

Emodin Inhibits Colon Cancer Cell Invasion and Migration by Suppressing Epithelial–Mesenchymal Transition via the Wnt/ β -Catenin Pathway

Juan Gu,* Chang-fu Cui,† Li Yang,‡ Ling Wang,* and Xue-hua Jiang*

*Department of Clinical Pharmacy, West China School of Pharmacy, Sichuan University, Sichuan, P.R. China

†Department of Neurology, Research Institute of China Weapons Industry, 521 Hospital, Shanxi, P.R. China

‡Microbiological Laboratory, Xinyang Vocational and Technical College, Henan, P.R. China

Colon cancer (CC) is the third most common cancer worldwide. Emodin is an anthraquinone-active substance that has the ability to affect tumor progression. Our study aims to explore the effects and the relevant mechanism of emodin on the invasion and migration of CC in vitro and in vivo. In our study, we found that emodin inhibited the invasion and migration abilities of RKO cells and decreased the expression of matrix metalloproteinase-7 (MMP-7), MMP-9, and vascular endothelial growth factor (VEGF) in a dose-dependent manner. Further research suggested that emodin inhibited EMT by increasing the mRNA level of E-cadherin and decreasing the expression of N-cadherin, Snail, and β -catenin. Emodin also significantly inhibited the activation of the Wnt/ β -catenin signaling pathway by downregulating the expression of related downstream target genes, including TCF4, cyclin D1, and c-Myc. A Wnt/ β -catenin signaling pathway agonist abolished the effect of emodin on EMT and cell mobility, suggesting that emodin exerted its regulating role through the Wnt/ β -catenin pathway. The CC xenograft model was established to study the antitumor efficiency of emodin in vivo. The in vivo study further demonstrated that emodin (40 mg/kg) suppressed tumor growth by inhibiting EMT via the Wnt/ β -catenin signaling pathway in vivo. Taken together, we suggest that emodin inhibits the invasion and migration of CC cells in vitro and in vivo by blocking EMT, which is related with the inhibition of the Wnt/ β -catenin signaling pathway.

Key words: Emodin; Colon cancer (CC); Invasion; Migration; Epithelial–mesenchymal transition (EMT); Wnt/ β -catenin

INTRODUCTION

Colon cancer (CC) is the third most common tumor in the world¹. In 2014, 136,830 new cases of CC were identified worldwide, and 50,310 patients died¹. CC can be cured in the early stages with a 5-year survival rate of over 90%; however, the 5-year survival rate was less than 10% for late stage CC². The mortality of CC is increasing yearly in our country due to its long latent period and lack of effective treatment³. Hence, searching for efficient therapeutics is the key for the prevention and treatment of CC.

Studies show that about 90% of all CC deaths are associated with cancer metastasis, which is commonly relevant with epithelial–mesenchymal transition (EMT)^{4–6}. EMT has been increasingly demonstrated to play important roles in promoting carcinoma invasion and metastasis⁷. The intercellular tight junction disappears, the cellular backbone is reconstituted, cellular morphology is changed, and the genotype is recombinant during the

process of EMT. The series of alterations increase the movement and invasion of cancer cells, promote cancer cells to separate from the solid tumor tissue, and enter into the systemic circulation, thus endowing the tumor cells with the capability of invasion and migration^{5,8,9}. The expressions of E-cadherin, claudin, β -catenin, and cytokeratin are reduced, and the expression of mesenchymal cell markers Snail, vimentin, and N-cadherin are increased during EMT¹⁰. Previous research revealed that eribulin mesilate suppressed experimental metastasis of breast cancer cells by regulating EMT and mesenchymal–epithelial transition (MET)¹¹. Others have demonstrated that geraniin inhibits transforming growth factor- β (TGF- β)-induced EMT to suppress the migration, invasion, and anoikis resistance of A549 lung cancer¹². Kim et al. also revealed that dexamethasone has inhibitory effects on cell migration and invasion by suppressing EMT of CC cell lines in hypoxic condition¹³.

Address correspondence to Xue-hua Jiang, Department of Clinical Pharmacy, West China School of Pharmacy, Sichuan University, No. 3, Section 17, Renmin South Road, Wuhou District, Chengdu, Sichuan 646000, P.R. China. Tel: 028-85501370; E-mail: juanjuan20172018@163.com

Multiple signaling pathways are involved in the activation of EMT, including NF- κ B, Notch, RTK/Ras, and Wnt/ β -catenin signal pathways^{14,15}. Huynh et al. reported that destruxin B inhibited the growth of hepatocellular carcinoma cells by regulating EMT via the Wnt/ β -catenin signaling pathway¹⁶. Another report revealed that nicotine induced EMT via Wnt/ β -catenin signaling in human airway epithelial cells¹⁷. It has also been documented that the activation of the Wnt/ β -catenin signaling pathway regulates the proliferation and growth of CC cells by promoting EMT^{18,19}.

