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ORIGINAL PAPER

Asiaticoside conveys an antifibrotic effect by inhibiting activation of hepatic stellate cells via the Jagged-1/Notch-1 pathway

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Abstract

The aim of this study was to investigate the underlying protective mechanisms of asiaticoside (AS) against liver fibrosis (LF) both in vivo and in vitro. A rat model with carbon tetrachloride (CCl_4)-induced liver fibrosis is employed to verify the effect and mechanism of AS on the process of liver fibrosis in vivo experiment. Hematoxylin/eosin and sirius red staining was conducted to assess the severity of liver injury and fibrosis. Further, the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), glutamyl transferase (GGT), and total bilirubin (TBil) were measured. In addition, LX2 cells were cultured for vitro experiment to investigate the influence of AS on hepatic stellate cells (HSCs). Overproduction of α-smooth muscle actin and type I collagen is characteristic of LF and HSCs, as determined by immunohistochemical and Western blot analyses. The expression levels of molecules associated with the Notch signaling pathway (i.e., Notch-1, Jagged-1, and Delta-like-4) were assessed by Western blot analysis. The results revealed that AS attenuated LF, as defined by reduced deposition of collagen, expression of α-smooth muscle actin and collagen type 1, and expression of biochemical parameters (alanine aminotransferase, aspartate aminotransferase, and hydroxyproline). Notably, AS suppressed the expression levels of Notch-1, Jagged-1, and Delta-like-4 in activated HSCs and LF. Collectively, these results demonstrate that AS prevented the progression of LF by modulating the Notch signaling pathway, indicating that AS has potential therapeutic effects against LF.

Keywords Asiaticoside · Notch signaling pathway · Liver fibrosis

Introduction

Liver fibrosis (LF) is a prevalent chronic liver disease that can result in cirrhosis if untreated [1]. As the major mesenchymal cell type in the liver, hepatic stellate cells (HSCs) play pivotal roles in the progression of LF and are transformed by external stimuli into myofibroblast-like cells, which are characterized by accelerated proliferation and overaccumulation of α-smooth muscle actin (α-SMA) and type I collagen (Col-1) upon activation [2–4]. Liver transplantation is the most efficacious treatment for patients with LF, but is constrained due to the limited number of potential donors. Therefore, continued research on the mechanisms of LF and activation of HSCs is important for the development of novel curative options.

As the main active ingredient of the herbaceous annual plant *Centella asiatica*, asiaticoside (AS) reportedly has multiple biological functions, including anti-inflammation and anti-cancer activities, the ability to inhibit apoptosis of neurons [5–7], antioxidative, wound healing, hepatoprotective, as well as antitumor properties [8, 9]. Ma et al. reported that AS inhibited the proliferation and induced apoptosis of hepatocellular carcinoma cells via the PI3K/Akt pathway [10], while Zhang et al. demonstrated that AS alleviated pulmonary fibrosis by reducing collagen deposition and inflammation [11]. Notably, AS exhibited low cytotoxicity and anti-hepatofibrotic effects in an immortalized line of rat liver stellate cells (HSC-T6) [12]. However, the mechanism underlying the anti-hepatofibrotic effects of AS remains unclear.

The Notch signaling pathway has been implicated in the regulation of cell differentiation, proliferation, survival, and apoptosis [13–15]. Notably, abnormal changes in the Notch

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signaling pathway components Notch 1–4 and Jagged-1 are strongly associated with the progression of LF [16, 17]. Upregulated expression of Notch-3 and Jagged-1 has been observed in the fibrotic liver [18]. Accordingly, inhibition of the Notch signaling pathway might be an effective strategy for the treatment of LF.

In previous studies, some plant active compounds were confirmed that could attenuate liver fibrosis by targeting Notch signaling, such as capsaicin [19] and natural sesquiterpene costunolide [20]. Therefore, the aim of the present study was to determine whether AS can inhibit LF and liver cirrhosis via the Notch signaling pathway. The expression levels of α-SMA and Col-1 in liver tissues were assessed by immunohistochemical and Western blot analyses. Meanwhile, the expression levels of proteins associated with the Notch signaling pathway (i.e., Notch-1, Delta-like [DLL]-4, and Jagged-1) were evaluated by Western blot analysis.

Material and methods

Chemicals and reagents

AS was acquired from Shanghai yuanye Bio-Technology Co., Ltd (Shanghai, China). CCl_4 was obtained from Jiangsu Qiangsheng Chemical (Jiangsu, China) and dissolved in olive oil to a final concentration of 50% (*v/v*). Monoclonal antibodies against Col-1 were purchased from Sigma (St. Louis, MO, USA), while those against α-SMA. Notch-1, DLL-4, Jagged-1, and glyceraldehyde-3-phosphate dehydrogenase (GADPH) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin, and streptomycin were purchased from Sigma.

