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Ivermectin inhibits cell proliferation and the expression levels of type I collagen, α-SMA and CCN2 in hypertrophic scar fibroblasts

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Abstract. A hypertrophic scar (HPS) is characterized by abnormal cell proliferation and the overproduction of extracellular matrix. Currently, the treatment options available for this remain unsatisfactory. Innovative treatments are required to attenuate or prevent hypertrophic scarring and the present study searched for a drug capable of becoming a new preventative and therapeutic strategy. Although the underlying mechanisms have not been fully clarified; it is widely accepted that the TGF-β1/SMAD3 signaling pathway serves an essential role in HPS formation. In the present study, a compound library consisting of clinically used drugs was screened for their inhibitory activity against the SMAD3 protein. The results indicated that ivermectin was able to suppress the phosphorylation of SMAD3. Therefore, the present study further investigated whether ivermectin exhibited antifibrotic effects on HPS fibroblasts. The results demonstrated that ivermectin inhibited the proliferation of HPS fibroblasts and significantly decreased the production of type I collagen, α-smooth muscle actin and cellular communication network factor 2, as determined by analyzing the mRNA and protein expression levels. In conclusion, the results of the present study suggested that ivermectin may be a promising therapeutic agent for HPS.

Introduction

A hypertrophic scar (HPS) is a serious dermal condition resulting from burn or traumatic injuries, which severely impairs the quality of life by causing cosmetic disturbance and functional deformities (1). Currently, no satisfactory therapeutics have been developed for the treatment of HPS. Therefore, the development of novel pharmacological agents for preventing and treating HPS is required.

HPS formation involves an abnormal fibrous wound healing process characterized by the over-proliferation of local fibroblasts and the excessive deposition of collagen and other extracellular matrix (ECM) proteins such as proteoglycans and fibronectin (2). During the development of HPS, fibroblasts differentiate into myofibroblasts, which increases ECM synthesis and tissue contraction (3). Myofibroblasts are a particular type of fibroblast characterized by the abundant expression of α-smooth muscle actin (α-SMA) (4). Cellular communication network factor 2 (CCN2) also serves an important role in the proliferation of HPS fibroblasts and ECM deposition (5). Currently, the underlying mechanisms of HPS are not fully understood. However, it is widely accepted that the TGF-β1/SMAD3 signaling pathway serves an essential role in HPS formation (6). SMAD3 is a major intracellular effector of TGF-β1 that is able to regulate the expression levels of collagen, α-SMA and CCN2 in the fibrotic response, indicating it is a potential target for HPS therapy (7). Previous studies have reported that the downregulation of SMAD3 expression or activation in HPS fibroblasts can significantly reduce the fibrotic reactivity (8-11). Therefore, pharmacological agents with inhibitory activity over SMAD3 may have a significant clinical potential in the treatment of HPS.

In the present study, the ability of specific compounds, derived from a library of existing therapeutics, was analyzed with regard to their inhibitory function towards SMAD3. Ivermectin was selected from the library, which is a semi-synthetic macrocyclic lactone derivative of the avermectin family used to treat parasitic diseases (12). Recently, ivermectin was discovered to be an inhibitor of cancer stem-like cells and to exhibit anti-inflammatory properties (13,14). However, to the best of our knowledge, the effects of ivermectin on HPS fibroblasts have not been previously reported. In the present *in vitro* study, the inhibitory effect of ivermectin on the phosphorylation of SMAD3 was examined in scar fibroblasts. Furthermore, the experiments performed in the current study aimed to determine whether ivermectin treatment could suppress the proliferation of scar fibroblasts and the expression levels of type I collagen, α-SMA and CCN2. The results indicated that ivermectin may be a potential therapeutic agent for HPS treatment.

