

# Treatment with the herbal formula Songyou Yin inhibits epithelial-mesenchymal transition in hepatocellular carcinoma through downregulation of TGF- $\beta$ 1 expression and inhibition of the SMAD2/3 signaling pathway

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**Abstract.** It was previously reported that treatment with the herbal formula Songyou Yin (SYY) may serve a role in attenuating epithelial-mesenchymal transition (EMT). In the present study, the effect of treatment with SYY on transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)-induced EMT was investigated and the potential underlying molecular mechanisms were evaluated. MHCC97H cells were pretreated with SYY for 4 weeks and subsequently named MHCC97HSYY cells. Simultaneously, MHCC97H cells were cultured for 4 weeks without SYY and used as a negative control. Western blot analysis and enzyme-linked immunosorbent assays demonstrated that treatment with SYY inhibited EMT-associated changes and TGF- $\beta$ 1 expression in MHCC97H cells. MHCC97H and MHCC97HSYY cells were treated with 10 ng/ml TGF- $\beta$ 1 to induce EMT. The results of the present study demonstrated that pretreatment with SYY markedly inhibited TGF- $\beta$ 1-induced morphological changes, and reversed the expression of the EMT markers E-cadherin and N-cadherin. In addition, expression levels of the TGF- $\beta$ 1 downstream proteins, phosphorylated mothers against decapentaplegic homologs (p-SMAD)2 and 3, were reduced. Transwell assays indicated that pretreatment with SYY inhibited TGF- $\beta$ 1-induced cancer cell invasion and migration. The results of the present study indicate that SYY inhibited EMT through attenuation of TGF- $\beta$ 1 expression, and downregulation of p-SMAD2 and 3.

## Introduction

Hepatocellular carcinoma (HCC) is the sixth most frequent cancer worldwide and the third leading cause of cancer-associated mortality (1). The survival rates of patients with HCC are unsatisfactory (2). Continuing problems in the management of HCC are tumor invasion and metastasis (3). Understanding the underlying molecular mechanisms of HCC invasion and metastasis may provide novel therapeutic targets. Increasing evidence indicates that epithelial-mesenchymal transition (EMT) serves an essential role in tumor invasion and metastasis (4,5).

EMT is a cellular process during which polarized epithelial cells become motile and exhibit mesenchymal characteristics. The features of EMT include the loss of cell-cell adhesion, reorganization of the actin cytoskeleton and acquisition of increased migratory characteristics (6,7). EMT is characterized by the upregulation of the mesenchymal marker N-cadherin, downregulation of the epithelial marker E-cadherin, and acquisition of fibroblast-like migratory and invasive phenotypes (8). Previous studies indicated that EMT serves a critical role in HCC invasion and metastasis (9-11).

Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is a multifunctional cytokine that regulates a range of cellular functions, including proliferation, differentiation, migration and apoptosis (12). In addition, TGF- $\beta$ 1 is the primary mediator of EMT and is involved in epithelial-mesenchymal interactions during HCC metastasis (13,14). The mothers against decapentaplegic homolog (SMAD) family of proteins has been demonstrated to be activated in response to members of the TGF- $\beta$  superfamily (15). During TGF- $\beta$ 1-mediated EMT, TGF- $\beta$ 1 binds to the TGF- $\beta$  type II receptor, and this binding induces the recruitment and phosphorylation of the type I TGF- $\beta$  receptor. The activated type I TGF- $\beta$  receptor subsequently induces the recruitment and phosphorylation of the receptor-regulated SMADs, SMAD2 and 3, which form heteromeric complexes with the common mediator SMAD, SMAD4 (16). The SMAD complexes translocate to the nucleus and mediate gene transcription by binding to SMAD binding elements contained within the promoter regions of target genes (17).

Traditional Chinese medicine has gained increasing attention in cancer therapy (18-20). Songyou Yin (SYY),

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**Abbreviations:** TGF- $\beta$ 1, transforming growth factor  $\beta$ -1; HCC, hepatocellular carcinoma; EMT, epithelial-mesenchymal transition

**Key words:** hepatocellular carcinoma, transforming growth factor- $\beta$ 1, Songyou Yin, epithelial-mesenchymal transition

a Chinese herbal formula consisting of four herbs: *Salvia miltiorrhiza*, *Astragalus membranaceus*, *Lycium barbarum*, *Crataegus pinnatifida*, and the *Trionyx sinensis* Wiegmann species of turtle, has been demonstrated to inhibit the development of HCC by inducing tumor cell apoptosis and reducing invasion (21). SY Y attenuates hepatoma cell invasion and metastasis through the downregulation of cytokine secretion by activated hepatic stellate cells (22). Treatment with SY Y has been demonstrated to inhibit the invasive and metastatic potential of HCC cells, and improve survival in nude mice models with chronic fibrosis, through the inhibition of cytokine secretion by activated hepatic stellate cells (23). Another report indicated that SY Y serves a role in attenuating EMT by inhibiting EMT-associated molecular changes in oxaliplatin-treated tumor tissues and cell lines (24). However, the underlying molecular mechanisms of SY Y-mediated EMT regulation have not been completely elucidated.

