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Trichosanthin inhibits the proliferation of cervical cancer cells and downregulates STAT-5/C-myc signaling pathway

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ABSTRACT

Background: Previous studies have indicated that Trichosanthin (TCS) exerts anti-virus, immunoregulation and a broad spectrum anti-tumor pharmacological activities. Trichosanthin is a promising agent for the treatment of cervical cancer. However, the exact effects and potential mechanism of TCS on cervical cancer are not well known.

Method: The cell viability of TCS on cervical cancer cell lines (HeLa and caski cells) were detected by a Cell Counting Kit-8 (CCK-8) assay. Cell proliferation was measured by Ki-67 staining and cell apoptosis was detected by flow cytometry. Cell migration and invasion were detected by wound assay and transwell assay, respectively. The levels of E-cadherin, N-cadherin, Snail, Bcl-2, Caspase-3, p-STAT5, STAT5, p-C-myc, C-myc were detected by western blot.

Results: The present study showed that TCS inhibited the proliferation of HeLa and caski cells and reduced Ki-67 and P-C-myc expression. In addition, flow cytometric analysis showed that TCS induced the apoptosis of HeLa and caski cells. The potent effect of TCS on cell apoptosis as determined by the increase the levels of caspase-3 and decrease the levels of Bcl-2. TCS also inhibited cervical cancer cell invasion, migration and epithelial-mesenchymal transition (EMT). Furthermore, TCS treatment markedly inhibited the activation of STAT5/C-myc signaling pathway.

Conclusion: In conclusion, the present study suggest that TCS inhibits the proliferation, migration and EMT of human cervical cancer cells, which maybe mediated by inhibiting the activation of STAT5/C-myc signaling pathway.

1. Introduction

Trichosanthin (TCS) is an active component purified from the root tubers of the Chinese medicinal herb Trichosanthes kirilowii [1], which has a broad spectrum pharmacological properties including immunomodulatory, antitumor and antiHIV activities [2–4]. TCS exhibits potent cytotoxicity in a variety of tumor cell lines but less cytotoxicity in normal cell lines [5]. TCS has been shown that exerts antitumor activities with different molecular mechanisms including anti-tumor cell proliferation, induce tumor cell apoptosis [6]. Previous studies has reported that TCS is a promising agent for the treatment of cervical cancer [7,8]. However, its anti-tumor mechanism against cervical cancer has not been fully elucidated.

Signal transducer and activator of transcription (STAT) proteins comprise a series of transcription factors latent in the cytoplasm. It is phosphorylated to form homo- or heterodimers that translocate to the nucleus, influencing different normal physiologic cell processes, including proliferation, differentiation, apoptosis, and angiogenesis [9]. Among STAT family members, STAT5 is considered directly contribute to oncogenesis because they increasing the levels of cyclin D1, C-myc and bcl-xl and promoting cell cycle progression, cell transformation and inhibiting cell apoptosis [10,11]. STAT5 is activated by phosphorylation of a specific tyrosine residue, after which phosphorylated STAT proteins dimerize and translocate to nucleus to regulate gene transcription [12]. However, excessive or constitutively activated STAT5 has been found in a variety of human primary tumors [13–15] and

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directly contribute to tumorigenesis. However, whether TCS inhibited the activation of STAT5 in cervical cancer until unclear.

In this study, we screened the proliferation, apoptosis and epithelial-mesenchymal transition (EMT) of the cervical cancer cell lines (HeLa and caski cells) in vitro following TCS treatment. In addition, we detected the activation status of the STAT5 signaling pathway underlying action of TCS on cervical cancer. The present study provides an experimental basis for further research, and indicates that TCS may be a protein antitumor agent for the treatment of cervical cancer.

2. Materials and methods

2.1. Cell culture and reagents

The human cervical cancer cell lines HeLa and caski were obtained from American Type Culture Collection (ATCC, USA). Cells were cultured in RPMI 1640 medium (Gibco BRL, USA) containing 10% fetal bovine serum (Gibco BRL, USA), 100 U/ml of penicillin and 100 μg/ml of streptomycin (GE Healthcare Life Sciences, Logan, UT, USA) in a 5% CO_2 incubator at 37℃. TCS was purchased from Shanghai Jinshan Pharmaceutical Co., Ltd. (Shanghai, China) and dissolved in dimethylsulfoxide (DMSO).

