Original Article
Preliminary study of the effect of abnormal savda munziq on TGF-β1 and Smad7 expression in hypertrophic scar fibroblasts

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Abstract: Background: To study the effect of abnormal savda munziq (ASMq) on TGF-β1 and Smad7 expression in hypertrophic scar fibroblasts (HSFs) and to preliminarily assess the function of abnormal savda munziq in hypertrophic scar formation at the molecular biology level. Methods: HSFs were cultured in vitro. RT-PCR and Western-blot were used to investigate the influence of 48-h treatment with ASMq at different concentrations (0 mg/mL, 0.1 mg/mL, 0.4 mg/mL, and 0.7 mg/mL) on TGF-β1 and Smad7 mRNA and protein expression levels. Results: After 48-h treatment with ASMq, the expression of TGF-β1 mRNA and protein gradually decreased in HSFs as the concentration increased. In contrary, Smad7 mRNA and protein expression were positively correlated with ASMq concentration. Conclusions: ASMq reduces TGF-β1, increases Smad7 mRNA and protein expression through regulating TGFβ-1/Smad signaling pathway, inhibiting HSFs proliferation and reducing extracellular collagen deposition.

Keywords: Abnormal savda munziq, hypertrophic scar fibroblasts, TGF-β1, Smad7

Introduction

Hypertrophic scars (HS) are a type of pathological scars involving the over-proliferation of fibroblasts and excessive deposition of collagen-dominated extracellular matrix [1]. The cytokine TGF-β1 plays an essential role in HS formation. It is secreted from fibroblasts and promotes cell proliferation and collagen synthesis in a positive feedback loop. Smad7 is a negative inhibitor of TGF-β1 activity. It is a downstream protein in the TGF-β1/Smad signaling pathway. The TGF-β type I receptor can bind Smad7, which inhibits phosphorylation of the receptor protein kinase R-TGF-β and blocks signal transduction. Therefore, increasing Smad7 expression in the pathway would indirectly inhibit TGF-β1 expression. According to experiments with Uighur medicine, ASMq (Patent No.: Z65020166) can be used to treat diseases induced by abnormal savda, such as tumor, diabetes mellitus, hypertension, and fibrotic diseases [2]. Previous data from our lab [3] have shown that ASMq can inhibit HSF proliferation at the cellular level and arrest the cell

cycle at G_2/M, promoting premature apoptosis. In this study, ASMq was studied for its effects on TGF-β1 and Smad7 mRNA and protein expressions in HSFs. This study also attempts to reveal in a preliminary manner the molecular mechanism by which ASMq exerts its influence on HS formation.

Materials and methods

Instruments and reagents

ASMq (Xinjiang Uygur Medicine Co, Ltd.); DMEM (Hyclone, USA); Neonatal bovine serum (Hyclone, USA); DNA Marker I (BioTeke Corporation); TRIzol reagent (Invitrogen, USA); Reverse transcriptase (TaKaRa Bio Company, Japan); TGF-β1 (National Engineering Research Center for Biotechnology, China); Smad7 (National Engineering Research Center for Biotechnology, China); Reference gene: GAPDH (National Engineering Research Center for Biotechnology, China); TEMED (Sigma, USA); SDS (Sigma, USA); Acrylamide (Amresco, USA); PVDF membrane (Millipore, USA); RIPA lysate

(BioTeke Corporation, Beijing); BCA protein assay kit (Beijing Biomed Biotechnology Company); TGF-β1 rabbit anti-human polyclonal antibody (Abcam, USA); Smad7 mouse anti-human monoclonal antibody (Santa, USA); GAPDH rabbit anti-human polyclonal antibody (Santa, USA); HRP-labelled goat anti-rabbit IgG antibody (ZSGB-Bio Origene, Beijing); DAB kit (ZSGB-Bio Origene, Beijing); Vilber Lourmat (BIO-RAD, USA); DYY-6C Electrophoresis System (Beijing Liuyi Instruments Company); FS-312 box-type Ultraviolet transmission analyzer (Fusun Group, Shanghai); PCR thermocycler (BIO-RAD, USA); Inverted phase-contrast microscope (LEICA, DM300, Germany); Vertical electrophoresis and transmembrane devices (BIO-RAD, USA).