In the present study, the ability of SY Y to inhibit HCC cell EMT was investigated, in addition to the molecular mechanism underlying the SY Y-mediated decrease in TGF- $\beta$ 1 expression.

## Materials and methods

**SY Y preparation.** The Chinese herbal formula SY Y, a dietary component authorized by the China Food and Drug Administration (Beijing, China; Chinese health food approval no. G20070160), includes five Chinese medicinal extracts whose proportions, 'fingerprints' and protocols of preparation have previously been reported (21). The SY Y used in the present study was obtained from Shanghai Fang Xin Pharmaceutical Technology Co., Ltd. (Shanghai, China; batch no. 20110401). An 800 mg/ml SY Y stock solution was sterilized twice by 0.22- $\mu$ m Millex-GP Filter Unit (Millipore Express PES Membrane; EMD Millipore, Billerica, MA, USA) for further use.

**Cell culture and reagents.** The human hepatocellular carcinoma cell line MHCC97H (established at Fudan University, Shanghai, China) was used in the present study due to its metastatic potential. The cell line was established from a subcutaneous xenograft of a metastatic model of human HCC in nude mice (25). Similarly to a previous study (22), MHCC97H cells pretreated with 2 mg/ml SY Y for 4 weeks were labeled as MHCC97HSY Y cells. Cells were grown in 75 cm<sup>2</sup> flasks and incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub>. All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.). TGF- $\beta$ 1 (PeproTech, Inc., Rocky Hill, NJ, USA) was used to induce EMT in MHCC97H cells. MHCC97H cells were treated with TGF- $\beta$ 1 (5, 10, 20 and 40 ng/ml) for 48 h at 37°C. It was established that 10 ng/ml TGF- $\beta$ 1 was sufficient to induce EMT in MHCC97H cells. MHCC97H cells treated with 10 ng/ml TGF- $\beta$ 1 for 48 h were labeled as the TGF- $\beta$ 1 group. MHCC97HSY Y cells treated with 10 ng/ml TGF- $\beta$ 1 and 2 mg/ml SY Y for 48 h were labeled as the SY Y+TGF- $\beta$ 1 group. The MHCC97HSY Y cells treated with 2 mg/ml SY Y were labeled as the SY Y group. MHCC97H cells cultured for 4 weeks without SY Y were used as a negative control.

**Enzyme-linked immunosorbent assay (ELISA).** MHCC97HSY Y and MHCC97H cells were transferred into T-75 flasks at a density of 1x10<sup>6</sup> cells/flask and cultured overnight. The culture medium was subsequently replaced with 12 ml serum-free DMEM and cells were incubated for a further 24 h. The flasks were centrifuged at 800 x g for 5 min at 4°C and the cell-free media were collected and stored at -80°C until required. The concentration of TGF- $\beta$ 1 secreted into the media was quantified using a human TGF- $\beta$ 1 ELISA kit (cat. no. DB100B; R&D Systems, Inc., Minneapolis, MN, USA), according to the protocol of the manufacturer. Optical density was measured using a microplate reader (Biotek Instruments, Inc., Winooski, VT, USA) at a wavelength of 450 nm. A standard curve was generated by TGF- $\beta$ 1 standard (13.72, 41.15, 123.5, 370.4, 1,111, 3,333 and 10,000 pg/ml) of the ELISA kits.

**Cell invasion and migration assays.** Transwell® plates (Corning Incorporated, Corning, NY, USA) were used to evaluate the invasion and migration of MHCC97H cells. The invasion assay was performed in 24-well Transwell culture plates coated with 25  $\mu$ l BD Matrigel™ (BD Biosciences, Franklin Lakes, NJ, USA). A total of 5x10<sup>4</sup> MHCC97H cells in serum-free DMEM were seeded into the upper chamber and 0.5 ml DMEM containing 20% FBS was added to the lower chamber, with or without 10 ng/ml TGF- $\beta$ 1. In total, 5x10<sup>4</sup> MHCC97HSY Y cells in serum-free DMEM containing 2 mg/ml SY Y were seeded into the upper chamber, and DMEM containing 20% FBS and 10 ng/ml TGF- $\beta$ 1 was added to the lower chamber. The plates were incubated for 48 h, and cells in the lower chamber were subsequently fixed with 4% paraformaldehyde for 30 min at room temperature. The cells were then stained with Giemsa (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) for 15 min and counted at magnification, x200 with a fluorescence microscope (DMI300B; Leica Microsystems GmbH, Wetzlar, Germany). The migration assay was performed as described above without the BD Matrigel coating.

