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Original Article

Association between CASK/Id1 signal pathway and keloid fibroblasts

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Abstract: Objective: To verify the existence and significance of calcium/calmodulin dependent serine protein kinase (*CASK)/*inhibitors of differentiation 1 (*Id1*) pathway in fibroblasts of human keloid. Methods: The expression and localization of *CASK* and inhibitors of differentiation 1 *Id1* in fibroblasts of the keloid and normal skin were detected by Immunofluorescence laser. The expression of both the *CASK* and the *Id1* at mRNA and protein levels in fibroblasts of keloid and normal skin were analyzed via reverse transcription-polymerase chain reaction (RT-PCR) and western-blot, respectively. The natural combination of *CASK* and *Id1* in keloid fibroblasts was tested by immunoprecipitation. Results: *CASK* and *Id1* were found mainly distributed in the cytoplasm and nucleus of fibroblasts. RT-PCR showed that the expression of *CASK* mRNA in the keloid group was lower than that in normal control group (0.658±0.024 vs. 1.076±0.008, $P < 0.05$) while the expression of *Id1* mRNA in case group was higher than that in normal control group (0.497±0.014 vs. 0.307±0.017, $P < 0.05$). Western-blot demonstrated that *CASK* protein expression in case group (0.057±0.006) was lower than that in control group (0.168±0.012, $P < 0.05$) and the *Id1* protein expression in case group (0.812±0.035) was higher than that in control group (0.368±0.031, $P < 0.05$). Immunoprecipitation indicated that *Id1* and *CASK* could be detected in their mutual precipitate, thus suggesting a natural binding of *CASK* and *Id1* in keloid fibroblasts. Conclusions: *CASK/Id1* signal pathway exists in the proliferation of keloid fibroblasts and is linked with the occurrence of keloid.

Keywords: Fibroblasts, immunoprecipitation, confocal laser, calcium

Introduction

The pathogenesis of keloid has always been the main focus in plastic surgery field [1]. Plenty of studies have shown that increased expression of transfer growth factor-β (TGF-β), increased synthesis of extracellular matrix components of fibroblasts such as collagenous fiber and fibronectin, and reduced synthesis of extracellular matrix degrading enzymes like metalloprotease may have all participated in the occurrence and development of keloid [2-5]. Calcium/calmodulin dependent serine protein kinase (*CASK*)/inhibitors of differentiation 1 (*Id1*) is a signal pathway for regulating cell proliferation, which has been discovered recently. In the meanwhile, studies on this pathway are mainly focused on vascular endothelial cells [6]. However, based on the fact that the effects of *Id1* in cell proliferation and tumor diseases have been frequently investigated in recent years and the pathogenesis of keloid is similar to that of tumors, we consider that there may also be abnormal *Id1* expression in the keloid [7]. In addition, *CASK/Id1* signal pathway probably plays a possible role in the occurrence and development of keloid if the combination between *Id1* and *CASK* existed. It has been shown by some studies that *CASK* and *Id1* have differential expressions in keloid and normal skin tissues [8], so we intended to verify the above hypothesis by the following experiments.

Materials and methods

Materials

Main reagents and instruments: The main reagents included: DMEM medium with high

glucose and FBS from American Gibco company); Trizol total RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR) kits and Taq enzyme from TaKaRa company, Dalian; primers for CASK, Id1 and reference gene GAPDH from Shanghai Sangon Biotech Co., Ltd; rabbit anti-human CASK polyclonal antibody and mice anti-human Id1 monoclonal antibody from American Santa Cruz company; goat anti-rabbit secondary antibody labeled with FITC green fluorescent and goat anti-mice secondary antibody labeled with Cy3 red fluorescent from Beijing CowWin Biotech Co., Ltd; protein lysis buffer (RIPA), bicinchoninic acid (BCA) protein quantification kits, gel preparation kits and enhanced chemiluminescence kits (ECL) from Shanghai Beyotime company; goat anti-rabbit secondary antibody labeled by horse radish peroxidase (HRP) and goat anti-mice secondary antibody labeled by HRP from MultiSciences Biotech Co., Ltd; β-actin from Beijing 4A Biotech Co., Ltd, and protein G agarose from Beyotime institute of biotechnology. Main instruments included: Western electrophoresis apparatus from Bio-Rad Company, America; cryogenic refrigerator from Sanyo Company, Japan, and micropipets from Eppendorf Company, Germany. Total protein extraction reagent, 5×SDS-PAGE protein loading buffer, SDS-PAGE gel preparation kit, pre-stained protein molecular weight, confining liquid, first and second antibody diluent and ECL detection kits were all purchased from Nanjing KeyGEN Biotech Co., Ltd and protein G agarose was supplied by Beyotime Institute of Biotechnology.