2.2. Cell morphology

HeLa and caski cells were seed in 6-plate wells to the logarithmic growth phase. Then, cells were treated with different concentrations of TCS (0, 10, 20, 40, 80, 120ug/ml) for 24 and 48 h according to the previous study [8]. The morphological changes of the cells were observed using an inverted microscope, and the cells were photographed after 24 h and 48 h of treatment.

2.3. Cell viability assay

Cell viability was measured by Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) following manuscript protocol. Briefly, HeLa and caski cells were plated in 96-well plates at a density of 5×10^3 cells/well and cultured overnight. Various concentrations of TCS (0, 10, 20, 40, 80, 120 μg/ml) were added to each well. After incubation for 24 h and 48 h, the medium was removed and CCK-8 reagent (10 μl) was added to each well and incubation for 4 h at 37℃. Subsequently, the absorbance at 450 nm was measured using a SpectraMax M5 plate reader (Molecular Devices LLC, Sunnyvale, CA, USA).

2.4. Flow cytometry

The apoptosis of HeLa and caski cells was analyzed using an Annexin V-FITC/PI apoptosis detection kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. HeLa and caski cells were cultured in 6-well plates (5×10^5/well) and treated with TCS (20 μg/ml) for 48 h. Then, cells (1×10^6 cells/ml) were harvested and resuspended in 195 μl of binding buffer, and 5 μl of Annexin V-FITC and 10 μl propidium iodide (PI) were added, following incubation at room temperature for 20 min in the dark. Cell apoptosis were analyzed by flow cytometry (BD FACS Calibur, Franklin Lakes).

2.5. Ki-67 staining

HeLa and caski cells were treated with TCS (20 μg/ml) for 48 h, then fixed in 4% formaldehyde. The cells were stained with anti-Ki-67 antibody (cat.no. ab15580, Abcam, USA) overnight at 4℃ followed by Goat Anti-Rabbit IgG secondary antibody (cat.no. ab205718, Abcam, USA). The morphologic changes in the nuclei were detected using a confocal laser scanning microscope (Olympus FluoviewFV1000; Olympus Corporation, Tokyo, Japan) (Microscopic magnification 200×).

2.6. Cell migration assay

The effect of TCS on HeLa and caski cells migration was measured by wound healing assays. HeLa and caski cells were grown in culture plates and reached 80% confluence. Subsequently, a 10-μl pipette tip was used to scratch the cell surface. Then, cells were treated with TCS (20 μg/ml) for 48 h. The width of the wound was photographed at 0 and 24 h under a microscope.

2.7. Cell invasion assay

The effect of TCS on HeLa and caski cells invasion was measured by transwell assays. HeLa and caski cells were grown in culture plates, and treated with TCS (20 μg/ml) for 48 h. Then, the cells (1×10^5) were seeded into the upper chamber of a Transwell insert and the lower chamber was added medium containing 20% FBS, and the cells were cultured for 24 h. The cells that invaded into the outer surface of the Transwell insert were stained with 0.5% crystal violet solution and counted using an inverted microscope.