Culture and grouping of ASMq-originated HSFs

Specimen origin

The HS tissues were collected from HS patients in the Department of Orthopaedics at the First Affiliated Hospital of Xinjiang Medical University. This experiment was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University, and all patients signed the informed consent form. Five patients were included in this study (3 males and 2 females), and they were all of the Han nationality. Their age ranged from 25-38 yr. All patients suffered from scar proliferation 3-12 months after experiencing burn wounds. The presence of HS was also confirmed by pathological examination.

Culture and grouping of fibroblasts

The HS tissues were placed on an aseptic bench, and rinsed three times with PBS to remove remaining blood and other foreign materials. The epidermis and any attached scar tissues were excised to prevent contamination from surface epidermic cells. HS tissues were trimmed into 1 mm × 1 mm × 1 mm pieces and cultured under uniform conditions in culture flasks. DMEM containing 10% fetal bovine serum was slowly added into the culture flask and the tissues were cultured under 37°C, 5% CO_2, and saturated humidity. The media were changed twice per week. When cells around the tissues grew into a dense monolayer, trypsin digest and subculture were used to propagate the cells and cells that had been

passaged 3-6 times were selected for further experiments.

Experimental design

The fibroblasts were divided into 2 groups: (1) Control group: supplemented with identical volumes of culture medium. (2) ASMq treatment group: subdivided into 4 groups and treated with either 0.1, 0.4, 0.7, or 1.0 mg/mL ASMq.

Measuring TGF-β1 and Smad7 mRNA expression by RT-PCR