Animals

Male Wistar rats (body weight, 150–200 g) were raised under a 12-h light/dark cycle at room temperature with ad libitum access to food and water. All animal procedures were approved by the Animal Experimental Ethics committee of our hospital and conducted in accordance with the "Guide for the Care and Use of Laboratory Animals." The rats were randomly allocated to one of three groups ($n = 8$/group): a sham control group; a model group, which was intragastrically administered CCl_4 (1 ml/kg, dissolved in olive oil, 1:1 *v/v*) twice per week for 8 weeks; or the AS group, which was intragastrically administered CCl_4 (1 ml/kg, dissolved in olive oil, 1:1 *v/v*) twice per week for 8 weeks (9) plus AS at 5, 10, and 15 mg/kg body weight. At the end of the treatment period, all animals were overnight fasted, weighed, and anesthetized by deep isoflurane inhalation. Serum and liver tissues were collected and stored at −80 °C until analysis.

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), glutamyl transferase (GGT), and total bilirubin (TBil)

Serum levels of ALT, AST, ALB, GGT, and TBil were measured using a commercial kit (Abbkine Scientific Co., Ltd., Wuhan, China) in accordance with the manufacturer's instructions.

Histological staining

Liver tissues were fixed in 10% formalin, embedded in paraffin, and cut into sections, which were stained with hematoxylin and eosin. Moreover, the extent of LF was determined by staining with Sirius Red azo dye. All sections were photographed under a microscope equipped with a digital camera, and the extent of LF was calculated using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Immunohistochemical analysis

For immunohistochemical analysis, the tissue sections were mounted on slides and incubated with primary antibodies against Col-1 and α-SMA, washed, then incubated with secondary antibodies, and visualized with a 3,3′-diaminobenzidine chromogen kit (Shanghai Enzyme-linked Biotechnology Co., Ltd, Shanghai, China). Quantification of immunohistochemical staining was performed using Image-Pro plus 6.0 software.

Cell culture

LX2 cells (American Type Culture Collection, Manassas, VA, USA), as a HSC line, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin at 37 °C under an atmosphere of 5% CO_2 and 95% air. In addition, the LX2 cells were transfected with small interfering RNA (siRNA) against Jagged-1 using Lipofectamine™ 2000 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's protocol and then incubated for 24 h. LX2 cells were divided into four groups: a control group and three treatment groups treated with, transforming growth factor (TGF-β, 2 ng/ml), AS (TGF-β plus 150 nM AS), or siRNA-Jagged-1 (TGF-β plus siRNA-Jagged-1).

The viability of LX2 cells (3×10^4 cells/well) following treatment with AS and siRNA-Jagged-1 was assessed using the CCK8 assay (Abbkine Scientific Co., Ltd., Wuhan,

China) in accordance with the manufacturer's instructions. The wound healing and transwell assays were performed to explore the migration and invasion capabilities of AS-treated LX2 cells, as previously reported.

Western blot analysis

The cells were lysed with radioimmunoprecipitation assay buffer containing a protease inhibitor on ice, and the total protein content was quantified using a bicinchoninic acid assay kit (Pierce Biotechnology, Waltham, MA, USA) in accordance with the manufacturer's instructions. Afterward, the proteins were separated by electrophoresis using 10% sodium dodecyl sulfate–polyacrylamide gels and then transferred onto nitrocellulose membranes, which were blocked with 5% skim milk for 2 h and probed with primary antibodies against GADPH, α-SMA, Col-1, Jagged-1, Notch-1, and DLL-4 at 4 °C overnight and then with secondary antibodies for 2 h at room temperature.

Statistical analysis

Data are presented as the mean ± standard error of the mean. The Dunnet's multiple *t*-test was used to identify differences between groups. For comparisons among multiple groups, one-way ANOVA statistical test was used, and Tukey test was used for post hoc analysis. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). A probability (p) value of < 0.05 was considered statistically significant.

Results

AS protected against CCl_4-induced liver injury in vivo experiment

As shown in Fig. 1B, the liver/body weight ratio was increased in the model group as compared to the control group (0.032 ± 0.004 vs 0.054 ± 0.005, respectively,