Sample collection: Here follows the inclusion criteria of keloid patients: (1) no spontaneous regression signs after 9 months of disease course; (2) lesions with red color and tough texture as well as pain and itching feelings; (3) greater scope of the damaged skin greater than invasions to the surrounding normal skin tissues; (4) no radiation or other treatments received before surgery; (5) pathological confirmation of the disease. The 10 selected samples (6 males and 4 females) aged 18-45 years were keloid patients who received plastic surgeries in Renmin Hospital and all of them signed an informed consent before the surgery. Six of them had lesions on their trunk and extremities while the rest four had lesions on their earlobes. 6 normal skin tissue samples were collected from the normal skin around the operative area.

Methods

Keloid fibroblast cultivation: The primary culture of keloid fibroblasts was performed by tissue block adhering means. The passage was started when cell growth density reached 80%, additionally, we applied fibroblasts belonging to the 3rd to 6th generation in this study. The normal skin tissues as well as their lesion parts were respectively put into control group and case group.

Identification of fibroblast types with hematoxylin eosin (HE) staining: The fibroblasts were absorbed and then diluted into suspension. After being counted, the cells were inoculated into a 24-well culture plate with sterile cell slides. Each well contained 200 μL cell suspension and 800 μL culture solution, then the mixture was cultured in 5% CO_2 at 37°C for 24 h. The cell slides were took out when the cell fusion reached 60%, and then washed for 3 times with pre-cooled PBS for 5 min each time. After that, the samples were fixed with 4% paraformaldehyde at room temperature for 15 min and HE staining method was used to identify the cell types.

Drawing the growth curve of fibroblasts using thiazolyl blue (MTT) method: Firstly, the fibroblasts of the case group and the control group were respectively collected then counted after being digested with 0.2% trypsin. The cell concentration of the two groups was adjusted to be same then the cells were inoculated into a 96-well plate with 2×10^3 cells for each well. The cells were inoculated into 6 wells of each plate and a total of 8 plates were used. One of the plates was utilized to determine the original absorptance values (A values) of the cells and the rest 7 plates were cultivated in 5% CO_2 at 37°C. We took one culture plate out for detection of the absorptance values (A values) of the cells with MTT method every 24 hours. Secondly, after the above procedures, the growth curves were drawn.

Confirmation of the CASK, Id1 protein expression and localization with immunofluorescence laser confocal microscope: Firstly, some keloid fibroblasts in logarithmic growth phase were selected then digested and counted. Subsequently, the cells were inoculated into a 12-well culture plate with sterile cell slides. Each well contained 1 ml mixture (500 μL cell suspension

and 500 μL culture medium) with the plate placed in 5% CO_2 for cultivation at 37°C for 24 h.

Secondly, we took the cell slides out when the cell confluency was up to 60% and left the culture medium. Later, we washed the samples with Cold PBS for 3 times then put them into 4% paraformaldehyde for fixation at room temperature for 10 min.

Thirdly, cold PBS was used to wash the samples for 3 times with 5 minutes each time. Then, they were placed in PBS with 0.2% TritonX-100 for incubation for 10 min. After that, another 3 times of sample washing with PBS were performed for 5 minutes each time.

Fourthly, the cells were incubated in the PBS containing 10% goat serum at 37°C for 30 minutes to eliminate nonspecific binding.

Fifthly, the fibroblast samples were respectively incubated overnight with the first antibody of *CASK* and *Id1* (diluted concentration: 1:100) at 4°C. The culture medium was then abandoned and the fibroblasts were again washed by PBS for 3 times with each wash taking 5 minutes.

Sixthly, the cells were respectively incubated in the dark with the second antibody fluorescently labeled by FITC and Cy3 (diluted concentration: 1:100) at 37°C for 1 h. The second antibody solution was discarded and three times of washing of the cells utilizing PBS was carried out, each washing lasting for 5 min.

Finally, the cell slides were mounted by 50% glycerol and put under an inverted immunofluorescence laser confocal microscope so that they could be observed, photographed and analyzed.