Log growth phase cells were inoculated at a concentration of 1.0×10^6 cells per dish into a 100 mm culture dish. Adherent HSF cells were grown to logarithmic phase treated with ASMq at concentrations of 0.1 mg/mL, 0.4 mg/mL, 0.7 mg/mL, or 1.0 mg/mL. The culture media were aspirated, and pre-cooled PBS was used to rinse the cells twice. Total RNA was extracted with the Trizol total RNA extraction kit, and total RNA concentration and purity were determined using an ultraviolet spectrophotometer (measured in triplicate). The OD_{260}/OD_{280} absorbance ratio of the specimen was >1.8 and the total RNA concentration in each sample was calculated. Primers sequences for RT-PCR were: TGF-β1, Forward: CAAGA-GGCTGTGTTGVTGTGAATC; Reverse: GTTGGTT-TGAGAAAATCCATCGG; Smad7, Forward: CAAG-AGGCTGTGTTGVTGTGAATC; Reverse: GTTGGT-TTGAG AAAATCCATCGG; GAPDH, Forward: CG-TCTTCACCACCATGGAGA; Reverse: CGGCCAT-CGCCACAGTTT. Two micrograms total RNA and 1 μg Oligo dT in 12 μL 0.1% DEPC were denatured at 70°C for 5 min. Then, 4 μL 5 × reverse transcription buffer, 2 μL of 10 mmol/L dNTP, and I μL of 20 μg/μL ribonuclease inhibitor were added. After 5 min incubation at 37°C, 1 μL of 200 μg/μL MuLV reverse transcriptase was added for a final reaction volume of 20 μL. Then, after denaturation at 45°C for 60 min, the reaction mixture was heated at 70°C for 10 min to terminate the reaction. The total volume of PCR reactions was 50 μL and the cycles proceeded as follows: pre-denaturation at 94°C for 10 min, 30 cycles of amplification (denature at 94°C for 1 min, annealing at 56°C for 0.5 min, extension at 72°C for 0.5 min), final extension at 72°C for 10 min. After the reaction was terminated, 15 μL of the PCR reaction was electrophoresed through 2% agarose gel using the PCR marker as molecular weight standard.
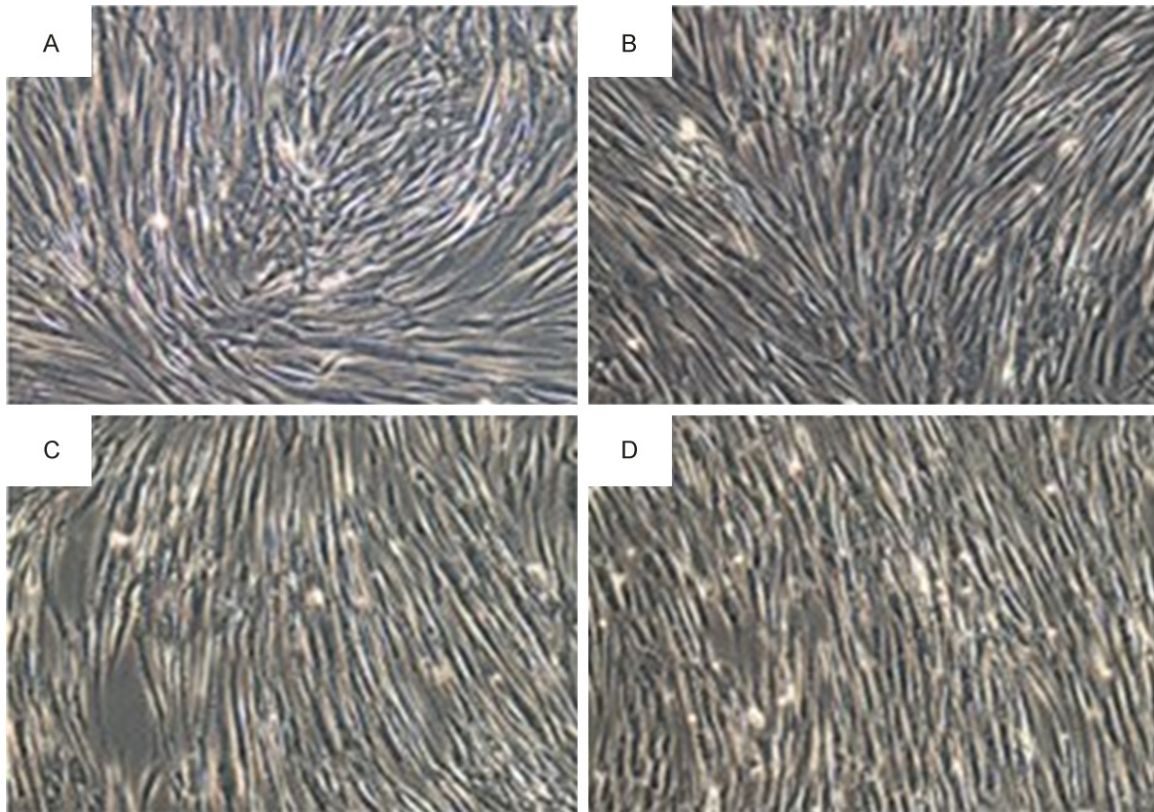
Figure 1. Influence of ASMq on cell morphology of cultured HSFs *in vitro*. Compared to the control group (A), HSFs in groups (B-D) demonstrate smaller cell volume, rounded shape, shrunken nucleus, decreased nuclear-cytoplasmic ratio, muddy cytoplasm, and the appearance of vesicles. Part of the cell nuclear membrane disappeared and rounded dead cells could be observed.

