Compound *Astragalus* and *Salvia miltiorrhiza* extract inhibits hepatocarcinogenesis via modulating TGF-β/TβR and Imp7/8

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Abstract. Compound *Astragalus* and *Salvia miltiorrhiza* extract (CASE) is a Chinese herbal formula consisting of astragalosides, astragalus polysaccharide and salvianolic acids extracted from *Astragalus membranaceus* and *Salvia miltiorhiza*. Previous studies by our group have demonstrated that CASE effectively suppresses diethylinitrosamine (DEN)-induced hepatocellular carcinoma (HCC) in rats via modulating transforming growth factor β/Mothers against decapentaplegic (TGFβ/Smad) signaling. To further elucidate the mechanism of CASE, the effects of CASE on TGF-β_1, the serine/threonine kinase receptors of TGF-β [TGF-β receptor type-I (TβRI) and TβRII] and karyopherins [Importin 7 (Imp7) and Imp8], which are crucial for TGF-β/Smad signaling in fibro-hepatocarcinogenesis, were assessed in the present study using *in vivo* (DEN-induced HCC in rats) and *in vitro* [TGF-β_1-stimulated rat myofibroblasts (MFBs) and HepG2 cells] models of fibro-hepatocarcinogenesis. Hematoxylin and eosin staining revealed that CASE may suppress inflammatory reactions and fibrosis in HCC as well as increasing the differentiation of HCC cells. Positive TGF-β_1 staining was increased in HCC nodule areas and in adjacent normal liver tissues in DEN-treated rats, while TβRI staining was increased only in normal adjacent liver tissues. The elevated expression of TGF-β_1, TβRI and TβRII was suppressed by CASE. CASE treatment also reduced glutathione S-transferase P 1 and Imp7/8 protein expression in fibro-hepatocarcinogenesis. *In vitro* experiments confirmed that CASE was able to decrease the expression of TβRI and TβRII in TGF-β_1-stimulated MFBs and HepG2 cells. These results indicate that the anti-HCC effect of CASE may be achieved by mediating TGF-β/TβR and Imp7/8 protein expression, suggesting that CASE has multiple targets in HCC treatment.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most threatening types of cancer, with high malignancy, poor prognosis and high morbidity (1). It is ranked as the fifth most common type of cancer and the third cause of cancer-associated mortality worldwide (1). HCC is derived from chronic liver injury with persistent inflammation leading to a progressive disease in which the liver undergoes pathological changes, spanning hepatitis, hepatic fibrosis, cirrhosis and finally HCC (2). However, at present there is no effective treatment for HCC, as current treatment regimes are accompanied by high recurrence rates and serious adverse reactions (3). It would therefore be beneficial to identify an effective, safe herbal medicine, whose mechanism of action is well-characterized, to be used as an adjunct therapy for HCC.

Pre-clinical and clinical studies have reported that treatment with *Astragalus membranaceus* or *Salvia miltiorhiza* effectively improves liver function and suppresses hepatic fibrosis and cirrhosis (4-6). Based on these findings and traditional Chinese medical theory, a formula termed Compound *Astragalus* and *Salvia miltiorrhiza* extract (CASE) was developed, comprising astragalosides, astragalus polysaccharide and salvianolic acids extracted from *Astragalus membranaceus* and *Salvia miltiorhiza* (7). Previous studies have revealed that CASE has an anti-fibrotic effect in rats with carbon tetrachloride-induced fibrosis and that the underlying mechanisms are associated with modulation of the transforming growth factor-β (TGF-β)/Smad signaling pathway (7,8). CASE inhibits HepG2 cell proliferation and invasion by regulating the TGF-β/Smad/plasminogen activator inhibitor 1 (PAI-1) signaling pathway (9). Furthermore, CASE has been demonstrated to have anti-cancer effects in rats with HCC induced by diethylinitrosamine (DEN), which are achieved by

inhibiting fibrosis as well as modulating Smad protein expression and PAI-1 transcription (6,10). However, it remains to be elucidated how CASE modulates the expression of TGF-β_1, specific membrane receptors [TGF-β receptor type-I (TβRI) and TβRII] and karyopherins [Importin (Imp)7 and Imp8] in the TGF-β/Smad signaling pathway. The aim of the present study was to investigate the effects of CASE on the expression of TGF-β_1, TβRI, TβRII and Imp7/8 during the development of HCC using DEN-induced hepatocarcinogenesis in rats, rat myofibroblasts (MFBs, key fibrogenic cells implicated in liver fibrosis) and the human hepatoblastoma cell line HepG2.

Materials and methods

Preparation of CASE. The herbs of *Astragalus membranaceus* Bunge (Leguminosae) and *Salvia miltiorhiza* Bunge (Lamiaceae) were purchased from Bozhou Huqiao Pharmaceutical Co., Ltd. (Bozhou, China) and authenticated by Professor Xiaoxiang Zhang (Department of Pharmaceutical Engineering, Hefei University of Technology, Hefei, China), a specialist in traditional Chinese herbal medicine. Voucher specimens were deposited in the specimen room of traditional Chinese medicine (Anhui University of Chinese Traditional Medicine, Hefei, China). The processes of extracting and preparing the three CASE components were performed as previously described (7). Briefly, astragalosides, astragalus polysaccharide and salvianolic acids were made into powders, weighed and dissolved in 0.5% sodium carboxymethylcellulose (CMC-Na) with distilled water according to a standard ratio of 70:1:1.85.

DEN-induced hepatocarcinogenesis in rats. A total of 150 male Sprague-Dawley rats (age, 6-7 weeks) weighing 180-200 g were purchased from Shanghai Xipuer-Bikai Laboratory Animal Ltd., Co. (Shanghai, China) and housed in conventional cages at 20-22˚C with a 12-h light-dark cycle and a 40-70% relative humidity. Rats were supplied with laboratory chow and water *ad libitum*. The rats were kept under these conditions for ≥1 week prior to the experiment. The current study was performed in accordance with the guidelines for the humane treatment of animals set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University based on Health Guide for the Care and Use of Laboratory Animals from National and International Institutions (11) and approved by the Experimental Animal Ethics Committee of Anhui Medical University (Hefei, China).

