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MiR-6836-3p promotes proliferation of hypertrophic scar fibroblasts by targeting CTGF

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Abstract. – OBJECTIVE: To explore the role of micro ribonucleic acid (miR)-6836-3p in regulating hypertrophic scar (HS) and its potential mechanism.

PATIENTS AND METHODS: The level of miR-6836-3p in HS or normal skin was determined by Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Cell counting kit-8 (CCK8) and flow cytometry were applied to explore the effect of miR-98 on the growth and apoptosis of HS fibroblasts (HSFBs). Luciferase assay was employed to acknowledge whether connective tissue growth factor (CTGF) is a target of miR-6836-3p. Western blotting was used to detect the protein expression of CTGF after transfection with miR-6836-3p mimic or inhibitor.

RESULTS: MiR-6836-3p expression was much higher in HS than that in normal skin. MiR-6836-3p mimic promoted fibroblast growth, and CTGF was confirmed to be a direct target of miR-6836-3p. The results of Western blotting clarified that miR-6836-3p mimic raised the level of CTGF, and its expression was positively correlated with that of CTGF.

CONCLUSIONS: MiR-6836-3p promoted the development of HS by increasing the expression of CTGF. MiR-6836-3p may be a potential novel molecular target for the treatment of HS.

Key Words:
miR-6836-3p, CTGF, Hypertrophic scar.

Introduction

Hypertrophic scar (HS) caused by various traumas is a product in the healing process in the body[1-4]. Besides, it is a general term denoting the changes in the appearance and pathology of normal skin. The formation of HS is indispensable for the repair in trauma. However, the growth of a scar exceeding a certain limit will cause problems such as damage to the appearance and dysfunction of tissues[5,6]. In addition, pathological scars result from the broken balance between anabolism and catabolism of collagens during the repair in trauma. The research on the formation and prevention of scars is one of the important fields in medical research[7]. Scars have irregular shapes on the skin and even protrude from the skin. Moreover, they have tough texture and are itching and causalgic, resulting in serious consequences (deformity and dysfunction) to patients in both appearance and function and affecting the mental status and quality of life of patients. Currently, it is generally considered that the direct cause of scar formation is the abnormal accumulation of the extracellular matrix (ECM) in the dermis, during which fibroblasts play key roles. Fibroblasts directly synthesize and secrete Collagen I, Collagen III, and connective tissue growth factor (CTGF) to form ECM and then regulate the metabolism of ECM by releasing various cytokines including transforming growth factor-beta 1 (TGF-β1) and matrix metalloproteinases (MMPs)[8]. The disordered functional activity of fibroblasts is sure to result in ECM accumulation and cause abnormal proliferation of scars at the same time.

Micro ribonucleic acids (miRNAs) refer to endogenous and non-coding single-stranded miRNAs with 21-25 nt in length, which are widely involved in the regulation of eukaryotic cells[9]. MiRNAs regulate cell proliferation, differentiation, and apoptosis by acting on target messenger RNAs (mRNAs) to degrade them or inhibit their translation, participating in body metabolism. Mature miRNAs can bind to complementary mRNA sites and suppress or degrade target genes *via* base pairing, thus exerting their regulatory effects[10]. At present, it has been extensively proved that miRNAs participate in the repair in skin trauma and the occurrence of development

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of dermatoses. Previous studies[11,12] have used miRNA microarrays to detect miRNA differential expression profiles of HS and normal skin and found that miRNAs are significantly up-regulated or down-regulated in HS. Their target genes are closely related to the growth, differentiation and proliferation of cells, growth of the epidermis, and formation of collagen fibers.

Patients and Methods

Tissue Samples

In this study, 20 pairs of HS and normal skin tissue samples were collected from 20 patients clinically diagnosed with proliferative scars in our hospital from May 2016 to December 2017. These 20 subjects signed the informed consent. This study was approved by the Ethics Committee of Affiliated Hospital of Jining Medical University.

