

Original Article

Emodin reverses 5-Fu resistance in human colorectal cancer via downregulation of PI3K/Akt signaling pathway

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Abstract: Background: 5-Fu resistance is a major obstacle in the treatment of malignant tumors. Therefore, combination therapy is employed to overcome this limitation. Since it was demonstrated that emodin could resensitize breast cancer to 5-Fu treatment, we aimed to investigate if emodin could reverse 5-Fu resistant colorectal cancer (CRC) in the current study. Methods: For the aim to explore the effect of emodin on 5-Fu resistant CRC, 5-Fu-resistant cell line (SW480/5-Fu) was established. CCK-8 assay and Ki67 staining were performed to evaluate the effects of emodin in combination with 5-Fu on cell proliferation. Flow cytometry was used to detect the apoptosis of SW480/5-Fu cells. Additionally, the invasion and migration of SW480/5-Fu cells were tested by transwell assay and wound healing, respectively. Western-blot was performed to examine the protein expressions in SW480/5-Fu cells. Moreover, xenograft mice model was established to test the anti-tumor effect of emodin in combination with 5-Fu *in vivo*. Results: Emodin notably increased the anti-proliferation effect of 5-Fu in SW480/5-Fu cells. Similarly, the invasion and migration of SW480/5-Fu cells were further inhibited in the presence of emodin. In addition, the combination treatment (emodin plus 5-Fu) induced cell apoptosis via inhibiting Bcl-2 and activating cleaved caspase3 and Bax. Moreover, emodin reduced 5-Fu resistance in CRC via downregulation of PI3K/Akt signaling. Finally, *in vivo* study indicated that emodin could notably reverse 5-Fu resistance in CRC xenograft. Conclusion: Our research revealed that emodin could reverse 5-Fu resistance in CRC through inactivating PI3K/Akt signaling pathway *in vitro* and *in vivo*. Thus, this finding might provide a molecular basis for treating 5-Fu resistant CRC.

Keywords: Colorectal cancer, resistance, emodin, PI3K/Akt signaling pathway

Introduction

Colorectal cancer (CRC) is one of the most lethal human cancers all over the world. Moreover, it ranks fourth for cancer-related deaths among males [1]. The previous report indicated that over 1 million new cases of CRC occur every year, and the mortality rate of patients was about 33% [2]. Over 100,000 new cases of CRC occurred in USA in 2017 according to a previous report from the National Cancer Institute [3]. However, a recent study has indicated that the overall survival of advanced patients with CRC were only 23.7 months [4]. The major cause of the poor prognosis is 5-Fu resistant, which occurs in 50% patients with advanced CRC [5]. Since resistance to 5-Fu has been regarded as a major obstacle in chemotherapy of advanced CRC,

novel and safe treatment strategies could contribute to 5-Fu-based chemotherapies are urgently needed.

Emodin (1,3,8-trihydroxy-6-methylantra-quinone) is a natural product isolated from *Rheum palmatum* L. Previous study has reported that Emodin exhibited anti-tumor effect [6]. Additionally, emodin could resensitized HL-60/ADR cells to MDR [7], and emodin in combination with AZT exhibited inhibitory effects on cell growth of K562/ADM [8]. Besides, emodin has been reported to have the ability in reversing 5-Fu resistant breast cancer [9]. However, if emodin could reverse 5-Fu resistance in CRC remains unclear.

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is recognized as a key pathway in car-

cinogenesis [10]. Moreover, tyrosine kinase receptors, G-coupled protein receptors, or mutant RAS can drive PI3K/Akt pathway. Besides, the production of the lipid second messenger has been confirmed to be catalyzed by PI3K [11-14]. Recent study has found that PI3K could act as an important inducer of chemoresistance in CRC [15]. On the other hand, previous studies have reported that emodin downregulated PI3K/Akt signaling in cancer cells [6, 16, 17]. Therefore, we aimed to investigate if emodin could reverse 5-Fu resistance in CRC in the current study.

Material and methods

Cell culture and establishment of 5-Fu resistant cell line

SW480 cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI1640 (Thermo Fischer Scientific) with 10% FBS (Thermo Fischer Scientific), 1% penicillin (Thermo Fischer Scientific) and streptomycin (Thermo Fischer Scientific) at 37°C, 5% CO₂. In order to establish stable 5-Fu resistant cells, cells were treated with gradually increased concentration of 5-Fu (Sigma Aldrich, St. Louis, MO, USA). The stable 5-Fu-resistant cell line SW480/5-Fu was established according to the previous reference [18].