**Western blot analysis.** Following a 4-week incubation, 5x10<sup>6</sup> MHCC97H and 5x10<sup>6</sup> MHCC97HSY Y cells were washed twice with PBS and lysed in radioimmunoprecipitation assay buffer, containing 150 mM NaCl, 1% Triton™ X-100 (Beyotime Institute of Biotechnology, Shanghai, China), 0.1% SDS, 50 mM Tris-HCl (pH 7.4), 1 mM ethylenediaminetetraacetic acid and 1 mM phenylmethylsulfonyl fluoride. The supernatants were collected following centrifugation at 10,000 x g for 15 min at 4°C. Total protein concentration was determined using a BCA Protein Assay kit. Equal amounts of protein (50  $\mu$ g) were incubated at 100°C for 10 min and separated using SDS-PAGE on a 10% gel. The proteins were subsequently transferred to polyvinylidene fluoride membranes. Following blocking in 10% milk for 2 h at room temperature, the membranes were incubated with the primary antibodies at 4°C overnight. The membranes were subsequently washed three times with TBS/Tween (0.05% Tween-20 in TBS) and incubated with the horseradish peroxidase-conjugated secondary antibody (cat. no. 7074; dilution, 1:5,000; Cell Signaling Technology, Inc., Danvers, MA, USA) at room temperature for 1 h. Protein bands were visualized using the enhanced chemiluminescent substrate with SuperSignal West Pico Chemiluminescent Substrate kit (Pierce; Thermo

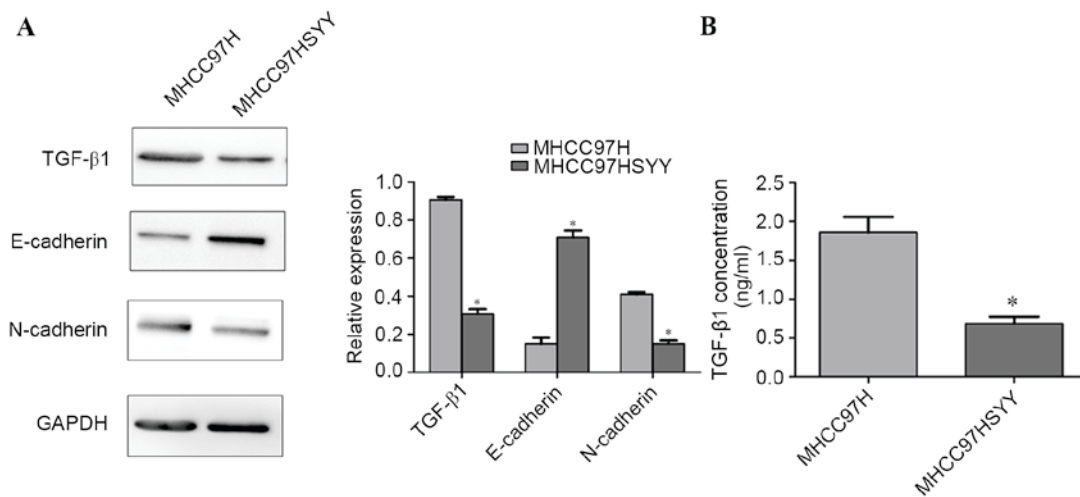


Figure 1. Effect of treatment with SY on TGF- $\beta$ 1, E-cadherin and N-cadherin protein expression. (A) Western blotting analysis of TGF- $\beta$ 1, E-cadherin and N-cadherin expression demonstrated altered protein expression following treatment with SY. Protein bands were normalized to GAPDH. (B) Results of the enzyme-linked immunosorbent assay demonstrated decreased TGF- $\beta$ 1 secretion following treatment with SY. \* $P<0.05$  vs. untreated MHCC97H cells. SY, Songyou Yin; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1.