Emodin, an active substance categorized to anthraquinone, is widely found in *Rheum*, *Polygonum*, and *Cassia*^{20,21}. Reports suggest that emodin has various biological activities such as anti-inflammation, antiapoptosis, and liver protection^{22,23}. It is also reported that emodin shows antitumor efficiency in several kinds of cancers, such as pancreatic cancer²⁴, prostate cancer, and lung cancer²⁵. Emodin was reported to inhibit the proliferation of CC cells and promote the apoptosis of tumor cells by modulating the synthesis of fatty acids²¹. Hu et al. reported that emodin inhibits EMT in epithelial ovarian cancer cells by regulating the GSK-3 epithelial ovarian cancer pathway²⁶. However, it remains unknown whether emodin could affect the invasion and migration of CC cells by regulating EMT.

Our study examined the effects of emodin on the invasion and migration abilities of CC cells and the related mechanism. Our results suggested that emodin inhibited CC cell invasion and migration *in vitro* and *in vivo*. Further study revealed that emodin inhibited the invasion and migration of CC cells by suppressing EMT via the inactivation of the Wnt/ β -catenin signaling pathway, providing new horizons for CC treatment.

MATERIALS AND METHODS

Cell Culture

Human CC cell lines HT29 and RKO were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). HT29 and RKO cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) and were incubated at 37°C in the atmosphere of 5% CO₂.

MTT Assay

Cell viability was examined through the MTT assay. HT29 and RKO cells were seeded into 96-well plates with 2×10^3 cells per well in triplicate and were cultured overnight. Different concentrations of emodin (0, 5, 10, 20, 40, 60, 80, and 100 μ mol/L) were added into the wells, respectively. After incubation for 24 and 48 h, MTT reagent was added into the wells, and the cells were

cultured in the incubator for 4 h. Discarding the supernatant, dimethyl sulfoxide (DMSO) was used to solubilize the formazan. The absorbance was measured with a microplate reader.

Transwell Invasion Assay

The 6.5-mm-diameter chambers with pore size of 8.0 μ m (Corning, Corning, NY, USA) were used for the invasion assay. RKO cells were seeded in the upper Transwell chamber, and different concentrations of emodin (5, 10, and 20 μ mol/L) in DMEM containing 10% FBS were added into the reservoir. After culturing for another 24 h, the filter side of the upper chamber was cleaned with a cotton swab, and the filter was stained with hematoxylin. Ten visual fields were randomly selected for statistics under microscopy.

Wound Healing Assay

Cells were seeded into six-well plates (5×10^5 /well) and were cultured for 24 h. A sterile pipette tip was used to create a straight scratch to form a wound after the cells reached 90%–100% confluence. The destroyed cells were rinsed off with PBS three times gently, and then emodin was added to the cells at the indicated concentrations in serum-free medium, and the cells were cultured for another 24 h. Then the cells that migrated to the wounded area were visualized, and images were captured at 0 and 24 h, respectively.

Western Blot

Total protein from cells and tissues was extracted with phenylmethane sulfonyl fluoride (PMSF) containing protein lysis buffer. The protein concentrations were adjusted according to the total protein amount quantified with a BCA assay kit (Sangon, Shanghai, P.R. China). The same amount of protein was loaded for each sample and used for SDS-PAGE. Separated proteins were then transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Millipore, Boston, MA, USA). The membrane was blocked in 5% milk for 1 h and then incubated with primary antibody (from Abcam, Cambridge, MA, USA) at 4°C overnight. After incubation with enzyme-conjugated secondary antibodies at room temperature (RT) for 1 h, enhanced chemiluminescence (ECL) was added for the sensitizer and imaging, with GAPDH as the internal control.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA from CC cells was extracted by the TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized with a reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Then stem-loop qRT-PCR was performed using SYBR Premix Ex Taq™

(TaKaRa, Shiga, Japan) according to the manufacturer's protocol. The primers applied for E-cadherin were as follows: 5'-CGGACGATGATGTGAACACC-3' (forward) and 5'-TTGCTGTTGTGCTTAACCCC-3' (reverse). The relative level was calculated by the relative quantification ($2^{-\Delta\Delta Ct}$) method. U6 small nuclear RNA was used as an internal normalized reference.

In Vivo Antitumor Study

All animal experiments were carried out in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC). Balb.c nude mice (4 weeks, male) were purchased from Shanghai Experimental Animal Center, Chinese Academy of Science. After 7 full days to adjust, the nude mice were randomly divided into the control group and the emodin (40 mg/kg)-treated group (15 mice in each group). Emodin was administered by PO for 7 days, and then 2×10^6 RKO cells were subcutaneously inoculated into the inguinal region of the nude mice. The nude mice were followed for 25 days, and the tumor growth curve was monitored every 5 days.