CCK-8 assay

To test the effect of 5-Fu on the growth of SW480 and SW480/5-Fu cells, cells were cultured in RPMI1640 containing 0, 3, 6, 9, 12 or 15 µM 5-Fu for 72 h. Then, SW480/5-Fu cells were cultured with 0, 3, 6, 9, 12 or 15 µM emodin (MedChemExpress, Monmouth Junction, NJ, USA) for 72 h. CCK-8 assay (Sigma-Aldrich) was performed to assess the cytotoxicity of 5-Fu, emodin or combination treatment according to the manufacturer's protocol. Briefly, SW480 or SW480/5-Fu cells (in the logarithmic growth phase) were seeded into 96-well plate overnight (3.0×10³ cells/well). After treatments, CCK-8 reagent was added into each well. After incubation for 4 h, 150 µl DMSO (Thermo Fischer Scientific) was added into each well for 10 min. The absorbance at 450 nm (A₄₅₀) was tested by Thermo Fischer Multiskan FC (Thermo Fischer Scientific).

Trypan blue staining

The trypan blue staining was performed to observe viable cells in a cell suspension as previously described [19]. First of all, 90 µl of cell suspension was added into a cryo-vial. Next, an equal volume of 0.4% trypan blue staining buffer (10 µl) was mixed with cell suspension. Then, the mixture was incubated for 5 minutes at room temperature. Finally, the number of cells was counted by using a conventional light microscope. Dead cells (stained blue) were detected.

Cell apoptosis assay

SW480/5-Fu cells were cultured in RPMI-1640 medium supplemented with 10% FBS at 37°C, 5% CO₂. Cells were treated with 5-Fu or/and emodin for 72 h, and the apoptosis in SW480/5-Fu cells was analyzed by flow cytometry as previously described [20].

Transwell assay

For cell invasion analysis, transwell assay was performed in this study. The upper chamber is pre-treated with 100 µl of Matrigel. SW480/5-Fu cells were seeded into the upper chamber in media with 1% FBS, and the density was adjusted to about 1.0×10⁶ cells per chamber. RPMI1640 medium with 10% FBS was added in the lower chamber. After incubation for 48 h at 37°C, the non-invading cells in the upper chamber and on the Matrigel were removed with a cotton swab. Then, cells in the lower chamber were stained with 0.1% crystal violet and counted at 3 different fields under a microscope (LEICADMLB2, Frankfurt, Germany).

Wound-healing assay

SW480/5-Fu cells were plated into a 24-well Cell Culture Cluster, and were allowed to grow to 80-90% confluence. Then, cells were underlined perpendicular to the cell culture plate with a small pipette head. After washing with PBS 3 times, serum-free medium was used for further culture, and the scratch widths at 0 and 24 h were recorded under an optical microscope.

Immunofluorescence staining assay

SW480/5-Fu cells were cultured on glass coverslips until 80% confluent and then fixed with

4% paraformaldehyde for 30 min. Next, cells were blocked with 10% fetal bovine serum (FBS, Thermo Fischer Scientific) for 30 min at room temperature and then incubated with anti-Ki67 antibody (Abcam; 1:500) at 4°C overnight, followed by incubation with goat anti-rabbit IgG (Abcam; 1:2000) at 37°C for 1 h. Then, the nuclei were stained with DAPI (Beyotime, Shanghai, China) for 5 min. Finally, the cells were observed under a fluorescence microscope (Nikon, Tokyo, Japan).

TUNEL staining

Apoptosis was also determined by the TUNEL assay according to the manufacturer's instructions. Briefly, paraffin sections were permeabilized, and then incubated with 50 µl TUNEL reaction mixtures in a wet box for 60 min at 37°C in the dark. For signal conversion, slides were incubated with 50 µl of peroxidase (POD) for 30 min at 37°C, and then incubated with 50 µl diaminobenzidine (DAB) substrate solution for 10 min at 25°C. Finally, the expression of apoptotic cells was observed under an optical microscope.

