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The mechanisms of β-catenin on keloid fibroblast cells proliferation and apoptosis

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Abstract. – OBJECTIVE: To investigate the role of β-catenin siRNA on proliferation and apoptosis of keloid fibroblast cell.

PATIENTS AND METHODS: Real-time polymerase chain reaction (RT-PCR) and Western blot were performed to monitor the mRNA and protein expression levels of β-catenin in pathological scar tissue and adjacent normal tissue. Human keloid fibroblast cells (KFB) were isolated from the keloid's tissue by enzyme digestion assay and identified by immunocytochemistry assay. Keloid fibroblast cell lines *in vitro* were transfected with 3 pairs of specific β-catenin small interfering RNA (siRNA); RT-PCR and Western blot were performed to identify the best siRNA. The proliferation and apoptosis of KFB transfected with β-catenin were estimated by MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay and flow cytometry (FCM). In addition, the expression levels of Bcl-2, p53, and active-caspase-3 were detected by Western blot.

RESULTS: The RT-PCR and Western blot assay results showed that the expression levels of β-catenin mRNA and protein in pathological scar tissue were significantly higher than those in adjacent normal tissue ($p<0.05$). KFB were successfully separated from human pathological scar tissue, and immunofluorescence staining results showed that cells were spindle and positively stained with vimentin. The β-catenin siRNA2 remarkably inhibited the expression of β-catenin at mRNA and proteins levels in the human keloid fibroblasts. Compared with the control group, cell proliferation was decreased, and apoptotic rate was increased in β-catenin siRNA2 group.

CONCLUSIONS: Knockdown of β-catenin significantly decreased the proliferation and increased apoptosis of KFB, which could inhibit the formation of pathological scar.

Key Words:
Keloid, β-catenin, siRNA, Proliferation, Apoptosis.

Introduction

Pathologic keloid is a common disease of overgrowth in fibrous tissues with characteristics of excessive generation and aggregation of the extracellular matrix, like collagen[1-3]. Clinically, keloid is frequently identified in wound-healing after treatment of burns or other trauma operation. Fibroblasts constituting the keloid tissues have the ability of excessive proliferation, which, together with the insufficient apoptosis and abnormal synthesis in collagen, could induce the continuous hyperplasia in keloid tissues[4-6]. Thus, inhibiting the proliferation of fibroblasts and inducing the apoptosis in keloid tissues can reduce the hyperplasia in keloid tissues and delay the disease progression, which is important for improving the treatment of pathologic keloid.

Latest studies[7] have shown that in pathologic keloid tissues there exists the abnormal activation of Wnt/β-catenin signal pathway, suggesting the involvement of β-catenin in the pathogenesis of pathologic keloid. In tumor cells, the aberrant activation of β-catenin is also closely associated with the abnormal proliferation and apoptosis of cells[8,9]. Nowadays, it is recognized that pathologic keloid is essentially a benign fibrous tumor in skin, which, thus, has the tumor-like biological features[10]. Nevertheless, there are no study reporting the effect of β-catenin in proliferation and apoptosis of fibroblast in keloid. Thus, we detected the effect of β-catenin siRNA on proliferation and apoptosis of fibroblasts in keloid, results of which are expected to provide experimental basis for further investigation of the action mechanism of the β-catenin signal pathway in the pathogenesis of keloid.

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Patients and Methods

Patients

In this work, all clinical samples were collected from 12 patients aged between 17-59 years old who received the repair surgery for keloid in Plastic Surgery Department in our hospital between July 2015 and December 2016 named case group. During the same period, we selected 12 patients aged between 16-62 years old who received the surgical resection for treatment of other diseases for collecting the normal skin samples surrounding the surgical site named normal control group. All samples were collected from the skin samples in length and width of about 0.5 cm in shank. For samples collected from patients in the case group, these samples were identified and confirmed by the physicians in Pathology Department in our hospital. Patients with a history or suffering infection and ulcer in local skin, or with skin diseases, immune diseases, thyroid disease, and diseases involving pituitary and adrenal gland, were excluded. Before surgery, all patients were informed of the purpose of sample collection, and signed the written informed consent. This study was approved by the Ethic Committee of Fuling Central Hospital of Chongqing (Fuling, Chongqing, China).