Cell Culture

HS fibroblasts (HSFBs) and normal skin fibroblasts (NSFBs) were isolated from HS and normal skin tissue samples separately. Then, HSFBs and NSFBs were separately washed with chloramphenicol for 3 times and soaked for 15 min. Next, they were rinsed 3 times with phosphate-buffered saline (PBS) and cut into muddy flesh shape with a piece of sterile scissors. After that, cut tissues were transferred into a 50 mL centrifuge tube, added with 0.2% sterile compound collagenase digestion solution (approximately 5 times higher than the tissue volume), digested on a shaker at 37°C (for 3 h for scar tissue and 1.5 h for skin tissue) and filtered using a 200-mesh filter. Then, the filtrate was centrifuged at 1500 rpm for 10 min, and the supernatant was discarded. Thereafter, 10 mL high-glucose Dulbecco's modified Eagle medium (HG-DMEM) containing 10% fetal bovine serum was added, mixed and transferred to a culture dish with 100 mm in diameter for culture. After cells attached to the dish, medium was changed within 24 h for the first time. Then, the medium was replaced every other day. The 1-3 generations of fibroblasts were used for experiments.

Transfection of MiR-6836-3p Mimic and Inhibitor

MiR-6836-3p mimic and miR-6836-3p inhibitor were obtained from Genechem (Shanghai, China). The transfection experiment was performed as previously described[13]. Briefly, cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

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

Total RNA was obtained from tissues or cells using TRIzol (Invitrogen, Carlsbad, CA, USA). The expression of miR-6836-3p was measured by quantitative RT-PCR (qRT-PCR) according to the manufacturer's instructions on the TaqMan microRNA reverse transcription kit (Yifeixue Bio Tech, Nanjing, China). U6 was used as a control. Relative expression level of miRNA or mRNA was analyzed using the Bio-Rad C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The expression level was normalized using U6 small nuclear RNA by the $2^{-\Delta Ct}$ method. The Δ cycle threshold (Ct) values were normalized to U6 level.

Western Blotting

Tissues and cells were taken, added with radio immunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) and various protease inhibitors such as phenylmethanesulfonyl fluoride (PMSF) and incubated on ice for 30 min. During this period, continue purging was performed to avoid bubble generation. Then, tissues and cells were transferred to a centrifuge tube for centrifugation at 4°C and 15000 rpm for 15 min. All steps were done on ice to prevent protein degradation. After that, the concentration of samples was determined based on the instructions of the bicinchoninic acid (BCA) protein quantification kit, and equal loading of all samples was achieved. Next, samples were added with an equal volume of sodium dodecyl sulfate (SDS)-loading, mixed and boiled at 100°C for 10 min, followed by discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE). Prepared samples and standard samples were loaded separately. Standard samples were added to the first well and subjected to electrophoresis to separate proteins. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (sandwiched in three layers), followed by membrane transfer on ice at 100 V for 1 h. Transferred membrane was put in a box with suitable size, added with 5% non-fat milk and incubated on the shaker for 1 h. Then, the membrane was added with a primary antibody that directly bound to the target protein antigen and was washed with Tris-buffered saline and

Tween®20 (TBS-T) for 3 times (5 min/time). After that, the membrane was added with a secondary antibody that bound to the primary antibody and conjugated a horseradish peroxidase. When a right enzyme substrate was added, the mixture was converted into a substance with a chemiluminescent property *via* the oxidation reaction of the horseradish peroxidase, followed by testing. The mixture was washed with TBS-T for 4 times (8 min/time). Exposure: The substrate was added, and a film was used for signal collection and development.

Luciferase Reporter Assay

The CTGF 3'-untranslated region (3'UTR) luciferase reporter vector was purchased from Genechem (Shanghai, China). Luciferase reporter assay was done based on the previous research (28817807).