Fisher Scientific, Inc.). Positive immunoreactive bands were quantified using densitometry and normalized to GAPDH (Image lab software, version 4.1; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Primary antibodies were used as follows: Anti-E-cadherin (cat no. 3195; dilution, 1:1,000; Cell Signaling Technology, Inc.), anti-N-Cadherin (cat no. 13116; dilution, 1:1,000; Cell Signaling Technology, Inc.), anti-phosphorylated (p)-SMAD2 (cat. no. 3108; dilution, 1:1,000; Cell Signaling Technology, Inc.), anti-p-SMAD3 (cat. no. 9520; dilution, 1:1,000; Cell Signaling Technology, Inc.), anti-SMAD2 (cat. no. 5339; dilution, 1:1,000; Cell Signaling Technology, Inc.), anti-SMAD3 (cat. no. 9523; dilution, 1:1,000; Cell Signaling Technology, Inc.), anti-TGF- $\beta$ 1 (cat. no. ab92486; dilution, 1:1,000; Abcam) and anti-GAPDH (cat no. 5174; dilution, 1:5,000; Cell Signaling Technology, Inc.).

**Immunofluorescent staining.** E-cadherin expression in the TGF- $\beta$ 1, SY+TGF- $\beta$ 1 and SY groups was evaluated using immunofluorescent staining. Following 48 h of treatment with or without TGF- $\beta$ 1,  $5 \times 10^4$  cells were fixed with 4% paraformaldehyde in PBS at room temperature for 10 min. Nonspecific binding sites were blocked using 1% bovine serum albumin (BSA; Sigma-Aldrich; Merck Millipore) at room temperature for 30 min. A rabbit polyclonal antibody against E-cadherin (as aforementioned) was diluted in PBS containing 1% BSA (1:50) and incubated with the fixed cells at 40°C overnight. Cells were subsequently washed three times with 1% BSA prior to incubation with a fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G antibody (cat. no. 35562; dilution, 1:200; Thermo Fisher Scientific, Inc.) at room temperature for 30 min. The fixed cells were washed three times and subsequently stained with DAPI for 10 min, prior to the capturing of images. The images were captured at magnification, x200 using an inverted microscope (DMI300B; Leica Microsystems GmbH), whereby the anti-E-cadherin antibody was excited by green light and DAPI by ultraviolet light. In addition, changes in cell morphology were assessed with microscopy (DMI300B; Leica Microsystems GmbH). The

MHCC97H cells were treated with 0, 5, 10, 20 and 40 ng/ml TGF- $\beta$ 1 for 48 h and images were captured at magnification, x100 to evaluate the changes of the cell shapes. The typical feature of the mesenchymal phenotype were observed, which exhibited as scattered, lengthened and a spindle-like shape.

**Statistics analysis.** Statistical analysis was performed using the Student's t-test and SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA).  $P<0.05$  was considered to indicate a statistically significant difference. All experiments were repeated three times, and the values are presented as the mean  $\pm$  standard deviation of triplicate experiments.

## Results

**Effect of treatment with SY on EMT marker and TGF- $\beta$ 1 expression in MHCC97H cells.** To determine the effect of treatment with SY on EMT, E-cadherin and N-cadherin protein expression was evaluated using western blotting. The results demonstrated that treatment with SY significantly decreased N-cadherin expression and increased E-cadherin expression compared with the untreated MHCC97H cells ( $P=0.011$  and  $P=0.002$ , respectively; Fig. 1A). In addition, the effect of treatment with SY on TGF- $\beta$ 1 expression in MHCC97HSYY and MHCC97H cells was investigated using western blotting. Significantly decreased TGF- $\beta$ 1 protein expression was observed in the MHCC97HSYY cells compared with the MHCC97H cells ( $P=0.004$ ; Fig. 1A). To further determine whether treatment with SY regulated TGF- $\beta$ 1 secretion in HCC cells, the concentration of TGF- $\beta$ 1 secreted into the culture media was evaluated using an ELISA. MHCC97HSYY cells exhibited significantly decreased levels of secreted TGF- $\beta$ 1 compared with MHCC97H cells ( $P=0.03$ ; Fig. 1B). These results demonstrate that treatment with SY alters EMT marker expression and inhibits TGF- $\beta$ 1 expression in MHCC97H cells.