Materials

M199 culture medium (Gibco, Rockville, MD, USA); rabbit anti-human β-catenin and Caspase-3 polyclonal antibodies (Abcam, Cambridge, MA, USA); β-Actin antibody for internal reference (CST, Danvers, MA, USA); high molecular weight SDS calibration kit (Thermo Fisher, Waltham, MA, USA); enhanced chemiluminescence kit (ECL; Millipore, Billerica, MA, USA); fetal bovine serum (FBS), Gibco (Rockville, MD, USA); radio-immunoprecipitation assay (RIPA) and bicinchoninic acid (BCA) protein quantification kit (BCA kit; Beyotime, Beijing, China); TRIzol reagent, reverse transcription kit and SYBR Green (TaKaRa, Otsu, Shiga, Japan); RNase-free DEPC (Invitrogen, Carlsbad, CA, USA); β-catenin siRNA was constructed by Shanghai GenePharma Co., Ltd., (Shanghai, China).

Extraction of Primary Fibroblasts in Keloid

Direct adherent culture: the keloid tissues resected from the surgery were immersed into the M199 culture medium and removed from a super clean bench, where pre-warmed phosphate-buffered saline (PBS) was used to wash the keloid tissues to clean the blood followed by extraction of primary cells; thereafter, the keloid tissues were cut into tissue blocks (about 1 mm^3), and seeded into the M199 culture medium containing 20% fetal bovine serum (FBS) for incubation in a thermostat incubator (saturated humidity, 5% CO_2 and 37°C). After 5-7 days of inoculation, the emergence of cells in long fusiform and cambiform suggested the successful isolation of fibroblasts. Within 6 to 9 days after the fibroblasts were seen, tissue blocks were removed, and cells were digested using 0.25 trypsin for subculture when cell infusion reached 90%. Cell growth was regularly observed, and cells were prepared for following experiments.

Design, Synthesis of siRNA and Transfection of Target Gene

Interference sequences of β-catenin siRNA were designed and synthesized by Guangzhou RiboBio Co., Ltd., (Shanghai, China). Following siRNAs are specifically for gene encoding β-catenin: siRNA1 target sequence: 5'-CAGTTGTGGTTAAGCTCTT-3'; siRNA2 target sequence: 5'-GCCACAAGATTACAAGAAA-3'; siRNA3 target sequence: 5'-GCTGAAACATGCAGTTGTA-3'. In addition, a nonsense interference sequence, siN05815122147-1-5, was synthesized for transfection of the negative control group. In siRNA transfection experiment, cells were divided into the blank control group (no addition of reagent in addition to culture medium), the negative control group (addition of culture medium, β-catenin-negative-siRNA and transfection reagent), and the interference group (addition of culture medium, siRNA1, 2 and 3, and transfection reagent). 1 day before transfection, cells in logarithmic phase were seeded onto a 6-well plate at a density of 2×10^5/well; after the cell infusion reached about 40-50%, transfection experiment was started in accordance with the transfection kit instruction. In an incubator at 37°C and 5% CO_2, we calculated the transfection rate through statistics of the positive cells in a fluorescent microscope in the following formula: Transfection rate (%) = Count of positive cells/Count of all cells × 100%.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Primer sequences of β-actin: forward 5′-CGGAGTCAACGGATTTGGTCGTAT-3′, backward 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′.

Primer sequences of β-catenin: forward 5′-AGCTTCCAGACACGCTATCAT-3′, backward 5′- CGGTACAACGAGCTGTTTCTAC-3′. PCR reaction system: initial denaturation at 94°C for 2 min; 32 cycles of denaturation at 94°C for 20 s, and annealing at 56°C for 1 min; extension at 72°C for 30 s. Amplification product was preserved at 4°C. Statistical analysis was conducted for Real-time quantitative PCR results.