2.8. Western blotting

After culture with TCS (20ug/ml) for 48 h, HeLa and caski cells were lysed using RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA). The protein concentration was detected with the BCA reagent (KeyGEN Biotech). Equal amounts of protein were loaded on 10% SDS-polyacrylamide gels (SDS-PAGE; Bio-Rad Laboratories, Inc.) following transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 h at room temperature with 5% non-fat milk, then incubated with primary antibody : anti-caspase-3 (cat.no.9662, Cell Signaling Technology, Danvers, MA), anti-p-STAT5 (cat.no. 4322, Cell Signaling Technology, Danvers, MA), anti-STAT5 (cat.no. 94205, Cell Signaling Technology, Inc., Danvers, MA), anti-Bcl-2 (cat.no. ab32124, Abcam), anti-p-C-myc (cat.no. ab185656, Abcam), anti-C-myc (cat.no. ab32072, Abcam), E-Cadherin (cat.no. 14472, Cell Signaling Technology, Inc., Danvers, MA), N-Cadherin (cat.no. 13116, Cell Signaling Technology, Inc., Danvers, MA), Snail (cat.no. 3879, Cell Signaling Technology, Inc., Danvers, MA), anti-β-actin (cat.no. ab8226, Abcam) overnight at 4℃. Then, the membranes were incubated with a horseradish peroxidase-conjudated secondary antibody (Anti-rabbit IgG, cat.no. #7074, Cell Signaling Technology, Inc.; Anti-Mouse IgG H& L (HRP), cat.no. ab205719, Abcam) at room temperature for 2 h, and bands were detected by using ECL detection system (Amersham Life Science).

2.9. Statistical analysis

Statistical analysis was performed using the SPSS version 16.0 (SPSS,Inc., Chicago, IL, USA). The results were expressed as mean ± SEM. The difference between groups were analysis by Student's t-test or ANOVA. A P value < 0.05 was considered statistically significant.

3. Results

3.1. TCS inhibited the cell viability of cervical cancer cell lines in a time and dose dependent manner

To detect the effects of TCS on cervical cancer cells viability, the HeLa and caski cells were cultured with 0, 10, 20, 40, 80 and 120 μg/ml TCS for 24 and 48 h. CCK-8 assay results showed that TCS treatment inhibited the cell viability of HeLa (Fig. 1A) and caski cells (Fig. 1B) in a time and dose-dependent manner. Observed under a microscope, the morphological changes also confirmed the inhibition of HeLa and caski cells by TCS. As shown in Fig. 1C-D, cytoplasmic shrinkage, membrane blebbing and nuclear condensation was observed.