Rats were randomly divided into five groups (each, n=30): The control group, the DEN group and three CASE treatment groups. In the morning, rats in the DEN group and three CASE treatment groups received 0.2% DEN dissolved in 0.5% CMC-Na and distilled water at a dose of 10 mg/kg by gavage 5 times per week for 14 weeks to induce hepatocarcinogenesis, synchronously, the rats in control group administrated with equivalent 0.5% CMC-Na as control. In the afternoon, rats in the three CASE groups were concomitantly administered CASE at the doses of 60, 120 or 240 mg/kg/day by gavage for 16 weeks. Rats in the other two groups were treated with equivalent 0.5% CMC-Na as control. Rats were sacrificed 12 or 16 weeks following the start of DEN administration. One lobe from each rat liver was harvested and fixed in 10% formalin at room temperature for 3 days, dehydrated in a graded series of alcohol, embedded in paraffin and cut into 4-μm-thick sections for further histological analysis. Other tissue sections were preserved in liquid nitrogen for protein detection.

Cell models of liver fibrosis and liver cancer. Hepatic stellate cells (HSCs) were isolated from the normal rat liver using collagenase IV and pronase-E (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) digestion according to a method previously described (12). HSCs were cultured on plastic dishes and activated to give MFBs as previously described (13). The human hepatoblastoma HepG2 cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). MFBs and HepG2 cells were seeded at a density of $1x10^6$ cells in 25 cm^2 culture flasks and grown as sub-confluent monolayer cultures in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Zhejiang Tianhang Biological Technology Co., Ltd., Huzhou, Zhejiang, China) in a humidified 5% CO_2 incubator at 37˚C. Experiments were performed with cells in the log growth phase. Cells in the experimental groups were incubated in serum-free medium and CASE (20, 40 or 80 μg/ml) for 24 h, and subsequently treated with TGF-β_1 (40 pmol/l; R&D Systems, Inc., Minneapolis, MN, USA) for 1 h. Cells in the control group were incubated in serum-free medium. Total protein was extracted using Cell lysis buffer for Western and IP (cat. no. P0013; Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol. Each experiment was repeated three times.

Histopathological investigation. Pathological changes in the liver were assessed in each group using hematoxylin and eosin (H&E) staining, as previously described (14). In brief, paraffin sections were deparaffinized in xylene, rehydrated in a graded series of alcohol, and stained with hematoxylin for 8 min at room temperature. Sections were then washed in running water until a blue color was observed, following which they were stained using eosin for 30 sec at room temperature and mounted under cover slips. The pathological features were observed using a light microscope (Nikon 80i; Nikon Corporation, Tokyo, Japan; magnification, x100).

Immunohistochemical examination. Paraffin sections were deparaffinized in xylene and rehydrated in a decreasing graded alcohol series and distilled water. Non-enzymatic antigen retrieval was performed by heating the sections to 121˚C in 0.01 M sodium citrate buffer (pH 6.0) for 10 min. Sections were cooled, rinsed in Tris-buffered saline containing 0.1% Tween-20 (TBST) and incubated in methanol with 3% H_2O_2 for 30 min at 37˚C to quench endogenous peroxidase activity. After rinsing with TBST 3 times, sections were incubated with 5% bovine serum albumin (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) dissolved in TBST for 30 min at 37˚C to block non-specific antibody binding. The sections were then rinsed with TBST and incubated with primary antibodies (Abs) for 1 h at room

temperature in a humid chamber. Primary Abs used in the present study included rabbit anti-TGF-β_1 Ab (cat. no. sc-146; 1:100) and rabbit anti-TβRI Ab (cat. no. sc-398; 1:100; each Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Sections were rinsed in TBST again and incubated with peroxidase-labeled polymers conjugated to goat anti-rabbit immunoglobulin antibodies (cat. no. E0432; 1:2,500; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 1 h at room temperature. Finally, sections were developed with 3,3'-diaminobenzidine, counterstained with hematoxylin for 3 min at room temperature and mounted under cover slips. Images from each section were randomly collected using a light microscope (Nikon 80i; Nikon; magnification, x200). Results were evaluated using a semi-quantitative technique, assigning a score of 0-1 based on the percentage of positively stained cells (0, 0% stained cells; 0.1, <10% stained cells; 0.2, 10-20% stained cells; etc. and 1, >80% stained cells).

Western blot analysis. Frozen liver tissue specimens were homogenized in cell lysis buffer (cat. no. P0013; Beyotime Institute of Biotechnology) at 4˚C for the extraction of whole protein. Total protein was also extracted from MFBs and HepG2 cells. The protein concentration of samples were measured using a BCA Protein Assay kit (cat. no. P0012S; Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Proteins from each sample (50 μg/lane) were loaded on 10% polyacrylamide gels, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) by the wet transfer method. Non-specific antibody binding was blocked with 5% skim milk powder dissolved in TBST for 2 h at room temperature. The membranes were then incubated with the primary antibodies overnight at 4˚C, washed 3 times with TBST for 10 min each time and incubated with corresponding secondary antibodies for 2 h at room temperature. The membranes were washed 3 times with TBST for 10 min each time and developed using an ECL chemiluminescence system (GE Healthcare Life Sciences, Little Chalfont, UK). The primary antibodies utilized were as follows: Rabbit anti-TGF-β_1 Ab (cat. no. sc-146; 1:800; Santa Cruz Biotechnology, Inc.), rabbit anti-TβRI Ab (cat. no. sc-398; 1:800; Santa Cruz Biotechnology, Inc.) and goat anti-TβRII Ab (cat. no. sc-33929; 1:800; Santa Cruz Biotechnology, Inc.), mouse anti-GAPDH Ab (cat. no. TA-08; dilution, 1:5,000; Origene Technologies, Inc., Rockville, MD, USA), rabbit anti-GST-P1 Ab (cat. no. SAB3500265; 1:1,000; Sigma-Aldrich, Merck KGaA), rabbit anti-Imp7 Ab (cat. no. ab99273; 1:5,000; Abcam, Cambridge, UK) and rabbit anti-Imp8 Ab (cat. no. ab72109; 1:5,000; Abcam). Peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; H+L) (cat. no. ZB-2305; dilution, 1:10,000; Origene Technologies, Inc.), peroxidase-conjugated goat anti-rabbit lgG (H+L; cat. no. ZB-2301; 1:10,000; Origene Technologies, Inc.) and peroxidase-conjugated rabbit anti-goat IgG (H+L; cat. no. ZB-2306; 1:10,000; Origene Technologies, Inc.) were used as second Abs. GAPDH was used as the internal control. Results were densitometrically analyzed by ImageJ version 2 software (National Institutes of Health, Bethesda, MD, USA). These experiments were repeated three times.