Immunohistochemistry Analysis

Paraffin sections of the tumor tissue in the nude mice from the control group and the emodin-treated group were obtained, followed by deparaffinization and incubation with 5% goat serum for 2 h. Primary antibody (VEGF; 1:200; Abcam) was then added and incubated at 4°C overnight, followed by incubation with biotin-labeled secondary antibody (Abcam) for 1 h. After washing in Tris-hydrochloric acid buffer (TBS), the slides

were incubated with peroxidase-conjugated streptavidin complex reagent (Dako, Denmark) and developed with 3,3'-diaminobenzidine for 5 min. The slides were counterstained and dehydrated. Proteins were visualized using microscopy.

Statistics

All data were analyzed with GraphPad Prism 6.0, and the results were represented with mean value \pm standard deviation (SD). ANOVA analysis was applied if the data were compliant with normal distribution and variance homogeneity; otherwise, the Kruskal–Wallis test was used. A value of $p < 0.05$ suggests significant difference.

RESULTS

Cytotoxicity Detection for Emodin

To confirm the effects of emodin on CC cell viability, MTT assay was applied after HT29 and RKO cells were treated with different concentrations of emodin (5, 10, 20, 40, 60, 80, and 100 $\mu\text{mol/L}$) for 24 and 48 h, respectively. The structural formula of emodin is shown in Figure 1A. As shown in Figure 1B, emodin showed inhibitory effects on HT29 and RKO cells in a dose-dependent manner. The cytotoxicity of emodin on HT29 was higher than that on RKO cells, and there was more obvious cytotoxicity when the cells were treated for 48 h compared with 24 h. Based on preclinical study in vitro experimental guidelines, emodin at 5, 10, and 20 $\mu\text{mol/L}$ has no significant damage to cell viability, and the RKO cell line was selected for the following study.

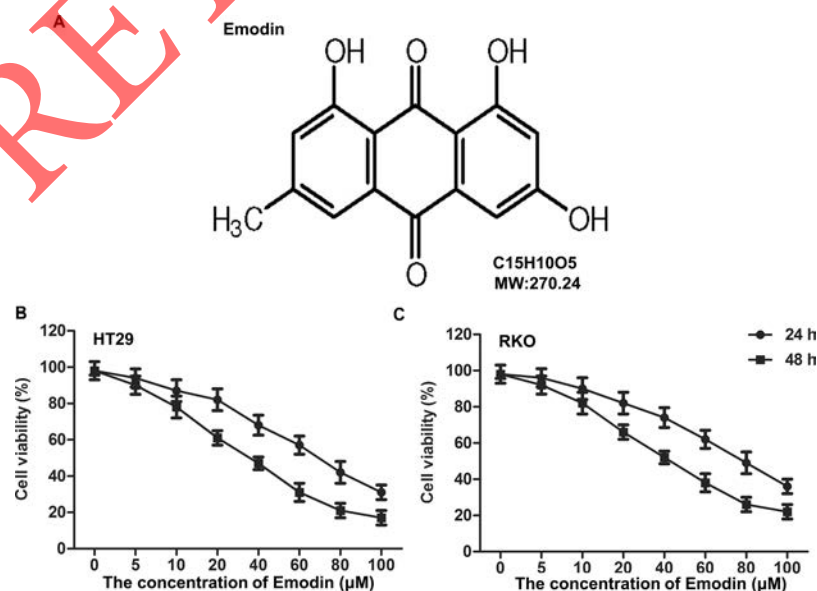


Figure 1. Cytotoxicity detection for emodin. Colon cancer (CC) cell lines HT29 and RKO were cultured and treated with different concentrations of emodin (0, 5, 10, 20, 40, 60, 80, and 100 $\mu\text{mol/L}$) for 24 and 48 h, respectively. (A) Chemical structure of emodin. The effects of emodin at different concentrations on HT29 (B) and RKO (C) cell viability were measured through the MTT assay.

The Inhibiting Effect of Emodin on Invasion and Migration of CC Cells

The inhibiting effect of emodin on cell invasion and migration has been reported in multiple cancer types^{25,27,28}, but how it works in CC cells has been rarely studied. The Transwell invasion assay and wound healing assay showed that emodin suppressed cell invasion and migration abilities in RKO cells in a dose-dependent manner ($p < 0.05$, $p < 0.01$) (Fig. 2A–C). The expressions of invasion and migration marker proteins [matrix metalloproteinase-7

(MMP-7), MMP-9, and VEGF] were also obviously inhibited by emodin, the inhibiting effect enhanced with emodin concentration increase ($p < 0.05$, $p < 0.01$) (Fig. 2D–G). The results suggested that emodin could inhibit the invasion and migration of CC cells in a dose-dependent manner.