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Materials and methods

Materials. Ivermectin was purchased from Sigma-Aldrich; Merck KGaA. DMEM, FBS and trypsin were purchased from Thermo Fisher Scientific, Inc. Anti-Collagen-I (cat. no. ab260043), anti-α-SMA (cat. no. ab119952), anti-CCN2 (cat. no. ab209780), anti-SMAD3 (cat. no. ab40854), anti-phosphorylated (p)SMAD3 (cat. no. ab52903) and anti-fibroblast surface protein (FSP; cat. no. ab11333) antibodies were purchased from Abcam. The anti-GAPDH antibody (cat. no. sc-365062) was purchased from Santa Cruz Biotechnology, Inc. The Cell Counting Kit-8 (CCK-8) was obtained from Beyotime Institute of Biotechnology. The Annexin V Apoptosis Detection kit I was purchased from BD Biosciences. TRIzol® reagent was purchased from Invitrogen; Thermo Fisher Scientific, Inc.

Isolation and culture of HPS fibroblasts. Human HPS fibroblasts were isolated from HPS tissues of five male patients who had undergone surgical excision 4-6 months after HPS occurred. The recruitment was started in April 2015 and ended in August 2015. The patients were 20-40 years old. HPS tissues were excised from shoulder and chest. The experiments were approved by the Ethics Committee of Changhai Hospital (approval no. CHEC2014-096). All HPS tissues were obtained with written informed consent according to the Declaration of Helsinki principles. The tissues in this study were from patients, and the informed consent was obtained and signed. All patients agreed to participate in this study. The nature of HPS was confirmed histologically using hematoxylin and eosin-stained sections of skin tissues. The tissues were fixed in 10% formaldehyde for 2 days at room temperature and sectioned at 1 cm^2 area and 5 μm thickness. The sections were stained in hematoxylin solution for 10 min and eosin solution for 10 sec at room temperature and observed under a light microscope (magnification, x40). The specimens were washed three times in sterile DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with an antibiotic/antimycotic (5% penicillin and streptomycin) preparation and were subsequently cut into 5x5 mm sections. The sections were incubated in DMEM with 0.2% dispase overnight at 4˚C. The epidermis was removed and the dermis was minced and digested in DMEM in the presence of 0.2% collagenase type I for 3 h at 37˚C with intermittent shaking. The digestive action was quenched with DMEM and the obtained cells were cultured in DMEM supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 0.1 g/ml streptomycin at 37˚C in a humidified incubator with 5% CO_2 (15). Only cells from the third to fifth passages were used in each experiment.

Ivermectin stimulation. The cells were seeded at a density of $1x10^5$ cells/ml into 6-well plates in DMEM containing 10% FBS (DMEM/10% FBS) for the analysis of mRNA and protein expression levels. Upon reaching 80% confluence, the medium was removed and the cells were incubated at 37˚C in serum-free DMEM for 24 h. Subsequently, different concentrations (0.1, 0.3, 1.0 and 3.0 μmol/l) of ivermectin were added simultaneously in DMEM/10% FBS to treat the cells at 37˚C for 48 h. The control cells were grown in DMEM/10% FBS without the addition of ivermectin.

Cell proliferation assay. The CCK-8 assay is a colorimetric assay that detects the metabolic activity of viable cells (16). In the present study, the CCK-8 assay was used according to the manufacturer's instructions to assess the proliferative activity of HPS fibroblasts. The cells were seeded at an initial density of 2,000 cells/well into 96-well plates and subsequently treated at 37˚C with ivermectin at different concentrations (0.1, 0.3, 1.0 and 3.0 μmol/l) for 1, 3 and 5 days. CCK-8 solution (10 μl) was added to the wells and incubated with the cells for 2 h at 37˚C. The absorbance of the supernatant was measured using a Multiskan spectrum microplate reader at 450 nm.

In vitro assessment of cell apoptosis via flow cytometry. Cell apoptosis was detected using the Annexin V-FITC Apoptosis Detection kit I. Briefly, HPS fibroblasts were treated at 37˚C with ivermectin at different concentrations (0.3 and 3.0 μmol/l) for 48 h. Subsequently, the cells were digested with 0.25% trypsin, washed twice with cold PBS, resuspended in binding buffer and adjusted to a final concentration of $1x10^6$ cells/100 μl. The cell suspension was incubated with Annexin V-FITC and PI for 20 min at room temperature in the dark and the samples were finally measured via flow cytometry (CytoFLEX; Beckman Coulter). The data was analyzed by Flowjo 10.0.7 (Tree Star FlowJo).