**TGF- $\beta$ 1 induces EMT in MHCC97H cells.** The optimum TGF- $\beta$ 1 concentration required to initiate EMT in MHCC97H



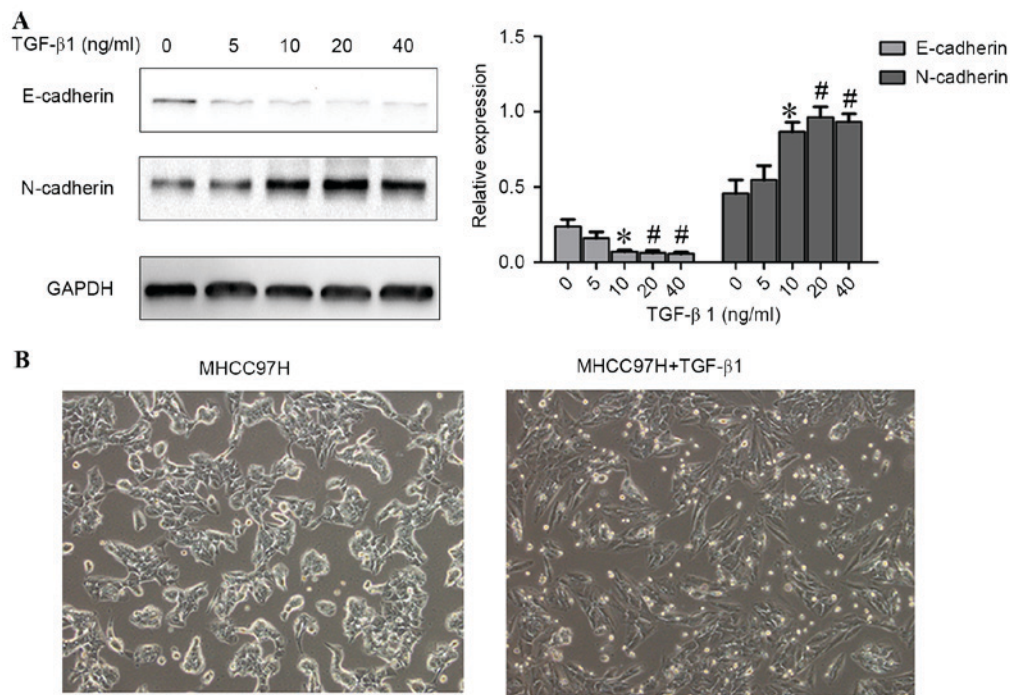


Figure 2. TGF- $\beta$ 1 induces epithelial-mesenchymal transition in MHCC97H cells. (A) MHCC97H cells were treated with 0, 5, 10, 20 and 40 ng/ml TGF- $\beta$ 1, and western blot analysis demonstrated altered E-cadherin and N-cadherin protein expression. Protein bands were normalized to GAPDH. \* $P$ <0.05 vs. untreated MHCC97H cells; # $P$ <0.05 vs. MHCC97H cells treated with 10 ng/ml TGF- $\beta$ 1. (B) Representative images of morphological changes in MHCC97H cells treated with or without 10 ng/ml TGF- $\beta$ 1. Images are shown at magnification, x100. TGF- $\beta$ 1, transforming growth factor- $\beta$ 1.

cells was initially determined. Changes in cell morphology were assessed following treatment with 0, 5, 10, 20 and 40 ng/ml TGF- $\beta$ 1 for 48 h. E-cadherin and N-cadherin expression was determined following treatment of MHCC97H cells with TGF- $\beta$ 1. Western blotting demonstrated a dose-dependent decrease in E-cadherin protein expression and a significant decrease following treatment with 10 ng/ml TGF- $\beta$ 1 ( $P$ =0.008; Fig. 2A). By contrast, N-cadherin expression was increased in a dose-dependent manner and was significantly increased following treatment with 10 ng/ml TGF- $\beta$ 1 ( $P$ =0.017; Fig. 2A). In addition, E- and N-cadherin expression levels were not significantly altered in MHCC97H cells following treatment with 20 and 40 ng/ml TGF- $\beta$ 1 compared with cells treated with 10 ng/ml TGF- $\beta$ 1 (for E-cadherin,  $P$ =0.497 and 0.369 respectively; for N-cadherin,  $P$ =0.1 and 0.116 respectively; Fig. 2A). Following treatment with 10 ng/ml TGF- $\beta$ 1, MHCC97H cells also underwent morphological changes, and gained an elongated and spindle-like morphology (Fig. 2B). These results indicate that TGF- $\beta$ 1 initiates EMT in MHCC97H cells.

