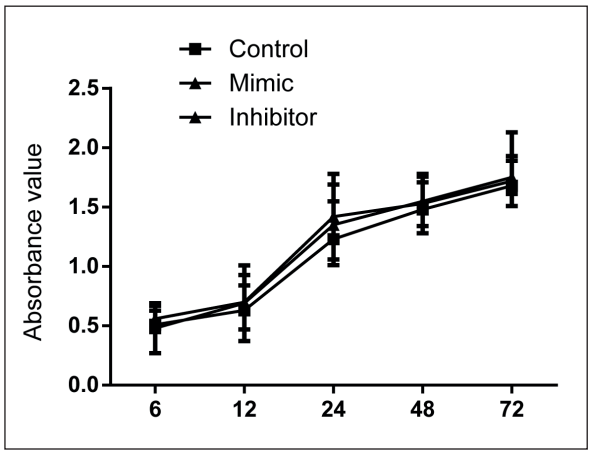

Figure 1. There was no significant difference between mimic and inhibitor group after transfection for 6, 12, 24, 48, and 72 h.

= 5 g albumin bovine V + 100 mL 1×PBS) at room temperature for 1-2 h, incubated with primary antibody (diluted with 5% BSA and calculated as 200 μL/well) at 4°C overnight (≥14 h) and rinsed with 1×PBS for 3 times (5 min/time). Thereafter, a fluorescent secondary antibody was added for incubation in a wet box at 37°C for 1 h, followed by washing with 1×PBS for 3 times (5 min/time). Cells were subjected to nucleus staining with 4',6-diamidino-2-phenylindole (DAPI) for 30 s to 1 min and washing with 1×PBS for 3 times (5 min/time). Next, mounting with Debico (6 μL/glass slide) was conducted, i.e., Debico was dripped on a glass slide, and the coverslip was placed over Debico with the side with cells down. Lastly, photographing and archiving were immediately performed under a fluorescence microscope.

Luciferase Reporter Assay

The collagen I A13'-untranslated region (3'UTR) luciferase reporter vector was purchased from Genechem (Shanghai, China). Luciferase reporter assay was done according to the previous research[13].

Statistical Analysis

In this study, data were collected and analyzed, and the results of experimental reports were means ± standard deviations (mean ± SD). In addition, Statistical Product and Service Solutions (SPSS) 19.0 software (IBM Corp., Armonk, NY, USA) was adopted for the statistical comparisons among groups. Statistical differences between different groups were analyzed *via* Student's *t*-test. $p<0.05$ suggested that the difference was statistically significant.

Results

MiR-382-5p Had no Significant Effect on Fibroblast Proliferation

The results of MTT assay showed that the proliferation rate of cells in the mimic transfection group at 6, 12, 24, 48, and 72 h was not significantly different from that in the inhibitor group and control group ($p>0.05$), suggesting that miR-382-5p did not have an apparent effect on fibroblast proliferation (Figure 1).

MiR-382-5p, Collagen I A1, Collagen I A2, α-SMA and CTGF Were Significantly Increased After Treated with TGF-β1

Primary cells were mainly arranged in spindle bundles (with an irregular shape in a small amount of them) and mutually cross-linked with elongated projections (Figure 2A). After cultured with TGF-β1, the expressions of miR-382-5p, collagen I A1, collagen I A2, α-SMA and CTGF were overtly increased ($p<0.05$) (Figure 2B and 2C)

Confirmation of Overexpression or Knockdown

RT-PCR was employed to examine the level after transfected with miR-382-5p mimic or miR-382-5p inhibitor, and it was detected that the expression level in the mimic group was significantly enhanced, while that in the inhibitor group was remarkably decreased ($p<0.05$) (Figure 3).