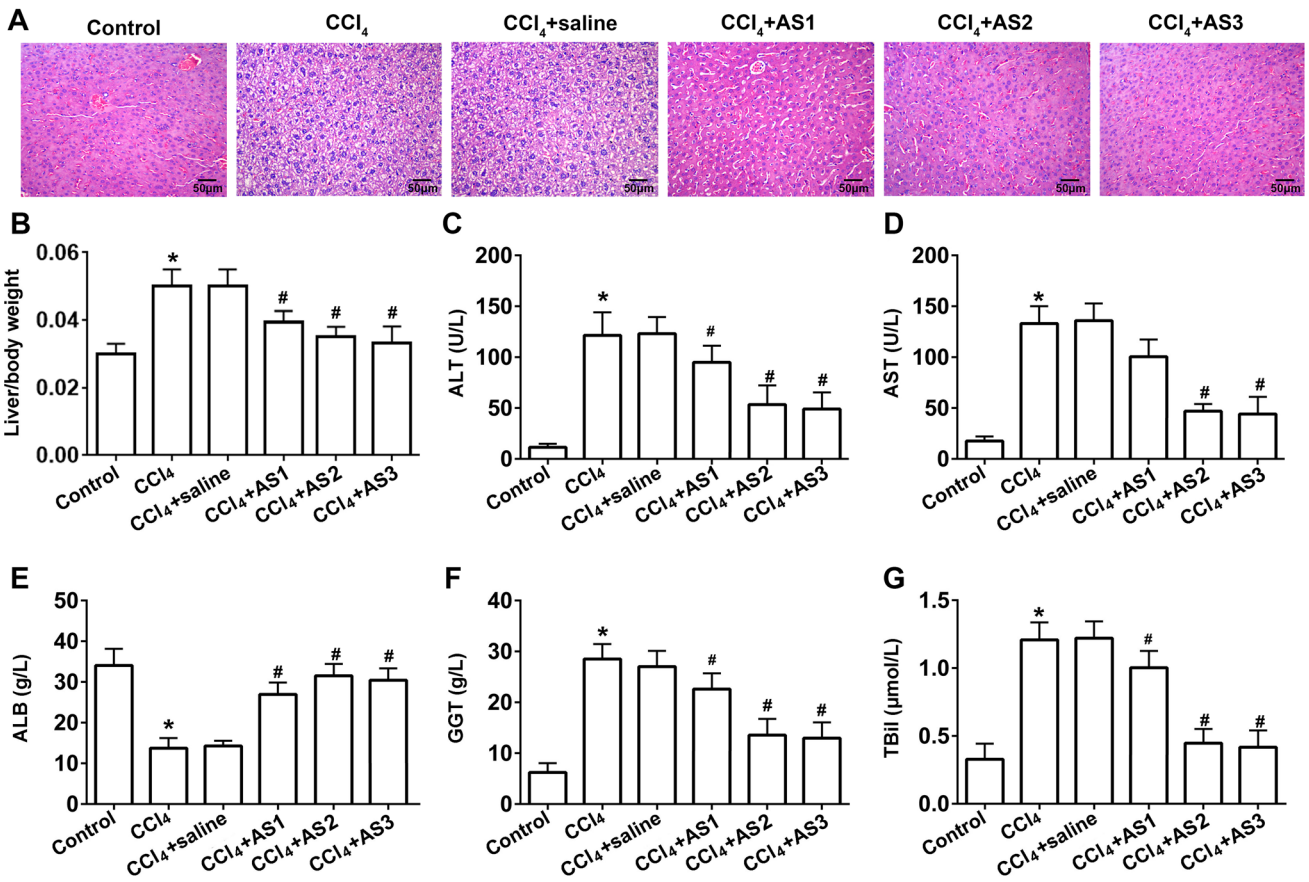

Fig. 1 Protective effect of asiaticoside (AS) in CCl_4-induced liver cirrhosis rats. **A** Hematoxylin and eosin staining of rat liver; **B** the ratio of liver/body weight; **C**–**G** The biochemical parameters in CCl_4-induced liver cirrhosis rats, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), glutamyl transferase (GGT) and total bilirubin (TBil). AS1 group (5 mg/kg), AS2 group (10 mg/kg) and AS3 group (15 mg/kg) (*$p < 0.05$, vs control; $^{\#}p < 0.05$ vs CCl_4)

$p < 0.05$). Serum levels of ALT and AST, as markers of severe liver injury, were significantly greater in the model group than the control group (Fig. 1C–D). Likewise, serum levels of glutamyl transpeptidase and total bilirubin were significantly higher in the CCl_4-treated group as compared to the control group (Fig. 1E–G). Notably, the hematoxylin and eosin staining results revealed hepatocyte degeneration in the model group (Fig. 1A). The data indicated that the liver injury model was successfully established. Afterward, changes to the above parameters were detected to assess the protective role of AS against liver injury. As shown in Fig. 1, AS treatment resulted in decreases in the measured parameters as compared to model group. When the concentration of AS was 15 mg/kg body weight, an obvious improvement in liver injury was observed compared to the concentration at 5 mg/kg body weight, which showed the best therapeutic effect. Hence, this concentration of AS was used in subsequent experiments. As shown in Fig. 2, AS efficiently attenuated CCl_4-induced pathological lesions. Collectively, these results demonstrated the protective role of AS against CCl_4-induced liver injury.