Western Blotting Assay

After cell treatment, we removed the culture medium followed by washing the adherent cells using phosphate-buffered saline (PBS) twice with the filter paper to clean the residual liquid. RIPA reagent was added into the cells followed by scrubbing using a cell scraper and collecting cells into a 1.5 mL Eppendorf tubes (EP). Lysis was followed for 30 min on ice; then, cells were centrifuged at 12000 rpm for 5 min, and the supernatant was collected and preserved at -20°C. Protein samples were loaded for aggregating the proteins in electrophoresis at 80 V, followed by isolation and member-transferring of proteins at 120 V. Membrane was blocked with 5% skim milk powder. Next, rabbit anti-human polyclonal β-catenin (1:1000), cleaved caspase-3 (1:750) and rabbit anti-human polyclonal β-actin antibodies were added onto the membrane for incubation at 4°*C* overnight, followed by washing the membrane on a decoloring shaker with Tris-buffer saline and Tween-20 (TBST) 3 times (5 min/time). Horseradish peroxidase (HRP) labeled goat anti-rabbit immunoglobulin G (IgG) for 1 h of incubation. The membrane was washed with TBST 3 times (5 min/time); enhanced chemiluminescence (ECL) reagent was added for reaction at room temperature for 1 min followed by exposure under X-ray, fixation, and development.

Detection of Cell Proliferation Rate via MTT Assay

Single cell suspensions were prepared using cells in 3 groups for inoculation onto a 96-well plated with 4 replicate wells in each group and 1 well for blank control followed by culture in a cell incubator (37°C, 5% CO_2 and saturated humidity). Culture in five wells was terminated at 24 h, 48 h, 72 h, 96 h, and 120 h, respectively; in each well, 10 μL MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0.5 mg/mL] were added for 4 h of reaction. With the culture medium being discarded, 150 μL dimethyl sulfoxide (DMSO) were added to dissolve the sediments followed by measurement of optical density at a wavelength of 492 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The MTT experiment was repeated three times independently.

Detecting the Cell Apoptosis Rate via Annexin V/PI

Cells in different group were digested with Ethylene Diamine Tetraacetic Acid (EDTA)-free trypsin and collected into a 1.5 mL EP tube for centrifugation at 1000 rpm for 5 min. Cells were sufficiently washed using septic PBS at 4°C twice for later detection. The cell suspension was prepared with 400 μL, where 5 μL Annexin V-FITC were added and well-mixed followed by incubation at 2-6°C for 15 min in a dark place. Cells were stained using 10 μL propidium iodide (PI) and well mixed for incubation at 2-8°C for 5 min in a dark place, and immediately loaded onto a flow cytometer for detection and analysis.

Statistical Analysis

All data were presented as (), and one-way analysis of variance (ANOVA) was performed using SPSS 19.0 software (IBM Corp. IBM SPSS Statistics for Windows, Armonk, NY, USA). Tukey's HSD (honestly significant difference) test was used in conjunction with an ANOVA to find means that are significantly different from each other. With $\alpha = 0.05$ as the inspection level, $p<0.05$ suggested that the difference had statistical significance.

Results

Expressions of β-catenin in Normal Tissues and Keloid Tissues

Compared with the normal tissues, mRNA and protein expressions of β-catenin in keloid tissues were significantly elevated with a statistically significant difference ($p<0.05$), suggesting the role of β-catenin in the development and progression of keloid (Figure 1).

Identification of Primary Fibroblasts of Human Keloid

Under the inverted microscope, the primary fibroblasts extracted from human keloid tissues were in long-fusiform and cambiform with long protuberances at two ends; the oval-shaped nuclear was paracentral in cytoplasm. The results of vimentin immunofluorescent analysis through cell chemical dyeing revealed that there were 95% cells with