After electrophoresis, the gel was observed and imaged under ultraviolet lamp, and density scanning was used to analyze the PCR products. The index of mRNA expression (EI) = (target mRNA band area × gray scale)/(reference mRNA band area × gray scale).

Level of TGF-β1 and Smad7 protein expression by Western blotting

HSF cells were inoculated during logarithmic growth phase into 100 mm culture dishes at 1.0×10^6 cells per dish. Adherent cells were grown past logarithmic phase ASMq was administered at concentrations of 0.1 mg/mL, 0.4 mg/mL, 0.7 mg/mL, and 1.0 mg/mL. The culture media were aspirated, and pre-cooled PBS was used to rinse the cells twice. One hundred microliters pre-cooled lysis buffer was added and the samples were incubated on an ice-bath for 30 min. Samples were collected with a spatula, and the supernatant was collected after centrifugal separation at 4°C and 12000 rpm

for 15 min. Protein concentrations were measured by Coomassie light blue technique. Fifteen microliters of the samples were loaded onto a 10% SDS-PAGE vertical gel and separated by electrophoresis. The samples were transferred onto a PVDF membrane by electrophoresis. Ponceau red staining was used to observe the efficiency of transfer. The membrane was then blocked using a 5% solution of skim milk at room temperature for 1 h or at 4°C overnight. TGF-β1 and Smad7 polyclonal antibodies were diluted at a ratio of 1:1000 into the blocking solution and allowed to incubate at room temperature for 1 h or at 4°C overnight. The membrane was rinsed with PBS-T buffer for 10 min thrice, and HRP-labelled secondary antibody (1:20000) was added. The membrane was incubated at room temperature for 1 h and rinsed with PBS-T buffer for 10 min thrice. A 1:1 mixture of solutions A and B from the ECL chemiluminescence kit was added to the transfer surface of the PVDF membrane, and the membrane was incubated at room temperature for 1
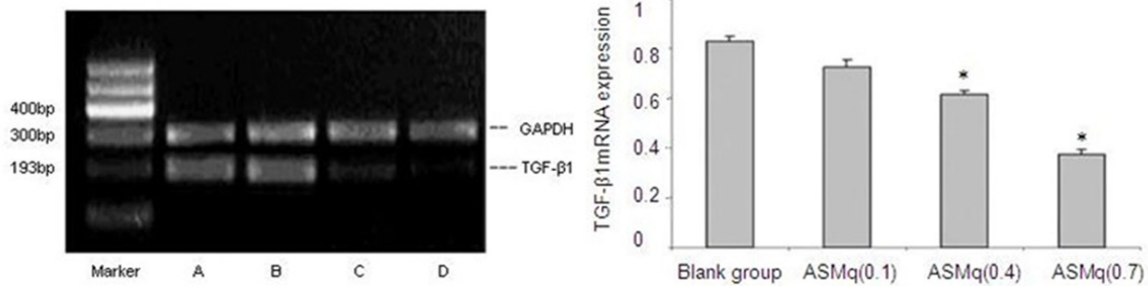
Figures 2. Effect of ASMq on TGF-β1 mRNA expression. Compared to the control group, as the concentration of ASMq increases, gradually decreases. The decrease in TGF-β1 mRNA was significantly reduced when 0.4 mg/mL and 0.7 mg/mL ASMq were used (P<0.05). A. Control; B. 0.1 mg/mL; C. 0.4 mg/mL; D. 0.7 mg/mL. *P<0.05.

h. Remaining buffer was removed and the membrane was wrapped in preservative film. In the darkroom, the X film was pressed onto the membrane and then exposed, developed, and fixed with fixing solution. Protein expression index EI = (target protein band area × gray scale)/(reference protein band area × gray scale).

Statistical analysis

Experimental data were processed using SPSS 11.5. The data are shown as $\bar{x} \pm s$, q-test was used for paired-comparisons, while ANOVA was performed for comparisons between groups. The statistical significance was set at α = 0.05.

Results

Influence of ASMq on cell morphology of in vitro cultured HSFs

After 48 h cell culture, inverted phase contrast microscope was used to observe the cell morphology. In the control group (A: 0 mg/mL), the cell morphology appeared as expected: elongated spindle, large cell body, abundant cytoplasm, many cellular protrusions, no nucleolus, and some cells showed 2-3 extended protrusions of varying lengths. Cells were uniformly attached to the culture surface and there were no apparent floating cells. The cytoplasm appeared clear. However, ASMq-treated cells (B: 0.1 mg/mL, C: 0.4 mg/mL, D: 0.7 mg/mL) showed smaller cell volume, shorter and rounded protrusions, shrunken nucleus leading to reduced nuclear-cytoplasmic ratios, and muddy cytoplasm. Cells had cytoplasmic vesicles and parts of the cytoplasmic structure were unclear. The nuclear membrane disappeared in some

cells, while rounded and floating dead cells were observed (**Figure 1**).