AS ameliorated CCl_4-induced LF in vivo experiment

Abnormal change in serum levels of collagen and α-SMA reflects the extent of CCl_4-induced LF. Sirius red staining was used to evaluate collagen fibers in the liver tissue sections. As shown in Fig. 2A–B, the intensity of staining of collagen fibers in the model group was threefold greater than that of the control group. However, treatment with AS markedly reduced collagen accumulation in the liver by 50% as compared with the model group. Moreover, immunohistochemical analysis revealed that treatment with AS reduced the contents of Col-1 and α-SMA (Fig. 2C–D). Finally, Western blot analysis was performed to assess the expression levels of Col-1 and α-SMA. As shown in Fig. 2E–G, AS suppressed the overexpression of Col-1 and α-SMA in the model group. Overall, these results demonstrated that AS ameliorated CCl-4-induced LF.

AS modulated CCl_4-induced LF via the Notch signaling pathway in vivo experiment

The Notch signaling pathway is thought to be a primary regulator of liver development. Western blot analysis was performed to determine whether AS activates the Notch signaling pathway. The results demonstrated that Notch-1 expression was extremely increased in the model group as compared to control group (0.90 ± 0.08 vs 2.12 ± 0.23, respectively, $p = 0.017$), but significantly decreased by AS administration (1.34 ± 0.16). Similarly, the expression levels of Jagged-1 and DLL-4 were increased in the model group, while decreased in the AS-treated group as compared to the model group (Fig. 3). These data demonstrate that AS might participate in the regulation of CCl_4-induced LF via the Notch signaling pathway.

AS inhibited TGF-β-mediated activation of LX2 cells in vitro experiment

As mentioned above, AS has the ability to strongly ameliorate CCl_4-induced LF. As reported in previous studies, overexpression of TGF-β leads to tissue fibrosis, which. involves activation of HSCs. First, the effects of AS on the viability of TGF-β-stimulated LX2 cells were determined using the CCK8 assay. As shown in Fig. 4A, the viability of LX2 cells after AS exposure was reduced by nearly 30% as compared with the TGF-β group, demonstrating that AS greatly inhibited TGF-β-mediated proliferation of LX2 cells. Next, the effects of AS on the migration and invasion capabilities of LX2 cells were evaluated using the wound healing and transwell assays, respectively. The results of the wound healing assay showed that the wound closure rate was greater in the TGF-β group than the control group, but was significantly lower in the AS-treated and siRNA-Jagged-1 groups (Fig. 4B). As compared to the TGF-β group, the invasion capability of the LX2 cells was decreased in the AS-treated and siRNA-Jagged-1 groups (Fig. 4C). Notably, AS combined with siRNA-Jagged-1 had a synergistic suppressive effect on the invasion capability of LX2 cells stimulated with TGF-β.

Activated LX2 cells regulate the synthesis and deposition of collagen in the liver. Therefore, the contents of collagen and α-SMA were detected to assess the effect of AS on the activation of LX2 cells after exposure to TGF-β. Consistent with the results of previous studies, TGF-β significantly increased the expression levels of Col-1 and α-SMA (Fig. 5). In contrast with the TGF-β group, the expression levels of α-SMA and Col-1 were decreased after AS treatment, suggesting that AS blocked TGF-β-mediated activation of LX2 cells. Transfection of LX2 cells with siRNA-Jagged-1 lowered the expression levels of Col-1 and α-SMA. Taken together, these results confirmed that AS and siRNA-Jagged-1 each lowered expression of α-SMA and Col-1. However, further studies are needed to determine whether the Notch signaling pathway is involved the mechanism underlying the effects of AS in LX2 cells.

AS inhibited TGF-β-mediated activation via Notch signaling molecules in vitro experiment

Western blot analysis was conducted to investigate the effect of AS on TGF-β-mediated activation of Notch signaling molecules. As shown in Fig. 6, the expression levels of Notch-1 and Jagged-1 were significantly higher in the TGF-β group than the control group, suggesting TGF-β-mediated