Detection of the mRNA expressions of CASK and Id1 with RT-PCR method: 1. The mRNA sequences of CASK and Id1 were looked up in the GeneBank database, and primers and internal primers of target genes were synthesized by Shanghai Sangon Biotech Co., Ltd. The forward and reverse sequences respectively for CASK, Id1, and the internal reference gene GAPDH were as follows: 5'-agaaatcaatggcatcagtgtg-3' and 5'-accatgtgcgcctaataagact-3' (622 bp); 5'-gcgctgtctgtctgagca-3' and 5'-ggtccctgatgtagtcgatga-3' (218 bp); and 5'-gtctactggcgtcttcaccac-3' and 5'-gcttcaccaccttcttgatgtc-3' (500 bp). 2. The total RNA of the fibroblasts in both groups were extracted with Trizol reagent, and the concentration and purity of the RNA were detected by an ultraviolet spectrophotometer at A260 nm and A280 nm positions respectively. Reverse transcription operations were conducted according to instructions of TAKARA kit. The amplification conditions were: *CASK* and *GAPDH*: 35 cycles of 94°C for 5 min, 94°C for 30 s, 62°C for 30 s, 72°C for 45 s and final 72°C for 10 min; Id1: 35 cycles of 95°C for 5 min, 95°C for 30 s, 57.5°C for 35 s, 72°C for 45 s and final 72°C for 10 min. After 4% agarose gel electrophoresis of the amplification products was performed, images were captured and analyzed by gel imaging analysis system, and the obtained bands were analyzed with Quantity One software to calculate the gene expression index.

Examination of protein expressions of CASK and Id1 using Western-blot: 8 bottles of fibroblasts in logarithmic growth phase from both groups were washed and centrifuged and then added with 320 μL cell lysis buffer RIPA. The mixture was then repeatedly sucked and blew, put on the ice at 4°C for 30 min, and centrifuged at 12000 r/min for 10 min with the centrifugal radius being 10 cm. We carefully removed the supernatant from the mixture and used 2 μL of remained protein for protein quantification. The rest protein was added with loading buffer (volume ratio: 4:1) and then boiled in boiling water for 15 min to achieve total protein degeneration. After all the above steps, the samples were saved at -20°C until ready to use. Based on the Beyotime gel preparation kit instructions, the samples were added with first antibodies of CASK, Id1, and β-actin respectively with the dilutability for the three being 1:400, 1:300, and 1:4000) and then preserved at 4°C for the night. Repeated membrane washing was performed and then the samples were incubated in goat anti-rabbit second antibody (dilutability: 1:5000) and goat anti-mice second antibody (dilutability: 1:5000) that were labeled by HRP for 1 h. After another time of repeated membrane washing, the samples were colored using ECL. Then, we collected images with gel imaging system and applied Quantity One software to analyze the imaged bands. The relative expression levels of CASK and Id1 were represented by the ratio between the A values of target protein bands and β-actin.

Examination of the natural combination of CASK and Id1 in keloid fibroblasts with immunoprecipitation (IP): First of all, protein samples extraction and preparation: We collected the keloid fibroblasts quantificationally and washed them with PBS once. Full lysis of the cells was shown using mild cell lysis buffer (300~400 μL/10^7). Then, we took an appropriate volume of the protein samples for calculation of concentration on the basis of standard curves.

Secondly, IP reaction: (1) 120 μg protein samples of *CASK* and *Id1* were respectively added with 1.0 μg *CASK*, *Id1* first antibody then shaken slowly at 4°C for the night. (2) The mixture was added with 20 μL Protein G Agarose that had been completely resuspended then shaken slowly at 4°C for 3 h. (3) The samples were centrifuged at 2500 r/min for 5 min with a 10 cm centrifugal radius. After the supernatant was removed carefully, the precipitates were washed with PBS. Then the second procedure was conducted for a second time. (4) When the washing procedure was over, the supernatant was removed for another time and the rest was resuspended using 1×SDS-PAGE loading buffer. After instantaneous super centrifugation, the samples were boiled for 5 min until ready for electrophoresis.