Emodin Inhibited Cell Mobility by Suppressing EMT in CC Cells

EMT is an indispensable process for tumor cells to realize invasion and migration¹⁴. The inhibitory effects of

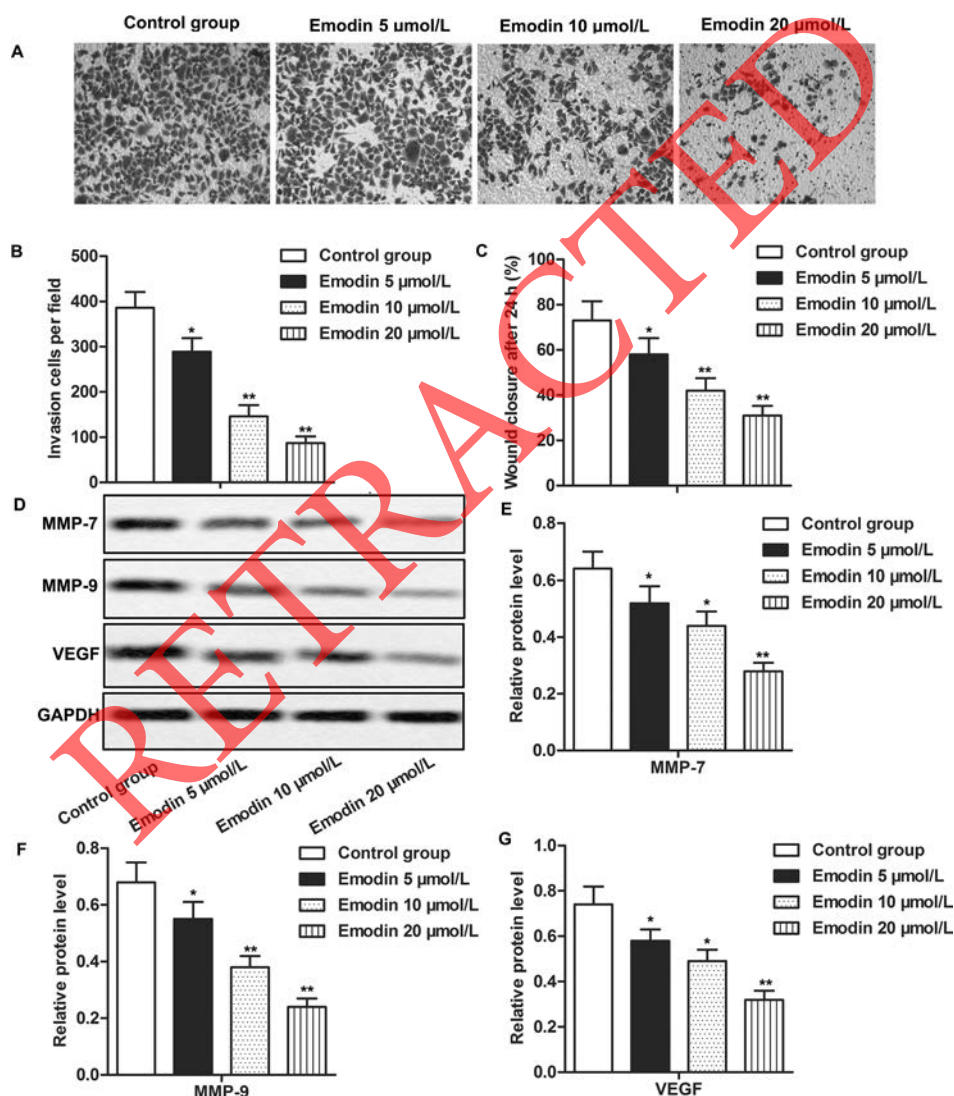


Figure 2. The inhibiting effect of emodin on invasion and migration of CC cells. RKO cells were divided into the control group and the emodin-treated groups. Cells in the control group were treated with vehicle, while cells in the emodin-treated groups were treated with different concentrations (5, 10, and 20 µmol/L) of emodin for 24 h. (A, B) The effects of emodin at different concentrations on the invasion of RKO cells were measured by Transwell invasion assay. (C) The effects of emodin at different concentrations on the migration of RKO cells as measured by wound healing assay. (D) The expression of MMP-7, MMP-9, and vascular endothelial growth factor (VEGF) was evaluated by Western blot. (E–G) Quantifications of (D). Each group was set up with triplicates, and all the experiments were repeated at least three times. GAPDH was used as an internal reference. * $p < 0.05$, ** $p < 0.01$ versus the control group.

emodin on CC cell mobility have been confirmed by the Transwell invasion assay and wound healing assay; thus, we then set out to detect whether emodin could affect cell invasion and migration through regulating EMT in CC. As depicted in Figure 3A, the treatment of emodin obviously increased the mRNA levels of E-cadherin and significantly decreased the expression of Snail, N-cadherin, and β -catenin ($p < 0.05$, $p < 0.01$) (Fig. 3B–E). The effects were enhanced with increased emodin concentrations. The results suggested that emodin inhibited cell mobility by suppressing EMT in CC cells.

The Treatment of Emodin Inactivated the Wnt/ β -Catenin Signaling Pathway

In order to explore the effects of emodin on the Wnt/ β -catenin signaling pathway, the expressions of related downstream targeted genes (TCF4, cyclin D1, and c-Myc) of the Wnt/ β -catenin signaling pathway in RKO cells were analyzed through Western blotting. Compared with the control group, the expression of TCF4, cyclin D1, and c-Myc in the emodin treatment groups was obviously reduced in a dose-dependent manner ($p < 0.05$, $p < 0.01$, $p < 0.001$) (Fig. 4A–D), indicating the inhibiting effect of

emodin on the activation of the Wnt/ β -catenin signaling pathway.