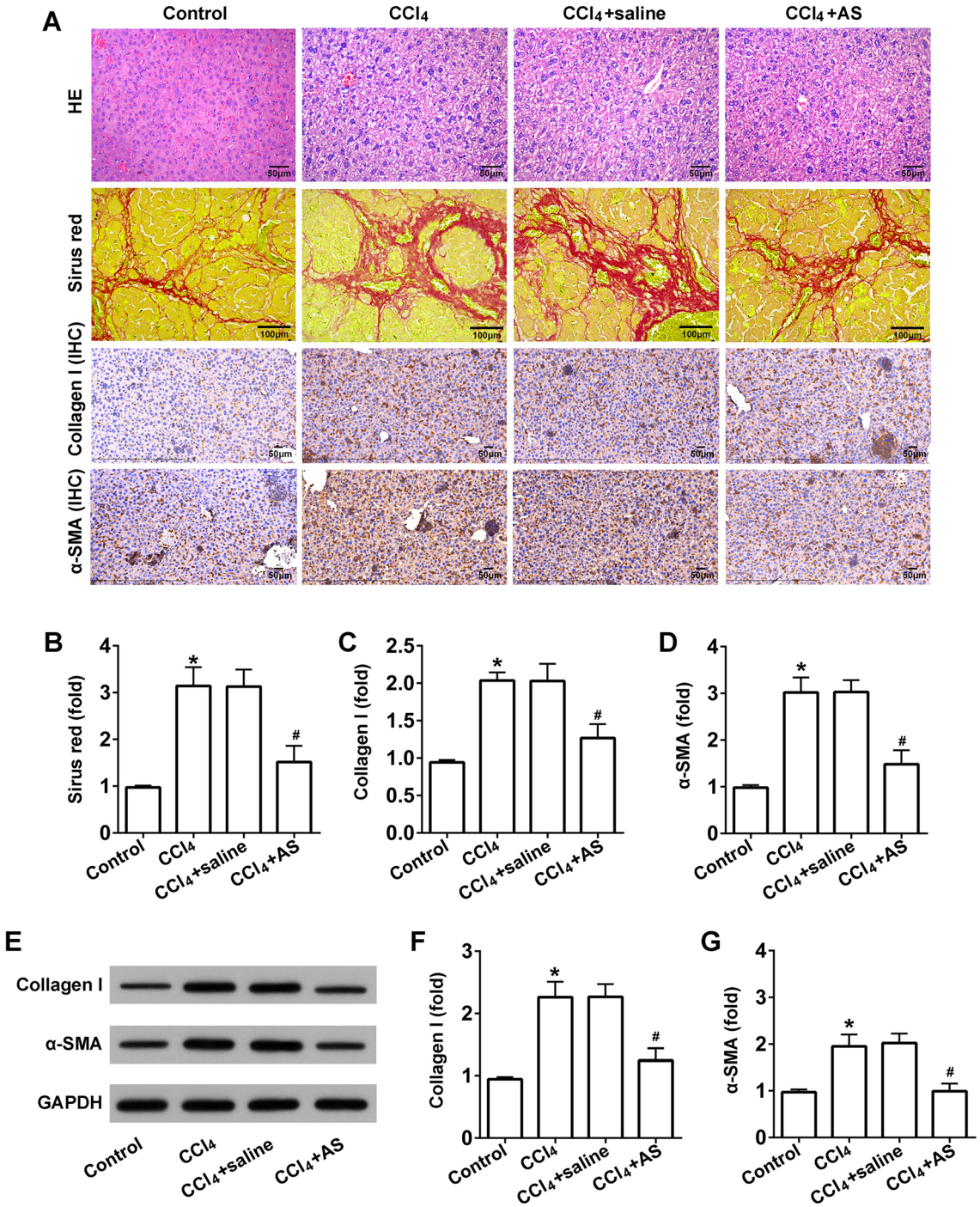

Fig. 2 Effect of asiaticoside on pathological changes and collagen deposition in liver tissue of CCl_4-induced liver cirrhosis rats. **A–D** Hematoxylin and eosin, sirus red staining of rat liver and the expression of collagen I (Col I) and α-smooth muscle actin (α-SMA) in rat liver by immunohistochemical staining; **E–G** the expression of Col I and α-SMA by Western blot (AS: 10 mg/kg; $^{*}p < 0.05$, vs control; $^{\#}p < 0.05$ vs CCl_4)

activation of LX2 cell occurred via modulation of the Notch signaling pathway. Next, changes to Notch-1 and Jagged-1 in response to AS treatment were investigated. The results showed that AS significantly decreased the expression levels of Notch-1 and Jagged-1. There was no obvious difference in the inhibition effect for Notch signaling pathway in TGFβ1 + AS and TGFβ1 + si-Jagged-1 group. These results suggested that AS inhibited TGF-β-mediated activation