Reverse transcription-quantitative PCR (RT-qPCR). Following treatment with ivermectin at different concentrations (0.1, 0.3, 1.0 and 3.0 μmol/l) for 48 h at 37˚C, the fibroblasts were lysed and total RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. First strand cDNA was synthesized from 1 μg RNA using Superscript™ reverse transcriptase and oligo (dT) primers and PrimeScript RT Master Mix (Takara Bio, Inc.) at 37˚C for 15 min, 85˚C for 5 sec and 4˚C 5 for sec (15). qPCR was subsequently performed using the SYBR Premix Ex Taq (Takara Bio, Inc.) assay for human collagen-I, α-SMA, CCN2 and the housekeeping gene GAPDH, following the manufacturer's instructions. Solutions were predenatured at 95˚C for 15 sec, denatured at 95˚C for 5 sec, annealed at 60˚C for 30 sec, extended at 72˚C for 30 sec, and amplified for 40 cycles. The relative quantification of the target gene levels was assessed using RT-qPCR and the ΔΔCt method (17). The following primer sequences were used: GAPDH forward, 5'-GGAGCG AGATCCCTCCAAAAT-3' and reverse, 5'-GGCTGTTGTCAT ACTTCTCATGG-3'; Collagen-I forward, 5'-GAGGGCCAA GACGAAGACATC-3' and reverse, 5'-CAGATCACGTCA TCGCACAAC-3'; α-SMA forward, 5'-GTGTTGCCCCTG AAGAGCAT-3' and reverse, 5'-GCTGGGACATTGAAAGTC TCA-3'; and CCN2, forward 5'-ACCGACTGGAAGACACGT TTG-3' and reverse, 5'-CCAGGTCAGCTTCGCAAGG-3'.

Western blot analysis. Following treatment with ivermectin at different concentrations (0.1, 0.3, 1.0 and 3.0 μmol/l) for 48 h at 37˚C, the cells were washed with ice-cold PBS and lysed in cell lysis buffer (cat. no. P0013; Beyotime Institute of Biotechnology). Protein concentrations were determined using a bicinchoninic acid kit (cat. no. P0012; Beyotime Institute of Biotechnology). The protein lysates (2 μg) were separated by SDS-PAGE (10% gel) and transferred to a PVDF membrane. Following blocking with TBS-Tween-20 (0.1% Tween-20)

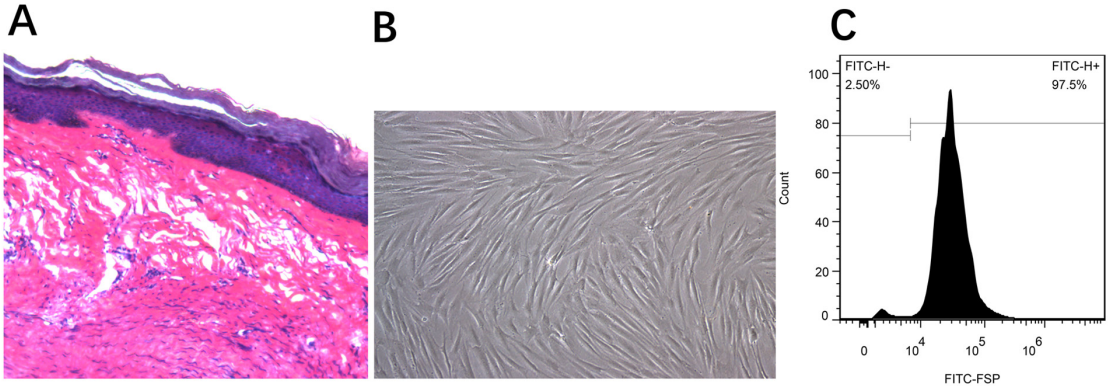

Figure 1. Identification of primary HPS fibroblasts. (A) Pathological section of HPS tissue using hematoxylin and eosin staining (magnification, x40). (B) Morphology of cultured HPS fibroblasts (magnification, x40). (C) Flow cytometric analysis indicated that 97.5% of the cells were fibroblasts. HPS, hypertrophic scar.