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical analyses were performed using SPSS 11.0 (SPSS, Inc., Chicago, IL, USA). Experimental and control groups were compared using one-way analysis of variance followed by a post-hoc Least-Significant-Difference test). $P<0.05$ was considered to indicate a statistically significant difference.

Results

CASE decreases the degree of histological changes in HCC. Representative hematoxylin and eosin-stained liver sections are presented in Fig. 1. In the DEN group, hepatic lobules were separated and/or encysted by collagen bundles. Inflammatory cell infiltration and typical pseudo lobule structures were observed in liver sections at week 12 (Fig. 1A). These pathological changes were improved in the CASE treatment groups compared with the DEN group. At week 16, HCC cells in the DEN group were poorly differentiated with marked atypia and arranged in cord- or crumby-like structures (Fig. 1B). However, the degree of differentiation in the CASE treatment groups increased in a dose-dependent manner, while the degree of HCC malignancy decreased compared with the DEN group.

CASE downregulates GST-P1 protein expression. DEN treatment significantly increased the expression of GST-P1 protein in HCC tissues compared with the control groups after week 12 (Fig. 2). CASE treatment ameliorated DEN-induced GST-P1 upregulation in a dose-dependent manner, especially CASE at the dose of 240 mg/kg, which markedly decreased the level of GST-P1.

CASE decreases the protein expression of TGF-β_1, TβRI and TβRII. The number of TGF-β_1- and TβRI-positive immunoreactive cells were markedly increased in the DEN group compared with the control group, but were decreased following CASE treatment in a dose-dependent manner when compared with the DEN group at week 12 (Fig. 3A). Positive TGF-β_1 staining was demonstrated in adjacent normal liver tissues and hepatoma nodule areas, whilst positive TβRI staining only occurred in adjacent normal liver tissues and was markedly higher in DEN-treated rats compared with the control group. Furthermore, the DEN-induced increase of TGF-β_1- and TβRI-positive cells was ameliorated by CASE treatment in a dose-dependent manner when compared with the DEN group at week 16 (Fig. 3B). Additionally, The number of TβRI-positive cells in the DEN group at week 16 was lower compared with those at week 12. Semi-quantitative integral optical density analysis revealed that there was significantly more TGF-β_1- and TβRI-positive staining in the DEN group compared with the control group and the elevated number of TGF-β_1- and TβRI-positive cells was significantly decreased following CASE treatment at week 12 (Fig. 3C) and week 16 (Fig. 3D).

The expression levels of TGF-β_1, TβRI and TβRII protein extracted from the liver tissues of rats at week 12 and 16 were measured. Compared with the control group, the expression of TGF-β_1 (Fig. 4A), TβRI (Fig. 4B) and

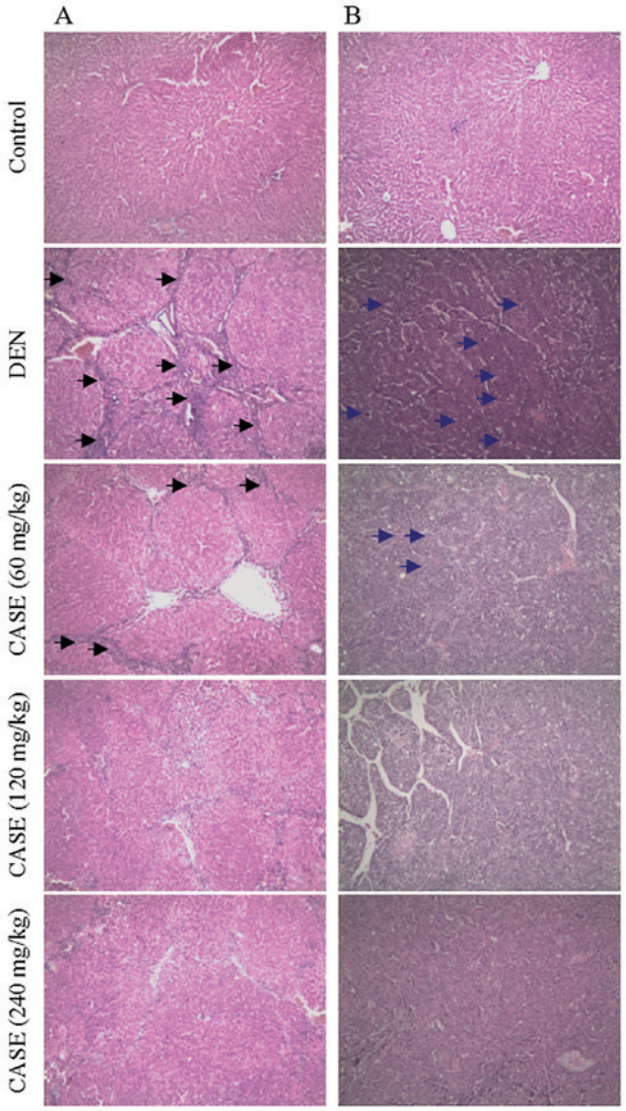

Figure 1. CASE decreases the degree of histological changes in HCC. Hepatic histopathology at (A) 12 and (B) 16 weeks after the induction of hepatocellular carcinoma by DEN. Liver sections from control rats, rats treated with DEN, and rats concomitantly treated with DEN and CASE (60, 120 or 240 mg/kg) were stained with hematoxylin and eosin. The typical collagen fibers in liver sections from week 12 are indicated with black arrows. Poorly differentiated tumor cells in liver sections from week 16 are indicated with blue arrows. Magnification, x100. DEN, diethylinitrosamine; CASE, compound *Astragalus* and *Salvia miltiorrhiza* extract.