**Treatment with SY Y suppresses TGF- $\beta$ 1-induced EMT in MHCC97H cells.** The effect of treatment with SY Y on TGF- $\beta$ 1-induced EMT was investigated. Cells in the SY Y group exhibited classical epithelial morphology, while cells in the TGF- $\beta$ 1 group exhibited a mesenchymal morphology. However, cells treated with SY Y and TGF- $\beta$ 1 seemed to change little, and still exhibited epithelial morphology without exhibiting an elongated and fibroblast-like phenotype (Fig. 3A). Western blotting was also used to evaluate changes in protein expression levels of the EMT markers E- and N-cadherin. As presented in Fig. 3B, E-cadherin expression was significantly decreased ( $P$ =0.016) and N-cadherin expression

was significantly increased ( $P$ =0.014) in the TGF- $\beta$ 1 group compared with the SY Y+TGF- $\beta$ 1 group. To determine the distribution of E-cadherin in HCC cells, immunofluorescent staining was performed. As presented in Fig. 3C, membrane E-cadherin expression was lost in MHCC97H cells treated with TGF- $\beta$ 1, whereas membrane E-cadherin expression was partially restored in the SY Y+TGF- $\beta$ 1 group. Furthermore, the effects of treatment with SY Y on TGF- $\beta$ 1-induced invasion and migration in MHCC97H cells were investigated. As presented in Fig. 3D, no significant difference in the numbers of migratory and invasive cells was observed between the SY Y and SY Y+TGF- $\beta$ 1 groups. However, the number of invasive and migratory cells in the SY Y+TGF- $\beta$ 1 group was significantly decreased compared with the TGF- $\beta$ 1 group ( $P$ =0.021 and 0.002, respectively). These results demonstrate that treatment with TGF- $\beta$ 1 is able to promote HCC cell invasion and migration, and that pretreatment with SY Y inhibits these effects, indicating that SY Y inhibits TGF- $\beta$ 1-induced EMT.

**Treatment with SY Y inhibits SMAD2 and 3 phosphorylation.** Phosphorylation of SMAD2 and 3 by the activated type I TGF- $\beta$  receptor is a critical step in the initiation of TGF- $\beta$ 1 signal transduction. Therefore, the effect of pretreatment with SY Y on SMAD expression was investigated. Cells in the SY Y, TGF- $\beta$ 1 and SY Y+TGF- $\beta$ 1 groups were compared. As presented in Fig. 4, no significant difference in total SMAD2 and 3 protein expression was observed between cell groups. However, p-SMAD2 and 3 expression was significantly increased in cells in the TGF- $\beta$ 1 group compared with cells in the SY Y+TGF- $\beta$ 1 group ( $P$ =0.014 and 0.002, respectively). No significant difference in p-SMAD2 and 3 expression was observed between the SY Y+TGF- $\beta$ 1 and SY Y groups. These