Western blot assay

Cells were lysed with RIPA plus buffer. Protein concentrations were quantified by the BCA protein assay kit (Beyotime). Proteins were resolved on 10% SDS-PAGE, then transferred to PVDF (Bio-Rad) membranes. After blocking with 5% skim milk for 1 h, the membranes were incubated with primary antibodies at 4°C overnight. Then, the membranes were incubated with secondary antibodies at room temperature for 1 h. Membranes were scanned by using an Odyssey Imaging System and analyzed with Odyssey v2.0 software (LICOR Biosciences, Lincoln, NE, USA). The primary antibodies used in this study as follows: anti-Bax (1:1000; Abcam), anti-Cleaved caspase 3 (1:1000; Abcam), anti-Bcl-2 (1:1000; Abcam), anti-ERK (1:1000; Abcam), anti-Akt (1:1000; Abcam) and anti-β-actin (1:1000; Abcam). β-actin was used as an internal control.

In vivo experiments

16 BALB/c nude mice (6-8 weeks old) were obtained from Vital River (Beijing, China). The mice were housed within a dedicated SPF facil-

ity. SW480/5-Fu cells were transplanted subcutaneously in each mouse according to the previous reference [21]. Once the tumor reached 200 mm³, treatments were initiated as follows: PEG400: ethanol: dextrose 5% in water (D5W) = 4:1:5 (vehicle); 5-Fu at 150 mg/kg of body weight (150 mg/kg 5-Fu) weekly, ip; emodin at 40 mg/kg of body weight (40 mg/kg emodin) daily (dissolved by PEG400), ip; combination of emodin with 5-Fu (5-Fu + emodin group) for 3 consecutive weeks. The tumor volume was measured weekly according to the reference [22]. Finally, body weight of each mouse was examined. Mice were sacrificed and the tumors were collected and weighted. Besides, immunohistochemistry (IHC) staining were performed. All *in vivo* experiments were performed in accordance with National Institutes of Health guide for the care and use of laboratory animals, following a protocol approved by the Ethics Committees of Shuyang Chinese Medicine Hospital.

Statistical analysis

To determine significant differences between multiple groups, measurement data were expressed as the mean ± standard deviation (SD). The comparisons among multiple groups were made with one-way analysis of variance (ANOVA) followed by Tukey's test. *P* < 0.05 was considered significant.

Results

Emodin resensitized SW480/5-Fu cells to 5-Fu

The chemical structure of emodin was showed in **Figure 1A**. First, 5-Fu resistant SW480 cell line (SW480/5-Fu) was constructed according to previous reference [8]. The result of CCK-8 assay indicated SW480/5-Fu exhibited chemoresistance to 5-Fu compared with the parental SW480 cells (**Figure 1B**). In addition, emodin dose-dependently decreased the proliferation of SW480/5-Fu cells (**Figure 1C**). Moreover, emodin notably resensitized SW480/5-Fu cells to 5-Fu, and 12 µg/mL 5-Fu plus 9 µM emodin induced about 50% growth inhibition (**Figure 1D**). Therefore, the combination of 12 µg/mL 5-Fu with 9 µM emodin was used in the following experiments. All these data demonstrated that emodin could resensitize SW480/5-Fu cells to 5-Fu.