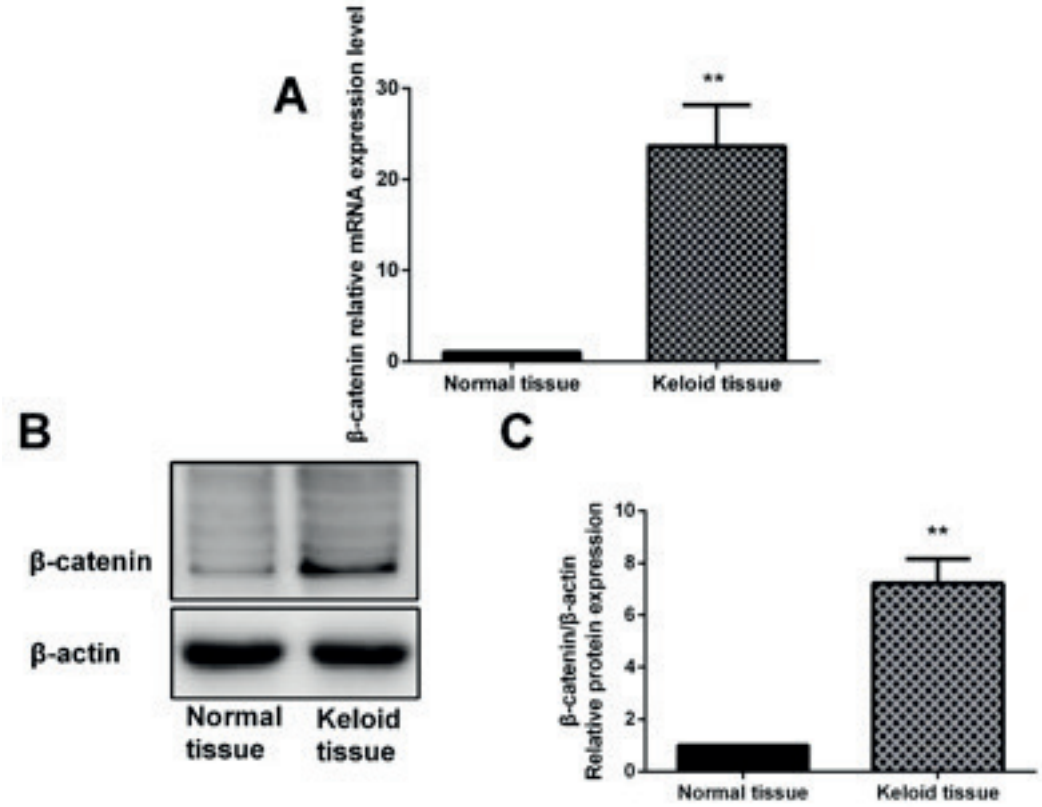

Figure 1. The mRNA and protein expression level of β-catenin in normal tissue and keloid tissue. ***A,*** The expression level of β-catenin mRNA in normal tissue and keloid tissue; ***B*** and ***C,*** The qualified expression level of β-catenin protein in normal tissue and keloid tissue.

positive response to vimentin dyeing, suggesting that those were fibroblasts of keloid (Figure 2).

Detection of siRNA Transfection Rate

In cytoplasm of positive cells, intensified green fluorescence was identified, suggesting that the transfection rate was above 90% (Figure 3).

Evaluation of the Silenced Expression of β-catenin

Results of RT-PCR and Western blotting assay showed that, in cells transfected by three β-catenin siRNAs, the mRNA and protein expressions of β-catenin were significantly downregulated, where the interference fragments of siRNA2 exhibited the most evident inhibitor effect on the protein expression of β-catenin. The differences in comparisons with the blank control group and the negative control group had statistical significance ($p<0.05$). Thus, the cells transfected by siRNA2 in the following experiment were set as the control group (Figure 4).

Effect of β-catenin siRNA on Proliferation of Keloid Fibroblasts

We further verified whether β-catenin siRNA could affect the proliferation of fibroblasts. The results of MTT assay revealed that, compared with the blank control group and negative control group, proliferation of fibroblasts transfected with β-catenin siRNA was evidently suppressed at 24 h, 48 h, and 72 h, respectively. The differences had statistical significance ($p<0.05$; Figure 5).

Effect of β-catenin siRNA on Apoptosis of Keloid Fibroblasts

We further verified whether β-catenin siRNA could affect the apoptosis of fibroblasts. Flow cytometry assay revealed that, compared with the blank control group and negative control group, the apoptotic rates of fibroblasts transfected with β-catenin siRNA were significantly elevated in early and advanced stages, respectively; the differences had statistical significance ($p<0.05$; Figure 6).