Effect of ASMq on TGF-β1 and Smad7 mRNA expression

Effect of ASMq on TGF-β1 mRNA expression

When compared to the control group, expression of TGF-β1 mRNA gradually decreased with increasing concentrations of ASMq. When ASMq was administered at concentrations of 0.4 mg/mL and 0.7 mg/mL, there was a significant decrease in TGF-β1 mRNA levels, and the decrease was dependent on the concentration of ASMq in the culture. The differences within groups and between groups are statistically significant (*P*<0.05). These data indicate that ASMq plays a role in lowering the expression of TGF-β1 mRNA in HSFs, as shown in **Figure 2**.
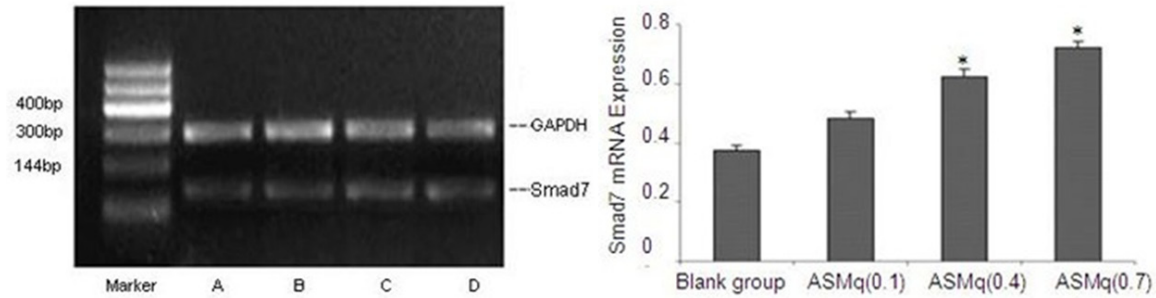
Effect of ASMq on Smad7 mRNA expression

When compared to the control group, Smad7 mRNA expression gradually increased as the concentration of ASMq increased in a concentration-dependent manner. The differences within groups and across groups were statistically significant (*P*<0.05). The data indicate that ASMq plays a role in increasing the expression of Smad7 mRNA in HSFs, as shown in **Figure 3**.
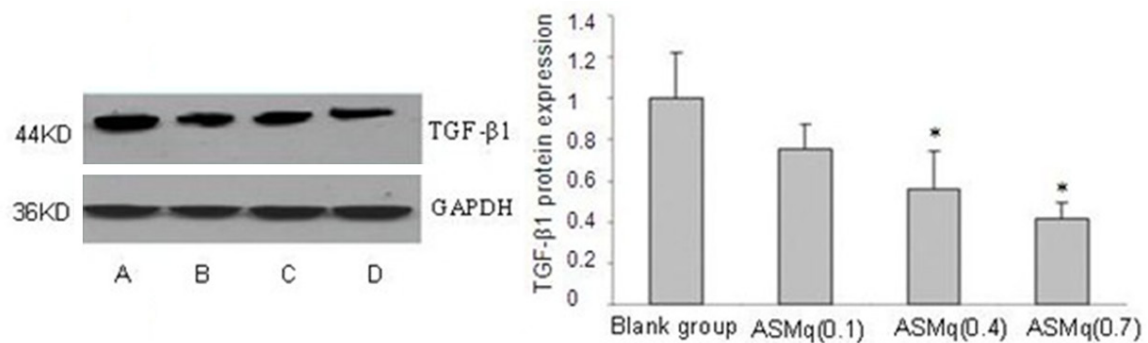
Effect of ASMq on TGF-β1 and Smad7 protein expression
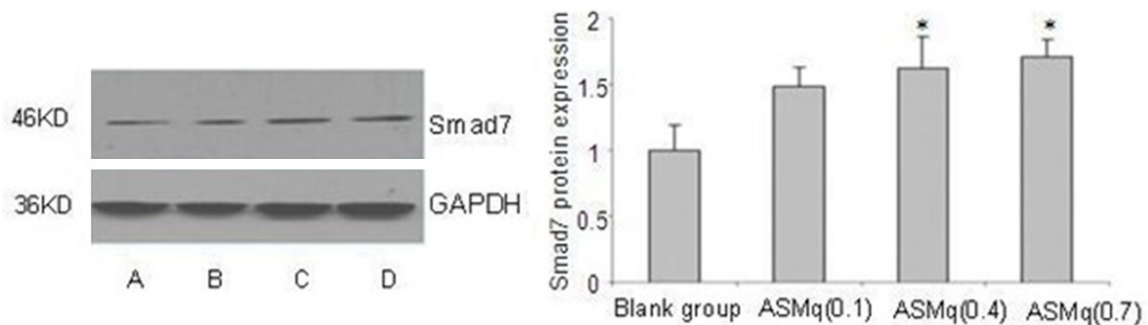
Effect of ASMq on TGF-β1 protein expression

When compared to the control group, the expression of TGF-β1 protein gradually decreased as the concentration of ASMq increased in a concentration-dependent manner.

Figure 3. Effect of ASMq on Smad7 mRNA Expression. Compared to the control group, as the concentration of ASMq increases, Smad7 mRNA expression also increases. A. Control; B. 0.1 mg/mL; C. 0.4 mg/mL; D. 0.7 mg/mL. *$P<0.05$.