Acknowledgment of Collagen I A1 as a Target of miR-382-5p

To explore the further molecular mechanism of miR-382-5p in regulating the process of fibrosis, databases were used in this study to search for potential target gene shaving clarified functions in fibrosis regulation. Among the fibrosis-associated target genes, it was found that there was complementarity between miR-382-5p and collagen I A1 3'UTR (Figure 4A). Results of the luciferase assay performed above (Figure 4B) also verified it.

MiR-382-5p Promoted Expression of Collagen I A1

To further explore the effect of miR-382-5p on collagen I A1, an immunofluorescence assay was performed in this study, and the results indicated that the fluorescence intensity in the miR-382-5p mimic group was clearly higher than that in the inhibitor group, revealing that miR-382-5p promoted the expression of collagen I A1 (Figure 5).

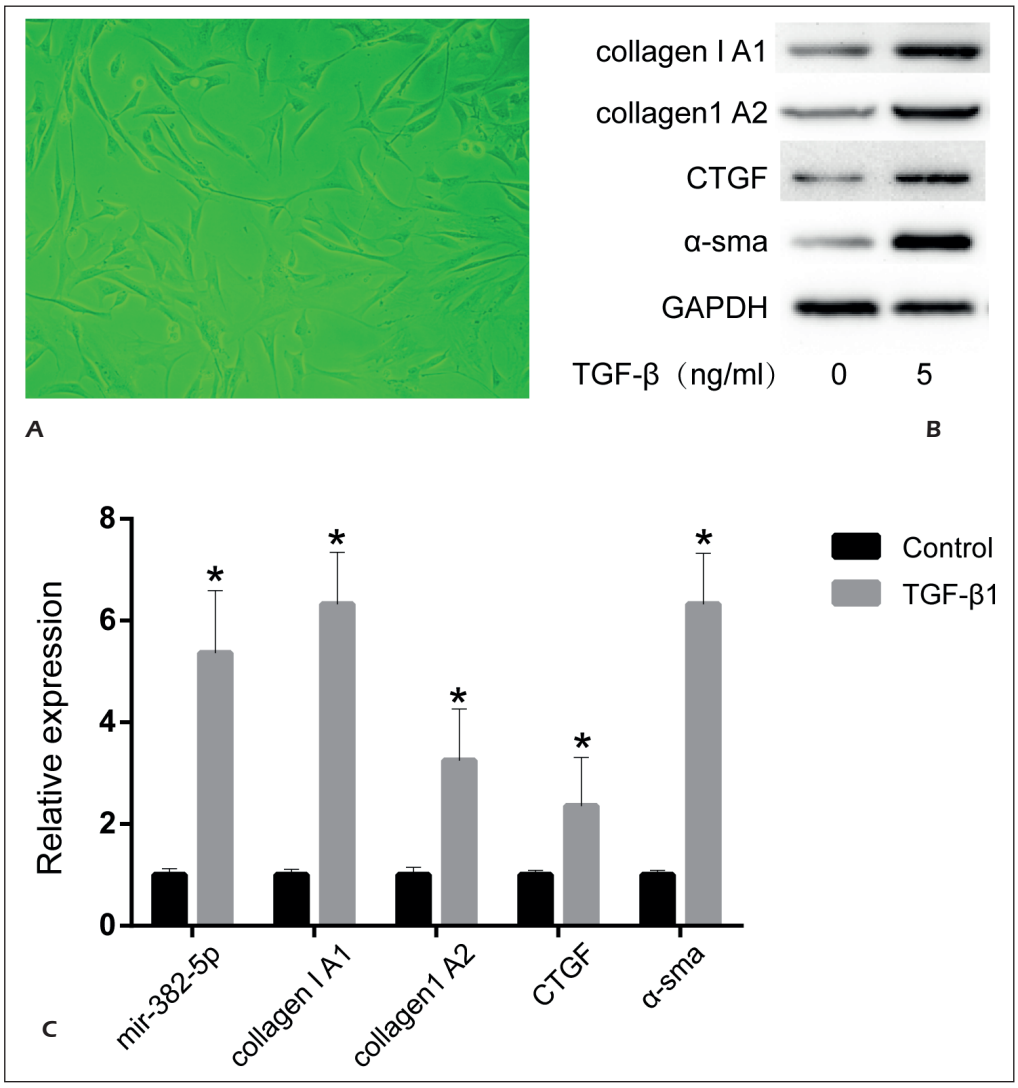

Figure 2. A, Morphology of fibroblasts. **B-C**, MiR-382-5p, collagen I A1, collagen I A2, α-SMA and CTGF were significantly increased (*p<0.05, compared with control group).