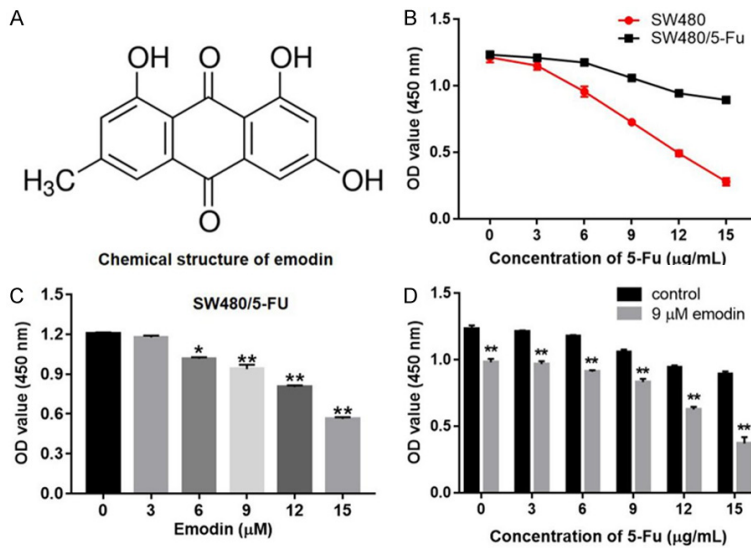


Figure 1. SW480/5-Fu cell line was successfully established. A. Chemical structure of emodin. B. SW480 and SW480/5-Fu cells were treated with 0, 3, 6, 9, 12 or 15 μg/ml 5-Fu for 72 h. C. SW480/5-Fu cells were treated with 0, 3, 6, 9, 12 or 15 μM emodin for 72 h. Then, the cell viability of SW480/5-Fu cells was measured by CCK-8 assay. D. SW480/5-Fu cells were treated with 0, 3, 6, 9, 12 or 15 μg/ml 5-Fu for 72 h with or without 9 μM emodin. The cell viability of SW480 and SW480/5-Fu cells was measured by CCK-8 assay. *P<0.05, **P<0.01 compared to 0 μM emodin.

Emodin resensitized SW480/5-Fu cells to 5-Fu via inducing cell apoptosis

Next, to confirm the effect of emodin in combination with 5-Fu on SW480/5-Fu cells, Ki67 immunofluorescence and trypan blue staining were used. The result suggested 5-Fu (12 μg/ml) had limited effect on cell proliferation of SW480/5-Fu, while emodin (9 μM) or emodin plus 5-Fu notably induced growth inhibition in SW480/5-Fu (Figure 2A and 2B). Similarly, trypan blue positive cell rate was significantly enhanced by the treatment of 5-Fu plus emodin (Figure 2C and 2D). In addition, the results of flow cytometry showed that 5-Fu alone slightly induced cell apoptosis, while the combination of emodin with 5-Fu significantly enhanced the apoptosis of SW480/5-Fu cells (Figure 3A). Moreover, the invasive ability of SW480/5-Fu cells was notably inhibited in the presence of 5-Fu or emodin (Figure 3B). As expected, the combination treatment exhibited better inhibitory effect on invasive ability of SW480/5-Fu cells, compared with 5-Fu or emodin alone (Figure 3B). In consistent, 5-Fu or emodin significantly suppressed the migration of SW480/5-Fu cells, while the combination treatment exhibited better inhibitory effect (Figure 3C and 3D). Briefly, these results indicated that

emodin resensitized SW480/5-Fu cells to 5-Fu via inducing cell apoptosis.

Emodin in combination with 5-Fu induced apoptosis via modulating apoptosis-related proteins in SW480/5-Fu cells

To further confirm the pro-apoptotic effect of emodin in combination with 5-Fu in SW480/5-Fu cells, apoptosis-related proteins were detected by western-blot. As showed in Figure 4A-D, the combination treatment significantly down-regulated the expression of Bcl-2 in SW480/5-Fu cells, compared with 5-Fu or emodin alone. Meanwhile, the expressions of cleaved caspase 3 and Bax in SW480/5-Fu cells were significantly increased by 5-Fu, which were further enhanced in the presence of emodin (Figure 4A-D). In addition,

emodin alone increased the expressions of Bax and cleaved caspase3 and decreased the protein level of Bcl-2 (Figure 4A-D). Taken together, all these results suggested that emodin in combination with 5-Fu notably increased pro-apoptosis proteins in SW480/5-Fu cells.

Emodin resensitized SW480/5-Fu cells to 5-Fu through inactivating PI3K/Akt pathway

To further explore the mechanism by which emodin resensitized SW480/5-Fu cells to 5-Fu, the expressions of phosphorylated ERK (p-ERK) and phosphorylated Akt (p-Akt) in were detected. As showed in Figure 4A, 4E and 4F, the expressions of p-ERK and p-Akt were slightly decreased by 5-Fu, while emodin alone or combination treatment significantly down-regulated these two proteins. All these data suggested that emodin resensitized SW480/5-Fu cells to 5-Fu through downregulating PI3K/Akt signaling.