Emodin Inhibits EMT by Modulating the Wnt/ β -Catenin Signaling Pathway

The Wnt/ β -catenin signaling pathway was reported to be involved in the modulation of mostly life processes and is one of the important pathways involved in the induction of EMT²⁹. We have demonstrated that emodin could inhibit CC cell invasion and migration, suppress EMT of cancer cells, and inactivate the Wnt/ β -catenin pathway. To further prove that emodin affects CC cell EMT and mobility by modulating the Wnt/ β -catenin pathway, agonist LiCl was used in our study to activate the Wnt/ β -catenin pathway. As shown in Figure 5A and B, emodin inhibited EMT by increasing the expression of E-cadherin and decreasing the expression of N-cadherin, but the addition of LiCl obviously reversed the effects of emodin on EMT marker proteins ($p < 0.01$, $p < 0.05$). LiCl significantly weakened the inhibitory effects of emodin on the expressions of MMP-7 and VEGF ($p < 0.01$, $p < 0.05$) (Fig. 5C and D). These above results further demonstrated that emodin

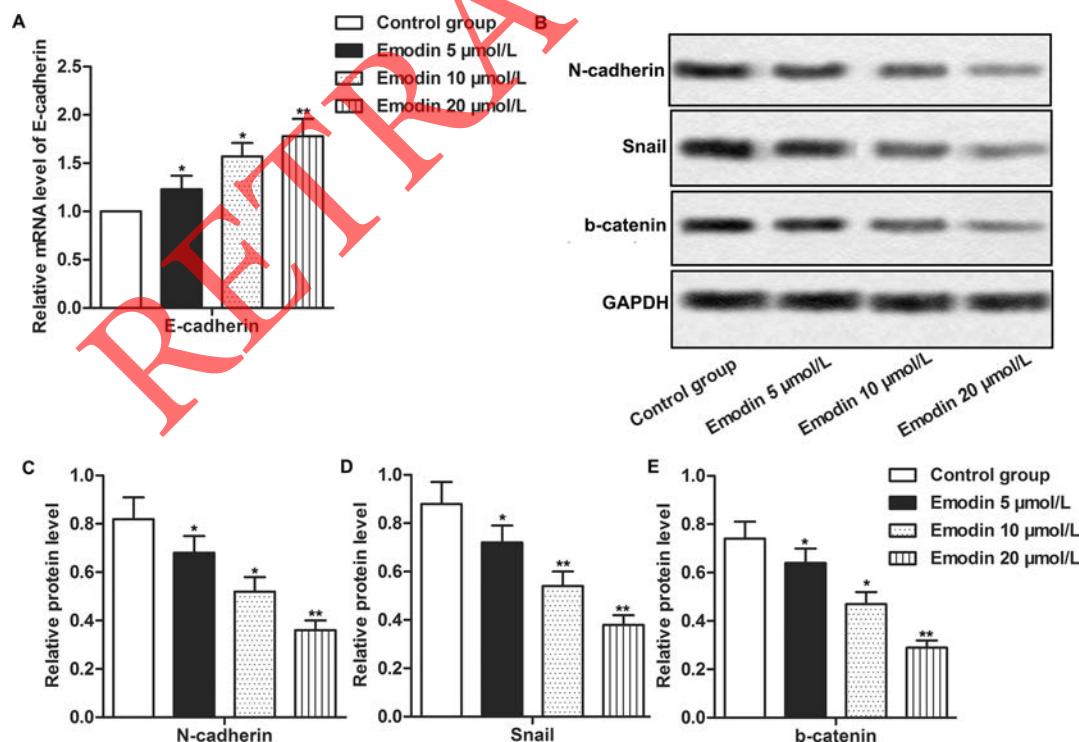


Figure 3. Emodin inhibited cell mobility by suppressing epithelial–mesenchymal transition (EMT) in CC cells. The cells in different groups were treated as previously described. (A) The mRNA expression of E-cadherin was measured by quantitative real-time polymerase chain reaction (qRT-PCR). (B) The expression of N-cadherin, Snail, and β -catenin was evaluated by Western blot. (C–E) Quantification of (B). All the experiments were repeated at least three times. GAPDH was used as an internal reference. * $p < 0.05$, ** $p < 0.01$ versus the control group.