Cell Viability Assay

Cells were inoculated on a 96-well plate at a density of 5×10^4/mL, 100 µL in each well. 10 µL cell counting kit-8 (CCK8, Dojindo, Kumamoto, Japan) solution mixed with 90 µL DMEM was added ito each well, and then, the plate was incubated at 37°C for 2 h. Absorbance was measured at 450 nm.

Detection of Cell Cycle and Apoptosis Rate

Cells were collected and made into single-cell suspensions. Suspensions were washed twice with PBS and fixed in 70% ethanol overnight. Then, reagents were added according to the operating instructions, and a flow cytometer was used to detect the cell cycle in each group. After that, the same steps were performed. Then, Annexin V/propidium iodide (AV/PI) double staining of cells was carried out according to the instructions, and the apoptosis rate in each group was detected by the flow cytometer. Each experiment was repeated for three times.

Statistical Analysis

In this study, Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze the experimental results. Comparison between groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). All quantitative data were expressed as mean ± standard deviation ($\bar{x} \pm s$). $p < 0.05$ suggested that the difference was statistically significant.

Results

MiR-6836-3p Expression Was Increased in HS

The expression level of miR-6836-3p in HS and normal skin was determined by RT-PCR. The expression of miR-6836-3p in HS was significantly increased compared with that in normal skin (Figure 1), which indicated that miR-6836-3p participate in the progress of scar formation.

MiR-6836-3p Regulated Fibroblast Growth

To further examine the effect of miR-6836-3p on HS, the miR-6836-3p mimic or inhibitor was respectively transfected with HSFBs. The RT-PCR was done to detect the expression level of miR-6836-3p after 24h of transfection. The level was significantly increased in mimic group, and it was remarkably decreased in inhibitor group (Figure 2A). Then, CCK-8 assay was used to explore cell viability. Results suggested that the viability was markedly increased in mimic group and decreased in inhibitor group (Figure 2B). Flow cytometry was employed to explore the effect of miR-6863-3p on apoptosis. Apoptosis rate was remarkably raised after transfection with miR-6836-3p inhibitor, and the rate was significantly reduced in mimic group (Figure 2C).

CTGF Was Confirmed to be a Direct Target of MiR-6836-3p

Among fibrosis-associated target genes, it was found that there was complementarity between

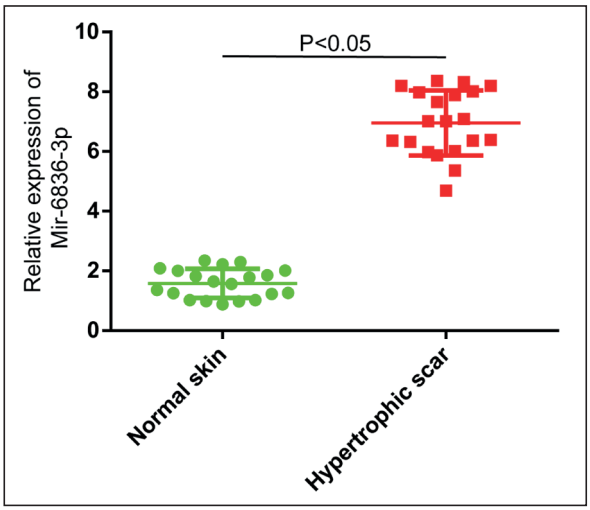

Figure 1. MiR-6836-3p was significantly raised in HS (*$p < 0.05$).