Emodin reduced 5-Fu chemoresistance in CRC in vivo

Finally, SW480/5-Fu xenograft model was used to explore if emodin could resensitized CRC to 5-Fu in vivo. As showed in Figure 5A and 5B,

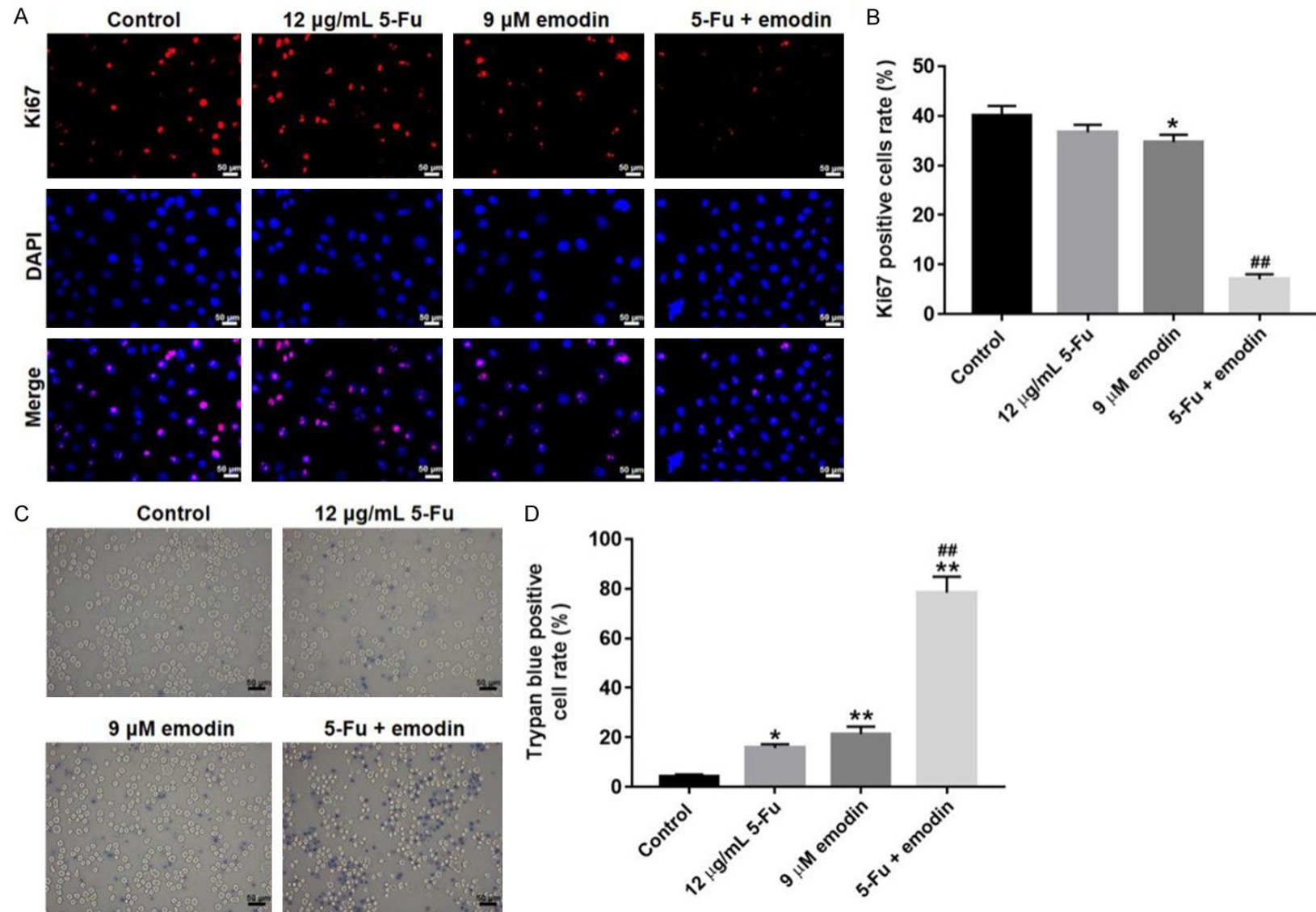


Figure 2. Emodin resensitized SW480/5-Fu cells to 5-Fu. SW480/5-Fu cells were treated with 5-Fu or/and emodin for 72 h. A. The proliferation of SW480/5-Fu cell was detected by Ki-67 staining. B. Ki-67 positive cell rate in each group was measured. C. SW480/5-Fu cell death was detected with trypan blue staining. D. Trypan blue positive cell rate in each group was calculated. * $P < 0.05$, ** $P < 0.01$ compared to control group. ** $P < 0.01$ compared to 5-Fu alone group.