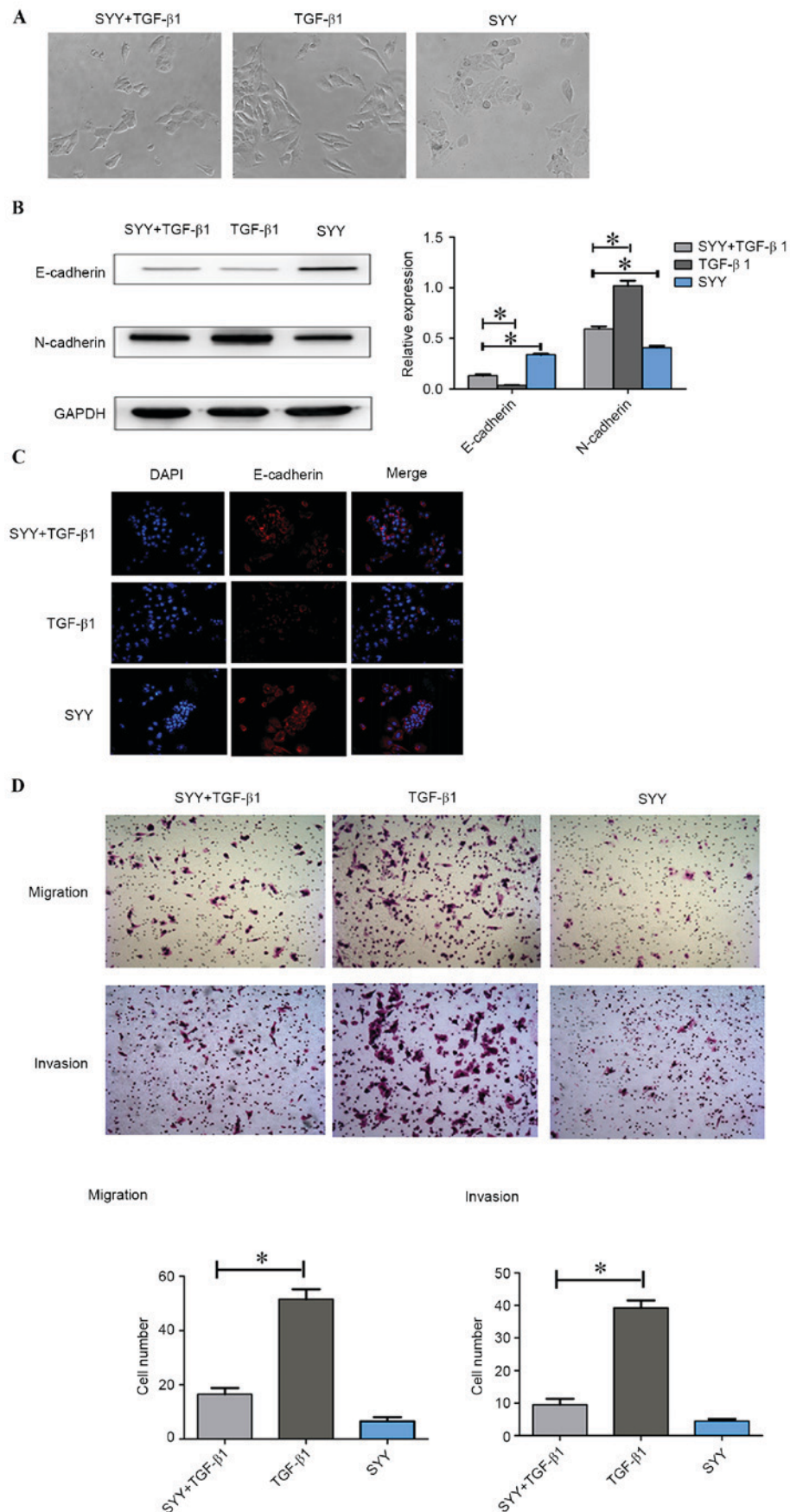


Figure 3. Treatment with SYY suppresses TGF- $\beta$ 1-induced epithelial-mesenchymal transition in MHCC97H cells. (A) Representative images of MHCC97H cell morphological changes in the SYY+TGF- $\beta$ 1, TGF- $\beta$ 1 and SYY groups. Magnification, x200. (B) Western blot analysis of E- and N-cadherin protein expression following treatment with SYY and TGF- $\beta$ 1. Protein bands were normalized to GAPDH. (C) Representative images of the immunofluorescent staining of membrane E-cadherin in the three groups. Magnification, x20. (D) Results from the invasion and migration assays demonstrating the increased invasive and migratory capacity of cells in the TGF- $\beta$ 1 group. Magnification, x200. \*P<0.05. SYY, Songyou Yin; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1.