containing 5% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) for 1 h at room temperature, the membranes were immunoblotted with monoclonal rabbit anti-type I collagen antibody (1:2,000; cat. no. ab260043; Abcam), monoclonal mouse anti-α-SMA antibody (1:250; cat. no. ab119952; Abcam), monoclonal rabbit anti-CCN2 antibody (1:500; cat. no. ab209780; Abcam), monoclonal rabbit anti-SMAD3 antibody (1:1,000; cat. no. ab40854; Abcam), monoclonal rabbit anti-pSMAD3 antibody (1:2,000; cat. no. ab52903; Abcam) and monoclonal mouse anti-GAPDH antibody (1:2,000; cat. no. sc-365062; Santa Cruz Biotechnology, Inc.) at 4˚C overnight. Subsequently, the bound antibodies were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h and visualized using an ECL chemiluminescence system (BeyoECL Star; Beyotime Institute of Biotechnology) (15). ImageJ software (v1.51; National Institutes of Health) was used for densitometry.

Statistical analysis. All experiments were repeated three times. Data are presented as the mean ± SD. The western blotting bands were semi-quantified via densitometry. The mRNA and protein expression levels of each target gene and protein were normalized according to the corresponding expression levels of GAPDH. SPSS software (v23; IBM Corp.) was used for the statistical analysis. A one-way ANOVA was used to compare the statistical differences between the groups and the post hoc test used the Tukey's method. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of primary HPS fibroblasts. Hematoxylin and eosin-stained sections from the tissues were histologically examined to confirm the diagnosis of HPS (Fig. 1A). The adherent fibroblasts were spindle or star-shaped squamous cells with multi-protrusions (Fig. 1B). Using flow cytometry, the cells were detected according to their FSP expression (anti-FSP antibody); the positive rate was estimated to be 97.5% (Fig. 1C).

Ivermectin inhibits the expression of pSMAD3 in HPS fibroblasts without affecting the production of SMAD3. Western blot analysis was performed to examine the effects of ivermectin on the expression levels of SMAD3 and pSMAD3 in HPS fibroblasts. The data indicated no significant differences in the expression of SMAD3 between the different groups (Fig. 2A), whereas the expression levels of pSMAD3 in the ivermectin treatment groups (0.1, 0.3, 1.0 and 3.0 μmol/l) were significantly downregulated compared with those in the blank control group (Fig. 2B). These results suggested that ivermectin may be able to inhibit the expression of pSMAD3 in HPS fibroblasts without affecting the production of SMAD3.

Ivermectin inhibits HPS fibroblast proliferation, but does not affect cell apoptosis. The CCK-8 assay was performed to detect cell proliferation. On day 1, no significant changes were observed with different concentrations of ivermectin treatment. On day 3, ivermectin treatment significantly diminished cell proliferation by 9, 14, 21 and 28%, at doses of 0.1, 0.3, 1.0 and 3.0 μmol/l, respectively, compared with the control group. On day 5, the proliferation rate following ivermectin treatment at 0.1, 0.3, 1.0 and 3.0 μmol/l decreased by 15, 31, 35 and 38%, respectively (Fig. 3).

HPS fibroblasts were treated with ivermectin for 48 h and flow cytometry was subsequently performed to assess cell apoptosis. Flow cytometric analysis indicated that the apoptotic rates in the groups receiving ivermectin treatment (0.3 and 3.0 μmol/l) were not significantly different compared with the blank control group (Fig. 4). These findings suggested that ivermectin could suppress the proliferation of HPS fibroblasts without affecting cell apoptosis.

Ivermectin downregulates the mRNA expression levels of type I collagen, α-SMA and CCN2 in HPS fibroblasts. The effects of ivermectin on the mRNA expression levels of type I collagen, α-SMA and CCN2 in HPS fibroblasts were assessed using RT-qPCR. The results demonstrated that the mRNA expression levels of all genes were significantly downregulated in the cell groups treated with ivermectin (0.1, 0.3, 1.0 and 3.0 μmol/l) compared with those in the control group (Fig. 5). The effects became stronger with the increase of concentration. These findings indicated that ivermectin may decrease the mRNA expression levels of type I collagen, α-SMA and CCN2 in HPS fibroblasts.