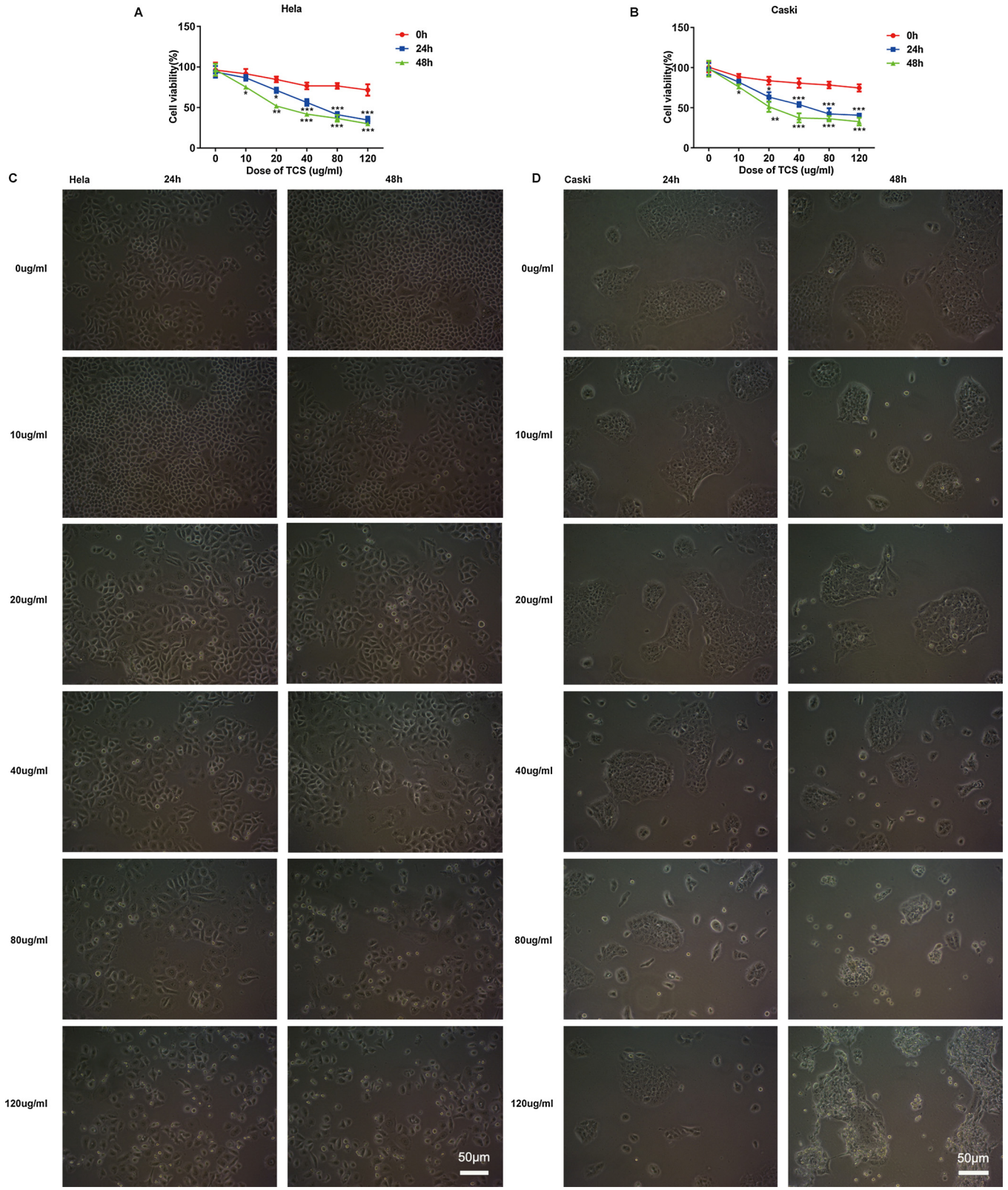

Fig. 1. TCS inhibited the cell viability of cervical cancer cell lines in a time and dose dependent manner. HeLa and caski cells were cultured with 0, 10, 20, 40, 80 and 120 μg/ml TCS for 24 and 48. HeLa (A) and caski cells (B) viability was detected by CCK-8 assay. Morphologic changes of HeLa (C) and caski cells (D) were observed under a microscope (Microscopic magnification 100×). Results are expressed as mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with non-treated group. TCS, Trichosanthin.