Figure 4. Effect of ASMq on. Compared to the control group, with increasing concentrations of ASMq, the expression of TGF-β1 protein gradually decreased, and the decrease in TGF-β1 protein expression was significant at ASMq concentrations of 0.4 mg/mL and 0.7 mg/mL. A. Control; B. 0.1 mg/mL; C. 0.4 mg/mL; D. 0.7 mg/mL. *$P<0.05$.

Figure 5. Effect of ASMq on. Compared to the control group, as the concentration of ASMq increases, expression of the suppressor protein Smad7 gradually increases. A. Control; B. 0.1 mg/mL; C. 0.4 mg/mL; D. 0.7 mg/mL. *$P<0.05$.

The decrease in TGF-β1 protein expression was significant at ASMq concentrations of 0.4 mg/mL and 0.7 mg/mL. The differences between groups and across groups were statistically significant ($P<0.05$). Our experiment showed that ASMq plays a role in reducing TGF-β1 protein expression in HSFs, as shown in **Figure 4**.

Effect of ASMq on Smad7 protein expression

When compared to the control group, expression of the suppressor protein Smad7 increased as the concentration of ASMq increased. The increase in Smad7 expression followed ASMq expression in a concentration-dependent manner. The differences within and across groups

were statistically significant ($P<0.05$). These data show that ASMq plays a role in increasing Smad7 protein expression in HSFs, as shown in **Figure 5**.

Discussion

TGF-β1 is a cytokine that plays multiple biological roles affecting the entire wound healing process, including the inflammatory reaction and extracellular matrix deposition [4]. As the key factor in the TGF-β/Smad signal transduction pathway, TGF-β1 is an important factor in HS [5]. Smad family proteins were discovered in recent years and are downstream signaling proteins regulated by the TGF-β receptor. Among them, Smad7 has been found to be an inhibitory protein [6, 7]. TGF-β1-mediated signal transduction is initiated when TGF-β type I and II receptors form a heterodimer and become activated. The activated TGF-β type I receptor phosphorylates Smad2 and Smad3, which form a heterodimer with Co-Smad that is translocated into the nucleus and binds to specific promoter sequences on TGF-β-inducible genes. This complex then indirectly regulates transcription through interaction with other nucleoproteins. In addition to activating R-Smad, TGF-β1 can also activate I-Smad (Smad7) and contribute to a negative feedback loop that interrupts TGF-β1 signal transduction. While resting I-Smad is found in the nucleus, it is translocated to the cytoplasm for its functions as a suppressor. Blocking signal transduction in fibroblasts help prevent the pathological effects triggered by TGF-β and may be an effective way to inhibit scar proliferation.

According to Uighur medicine theory, various nutrients produce various fluids in the liver. These form the so-called body fluids, which are part of the basic materials for human life and physiological activities. The body fluids consist of gallbladder hygroplasm, blood hygroplasm, lymphatic temperament, and savda [8], which are present throughout human life, are naturally formed *in vivo*, and play a key role in health and disease. They are continuously consumed and produced in the body and are maintained in a delicate equilibrium. When the four body fluids are not in proper balance (e.g. changes in quantity and quality), they may become detrimental to human body. Similarly, the abnormal body fluids consist of four components, namely abnormal gallbladder hygroplasm, abnormal blood hygroplasm, abnormal lymphatic temperament, and abnormal savda. Among these, abnormal savda results from changes in bile fluid quantity and quality due to the influence of various in vivo factors or the "burning" of bile fluids under the fundamental "heat" of the body. The presence of abnormal savda is a common characteristic for many diseases in Uighur medicine pathophysiology. ASMq is a mixture of thirteen Uighur medicinal herbs, namely Kiku Ko, celery root, chicory root, moldavica dragonhead seed, fennel flower seed, fennel root bark, camomile, liquorice, citronella, basil fruit, seed of hollyhock, anise fruit, Peganum harmala L [9]. Uighur medicine indicates that ASMq can help alleviate hepatic fibrosis and pulmonary fibrosis. According to Western medicine, HS is fibrosis of the skin and is one of the types of systemic fibrosis. We hypothesized that HS is induced by the abnormal deposition of abnormal savda on the skin wound surface.