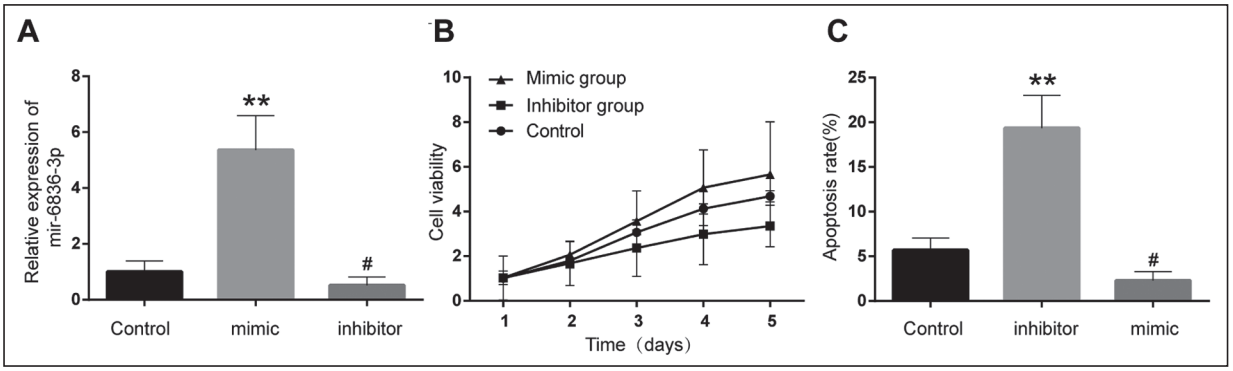

Figure 2. ***A,*** MiR-6836-3p was remarkable increased in mimic group, and it was remarkably decreased in inhibitor group (*$p < 0.05$), ***B,*** MiR-6836-3p mimic raised the cell viability and reduced the apoptosis rate (*$p < 0.05$).

miR-6836-3p and CTGF (Figure 3A). Results of the luciferase assays performed above (Figure 3B) also verified it.

MiR-6836-3p Enhanced the Level of CTGF

The protein expression of CTGF in mimic group and inhibitor group was explored by Western blotting. The level of CTGF was significantly increased in mimic group and decreased in inhibitor group (Figure 4).

MiR-6836-3p Was Positively Correlated With CTGF

To explore the further molecular mechanism of miR-382-5p in regulating CTGF, the relationship between them was detected. Results showed there was a positive correlation between miR-6836-3p and CTGF in HS (Figure 5).

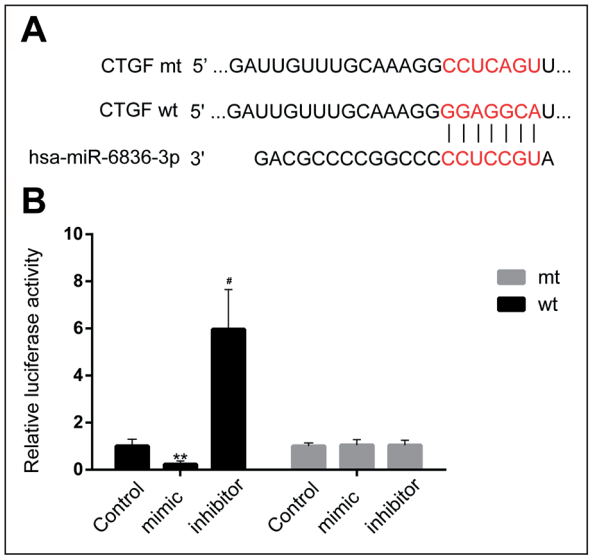

Figure 3. Luciferase assays clarified CTGF as a target as miR-6836-3p.