Thirdly, SDS-PAGE electrophoresis was as followings. (1) Sample preparation and electrophoresis: The samples were diluted with lysis buffer to have the same concentration. The same volume of loading buffer was put into test tubes and there were 70 μg proteins. Then, the samples were loaded after being cooled on the ice at 95~100°C for 5 min. The electrophoresis conditions were: at constant voltage 60 V for about 20 min for spacer gel and at 80 V voltages for about 80 min for separation gel. (2) Wet electrophoresis transfer: The gels were taken out and placed into transfer buffer to keep balance for 15 min. Then we put filter paper and PVDF membrane respectively into transfer buffer and deionized water. The positive electrode was laid flat meanwhile filter paper, PVDF membrane, gel and filter paper were respectively put upon it. After air bubbles on each layer were discharged, the negative electrode was placed on the top of the intercalations. The electrotransfer process was carried out at 200 mA constant current for 1 h. (3) Sealing: the PVDF membrane was sealed in 5% nonfat dried milk sealing fluid at room temperature for 1 h then the sealing fluid was removed, with the PVDF membrane not being washed. (4) Combination of antibodies with target proteins: the samples were added with sealing fluid (0.1 ml/cm^2) and an appropriate amount of *CASK* first antibody (1:400) and *Id1* first antibody (1:400). Then the mixture was incubated on the shaking table at 4°C for the night. PBST was applied to rinse the filter membrane for 4 times, each time taking 10 min. The filter membrane and second antibody combined with (labeled by) HRP (diluted by sealing fluid to a concentration at 1:5000) were incubated on the shaking table at room temperature for 1 h and then PBST was used to fully rinse the membrane for 4 times with each time lasting for 10 min. (5) Developing and image analyzing: the volume of developing solution was calculated according to 0.1 ml/cm^2. We added the developing solution on the PVDF membrane and then saved the membrane at room temperature for 1 min. The PVDF membrane was wrapped completely by plastic bags and the membrane protein we obtained was immediately attached on the X-ray film for exposure in the dark room. Development and filming processes were completed in a developing machine. The exposure time was adjusted until best bands were presented.

Statistical analysis

SPSS 17.0 software was adopted for statistical analysis. The metering results were shown as $\bar{x}$ ±s and assessed by t test. Statistically significant differences were considered to exist when $P < 0.05$.

Results

Fibroblasts types

Generally speaking, the keloid fibroblasts and normal skin fibroblasts cultivated in vitro had not clear differences in shape and size. They had clear cell boundary together with large cell volume and appeared as shuttles or irregular triangles. Some of the cells had 2~3 processes with different lengths. The nucleus of them was round or oval. However, when cell fusion occurred, the arrangements of normal skin fibroblasts were presented as radial pattern or swirling pattern. The arrangements of the keloid fibroblasts were disordered with clear

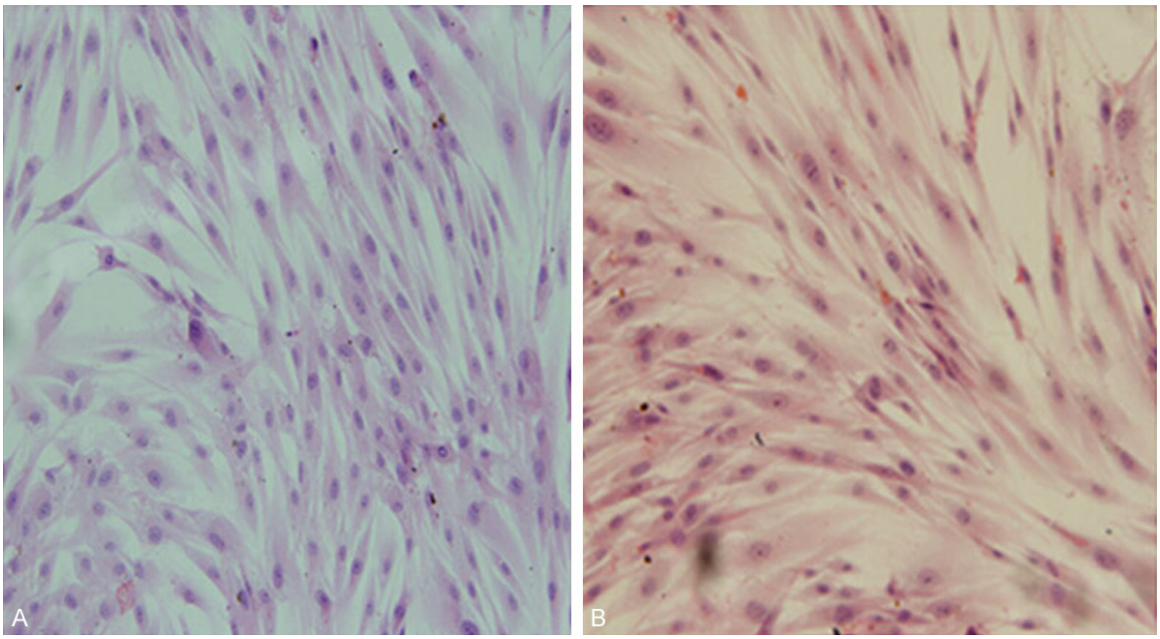

Figure 1. HE staining for the identification of fibroblast types. A. Keloid group; B. Normal control group.