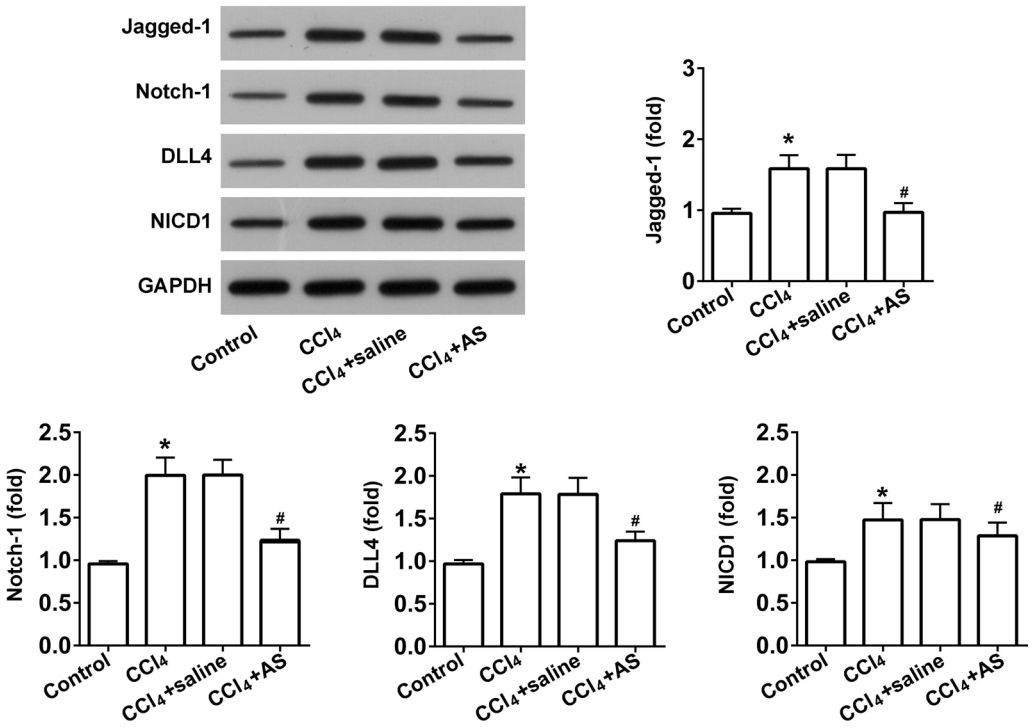

Fig. 3 Effect of asiaticoside on Jagged-1/Notch-1 pathway in liver tissue of CCL_4-induced liver cirrhosis rats (AS: 10 mg/kg; $^{*}p<0.05$, vs control; $^{\#}p<0.05$ vs CCL_4)

of LX2 cells via downregulation of the Notch signaling pathway.

Discussion

In the present study, AS alleviated CCl_4-induced LF in rats and played a role in inhibition of TGF-β-mediated activation of LX2 cells. The staining results showed that CCl_4 induced severe LF, as evidenced by the abundance of bridging fibrosis and extensive collagen deposition. Meanwhile, the levels of ALT, AST, and HYP, as markers of liver injury, were significantly elevated in CCl_4-treated rats. These results suggest that a rat model of LF was successful established.

As is extracted from the Apiaceae plant Centella asiatica and has antioxidative, antifibrotic, and antineoplastic actions [21], against inflammation [22], AS belongs to a triterpenoid saponin that plays a protective role in osteoporosis, Parkinson's disease, and various cancers [23–26]. Luo et al. found that AS plays a role in the BLM-induced pulmonary fibrosis [27]. In addition, the results of the present study showed that AS protected against LF. AS administration reduced CCl_4-induced LF, as demonstrated by the decreases in collagen deposition, α-SMA content, and the expression levels of several markers of liver injury (i.e., ALT, AST, and HYP), suggesting that AS might slow the progression of LF or recover liver structure in vivo. Zhang et al. showed that AS decreased the expression levels of inflammatory cytokines and ALT to protect against liver injury [28]. Zhang et al. reported that AS inhibited miR-142-5p expression and enhanced ACTN4 expression to alleviate fibrosis progression [29]. Zhao et al. demonstrated that AS decreased TGF-β-mediated mesothelial–mesenchymal transition and the generation of reactive oxygen species via regulation of Nrf signaling in the progression of fibrosis [30]. Tang et al. reported that AS suppressed the level of collagen stimulated by TGF-β [27].

As a major producer of extracellular matrix responsible for LF, activated HSCs are considered a valuable candidate for antifibrotic therapy [31–33]. Activated HSCs stimulated by TGF-β differentiate into myofibroblast-like cells, which are characterized by the accumulation of α-SMA and Col-1, and simultaneously secrete increased amounts of TGF-β, which acts as a positive feedback loop for the regulation of LF [34]. Yang et al. demonstrated that TGF-β-mediated LF involves activation of HSCs and overproduction of reactive oxygen species [35]. Inhibition of HSC activation was observed in dimethyl α-ketoglutarate- and CCl_4-induced LF [36]. In the present study, the immunohistochemical assay results clearly showed that AS reduced the number of hepatic myofibroblasts, as determined by decreased α-SMA levels in liver sections. Similarly, AS downregulated the protein expression levels of α-SMA and Col-1, as determined by Western blot analysis.