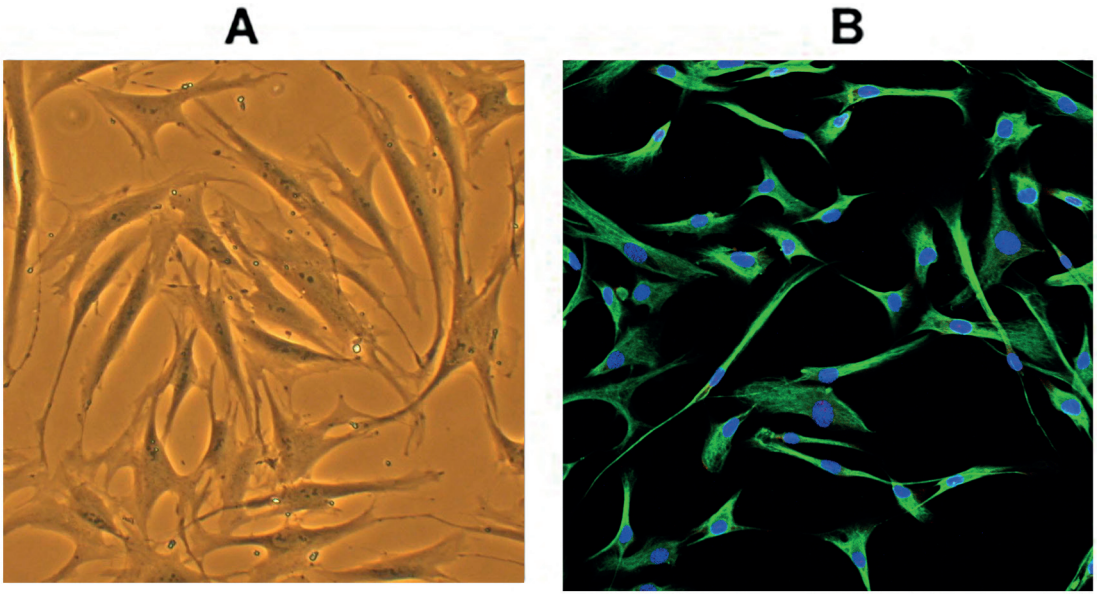

Figure 2. Identification of primary human keloid fibroblast cell. ***A,*** Light-microscopic morphology of keloid fibroblast cell; ***B,*** Immunofluorescence of Vimentin.

Discussion

Keloid is a special scar formed through the abnormal proliferation of cells in normal skin after trauma. The massive proliferation of scar-derived fibroblasts and excessive aggregation of collagen with aberrant proliferation, secretion, differentiation, and apoptosis of fibroblasts are the clinical features. Fibroblasts are the major effector cells involved in the wound-healing and formation of keloid after trauma, and have more potent proliferative and anti-apoptotic abilities. In terms of their functions, fibroblasts are one of the key-factors affecting the development, progression and clinical outcome of the pathologic keloid[11,12]. Latest studies[13,14] have revealed that aberrant cell apoptosis and proliferation are crucial to the formation of pathologic keloid. Some scholars inferred that during the development of keloid and wound-healing, the dysfunction of programmatic cell death in fibroblasts could result in the resistance to apoptosis, thereby leading to the abnormal proliferation of fibroblasts and massive secretion of collagen and extracellular matrix. In recent years, various studies on cell biology and molecular biology have deepened the understanding on the pathogenesis. The abnormal activation of many signal pathways is involved in the formation of keloid, where the role of Wnt/β-catenin is quite important and has attracted the attention of many researchers[15]. Wnt/β-catenin signal transduction pathway also plays important roles in development of a variety of embryonic tissues, like lung, kidney, and nerve tissues[16-18]. In addition, Wnt/β-catenin signal transduction pathway serves as a key regulatory factor in development of keloid. Classical Wnt signal pathway can block the degradation of β-catenin in cytoplasm through inhibiting the GSK3β-mediated phosphorylation effect. The massively aggregated β-catenin is delivered into the nuclear to bind with the TCF/LCF3 complex, thereby regulating the transcription and expression of downstream target gene[19,20]. Previous studies[21,22] have indicated the key role of Wnt/β-catenin signal transduction pathway in would-healing of skin, and its involvement in the pathogenesis of multiple fibrotic diseases. In keloid tissues,

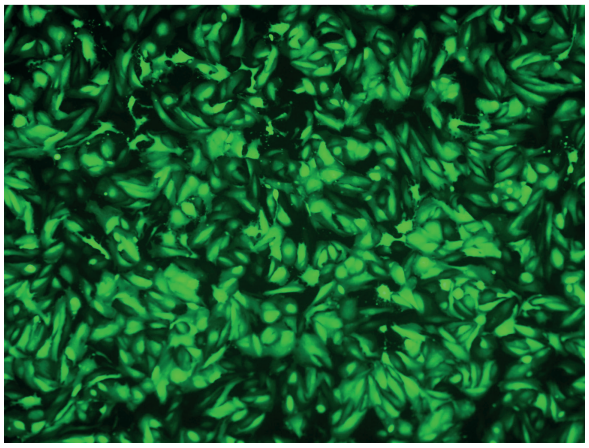

Figure 3. β-catenin siRNA transfection efficiency.