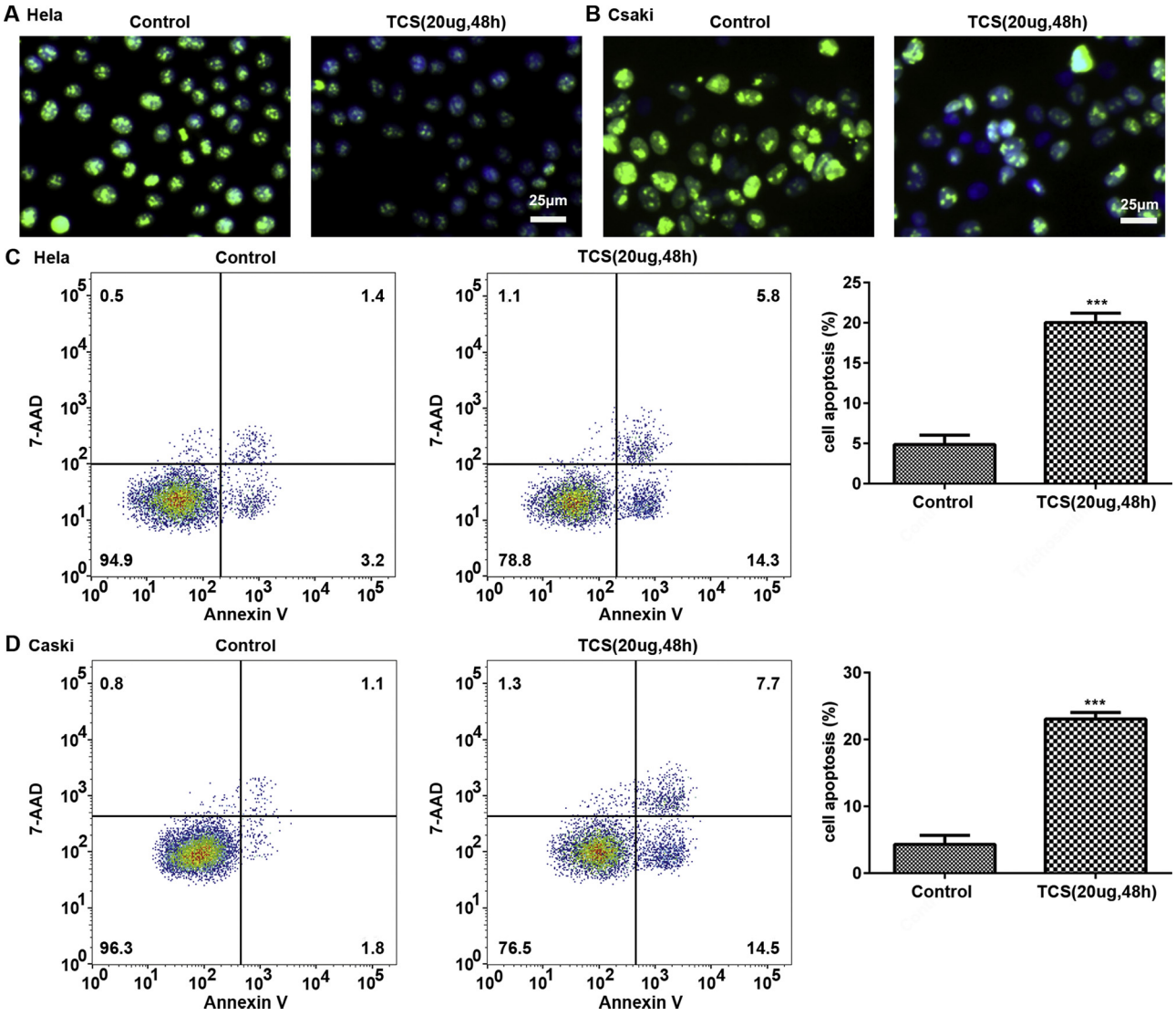

Fig. 2. TCS inhibited proliferation and induced apoptosis of cervical cancer cells. HeLa and caski cells were cultured with 20ug/ml TCS for 48 h. Representative pictures of Ki67 staining of HeLa (A) and caski cells (B) (Microscopic magnification 200×). Flow cytometric analysis of the cell apoptosis of HeLa (C) and caski cells (D). Results are expressed as mean ± SEM of three independent experiments. $^{***}p < 0.001$ compared with control. TCS, Trichosanthin.

3.2. TCS inhibited proliferation and induced apoptosis of cervical cancer cells

To investigate the effects of TCS on cervical cancer cells proliferation, the HeLa and caski cells were cultured with 20ug/ml TCS for 48 h and stained with anti-Ki-67. Ki67 staining results showed that compared with the control group, TCS treatment resulted in a significantly decrease of the number of Ki67-positive cell in HeLa (Fig. 2A) and caski cells (Fig. 2B). Then, we examined whether apoptosis was involved in the inhibition of proliferation. Flow cytometric analysis results showed that TCS treatment significantly increased the apoptotic percentages of HeLa (Fig. 2C) and caski cells (Fig. 2D) compared with the non-treated group.

3.3. TCS inhibited migration, invasion and EMT of cervical cancer cells

Wound healing assay results showed that compared with the control group, 20 μg/ml TCS treatment for 48 h significantly inhibited HeLa (Fig. 3A) and caski cells (Fig. 3B) migration. In addition, transwell assays was conducted to evaluate the effect of TCS on cell invasion. As shown in Fig. 3C-D, 20 μg/ml TCS treatment for 48 h significantly inhibited HeLa (Fig. 3C) and caski cells (Fig. 3D) invasion compared with the non-treated group. Furthermore, western bolt results showed that the protein levels of E-cadherin was significantly increased and the protein levels of N-cadherin and Snail were significantly decreased in HeLa (Fig. 3E) and caski cells (Fig. 3F) after treatment with 20 μg/ml TCS for 48 h, indicating that TCS could inhibit cervical cancer cells EMT.