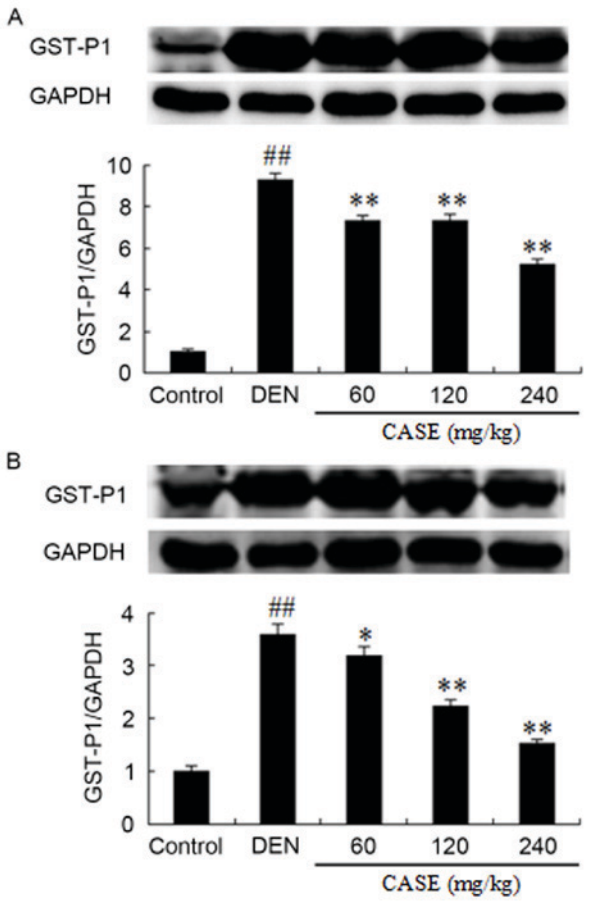

Figure 2. CASE decreases the protein expression of GST-P1. The proteins were extracted from frozen liver tissues (A) 12 and (B) 16 weeks after the induction of hepatocellular carcinoma by DEN. GST-P1 was analyzed by western blotting using anti-GST-P1 and -GAPDH antibodies. Intensities of GST-P1 bands were normalized to those of GAPDH in the corresponding treatment groups. The ratio of the GST-P1 protein to GAPDH in the normal group was assigned a value of 1. Data are expressed as mean ± standard deviation (n=3). $^{\#\#}$P<0.01 vs. the control group. *P<0.05 and **P<0.01 vs. the DEN group. DEN, diethylinitrosamine; CASE, Compound *Astragalus* and *Salvia miltiorrhiza* extract; GST-P1, glutathione S-transferase P 1.

TβRII (Fig. 4C) increased significantly in the DEN group, while these levels were downregulated following CASE treatment in a dose-dependent manner when compared with the DEN group at week 12. Additionally, CASE treatment significantly decreased the DEN-induced increase of TGF-β_1 (Fig. 4D), TβRI (Fig. 4E) and TβRII (Fig. 4F) expression compared with the DEN group at week 16.

CASE decreases the expression of Imp7/8. At week 12, DEN treatment induced a significant increase in Imp7/8 protein expression compared with the control group, while CASE treatment inhibited the DEN-induced overexpression of Imp7/8 proteins during hepatocarcinogenesis compared with the DEN group (Fig. 5A and B). At week 16, DEN treatment significantly increased Imp7/8 protein expression compared with the control group. Simultaneously, Imp7/8 expression was inhibited by CASE in the high dose (240 mg/kg) group, but not the low and middle dose (60 and 120 mg/kg, respectively) groups compared with the DEN group (Fig. 5C and D).

High and medium-dose CASE downregulates TβRI and TβRII expression in MFBs and HepG2 cells. The expression of TβRI and TβRII in TGF-β_1-stimulated MFBs was assessed using western blot analysis (Fig. 6A). Compared with the control group, TβRI and TβRII expression was significantly increased by TGF-β_1-stimulation. CASE treatment significantly decreased elevated TβRI protein levels at the highest dose (80 μg/ml) and elevated TβRII protein levels at the medium and high doses (40 and 80 μg/ml, respectively) compared with the TGF-β_1 group.

The expression of TβRI and TβRII protein in TGF-β_1-stimulated HepG2 cells was assessed using western blot analysis (Fig. 6B). TβRI and TβRII expression was significantly increased by TGF-β_1-stimulation compared with the control group. CASE treatment (40 and 80 μg/ml) significantly