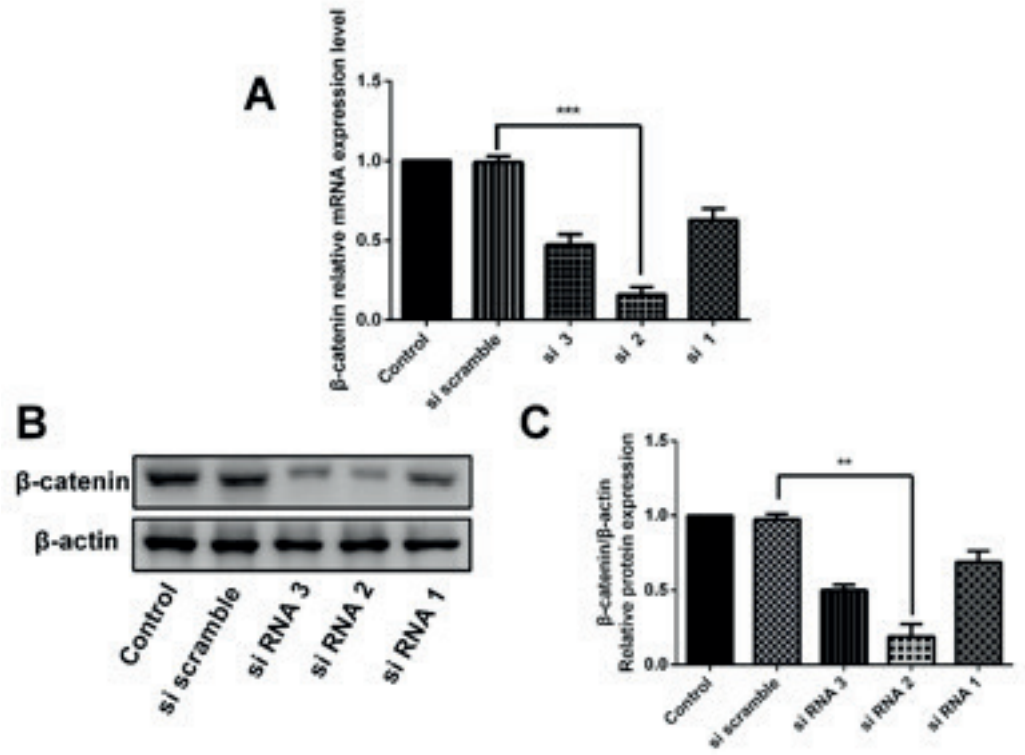

Figure 4. Identification of the silencing effect of different β-catenin siRNA. ***A,*** The silencing effect of different siRNA on β-catenin mRNA; ***B*** and ***C,*** The silencing effect of different siRNA on β-catenin protein.

β-catenin was significantly elevated, suggesting that dermal hyperplasia is caused by interaction between the β-catenin of high expression and hypodermal cells, thereby participating in the wound-healing process[23]. Li et al[24] discovered that signal transduction pathways, were mitogen-activated protein kinase and Wnt signal pathways, were key links in healing processes of various skin wounds. Moreover, through Smad3 and p38-MAPK signal pathways, TGF-β1 is also involved in the pathogenesis through activating the β-catenin-induced transcription in hypertrophic cicatrix and keloid tissues[7]. Igota et al[15] also reported that the mRNA and protein expressions of Wnt5a and β-catenin in keloid fibroblasts were higher than those in the fibroblast in normal skin. Zhang et al[16] postulated that high expression of β-catenin in pathologic scar can activate the Wnt/β-catenin signal transduction pathway, thereby facilitating the formation of keloid. Besides, β-catenin signal pathway also affects the proliferation and apoptosis in a variety of diseases[25,26]. Thus, we inferred that β-catenin may participate in the pathogenesis of keloid through regulating the proliferation and apoptosis of fibroblasts, which is scarcely reported in current studies. Here, the *in-vitro* keloid fibroblast models were established with silenced expression of β-catenin using specific siRNA to observe the effect on the proliferation and apoptosis levels in fibroblasts, thereby uncovering the correlation between expression of β-catenin and pathogenesis of keloid. We found that compared with the normal tissues, the expression of β-catenin in keloid tissues was significantly elevated,