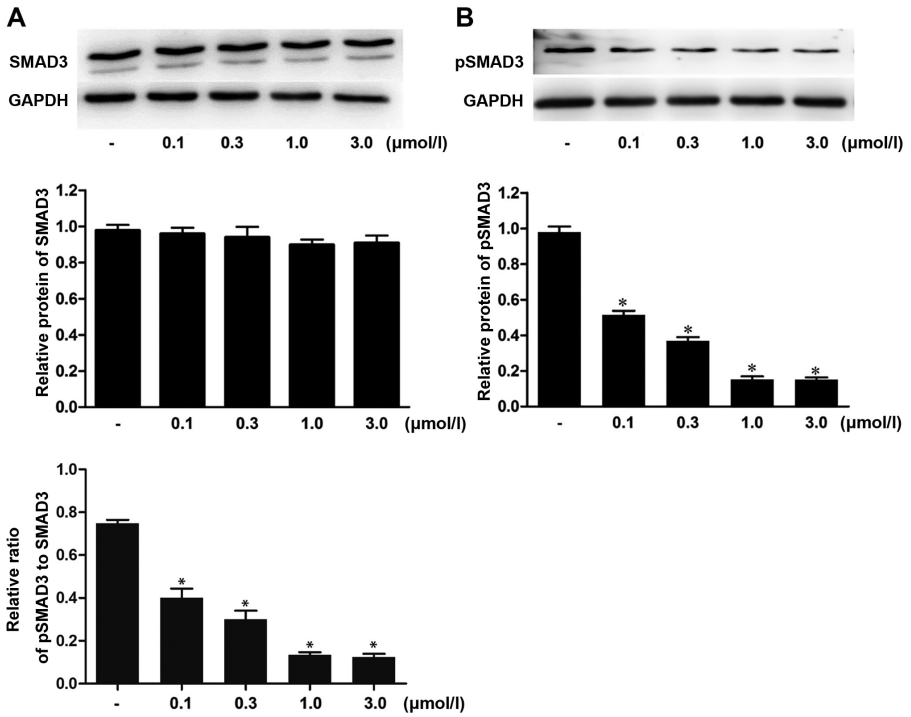

Figure 2. Ivermectin inhibits the expression of pSMAD3 in HPS fibroblasts without affecting the production of SMAD3. The cells were treated with or without different concentrations of ivermectin (0.1, 0.3, 1.0 and 3.0 μmol/l) in DMEM containing 10% FBS for 48 h. The cell lysates were prepared and analyzed via western blotting with antibodies against (A) SMAD3 and (B) pSMAD3. GAPDH was used as the loading control. The experiments were repeated thrice and similar results were obtained. *P<0.05 vs. control (without ivermectin treatment). P-, phosphorylated.

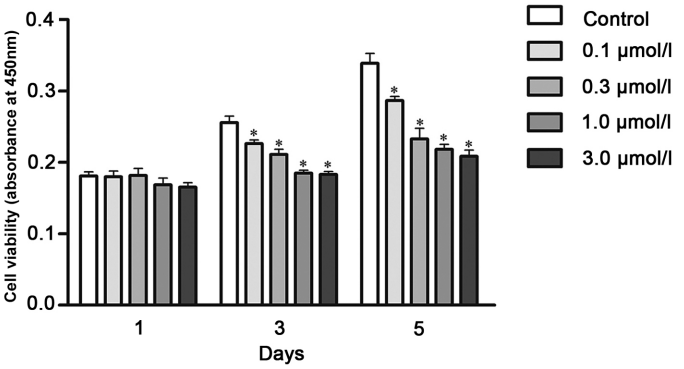

Figure 3. Ivermectin inhibits the proliferation of hypertrophic scar fibroblasts. The cells were treated with ivermectin at different concentrations (0.1, 0.3, 1.0 and 3.0 μmol/l) for 1, 3 and 5 days. The CCK-8 assay was used to assess the proliferation of HPS fibroblasts by detecting the metabolic activity of viable cells. A Cell Counting Kit-8 assay was performed to analyze cell viability on day 1, 3 and 5. Untreated fibroblasts were used as the control. The absorbance of the supernatant was measured using a Multiskan spectrum microplate reader at 450 nm. The data are presented as the mean ± SD of three independent experiments. *P<0.05 vs. control (without ivermectin treatment).