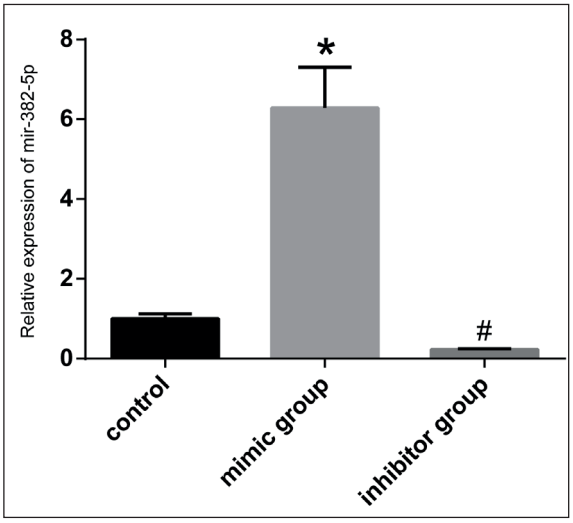

Figure 3. After transfection with mimic, the level of mir-382-5p was significant increased and remarkable decreased after inhibitor transfection (*p<0.05, #p<0.05, compared with control group).

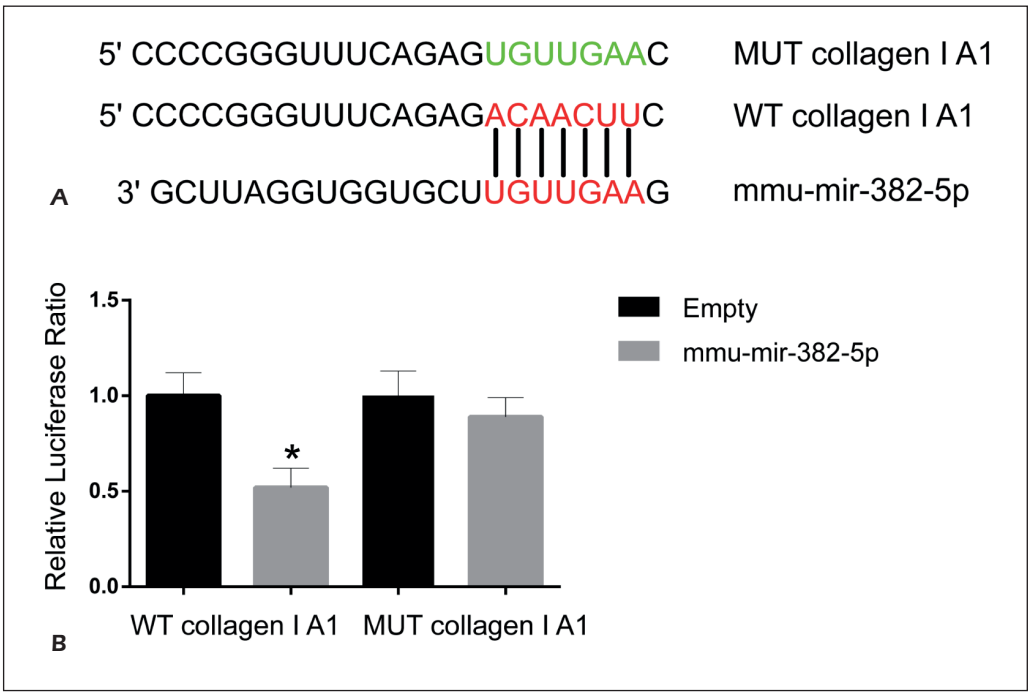

Figure 4. Luciferase assay confirmed collagen I A1 as a target of miR-382-5p (*p<0.05, compared with Empty in WT collagen I A1).