3.4. TCS inhibited the activation of STAT5/C-myc signaling pathway

To investigate the mechanism of TCS on cell apoptosis, we determined the expression of phosphorylated STAT5, phosphorylated C-myc, Bcl-2, and caspase-3. As shown in Fig. 4A-B, the protein levels of p-STAT5, p-C-myc and Bcl-2 were significantly decreased and the protein level of caspase-3 was significantly increased in HeLa (Fig. 4A) and caski cells (Fig. 4B) after treatment with 20 μg/ml TCS for 48 h. These results indicate that TCS significantly inhibited the activation of STAT5/C-myc signaling pathway.

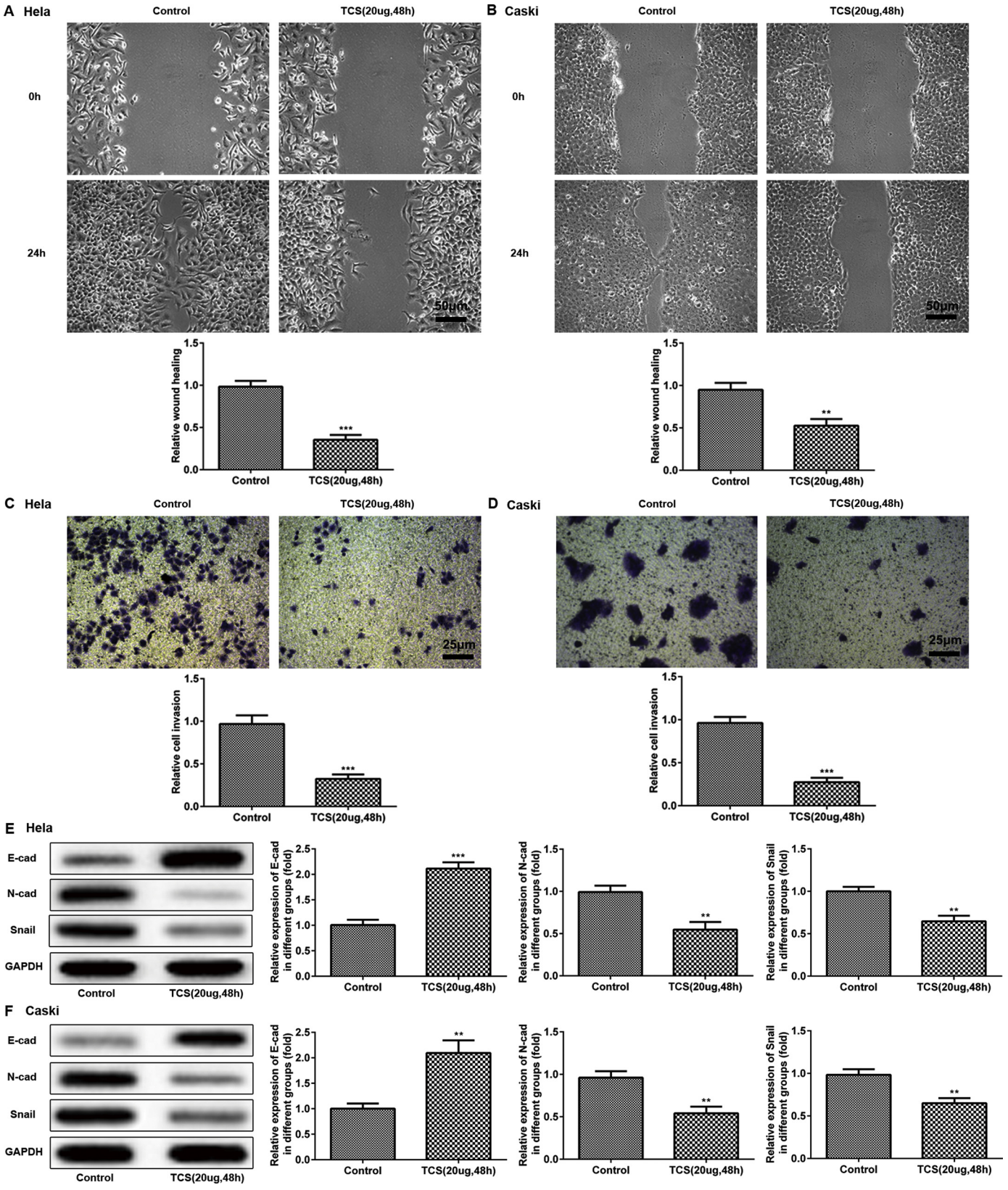

Fig. 3. TCS inhibited migration, invasion and EMT of cervical cancer cells. HeLa and caski cells were cultured with 20ug/ml TCS for 48 h. The effects of TCS on HeLa (A) and caski cells (B) migration were measured using wound healing assays. (Microscopic magnification 100×). The effects of TCS on HeLa (C) and caski cells (D) invasion were measured using Tanswell assays (Microscopic magnification 200×). The effects of TCS on the protein expression of E-cadherin, N-cadherin, and Snail in HeLa (E) and caski cells (F) were determined by western blotting. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control. TCS, Trichosanthin. E-cad, E-cadherin, N-cad, N-cadherin.