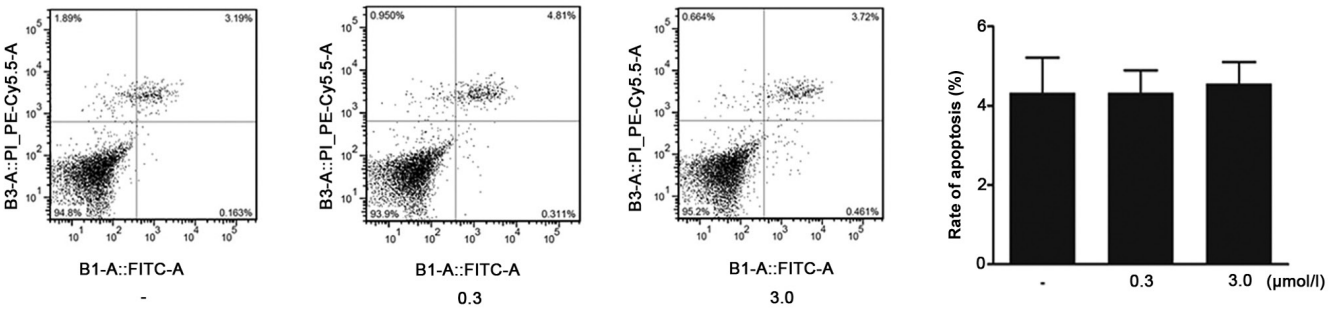

Figure 4. Ivermectin does not affect the apoptosis of hypertrophic scar fibroblasts. The cellular apoptotic rates were detected using the Annexin V-FITC Apoptosis Detection kit I. The cells were treated with ivermectin (0, 0.3 and 3.0 μmol/l) for 48 h. Subsequently, flow cytometry was performed to assess the level of cellular apoptosis.

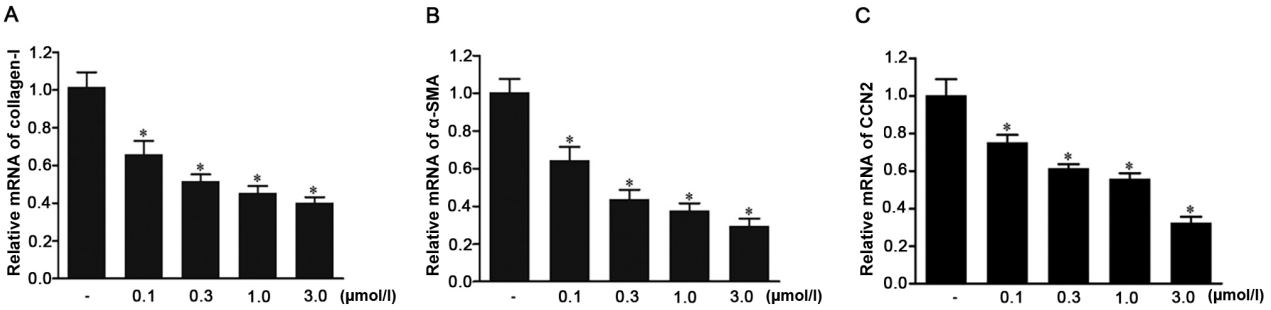

Figure 5. Ivermectin downregulates the mRNA expression levels of collagen-I, α-SMA and CCN2 in hypertrophic scar fibroblasts. The cells were treated without or with different concentrations (0.1, 0.3, 1.0 and 3.0 μmol/l) of ivermectin in DMEM containing 10% FBS for 48 h. Total mRNA was prepared and subjected to reverse transcription-quantitative PCR to detect (A) collagen-I, (B) α-SMA and (C) CCN2 expression levels. GAPDH was used as the loading control. The experiments were repeated thrice and similar results were obtained in all three experiments. *P<0.05 vs. control (without ivermectin treatment). α-SMA, α-smooth muscle actin; CCN2, cellular communication network factor 2.