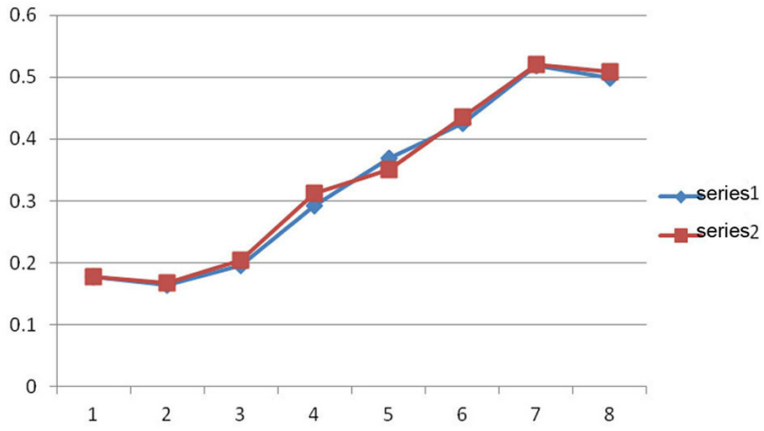

Figure 2. The growth curves of fibroblast determined by MTT method ($\bar{x}\pm s$). Horizontal axis: cell culture time; the vertical axis: absorbance value; series 1 and 2: keloid group and normal control group.

overlapping, in addition, the polarity disappeared as well (**Figure 1**).

Fibroblasts growth curves

Fibroblasts of the two groups both had an S-shaped growth curve. The cell number was reduced in 1 day after the passage; after 1 d~2 d of latent adaptation period, the cells went into a logarithmic phase; and at 6 d~7 d they reached the platform stage. It could be seen that no clear differences in the growth curves existed between case group (n=6) and control group (n=6). Differences in A values from the first day to the seventh day of the two groups were without statistical significance ($P > 0.05$) (**Figure 2**).

Protein expression and localization of CASK and Id1 in fibroblasts

Immunofluorescence laser confocal tests confirmed that *CASK* and *Id1* proteins were expressed in both keloid fibroblasts and normal skin fibroblasts cultivated in vitro. *CASK* was mainly localized in the cytoplasm, nucleus of the normal skin fibroblasts as well as in the cytoplasm of the keloid fibroblasts (**Figure 3**). *Id1* was mainly expressed in the cytoplasm of the normal skin fibroblasts and in the cytoplasm along with nucleus of the keloid fibroblasts (**Figure 4**).

mRNA expressions of CASK and Id1 in fibroblasts

After RT-PCR amplification and agarose gel electrophoresis, the expression level of *CASK* in