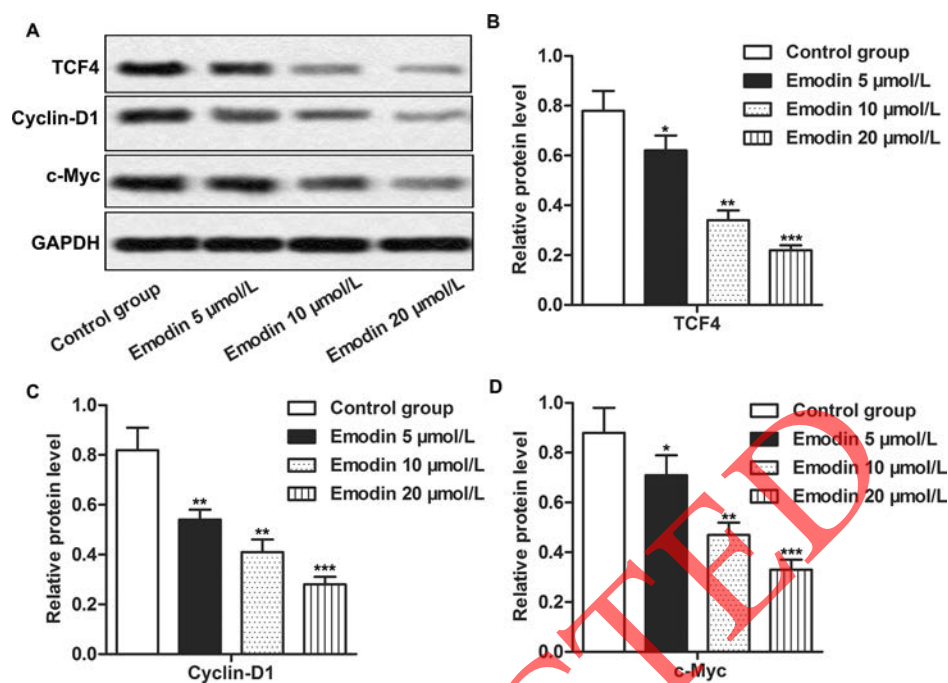


Figure 4. The treatment of emodin inactivated the Wnt/ β -catenin signaling pathway. Cells in each group were treated as previously described. (A) The expression of TCF4, cyclin D1, and c-Myc was measured by Western blot. (B–D) Quantification of (A). All the experiments were repeated at least three times. GAPDH was used as an internal reference. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the control group.

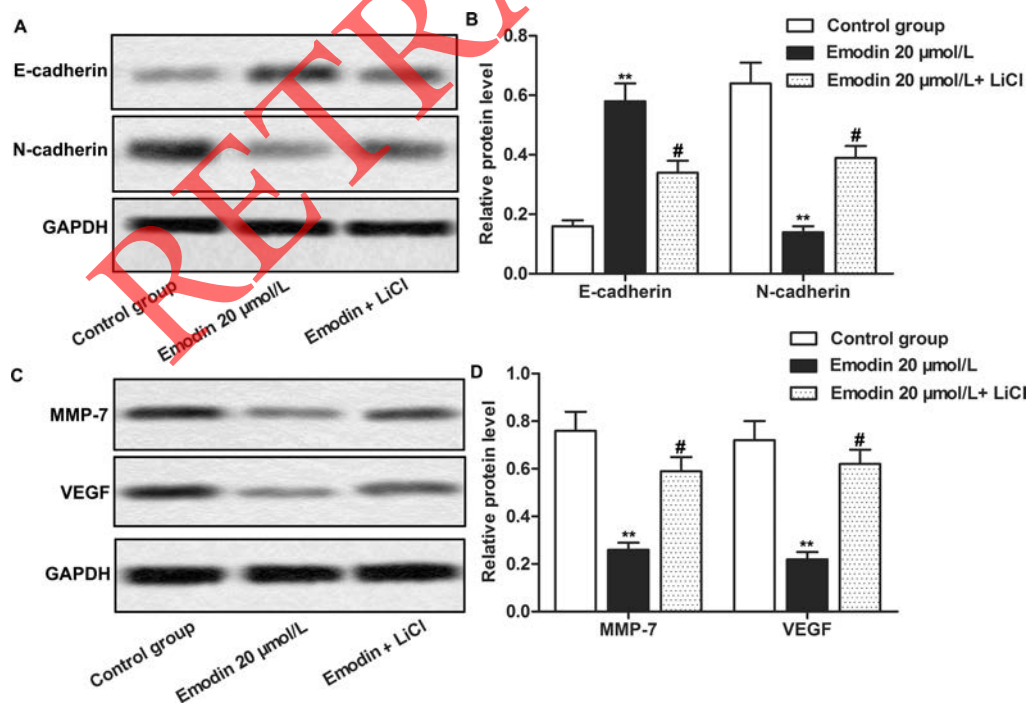


Figure 5. Emodin suppressed EMT by regulating the Wnt/ β -catenin signaling pathway. Cells in the control group were treated with vehicle. Cells in the experimental group were treated with 20 μmol/L emodin or with the combination of emodin (20 μmol/L) and LiCl (10 mmol/L) for 24 h. (A) The detection of E-cadherin and N-cadherin expression by Western blot. (B) Quantification of (A). (C) The detection of MMP-7 and VEGF expression by Western blot. (D) Quantification of (C). All the tests were repeated at least three times. GAPDH was used as an internal reference. ** $p < 0.01$ versus the control group, # $p < 0.05$ versus the emodin 20 μmol/L group.

inhibited the mobility and EMT of CC cells via inactivating the Wnt/ β -catenin signaling pathway.