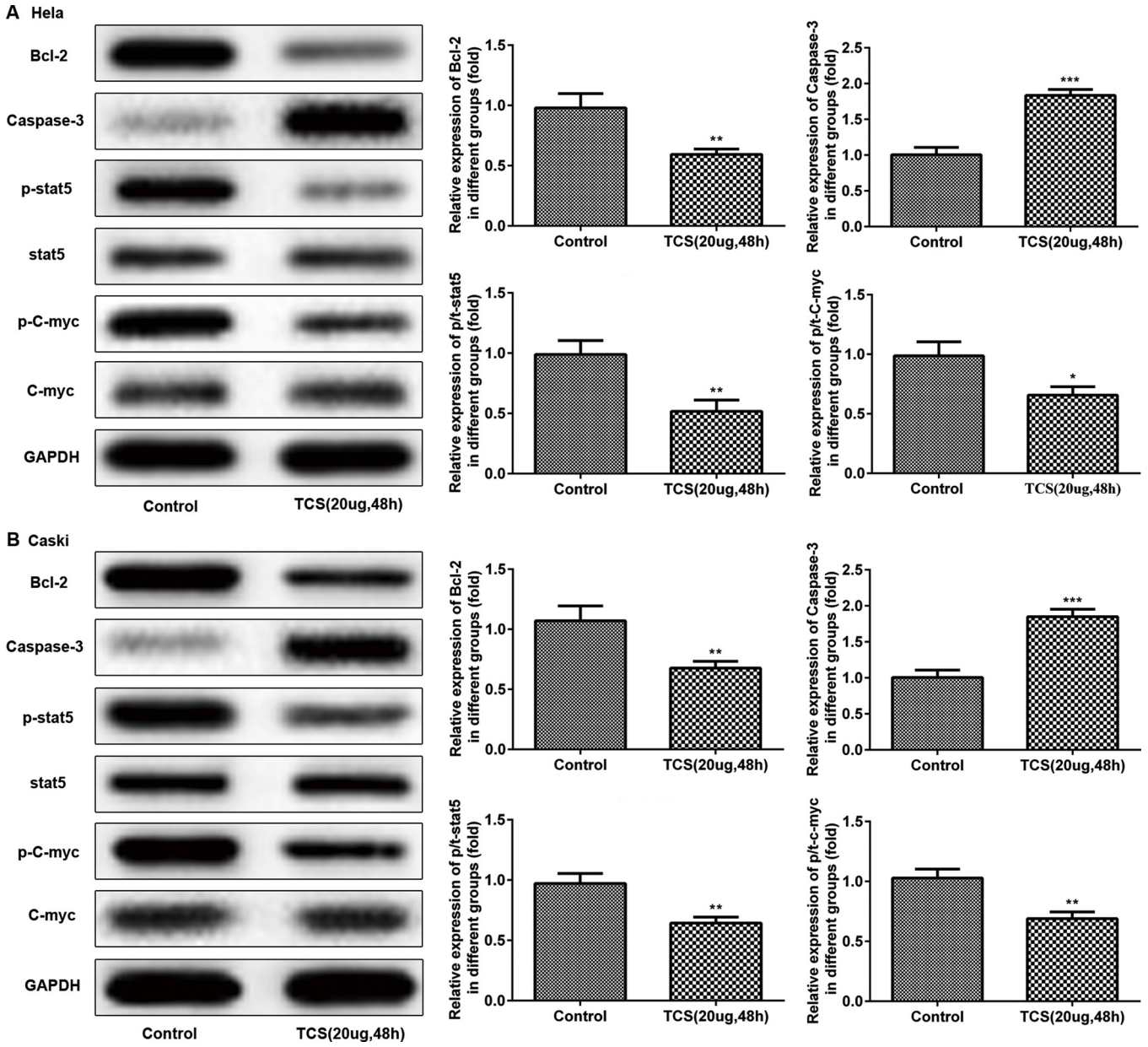

Fig. 4. TCS inhibited STAT5/C-myc signaling pathway in cervical cancer cells. HeLa and caski cells were cultured with 20ug/ml TCS for 48 h. The protein levels of Bcl-2, caspase-3, p-STAT5, STAT5, p-C-myc, C-myc in HeLa (A) and caski cells (B) were determined by western blotting. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control. TCS, Trichosanthin.

4. Discussion

Cervical cancer is a leading cause of cancer-related deaths in women worldwide [16]. Experimental evidence demonstrating that TCS has antitumor activity in several cancer, including cervical cancer [7]. The present study demonstrated that TCS was able to reduce HeLa and caski cells viability with a dose and concentration dependent manners. In addition, TCS inhibited HeLa and caski cells proliferation, migration and EMT. Furthermore, TCS induced HeLa and caski cells apoptosis was accompanied by decrease in protein levels of p-stat5, p-C-myc and Bcl-2 and increase in caspase-3 protein levels.

To date, the exact role and mechanisms of TCS antitumor activity in cervical cancer remains unclear. It has been reported that TCS exerts antitumor activity by inducing apoptosis and inhibiting cell proliferation [6,17]. Previous studies has reported that TCS could induce the death of Hela cells by increasing cytosolic calcium, accompanied by suppressing cAMP/protein kinase C levels [18] and inhibits Hela cells proliferation by suppressing the PKC/MAPK signaling pathway [19]. Consistent with previous study, the present study results showed that TCS significantly inhibited cell viability and proliferation of HeLa and caski cells. Previous study has demonstrated that induction of tumor cells apoptosis is an important mechanism of action for many antitumor