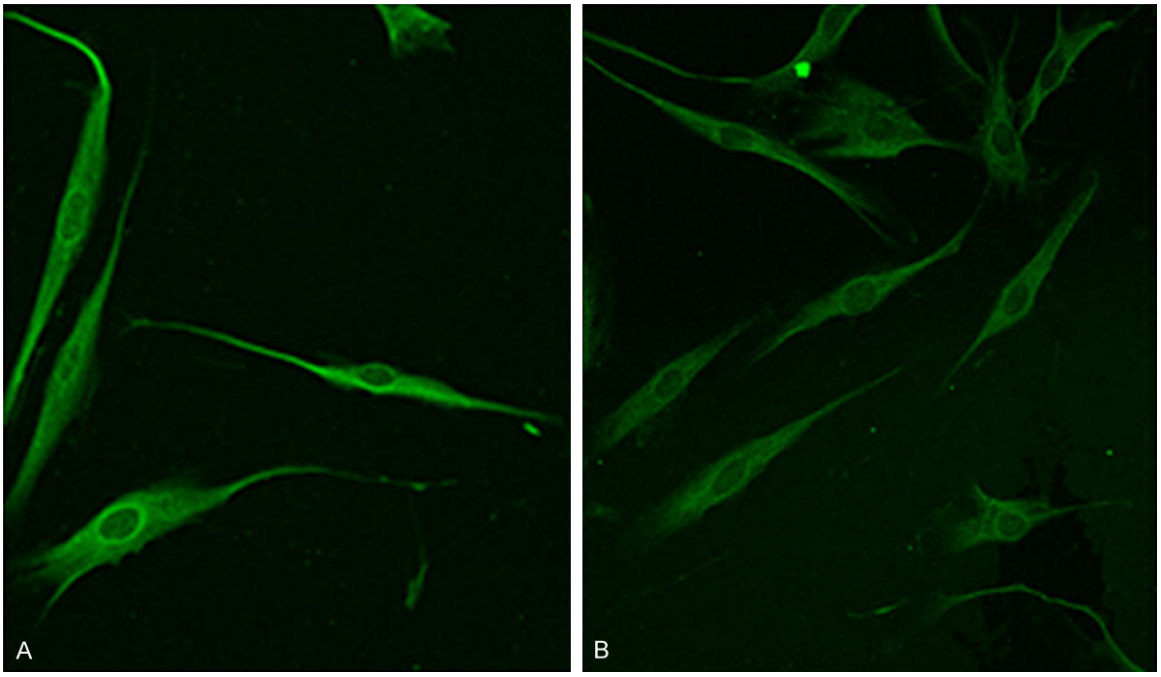

Figure 3. *CASK* expression in fibroblasts detected by laser confocal microscopy. A. Normal control group; B. Keloid group.

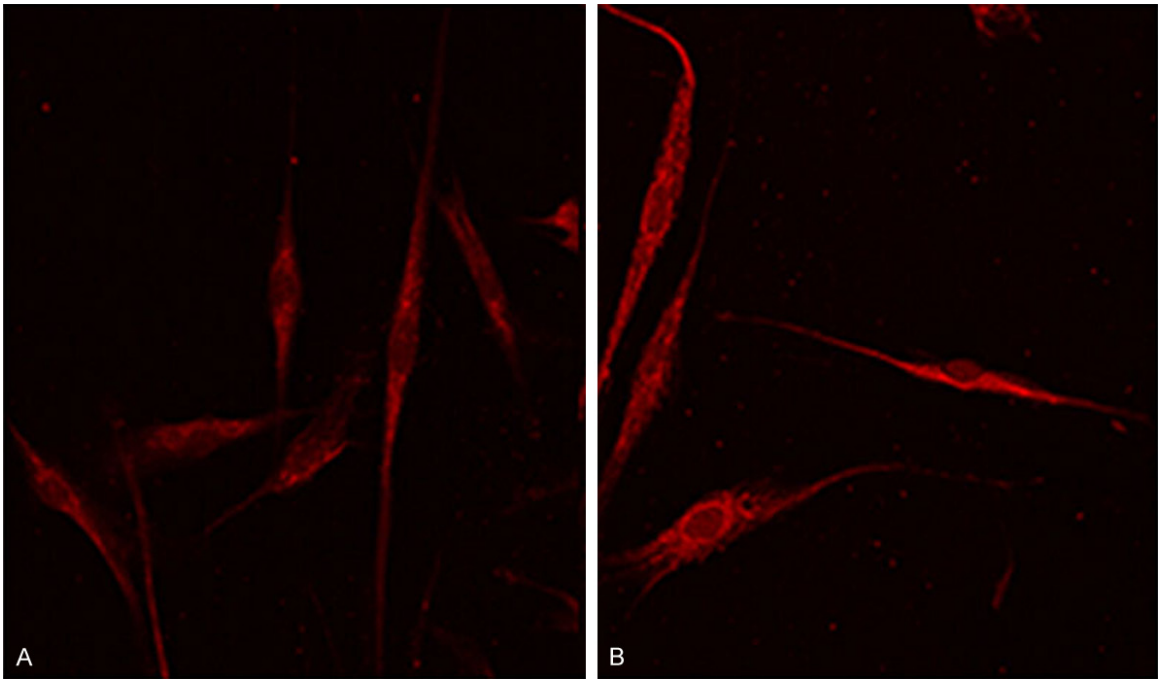

Figure 4. *Id1* expression in fibroblasts detected by laser confocal microscopy. A. Normal control group; B. Keloid group.

case group was 0.658±0.024, which was lower than that of the control group (1.076±0.008), and the difference was statistically significant (t=11.159, $P < 0.05$). The expression level of *Id1* in case group (0.497±0.014) was higher than that of the control group (0.307±0.017), and the difference had statistical significance (t=15.148, $P < 0.05$) (**Figures 5** and **6**).

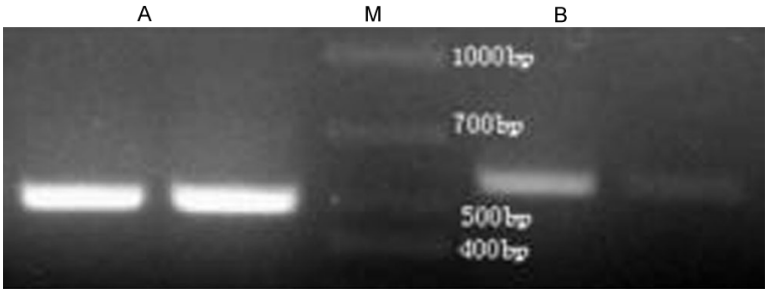

Figure 5. *CASK* mRNA expression. A: Normal control group; B: Keloid group; M: Marker.