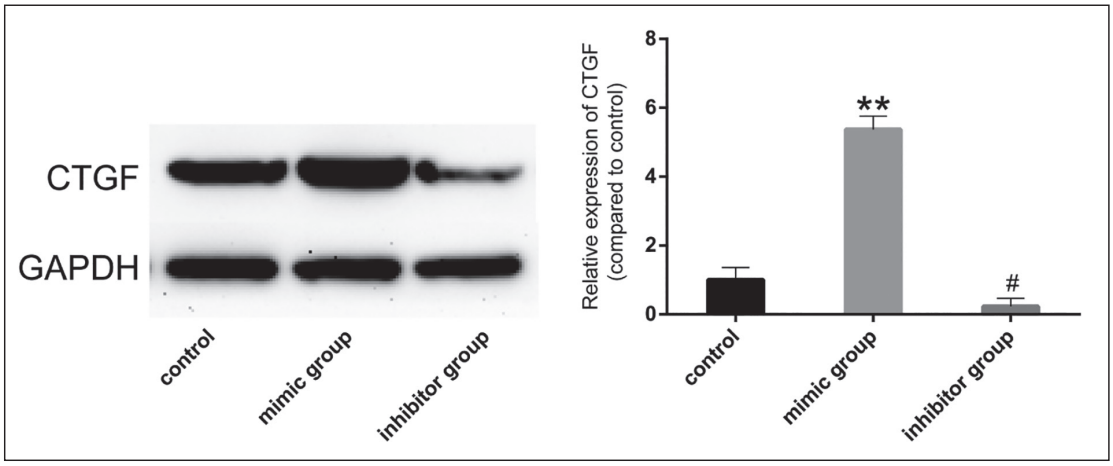

Figure 4. MiR-6836-3p significantly increased the expression of CTGF compared to the control group (*$p < 0.05$).

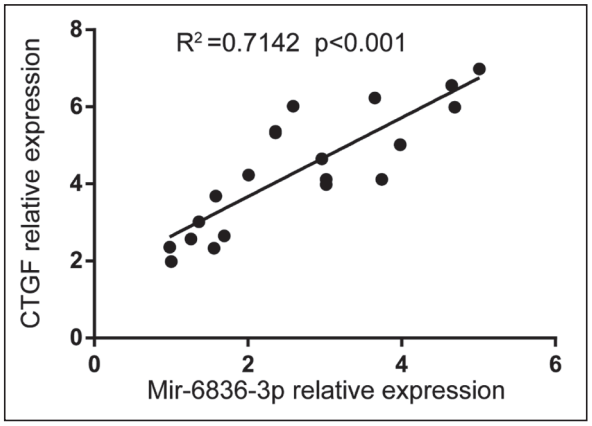

Figure 5. MiR-6836-3p was positively correlated with CTGF ($p < 0.001$).

Discussion

Scarring is a defensive physiological mechanism during the repair in human skin trauma[14]. Under normal circumstances, collagens maintain the dynamic balance between synthesis and degradation during the repair in trauma[15-17]. However, the normal dynamic balance in HS is broken, i.e., massive collagens are synthesized and secreted *via* the activation of fibroblasts, while the degradation of collagens is inhibited, thus causing the accumulation of a large amount of collagens in ECM and finally resulting in the pathological phenomena of HS. HS is a common complication after skin trauma, which not only affects the appearance of patients, but also leads to dysfunction or even disability of tissues and organs[18]. Therefore, HS is always a focus and hotspot of research on trauma and plastic. With the rapid development of related disciplines such as cell biology, molecular genetics, and immune metabolomics, it has been confirmed that the occurrence and development of HS are closely related to the accumulation of massive collagens that are synthesized and secreted after the abnormal activation of fibroblasts during the repair in trauma to ECM. ECM mainly consists of collagens and CTGFs[19,20]. Therefore, further exploration of the molecular mechanisms of the biological abnormalities of HSFBs and search of new effective therapeutic targets are focuses of current research on HS. Studies have shown that miR-6836-3p is closely related to HS, and the expression of miR-6836-3p in HS is significantly increased, which is also confirmed by the findings of this study. To further clarify the relationship between miR-6836-3p and CTGF, HS cells were subjected to transfection in this study, and the results suggested that after overexpression of miR-564, cell proliferation was decreased, and CTGF expression was enhanced, further proving that the two factors promote each other and synergistically and jointly regulate the synthesis and secretion of ECM in HS.

Conclusions

MiR-6836-3p gene evidently affects the proliferation of fibroblasts and the synthesis of ECM by changing the CTGF signaling pathway, and it can be used as a therapeutic target for the regulation of repair in trauma and scar hypertrophy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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