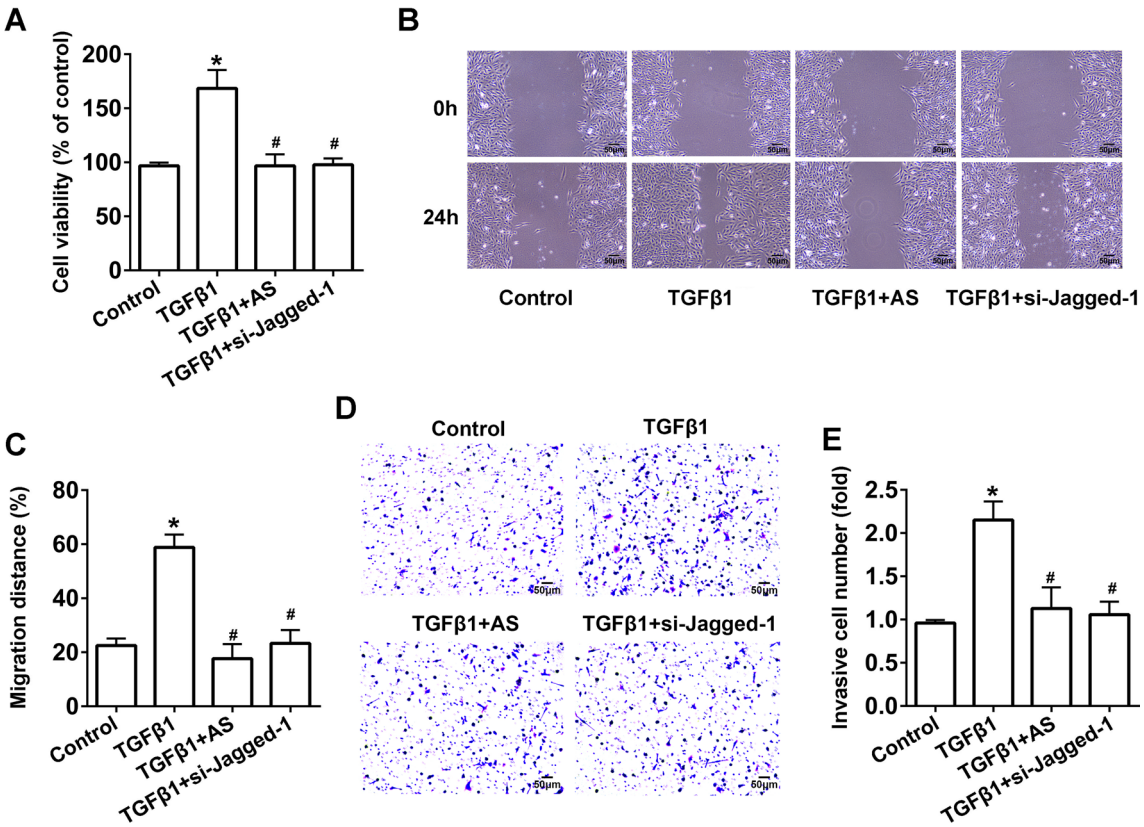

Fig. 4 Asiaticoside inhibits TGFβ1-induced hepatic stellate cell activation in LX-2 cells. **A** The viability of TGFβ1-induced LX-2 cells via CCK8 assay; **B** and **C** Migration ability experiment of TGFβ1-induced LX-2 cell; **D** and **E** invasion ability experiment of TGFβ1-induced LX-2 cell (AS: 10 mg/kg; $^{*}p<0.05$, vs control; $^{\#}p<0.05$ vs TGFβ1)

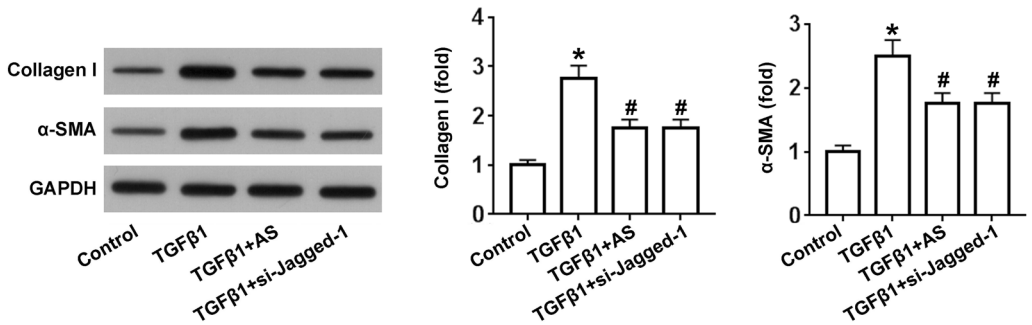

Fig. 5 Asiaticoside inhibits TGFβ1-induced collagen synthesis in LX-2 cells (AS: 10 mg/kg; $^{*}p<0.05$, vs control; $^{\#}p<0.05$ vs CCl_4)

Moreover, TGF-β was identified as a profibrogenic molecule involved in the development of LF. AS was found to inhibit the TGF-β-enhanced proliferation, migration, and invasion capabilities of LX2 cells. Hence, increased protein expression of α-SMA and Col-1 through TGF-β reflects activation of HSCs. However, these phenomena were reversed by AS treatment. The in vivo and in vitro results showed that AS protects against CCl_4-induced LF by inhibiting the activation of HSCs.