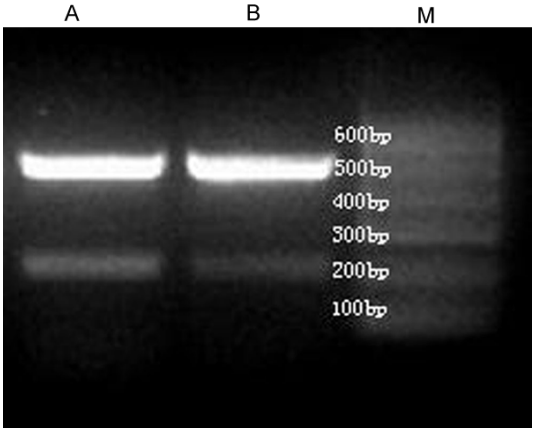

Figure 6. *Id1* mRNA expression. A: Keloid group; B: Normal control group; M: Marker.

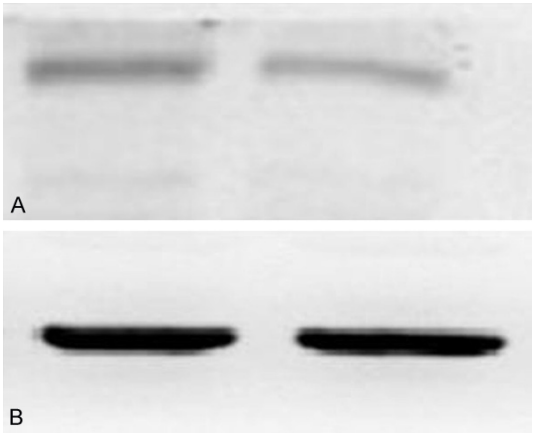

Figure 7. *CASK* protein expression. A: Keloid group; B: Normal control group; M: Marker.

Protein expressions of CASK and Id1

The protein expression level of *CASK* in case group (0.057±0.006) was lower than that in the control group (0.168±0.012) (t=13.524, $P < 0.05$). The protein expression level of *Id1* in case group (0.812±0.035) was higher than that in the control group (0.368±0.031) (t=16.356, $P < 0.05$) (**Figure 7**).

Natural combinations of CASK and Id1 proteins in keloid fibroblasts

Immunoprecipitation test results pointed out that *Id1* was able to be detected in the precipitates of *CASK* and *CASK* could also be detected in the precipitate of *Id1*, which proved that there was an interaction between the two proteins (**Figure 8**).

Discussion

The pathogenesis of keloid is very complex; moreover, multiple types of cells mainly represented by fibroblasts and a variety of substrates, pathways, as well as levels are involved. The relationship of TGF-β-Smad pathway with keloid has been most completely researched so far and abnormal expressions of TGF-β factor and Smad protein family have also been proved to have a close relationship with keloid occurrence [9]. Our previous studies have already confirmed that the occurrence of pathological scar may be correlated with the excessively high expression of *Id1* in hyperplastic scar and keloid [10]. Nevertheless, the expressions of Id1 in fibroblasts cultivated in vitro and the mechanism through which the cell proliferation can be affected by *Id1* are still unclear.

Id1 is an important member of helix-loop-helix type transcription factors family and also a critical transcriptional regulation factor associated with cell growth and differentiation. The *Id* proteins are closely related to the cell senescence, and the expressions of *Id1* and *Id2* will be obviously decreased with the aging of cells. It has been found that the over-expression of *Id1* is capable of delaying the cell senescence in fibroblasts, endothelial cells and breast epithelial cells, which results from the cell senescence associated with the transcriptional activation of its mediator *p16* and the expression of *p16* inhibited by the overexpression of *Id1*. At the same time, various studies have demonstrated that *Id* gene has some characteristics of biochemical cancer genes and was highly expressed in tumor samples as well as tumor