Discussion

Epidural fibrosis refers to the replacement of postoperative fibrotic tissue by normal epidural fat, which binds the dura and nerve root to the surrounding structures from the anterior and posterior, thus leading to poor outcomes of spinal operation. Previous studies[14-17] have suggested that epidural fibrosis is closely correlated with fibroblast activation and collagen overproduction. In this process, collagen I A1 is a key marker for the production of collagens in fibroblasts and plays an important role in the pathogenesis of hypertrophic scar. In addition, as far as we know, the increase in TGF-β production is one of the most important pathogeneses of fibrosis.

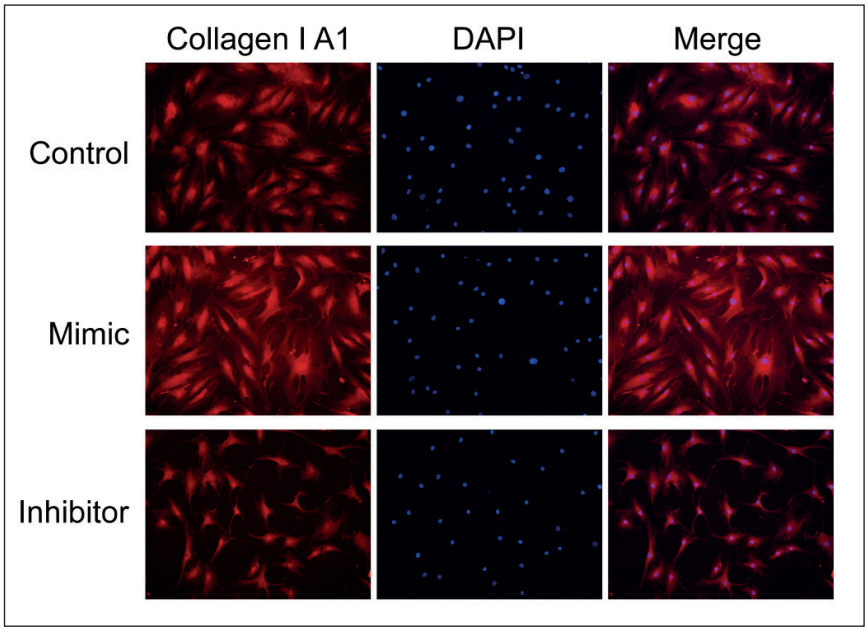

Figure 5. MiR-382-5p enhanced expression of collagen I A1.

Previous studies have shown that miRNAs play important regulatory roles in epidural fibrosis. Mature miRNAs are specifically paired with the base sequence in 3'-untranslated region (3'UTR) of the target gene mRNA to inhibit the translation of the mRNA or accelerate its degradation, thereby exerting the negative regulatory effects on gene expression at a post-transcriptional level[18]. A single miRNA molecule is able to regulate the expressions of multiple target genes, and a target gene can be regulated by multiple miRNA molecules. Furthermore, miRNAs mainly regulate transcriptional genes at the initial stage of translation, so that the transcription of target genes is unstable with the effects on 5'decapping and deadenylation[19,20].

After transfection with miR-382-5p mimic, it was found that, with the increase in miR-382-5p, collagen I A1 expression was increased at the same time, proving that miR-382-5p affects the occurrence and development of fibrosis by regulating collagen I A1 expression.

Conclusions

We demostrated that knockdown of miR-382-5p can inhibit the development of epidural fibrosis by reducing the expression of collagen I A1. It may be a target for treating renal interstitial fibrosis.

Conflict of Interest

The authors declare that they have no conflict of interest.

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