The Notch signaling pathway serves as an intercellular communication mediator that modulates multiple biological functions. A large body of evidence demonstrated that the Notch signaling pathway plays an active role in LF via regulation of myofibroblast differentiation. Mu et al. reported that inhibition of the Notch signaling activation prevented LF by inducing ligation of the common bile duct [37]. Gao et al. demonstrated that knockdown of Notch-1 reinforced the suppressive effect of emodin on epithelial–mesenchymal

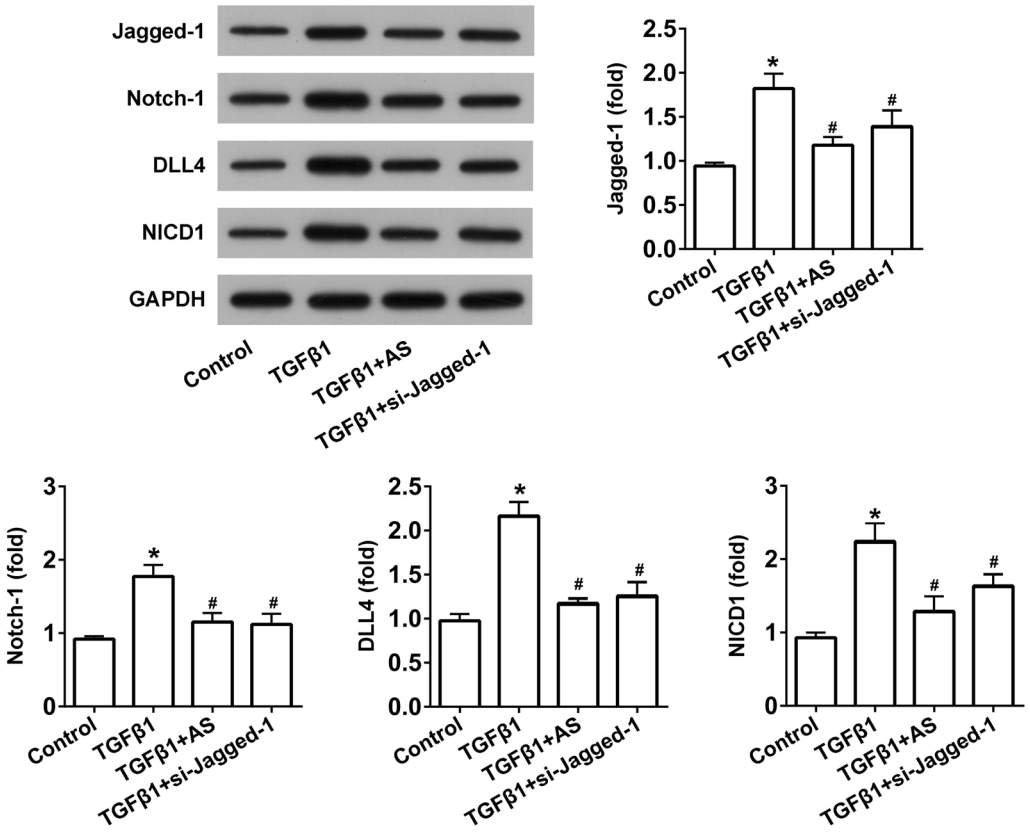

Fig. 6 Asiaticoside inhibits Jagged-1/Notch-1 pathway in TGFβ1-treated LX-2 cells. (AS: 10 mg/kg; $^{*}p < 0.05$, vs control; $^{\#}p < 0.05$ vs CCl_4)

transition, which is a key event in the onset of LF [38]. The results of the present study showed that the protein levels of Notch-1 and Jagged-1 were upregulated in the rat model of CCl_4-induced LF. In vitro, the protein expression levels of Notch-1 and Jagged-1 were significantly upregulated during TGF-β-mediated activation of HSCs. Here, Western blot analysis of proteins associated with the Notch signaling pathway (i.e., Notch-1 and Jagged-1) was performed to investigate the anti-fibrosis mechanism of AS. The results showed that AS inhibited Notch-1 and Jagged-1 expression both in vivo and in vitro. These data suggest that the anti-fibrotic properties of AS are closely related to inhibition of the Notch signaling pathway. The results of this study demonstrated that AS decreased collagen deposition, inhibited Col-1 and α-SMA expression, and ameliorated activation of HSCs via regulation of the Notch signaling pathway. These findings provide a new perspective that AS modulates the Notch signaling pathway to achieve a protective effect against LF as a possible clinical application in the future.

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Availability of data and material The data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Conflict of interest The authors have no conflict of interest to report.

Ethical approval and consent to participate All animal procedures were approved by The Animal Experimental Ethics committee of The People's Hospital of Yuhuan and conducted in accordance with the "Guide for the Care and Use of Laboratory Animals."

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