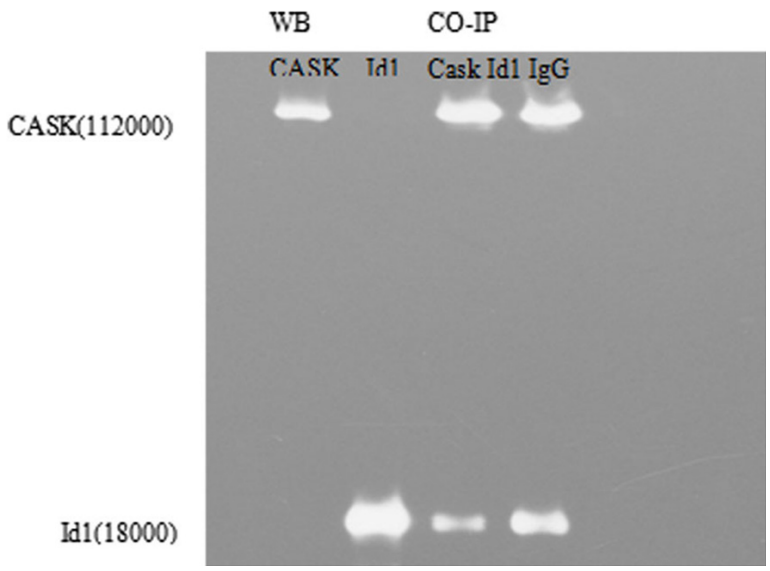

Figure 8. Interaction between *Id1* and *CASK* in keloid fibroblasts was detected by immunoprecipitation. 1, 2. Protein expression level of *CASK* and *Id1* detected by western blot; 3. Protein expression level of *CASK* and *Id1* detected by immunoprecipitation of *CASK*. 4. Protein expression level of *CASK* and *Id1* detected by immunoprecipitation of *Id1*; 5. Immunoprecipitation result of the independent control IgG antibody.

cells cultivated in vitro; and especially the role of *Id1* in the occurrence and development of tumors has been widely recognized [11-13]. Immunohistochemistry and in situ hybridization have both revealed the abnormal high expression of *Id1* in many tumors, besides, the proliferation speed of tumour cells in breast cancer and pancreatic cancer patients can be reduced through inhibiting the expression of *Id1* [14, 15]. The biological characteristics of keloid are similar to those of tumors, so *Id1* is likely to have abnormal expressions in keloid fibroblasts cultivated in vitro. The present study illustrated that the expression of *Id1* in case group was significantly higher than that in control group, which suggested that the over proliferation of fibroblasts might be related to the abnormal high expression of *Id1*.

CASK is an important member of the membrane-associated ornithine kinase family. Its main biological function is to take part in the important physiological process such as cell junction, signal transduction and gene regulation as a scaffolding protein. *CASK* can combine with the receptors of a variety of ECM proteins like laminin, fibronectin, and collagen protein to regulate cell morphology and participate in interactions between cells and ECMs [16-19]. The over deposition of ECMs of fibroblasts also contributes to the occurrence and development of keloid. Studies have reported that the combination of *Id1* with *CASK* in vascular endothellial cells can modulate cell proliferation through regulating the expression of tumor suppressor gene p16 and p21 [20]. The present study also explained that the expression of *CASK* was lower in case group than in control group.

The results of this study indicated that the keloid fibroblasts had lower expression of *CASK* and higher expression of *Id1* compared with normal skin fibroblasts; changes in expressions of the two genes were not isolated because immunoprecipitation reaction results showed natural combination of *CASK* and *Id1* proteins in keloid fibroblasts. Therefore, *CASK/Id1* signal pathway would be likely to play a role in the proliferation regulation of keloid fibroblasts.

In conclusion, *Id1* has an obvious function of promoting cell proliferation, more importantly, *CASK* can have effects on cell morphology and structure, signal transduction and the interaction of cells with ECMs. Keloid fibroblasts had normal expression of the two genes when compared with normal skin fibroblasts, which is very similar to the pathogenesis of keloid. Meanwhile, immunoprecipitation test manifested natural combination and interaction of *CASK* and *Id1* proteins in keloid fibroblasts. Hence, we believe that *CASK/Id1* signal pathway is perhaps involved in the proliferation regulation of keloid fibroblasts. The signal network responsible for the regulating of cell proliferation may be disrupted by some genetic, immune factors and tumor source mechanism, which possibly leads to decreased expression of *CASK* and increased expression of *Id1*. The combination of increased *Id1* with transcription factor E2A can reduce the transcriptional activity of E2A; consequently the expressions of cell proliferation cycle dependent kinase p16 and p21 are decreased. In this way, the cell cycle is inhibited and excessive proliferation of cells as well as ECM are caused, which may finally result in the

fibrosis change. However, further studies are needed to deeply investigate whether there are still other functional factors between *CASK* and *Id1* as well as the specific mechanism of downstream factors or not.

Disclosure of conflict of interest

None.

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