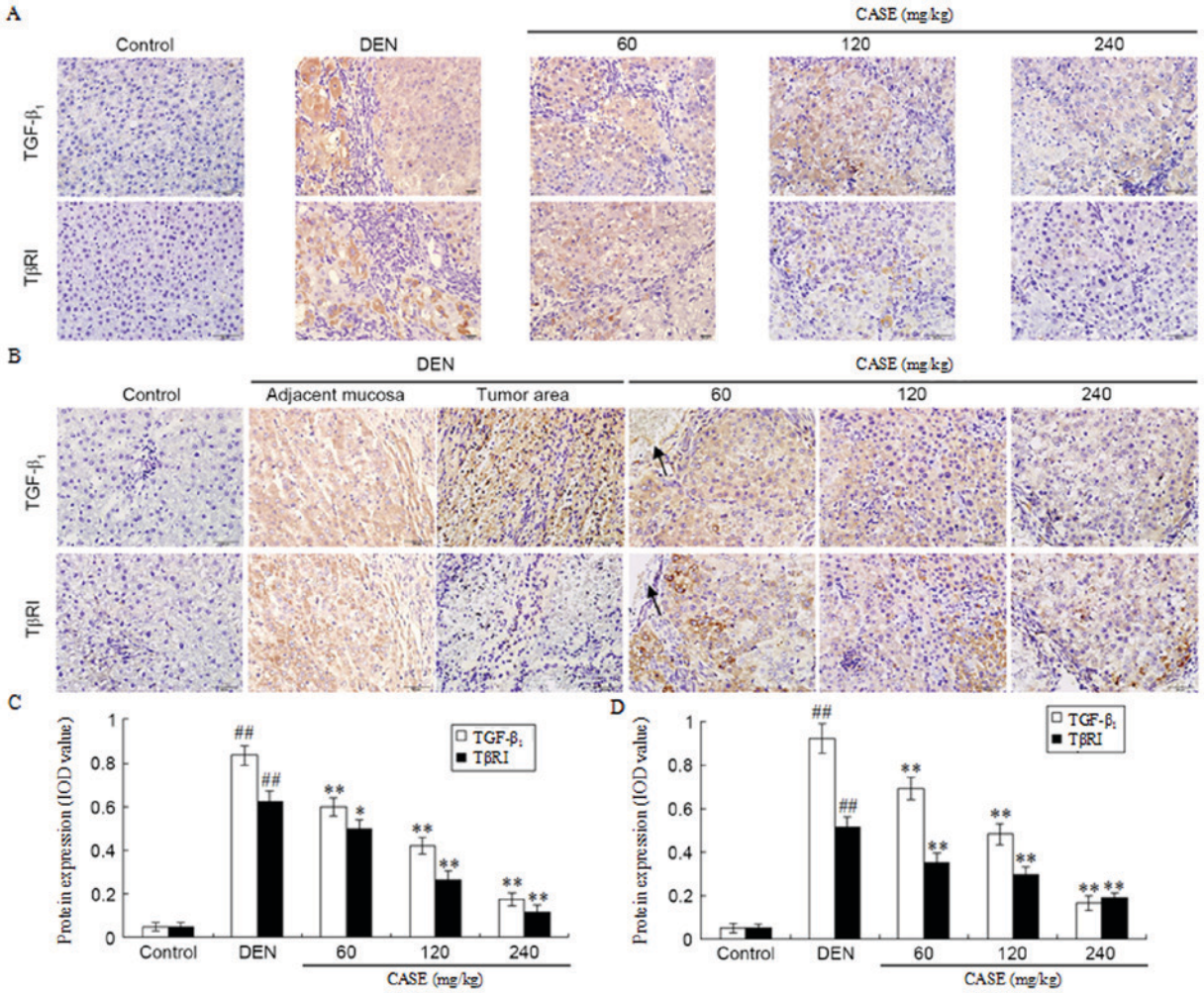

Figure 3. CASE decreases the protein expression of TGF-β_1 and TβRI. Immunohistochemical examination of TGF-β_1 and TβRI expression in the liver sections were examined (A) 12 and (B) 16 weeks after the induction of hepatocellular carcinoma by DEN. Liver sections from control rats, rats treated with DEN, rats concomitantly treated with DEN and CASE (60, 120 or 240 mg/kg) were incubated with anti-TGF-β1 and TβRI antibodies (brown) and counterstained with hematoxylin (blue). (B) Hepatocellular carcinoma nodules are indicated by black arrows. Magnification, x200. TGF-β_1 and TβRI expression level at (C) 12 and (D) 16 were semi-quantitatively analyzed. Data are expressed as mean ± standard deviation (n=8). $^{\#\#}$P<0.01 vs. the control group. *P<0.05 and **P<0.01 vs. the DEN group. DEN, diethylinitrosamine; CASE, Compound *Astragalus* and *Salvia miltiorrhiza* extract; TGF-β_1, transforming growth factor β-1; TβRI, transforming growth factor-β receptor type 1; IOD, integral optical density.

decreased the TGF-β_1-induced elevation in TβRI and TβRII expression in a dose-dependent manner compared with the TGF-β_1 group.

Discussion

The results of the present study suggest that CASE impedes DEN-induced hepatocarcinogenesis in rats via suppressing GST-P1 expression and modulating upstream (TGF-β_1, TβRI and TβRII) and downstream (Imp7 and Imp8) mediators of the TGF-β/Smad signaling pathway. Non-viral and non-alcohol-associated hepatocarcinogenesis, including DEN-induced hepatocarcinogenesis, typically progresses from chronic inflammation through fibrosis and cirrhosis, finally becoming hepatocarcinogenesis (15). A number of studies have acknowledged the link between chronic liver inflammation and hepatocarcinogenesis, particularly HCC (16,17). Similarly, other studies have indicated that the extent of fibrosis and cirrhosis is directly associated with HCC progression (18,19). Chronic inflammation, fibrosis and cirrhosis are therefore risk factors for hepatocarcinogenesis. Pharmacological interventions that are able to effectively disrupt or stop the collaboration between chronic liver inflammation, fibrosis and cirrhosis have become indispensable for reducing the risk factors of hepatocarcinogenesis as well as limiting the progression and severity of liver cancer.

Our research group previously demonstrated that CASE attenuates DEN-induced hepatocarcinogenesis in rats via modulating the TGF-β/Smad signaling pathway (6,10). CASE inhibited the downstream mediators of the TGF-β/Smad signaling pathway (Smad2 at the C-terminal and linker domains, Smad3 primarily at the linker phospho-domain and Smad2/3/4 complex) and a TGF-β target gene (PAI-1), while it also upregulated inhibitory Smad7 (6,10). However, it remained unclear whether CASE could also modulate the upstream mediators of TGF-β/Smad signaling (TGF-β_1, TβRI and TβRII).