ASMq is a strong free hydroxy radical scavenger, protects against DNA oxidative damage, inhibits tumor cell proliferation, and induces apoptosis [10]. Based on our previous experiments [3], ASMq is capable of inhibiting HSF proliferation at the cellular level and arrests the cell cycle at G_2/M, triggering premature apoptosis of cells. In this study, through *in vivo* culture of HSFs, RT-PCR, and Western-Blotting, we observed that Smad7 translocation from the cell nucleus to the cytoplasm increased significantly after treatment with ASMq. The results of this study indicate that in the TGF-β/Smad signal transduction pathway, ASMq can reduce the membrane expression of TGF-β type I receptor by decreasing TGF-β1 protein expression, and increasing the expression of the inhibitory Smad7 protein by translocating it from the cell nucleus to the cytoplasm and allowing it to act as part of the negative feedback loop. Smad7 competes with TGF-β1 for binding with TGF-β type I receptor, preventing the phosphorylation of Smad2 and Smad3, and further decreases the formation of the Co-Smad heterodimer. Thus, the formation of collagen-dominated extracellular matrix that makes up the HS is significantly reduced. These experiments demonstrate that ASMq can inhibit scar proliferation due to the decrease in TGF-β1 and increase in Smad7 protein expression. Promoting the inhibitory Smad transduction signal can effectively suppress the pathological

effects induced by TGF-β1, thereby playing a critical role in arresting HSF proliferation and reducing collagen secretion.

Disclosure of conflict of interest

None.

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References

[1]   Zhang Z, Finnerty CC, He J, Herndon DN. Smad ubiquitination regulatory factor 2 expression is enhanced in hypertrophic scar fibroblasts from burned children. Burns 2012; 38: 236-246.

[2]   Hu Hh, Sheng L, Halmurat U, Liu C, Xiong WJ, Wu CY, Guliniger·Kulaxi. Anti- cancer Effects of Uighur Medicine Abnormal Savda Munzip and Its Influence on Cell Migration. Science & Technology Review 2011; 29: 62-65.

[3]   Gao WC, Wang HJ, Qiao X, Ma J, Du J, Ma SL. Effect of uighur medicine abnormal savda munzip on human hypertrophic scar fibroblasts in vitro. Zhonghua Zheng Xing Wai Ke Za Zhi 2013; 29: 418-421.

[4]   Song R, Bian HN, Lai W, Chen HD, Zhao KS. Normal skin and hypertrophic scar fibroblasts differentially regulate collagen and fibronectin expression as well as mitochondrial membrane potential in response to basic fibroblast growth factor. Braz J Med Biol Res 2011; 44: 402-410.

[5]   Zhang GY. Caveolin I inhibits transforming growth factor-IM activity via inhibition of Smad signaling by hypertrophic scar derived fibroblasts invitro. J Dermatol 2011; 62: 128-131.

[6]   Kamiya Y, Miyazono K, Miyazawa K. Smad7 inhibits trans-forming growth factor beta family type I receptors through two distinct modes of interaction. J Biol Chem 2010; 285: 30804-20813.

[7]   Tang B, Zhu B, Liang Y, Bi L, Hu Z, Chen B, Zhang K, Zhu J. Asiaticoside suppresses collagen expression and TGF-β/Smad signaling through inducing Smad7 and inhibiting TGF-βRI and TGF-βRII in keloid fibroblasts. Arch Dermatology Res 2011; 303: 563-572.

[8]   Li L, Harmurat U, Chen Y, Ji YL, Wu GZ, Zhao XX. Influence of Abnormal Savda Munziq and Mushil on oxidative Stress Induced Damage and Bcl-2 Expression in Lymphocyte. Science & Technology Review 2008; 26: 43-48.

[9]   Kizaibek Murat, Liu B, Shi HH, Harmurat U. Preliminary investigation on the chemical constituents of abnormal Savda Munziq. Lishizhen Medicine And Materia Medica Research 2010; 21: 3139-3141.

[10]   Upur H, Yusup A, Baudrimont I, Umar A, Berke B, Yimit D, Lapham JC, Creppy EE, Moore N. Inhibition of cell growth and cellular protein, DNA and RNA synthesis in human hepatoma (HepG2) cells by ethanol extract of abnormal Savda Munziq of traditional Uighur medicine. Evid Based Complement Alternat Med 2011; 2011: 251424.