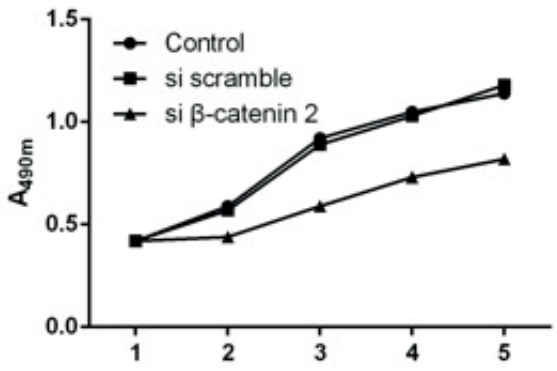

Figure 5. The effect of β-catenin siRNA on the proliferation of keloid fibroblast cell.

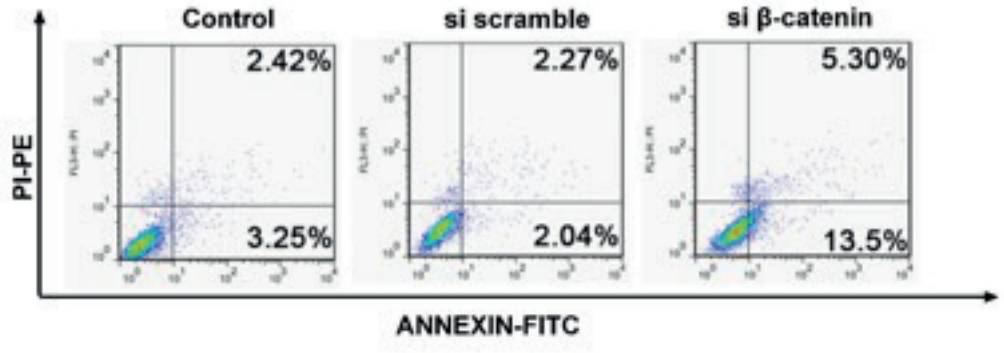

Figure 6. The effect of β-catenin siRNA on the apoptosis of keloid fibroblast cell.

suggesting that aberrant activation of Wnt/β-catenin signal pathway was involved in the pathogenesis of keloid. Sequentially, direct adherent culture method was applied to extract the primary fibroblasts from keloid tissues, and the extracts were identified in immunofluorescent assay. The data showed that cells presented positive expressions, which laid the foundation for following cell experiments. Next, RNA interference technique was also adopted for transfection of fibroblasts with 3 specific siRNAs, and the findings of identification of the interference efficiency through mRNA and protein levels showed that those siRNAs could significantly downregulate the β-catenin. Further MTT assay was performed to test the effect of β-catenin siRNA on proliferative ability of fibroblasts. The results showed that compared with the blank control group and negative control group, transfection with β-catenin siRNA could significantly decrease the proliferative ability of fibroblasts. Also, the results of RT-PCR and Western blotting assay indicated that β-catenin siRNA could evidently downregulate the caspase-3. FCM findings showed that a much higher apoptotic level was identified in the fibroblasts of β-catenin siRNA group.

Conclusions

We preliminarily confirmed that β-catenin was abnormally expressed in keloid tissues, and that silencing the expression of β-catenin in fibroblasts significantly inhibited cell proliferation and apoptosis, which are conducive to enhancing the understanding on the pathogenesis of keloid. Thus, in-depth research on the role and mechanism of β-catenin in development and progression of keloid is required to develop a new method for blocking the expression of β-catenin, increasing the apoptosis and decreasing the proliferation of fibroblasts, which, with widespread clinical significance and promising application prospect, would provide new ideas and evidence for specific treatment for keloid.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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