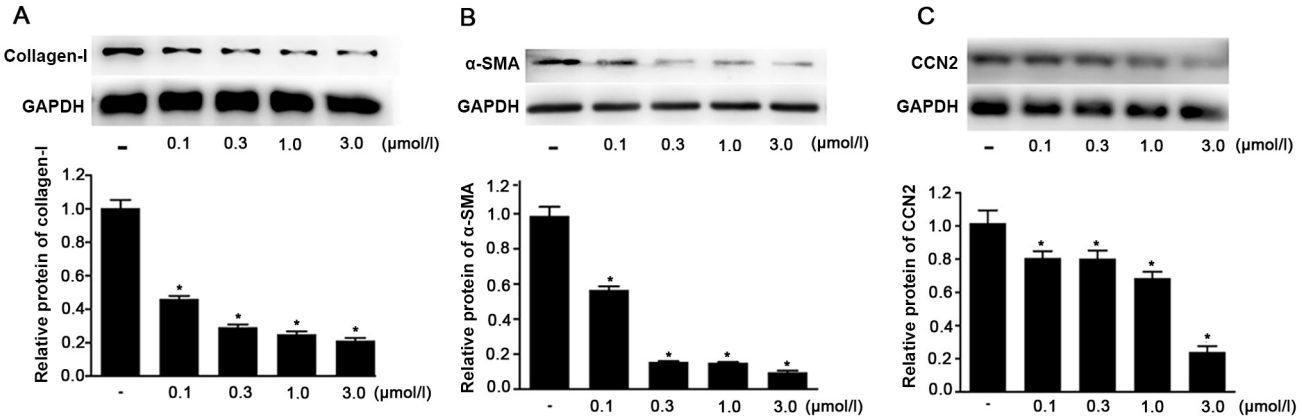

Figure 6. Ivermectin decreases the protein expression levels of collagen-I, α-SMA and CCN2 in HPS fibroblasts. The cells were treated with or without different concentrations (0.1, 0.3, 1.0 and 3.0 μmol/l) of ivermectin in DMEM containing 10% FBS for 48 h. Cell lysates were prepared and subjected to western blotting to analyze the protein expression levels of (A) collagen-I, (B) α-SMA and (C) CCN2. GAPDH was used as the loading control. The experiments were repeated thrice and similar results were obtained. *P<0.05 vs. control (without ivermectin treatment). α-SMA, α-smooth muscle actin; CCN2, cellular communication network factor 2.

Ivermectin downregulates the protein expression levels of type I collagen, α-SMA and CCN2 in HPS fibroblasts. Western blot analysis was performed to assess the effects of ivermectin on the protein expression of type I collagen, α-SMA and CCN2 in HPS fibroblasts. It was identified that the protein expression levels of all these factors were significantly decreased in the ivermectin treatment groups (0.1, 0.3, 1.0 and 3.0 μmol/l) compared with the control group (Fig. 6). The effects became stronger with the increase of concentration. These data suggested that ivermectin may significantly decrease the protein expression levels of type I collagen, α-SMA and CCN2 in HPS fibroblasts.

Discussion

Ivermectin is a type of macrocyclic lactone parasiticide that displays anti-inflammatory properties (14). In the present study, the inhibitory effects of ivermectin on fibrotic reactivity in HPS fibroblasts were investigated. The main findings of the study demonstrated that ivermectin could inhibit the phosphorylation of SMAD3 in HPS fibroblasts. In addition, it was identified that ivermectin could inhibit cell proliferation and decrease the expression levels of type I collagen, α-SMA and CCN2. To the best of our knowledge, these effects of ivermectin on HPS fibroblasts have not been previously reported.