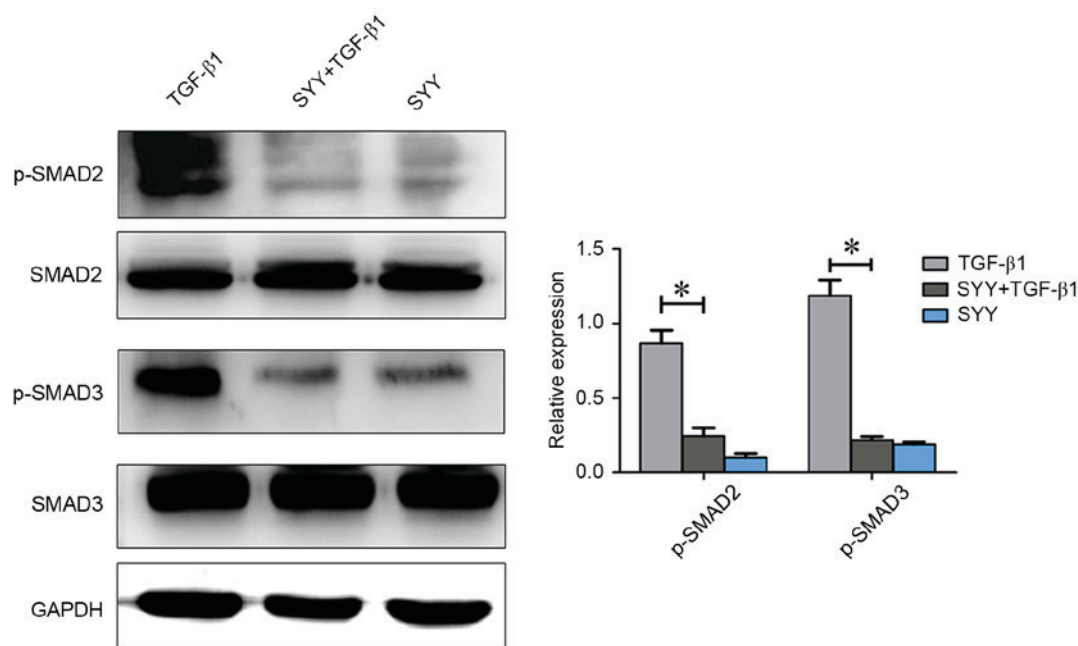


Figure 4. Western blot analysis of SMAD2 and 3, and p-SMAD2 and 3 expression in the three groups. Protein bands were normalized to GAPDH. \*P<0.05. SMAD, mothers against decapentaplegic homolog; p, phosphorylated.

results suggest that treatment with SYY decreases SMAD2 and 3 phosphorylation.

## Discussion

Metastasis is the primary obstacle in the management of patients with HCC. EMT significantly contributes to the conversion of early stage tumors into invasive malignancies. EMT is triggered by various external signals, including TGF- $\beta$ 1, hepatocyte growth factor and fibroblast growth factor (26). Furthermore, TGF- $\beta$ 1 is involved in the EMT of HCC metastases (13). In the present study, it was observed that TGF- $\beta$ 1 induced EMT in MHCC97H cells, suggesting that MHCC97H cells are sensitive to inducers of EMT. Treatment with TGF- $\beta$ 1 caused MHCC97H cells to lose their polygonal appearance, and altered the expression of cell adhesion molecules, including E- and N-cadherin, which are characteristics of EMT. Furthermore, TGF- $\beta$ 1 was demonstrated to be able to induce EMT in HCC cells in a dose-dependent manner. A total of 10 ng/ml TGF- $\beta$ 1 was used in to induce EMT in the subsequent *in vitro* experiments. In addition, the results of the present study demonstrated that TGF- $\beta$ 1 was a strong inducer of tumor cell metastasis.

SYY is a Chinese herbal formula and certain components have been recognized to be effective in the treatment of cancer (27,28). Astragaloside IV controls collagen reduction in photoaging skin by improving suppression of the TGF- $\beta$ /SMAD signaling pathway (29). Combined treatment with *A. membranaceus* and *S. miltiorrhiza* extracts significantly suppressed HCC progression, by mediating TGF- $\beta$ /SMAD signaling and modulating SMAD3 phosphorylation (30). In a previous study, it was reported that treatment with SYY inhibited the molecular changes characteristic of EMT in oxaliplatin-treated tumor tissues and cell lines (24). In the present study, it was investigated whether SYY inhibited TGF- $\beta$ 1-induced EMT

in HCC cells. Treatment with SYY was demonstrated to inhibit TGF- $\beta$ 1 expression and secretion in MHCC97H cells. Treatment with SYY was also able to inhibit TGF- $\beta$ 1-induced morphological changes in HCC cells. Western blot analysis demonstrated that treatment with SYY significantly increased E-cadherin protein expression and significantly decreased N-cadherin expression. Furthermore, the number of invasive and migratory cells in the SYY+TGF- $\beta$ 1 group was significantly decreased compared with the TGF- $\beta$ 1 group, suggesting that SYY effectively inhibits TGF- $\beta$ 1-induced MHCC97H cell invasion and migration. The results of the present study demonstrate that TGF- $\beta$ 1 induces EMT in MHCC97H cells and that this process is effectively inhibited by pretreatment with SYY.

TGF- $\beta$ 1 signaling begins with the binding of TGF- $\beta$  receptors, and phosphorylation of SMAD2 and 3, thereby activating multiple downstream signaling pathways (31,32). As treatment with SYY was demonstrated to inhibit TGF- $\beta$ 1 expression and TGF- $\beta$ 1-induced morphological changes, the effect of treatment with SYY on TGF- $\beta$ 1 signaling elements was subsequently investigated. In the present study, it was observed that TGF- $\beta$ 1 induced upregulation of p-SMAD2 and 3 expression, and that these changes were significantly inhibited by pretreatment with SYY. SYY appears to inhibit EMT via downregulation of TGF- $\beta$ 1-induced p-SMAD2 and 3 expression, which is consistent with previous studies (33,34).

In conclusion, it was demonstrated that treatment with SYY inhibits TGF- $\beta$ 1-induced EMT through attenuation of TGF- $\beta$ 1 expression, and downregulation of SMAD2 and 3 phosphorylation. These results further support the therapeutic potential of SYY in the treatment of HCC and inhibition of EMT. Therefore, future research into the clinical efficacy of SYY treatment in patients with HCC is warranted.



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