In the present study, a DEN-induced hepatocarcinogenesis model was established in rats to imitate the progression

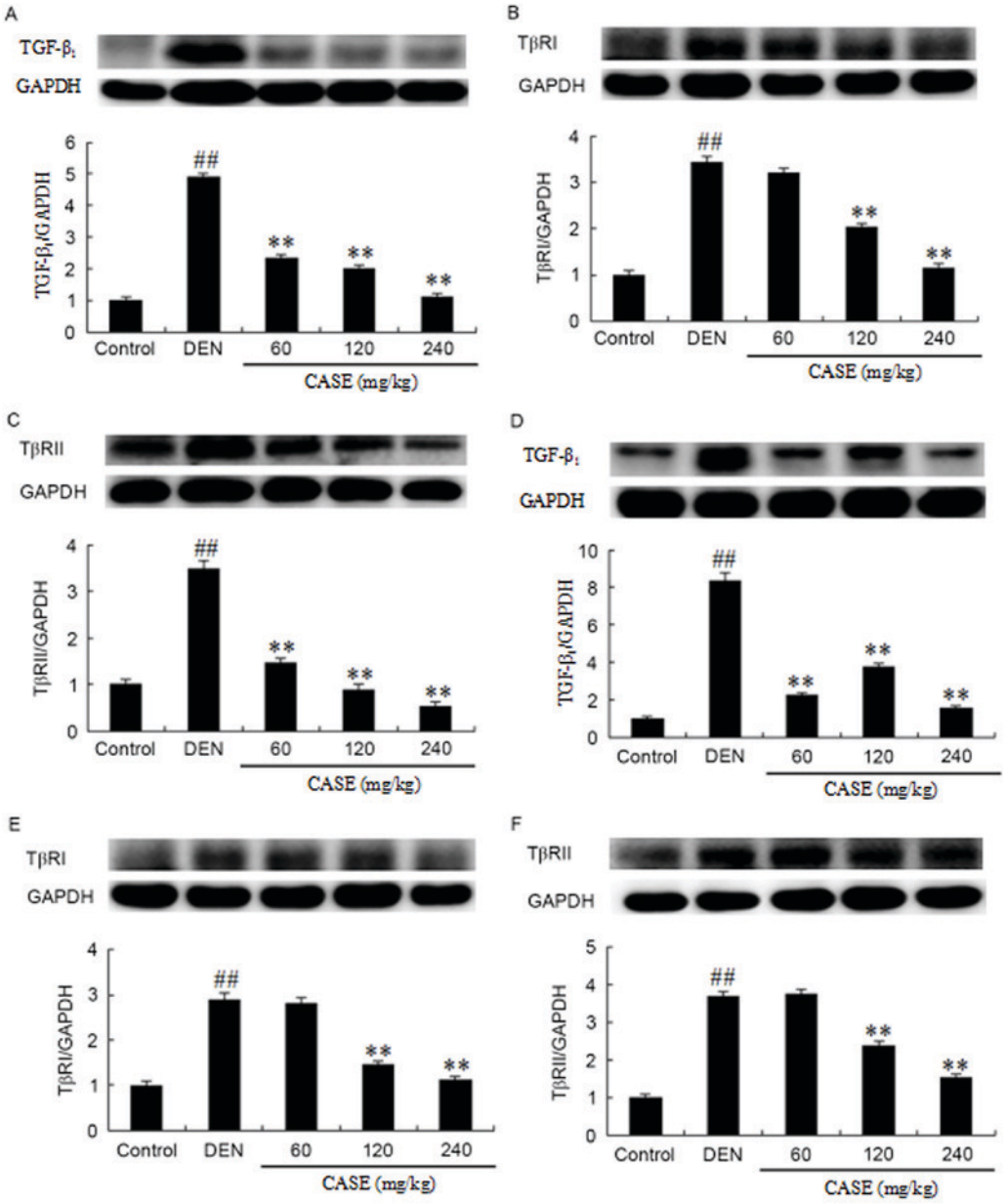

Figure 4. CASE decreases the protein expression of TGF-β_1, TβRI and TβRII. The effects of CASE on TGF-β_1, TβRI and TβRII expression in rats treated with DEN were assessed by western blotting (A-C) 12 and (D-F) 16 weeks after the induction of hepatocellular carcinoma by DEN. The proteins were extracted from frozen liver tissues. TGF-β_1, TβRI and TβRII proteins were analyzed using anti-TGF-β_1, -TβRI, -TβRII and -GAPDH antibodies. Intensities of TGF-β_1, TβRI and TβRII bands were normalized to those of GAPDH in the corresponding treatment groups. The ratios of the TGF-β_1, TβRI or TβRII protein to GAPDH in the normal groups were assigned a value of 1. Data are expressed as mean ± standard deviation (n=3). $^{\#\#}$P<0.01 vs. the control group. **P<0.01 vs. the DEN group. DEN, diethylinitrosamine; CASE, Compound Astragalus and Salvia miltiorrhiza extract; TGF-β_1, transforming growth factor β-1; TβR, transforming growth factor-β receptor.

of liver cancer in humans and further investigate the effects of CASE and its possible mechanisms. The preliminary results revealed that CASE was able to significantly reduce the incidence and multiplicity of HCC as well as levels of serum biochemical indices, including alanine transaminase, aspartate aminotransferase, albumin, alkaline phosphatase, total bilirubin, direct bilirubin and gamma-glutamyltransferase (data not shown), which are predictive of hepatic function. This confirmed the results of an earlier study by our group (6). GST-P1 is a biomarker of neoplastic cells (20) and has been used to provide accurate assessments of HCC risk (21).