The accumulation of ECM in fibrotic diseases usually results from elevated mRNA expression levels due to increased transcriptional activation (18). It has been shown that TGF-β1 serves an essential role in modulating ECM gene expression and that this process is SMAD3-dependent (19). Following the binding of the receptor on the cell membrane, TGF-β1 promotes TGF-β1 receptor kinase to phosphorylate SMAD3 (20). Subsequently, pSMAD3 translocates into the nucleus and activates gene transcription to mediate collagen production (21,22). pSMAD3 is the activated version of SMAD3 and has been reported to be an important modulator of ECM expression (23). Studies have revealed that SMAD3 is upregulated and phosphorylated in HPS fibroblasts (24,25). Thus, the inhibition of the production or phosphorylation of SMAD3 may be used as a potential treatment strategy for HPS. Small interfering RNA targeting SMAD3 can decrease ECM deposition and attenuate the process of fibrosis (26). In the present study, the results demonstrated that ivermectin treatment significantly decreased the expression of pSMAD3 in HPS fibroblasts in a dose-dependent manner. However, the effects on SMAD3 production were not investigated.

Collectively, the current findings indicated that ivermectin could suppress the phosphorylation of SMAD3 and that it may be able to inhibit the fibrotic reactivity of HPS fibroblasts.

The aberrant proliferation of fibroblasts is usually observed in pathological scars compared with scarless healing (27). Previous studies have revealed that the regulation of the proliferation and apoptosis of fibroblasts are altered in HPS (4,28). HPS fibroblasts exhibit a higher proliferation rate compared with normal fibroblasts (29). The inhibition of the proliferation of HPS fibroblasts may be a potential treatment for HPS. The present results suggested that ivermectin could significantly inhibit HPS fibroblast proliferation, but it did not affect cell apoptosis. The inhibitory effect was enhanced following an increase in the dose and treatment duration of ivermectin. It has been previously reported that TGF-β1 was able to promote the proliferation of fibroblasts via the SMAD3 signaling pathway (24). Although, the precise mechanism remains unknown, it was suggested that it may be associated with the inhibitory effect caused on SMAD3.

Overproduction and aggregation of ECM is the principal feature of HPS (4). Collagen is the key component of ECM and is mainly synthesized by fibroblasts (30). Fibroblasts can differentiate into myofibroblasts following stimulation with TGF-β1; this process also depends on the action of SMAD3 (31). Myofibroblasts exhibit increased proliferative and secretory properties, as well as serve a major role in HPS formation by persistently synthesizing collagen (32). These myofibroblasts are contractile cells promoting scar contraction; they also overexpress α-SMA, which is a well-known marker of HPS (33,34). CCN2 is a type of profibrogenic cytokine that can improve the proliferation of fibroblasts and ECM deposition; its expression is also regulated by the TGF-β1/SMAD3 signaling pathway (35). The inhibition of the expression levels of type I collagen, α-SMA and CCN2 is the main mechanism of HPS treatment. The present study demonstrated that ivermectin could significantly decrease the protein and mRNA expression levels of type I collagen, α-SMA and CCN2 in HPS fibroblasts using RT-qPCR and western blot analyses. These findings are important in considering that ivermectin may be able to decrease the deposition of the ECM and diminish tissue contraction. Ivermectin has been used as an antiparasitic agent for several years and has been identified to be safe for human use (14). Since SMAD3 serves an essential role in HPS formation, the effects of ivermectin on suppressing type I collagen, α-SMA and CCN2 expression may be associated with the inhibition of SMAD3 phosphorylation.

In conclusion, the present study demonstrated that ivermectin was able to inhibit the proliferation of HPS fibroblasts. Ivermectin could also suppress the phosphorylation of SMAD3 and decrease the production of type I collagen, α-SMA and CCN2, which are phenotypic and functional markers of fibrogenesis. The results suggested that ivermectin may be a promising therapeutic agent for HPS. However, further studies using animal models of dermal fibrosis and placebo-controlled clinical studies are required to conclusively identify the effects of ivermectin.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZX, RS and JL supervised the study. ZX and ST conceived and designed the experiments. ST conceived the current study, analyzed the data and drafted the manuscript. YZ, SX and PL advised on the design of the experiments and guidance on the process. YZ, SX and PL also made manuscript revisions and helped with the experiments. RS and JL selected ivermectin from the library, designed the current study and supervised the experiments. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Changhai Hospital (approval no. CHEC2014-096). The tissues in this study were from patients, and the informed consent was obtained and signed. All patients agreed to participate in this study.

Patient consent for publication

Consent for publication was obtained from all participants.

Competing interest

The authors declare that they have no competing interests.

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