Emodin Inhibited Tumor Growth by Suppressing EMT via Inactivating the Wnt/ β -Catenin Pathway In Vivo

Having explored the effect of emodin in CC cells, we further confirmed the above results in vivo; the CC xenograft model was established as described and was divided into the control group and the emodin-treated group. The significant inhibiting effect of emodin on tumor growth was shown in the emodin-treated group compared with the control group ($p < 0.05$, $p < 0.01$) (Fig. 6A).

Immunohistochemistry results showed that the proportion of VEGF⁺ cells was obviously decreased in the emodin-treated groups compared with the control group (Fig. 6B). Emodin obviously inhibited the expression of N-cadherin and promoted the expression of E-cadherin in CC tissues of the nude mice in the emodin-treated group ($p < 0.05$) (Fig. 6C and D). The expressions of TCF4, cyclin D1, and c-Myc were also reduced significantly in the emodin-treated groups compared with the control group ($p < 0.05$) (Fig. 6E and F). The results suggested that emodin could inhibit the invasion and migration of CC cells by suppressing EMT via inactivating the Wnt/ β -catenin signaling pathway in vivo.

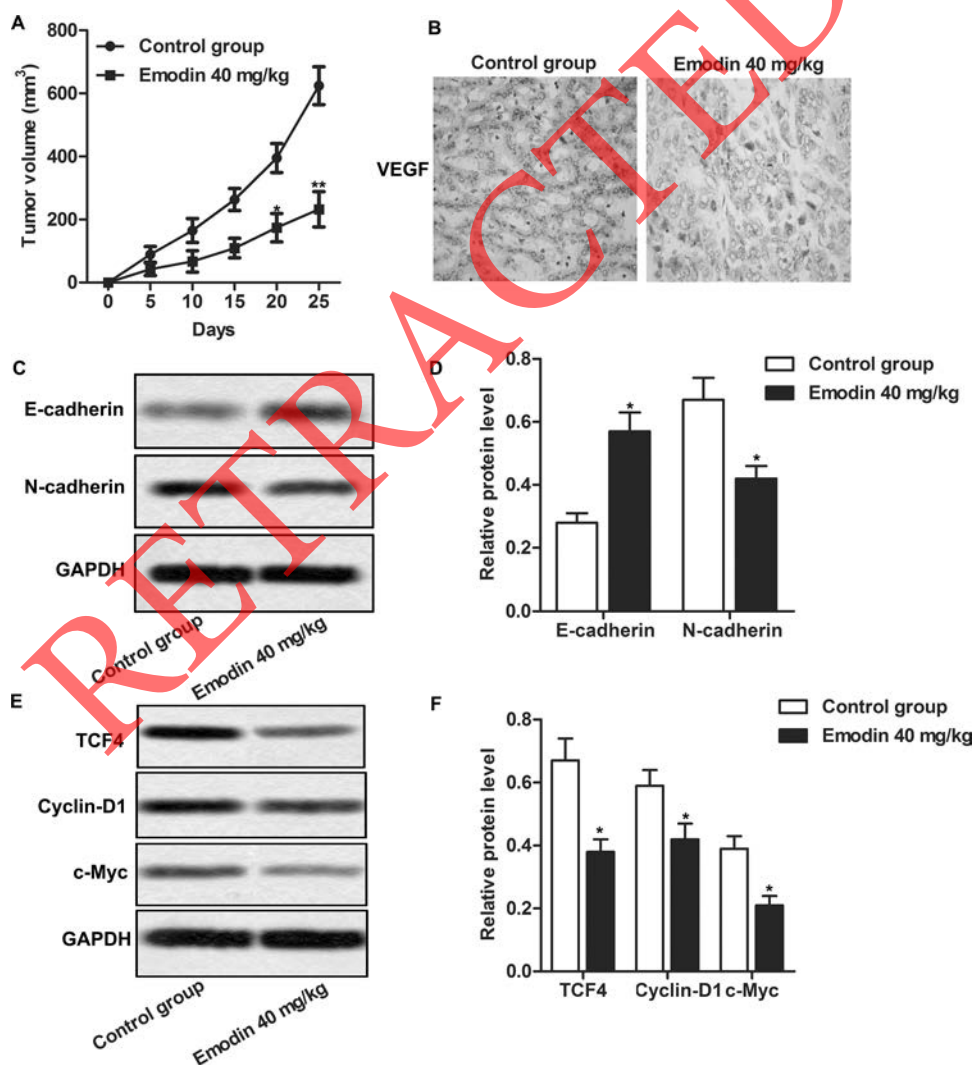


Figure 6. Emodin inhibited tumor growth by suppressing EMT via inactivating the Wnt/ β -catenin pathway in vivo. Animals were randomly divided into the control and emodin (40 mg/kg)-treated groups. After 7-day dosing of emodin by PO, 2×10^6 RKO cells were subcutaneously inoculated to the inguinal region of the mice to establish the CC xenograft model. (A) Tumor growth curves. (B) VEGF expression in tumor tissues by immunohistochemistry analysis. (C) Detection of E-cadherin and N-cadherin expression by Western blot. (D) Quantification of (C). (E) The effect of emodin on the expression of TCF4, cyclin D1, and c-Myc was valued by Western blot. (F) Quantification of (E). GAPDH was used as an internal reference. * $p < 0.05$ versus the control group.