drugs [20]. In the present study, the percentage of apoptotic cell was consistent with the proliferation of cervical cancer cells with the treatment of TCS. These results indicated that the inhibitory effect of TCS on HeLa and caski cells was mediated by inducing apoptosis.

STAT5 is a versatile transcription, which has been reported involve in promoting cell cycle progression, cell transformation and inhibiting cell apoptosis and plays important roles in development and progression of tumors [21]. Previous study has demonstrated that constitutively activated STAT5 in several cancer including cervical cancer [7,8]. It was also found that constitutive STAT5 activation correlates with better survival in cervical cancer patients treated with radiation therapy [22]. Previous study has reported that Epo-induced cervical

cancer cells proliferation was associated with the activation STAT5 [23]. In order to further investigate mechanism of TCS-induced apoptosis, we detected the effects of TCS on STAT5 activation. We observed that TCS treatment significantly downregulated the phosphorylation of STAT5 and causes downregulation of target genes that control several key events such as cell proliferation, apoptosis as a result of deregulation of p-C-myc and Bcl-2 proteins and upregulation of caspase-3.

In conclusion, we found that TCS inhibited proliferation and induced apoptosis in HeLa and caski cells, and inhibited the activation STAT5/C-myc pathway. These results indicate that inhibition of STAT5/C-myc signaling pathway may be a mechanism of TCS inhibition of cervical cancer growth

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None.

Conflict of interest

The authors declare no conflict of interest.

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