H&E staining and immunoblotting were used in the present study to assess the expression of GST-P1. Liver tissues from DEN-treated rats revealed increased GST-P1 protein expression, which was associated with an increase in inflammatory cell infiltration and the degree of fibrosis, as well as poor HCC cell differentiation. However, CASE treatment significantly reversed the effects of DEN treatment. Notably, CASE decreased GST-P1 protein expression and the degree of fibrosis, as well as improving HCC differentiation in a dose- and time-dependent manner. These findings suggest that CASE exhibits a clear protective effect in HCC and the mechanism involves inhibiting the expression of GST-P1 protein, the inflammatory reaction

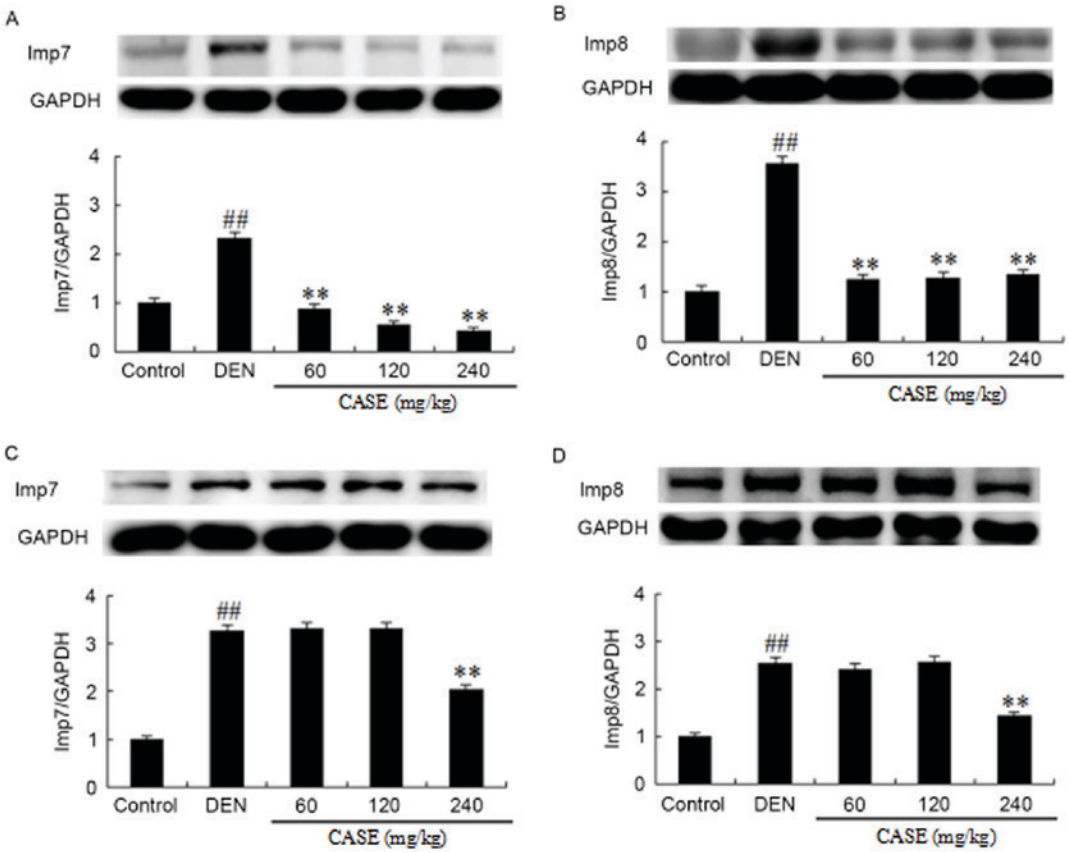

Figure 5. CASE decreases the protein expression levels of Imp7/8. The effects of CASE on Imp7/8 expression in rats treated with DEN were assessed by western blotting (A and B) 12 and (C and D) 16 weeks after the induction of hepatocellular carcinoma by DEN. The proteins were extracted from frozen liver tissues. Imp7 and Imp8 proteins were analyzed by western blotting using anti-Imp7, -Imp8 -GAPDH antibodies. Intensities of Imp7 and Imp8 bands were normalized to those of GAPDH in the corresponding treatment groups. The ratios of the Imp7 or Imp8 protein to GAPDH in the normal groups were assigned a value of 1. Data are expressed as mean ± standard deviation (n=3). $^{\#\#}$P<0.01 vs. the control group. **P<0.01 vs. the DEN group. DEN, diethylinitrosamine; CASE, Compound *Astragalus* and *Salvia miltiorrhiza* extract; Imp, Importin.

and fibrosis. Such inhibition is achieved possibly via decreasing the synthesis and release of pro-inflammatory and fibrogenic factors, including TGF-β_1.

The pathophysiological functions of TGF-β_1, particularly in hepatocarcinogenesis, are directly associated with its dysregulated biosynthesis and over secretion by cancerous cells (20). Among the TGF-β family of cytokines, the TGF-β_1 isoform has been implicated as a crucial regulator of HCC progression (22,23). TGF-β_1 overexpression has been reported in patients with cirrhosis (24) and HCC (25). However, TGF-β and its specific serine/threonine kinase receptors (TβRI and TβRII) are crucial for the TGF-β signaling cascade. Tan *et al* (26) demonstrated that TβRI and TβRII are important molecules in TGF-β signaling. Accordingly, TβRI and TβRII regulation was demonstrated to alter cellular responses to TGF-β stimulation (27). The upstream mediators of TGF-β serve roles in canonical TGF-β/Smad signaling to regulate the transcription of target specific genes, which in turn mediate the oncogenic roles of TGF-β (27). PAI-1 acts as the main inhibitor of the urokinase-type plasminogen activator system; it also stimulates cell migration and invasion by inhibiting cellular adhesion and enhancing basement membrane degradation (28).

Canonical TGF-β signaling begins as a ligand activation of constitutive transmembrane TβRII, which transphosphorylates TβRI, leading to the TβRI-dependent phosphorylation of Smad2 and Smad3 (23). Phosphorylated Smad2 and Smad3 oligomerize with Smad4 to form the Smad2/3/4 complex, which is translocated to the nucleus via nuclear Imp7/8 proteins in order to accurately target specific gene transcription (29,30). Smad4 is crucial for the whole canonical signaling pathway, particularly in terms spatio-temporal distribution (31). Smad4 is translocated into the nucleus in of hetero-complex forms (typically the Smad3/4 and Smad2/3/4 complexes), where it modulates target gene expression by directly binding with DNA or interacting with transcription factors, co-activators and co-repressors (29). Yao *et al* (30) reported that nuclear import proteins, including Imp7 and Imp8, were indispensable for the migration of Smad4 from the cytoplasm to nucleus in TGF-β-stimulated Hela cells.