DISCUSSION

Emodin is the active ingredient of the Chinese traditional drug rhubarb, which is usually applied for the treatment of diseases like sores and diarrhea in Chinese medicine³⁰. Modern science found that emodin showed relatively strong antibacterial efficiency, and it is applied in the treatment of infections caused by bacteria and fungi. The purified emodin is also used for the treatment of cancer including leukemia and gastric cancer³¹. Emodin was reported to promote apoptosis and inhibit the proliferation and metastasis of breast cancer cells^{32,33}. Emodin also showed antitumor efficiency on pancreatic cancer by inhibiting angiogenesis and promoting cell apoptosis³⁴. Our present study suggested that emodin showed antitumor efficiency on CC tumors by inhibiting EMT via the Wnt/ β -catenin pathway.

Tumor metastasis is the major cause of tumor development, so to try to find an effective way to inhibit the metastasis of cancer cells would be beneficial for anti-tumor treatments³⁵. It is reported that emodin inhibited cell invasion and migration in several tumor types^{25,27,36}. In support of previous research, our study showed that emodin inhibited the invasion and migration of CC cells significantly. MMP-7 and MMP-9 are important proteins that belong to the MMP family, which promote the cells' movement by degradation of the extracellular matrix, thus playing an important role in the invasion and migration of CC cells³⁷. As an important angiogenesis factor, VEGF is relevant with the angiogenesis of tumor tissue and could promote tumor metastasis by degrading the tumor cells' extracellular matrix via the activation of proteinase³⁸. Our study showed that emodin significantly inhibited the expression of VEGF in RKO cells and CC tumor tissue and also decreased the expression of MMP-7 and MMP-9 in RKO cells, indicating the inhibitory effects of emodin on CC cell metastasis.

Multibiological behaviors and signaling pathways are involved in the regulation of tumor metastasis, including EMT, CXCL12–CXCR4 signaling pathway, NF- κ B signaling pathway, and ILK/GSK-3 β /Slug signaling pathway^{28,39–41}. Therein EMT plays an important role in the metastasis of tumor cells. During EMT, tumor cells lose epithelial characteristics and reduce their expression of E-cadherin, tight junction protein, β -catenin, and other epithelial marker proteins; in contrast, they act as mesenchymal cells to obtain the invasive and migration abilities and stem cell characteristics⁴². Stress like hypoxia, strong acid, and lack of nutrition would induce EMT and promote the metastasis of tumor cells, thus deteriorating the disease⁴³. It was reported that emodin could inhibit the metastasis of ovary cancer cells and squamous cell carcinoma cells by modulating the EMT process^{26,44}. Our study showed that emodin suppressed EMT by upregulating

the mRNA level of epithelial marker protein E-cadherin and by reducing the expression of mesenchymal cell marker proteins, suggesting that emodin may inhibit CC cell invasion and migration by blocking EMT.

It is also reported that mutations of key modulators of the Wnt/ β -catenin signaling pathway are involved in approximately 90% of all CCs, indicating that abnormal activation of the Wnt/ β -catenin signaling pathway is closely involved in CC development⁴⁵. The Wnt/ β -catenin signaling pathway is involved in many different kinds of biological processes and is also an essential pathway to induce EMT. Blocking the activation of the Wnt/ β -catenin signaling pathway inhibited EMT by inducing epithelial differentiation⁴⁶. Therefore, searching for new drugs and effective methods to inhibit the activation of the Wnt/ β -catenin signaling pathway and inhibit the occurrence of EMT is the key to controlling the metastasis of cancer cells. It has been reported that emodin inhibited the activation of the Wnt signaling pathway in colorectal cancer cell lines⁴⁷. Our study found that emodin inhibited the expression of the Wnt/ β -catenin pathway downstream target genes including TCF4, cyclin D1, and c-Myc in both RKO cells and CC tumor tissues, identifying the inhibitory effect of emodin on the Wnt/ β -catenin pathway. The activation of the Wnt/ β -catenin pathway by the activator LiCl abolished the inhibitory effect of emodin on EMT and cell mobility. The above results further demonstrated that the activation of the Wnt/ β -catenin pathway benefited EMT and metastasis of CC cells, and emodin inhibited EMT and migration of CC cells by inactivating the Wnt/ β -catenin pathway.

In summary, we demonstrated that emodin could inhibit the invasion and migration of CC cells by suppressing EMT via the Wnt/ β -catenin signaling pathway, providing new rationale for the application of emodin in CC treatments.

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