The results of the present study suggest that CASE significantly decreases the expression of TGF-β_1 in the livers of DEN-treated rats compared with those treated with DEN alone and that this was associated with reduced inflammatory cell infiltration and improved HCC differentiation. These results agree in part with an earlier report by Liu *et al* (4), which revealed that Astragaloside IV, the major active component of *Astragalus membranaceus*, could decrease the level of TGF-β_1. At the TGF-β-specific receptor level, livers

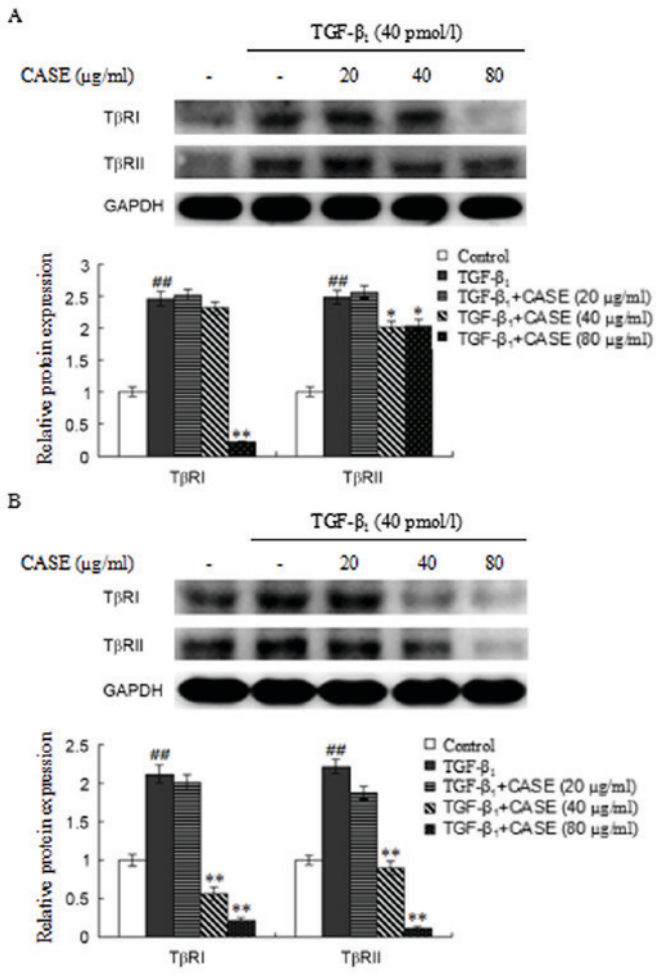

Figure 6. High and medium CASE doses decrease TβRI and TβRII protein expression in MFBs and HepG2 cells. Cells were starved for 24 h in serum-free medium and in the absence or presence of CASE (20, 40 or 80 μg/ml), subsequently treated with TGF-β_1. Total proteins were extracted from (A) rat myofibroblasts and (B) HepG2 cells. The expression of TβRI and TβRII proteins were analyzed by western blotting using anti-TβRI, -TβRII and -GAPDH antibodies. Intensities of TβRI and TβRII bands were normalized to those of GAPDH in the corresponding treatment groups. The ratios of the TβRI and TβRII protein to GAPDH without exogenous TGF-β_1 were assigned a value of 1. Data are expressed as mean ± standard deviation (n=3). $^{\#\#}$P<0.01 vs. the control group. *P<0.05 and **P<0.01 vs. the TGF-β_1 group. CASE, Compound *Astragalus* and *Salvia miltiorrhiza* extract; TGF-β_1, transforming growth factor β-1; TβR, transforming growth factor-β receptor.

of DEN-treated rats exhibited increased TβRI and TβRII staining, while CASE treatment significantly decreased the expression of TβRI and TβRII proteins. Coincidentally, DEN increased the protein expression of Imp7 and Imp8, while this change was reversed by CASE treatment, particularly in the high dose group (240 mg/kg), which is paralleled by the level of Smad4 protein in DEN-induced HCC reported in a previous study by our group (10). The aforementioned results demonstrate the ability of CASE to negatively modulate the TGF-β/Smad signaling pathway in DEN-induced hepatocarcinogenesis at multi-target levels and CASE's potential as an effective hepatoprotective candidate drug.

To confirm the observed *in vivo* repression of TβRI and TβRII by CASE, *in vitro* experiments were performed. HSCs have been verified as a cell type vital to liver fibrogenesis (32). The activation and trans-differentiation of HSCs into MFBs by cytokines (primarily TGF-β_1) are considered to be central events in liver fibrogenesis (33). MFBs directly generate hepatocarcinogenesis by inducing autocrine TGF-β signaling and nuclear β-catenin accumulation in neoplastic hepatocytes (34). The HepG2 cell line is one of the most common *in vitro* experimental models used in liver cancer research and drug development (35). Therefore, in order to investigate the effects of CASE on TβRI and TβRII, which are important targets of the TGF-β signaling pathway, MFBs and HepG2 cells were used. The results revealed that CASE decreased TβRI and TβRII expression in MFBs and HepG2 cells. This coincides with the *in vivo* results of the present study, implying that the receptors of TGF-β may be likely direct targets in CASE's anti-HCC effects. Collectively, the results of the current study indicate that CASE suppresses DEN-induced hepatocarcinogenesis by downregulating GST-P1 protein expression and negatively modulating the upstream and downstream mediators of the TGF-β/Smad signaling pathway. These results highlight that the multiple-target effects of CASE in the prevention and treatment of HCC is a potential drug candidature.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YY designed and supervised the current study; CW, HWK, MH, YFJ, JJW, JYW, XCY and AB conducted the experiments; CW, HWK, MH and XL collected, analyzed and interpreted the data; CW, XL and AB drafted the manuscript; and CW and YY were responsible for the final revision of the manuscript.

Ethics approval and consent to participate

The current study was approved by the Experimental Animal Ethics Committee of Anhui Medical University (Hefei, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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