Emodin reverses 5-Fu resistance via inhibiting PI3K/Akt signaling

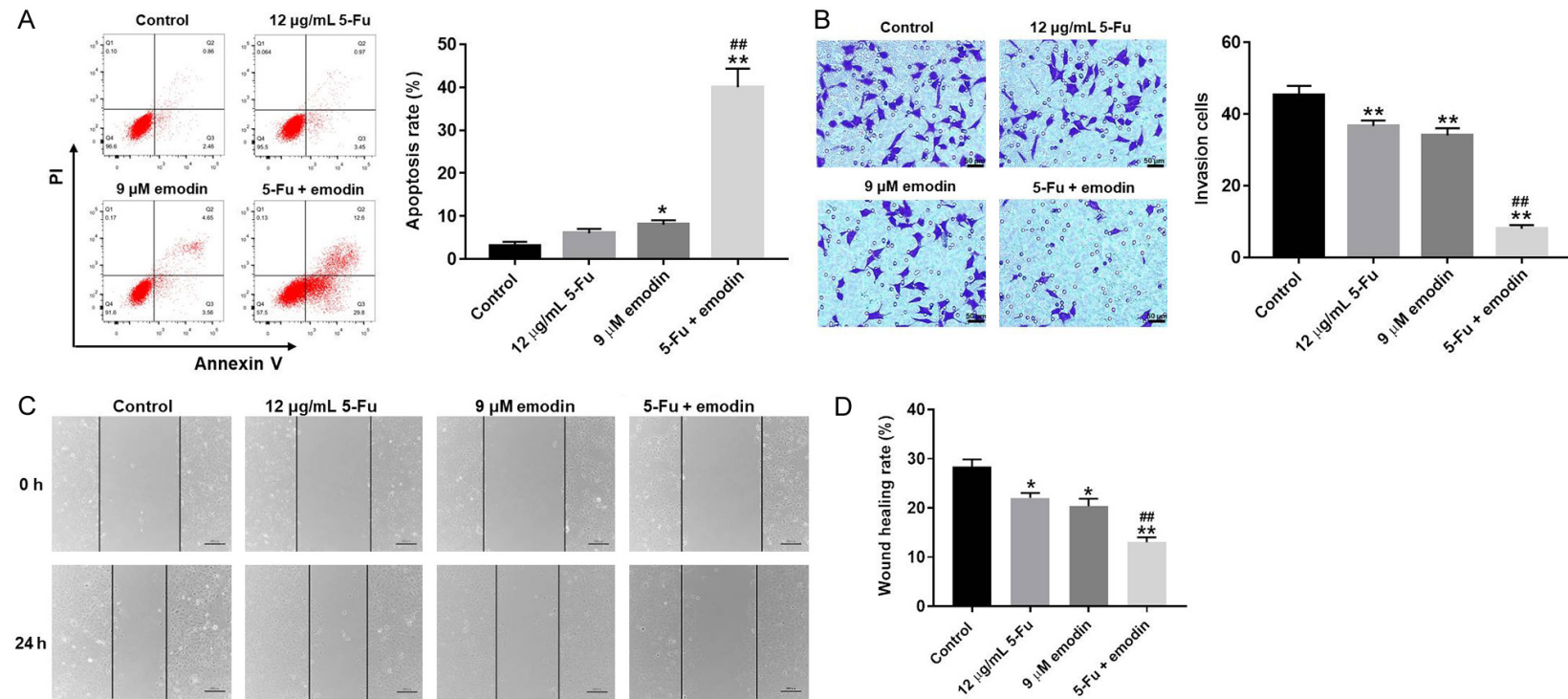


Figure 3. Emodin enhanced 5-Fu-induced apoptosis in SW480/5-Fu cells. A. SW480/5-Fu cells were treated with 5-Fu or/and emodin for 72 h, cell apoptosis was detected with Annexin V/PI staining. The rate of apoptotic cells was detected by FACS. X axis: the level of Annexin-V FITC fluorescence; Y axis: the PI fluorescence. B. SW480/5-Fu cells were treated with 5-Fu or/and emodin for 24 h, cell invasion ability was analyzed by transwell assay. C. Cell invasion ability was analyzed by wound healing. D. The wound healing rate was calculated. * $P < 0.05$, ** $P < 0.01$ compared to control group; ## $P < 0.01$ compared to 5-Fu alone group.

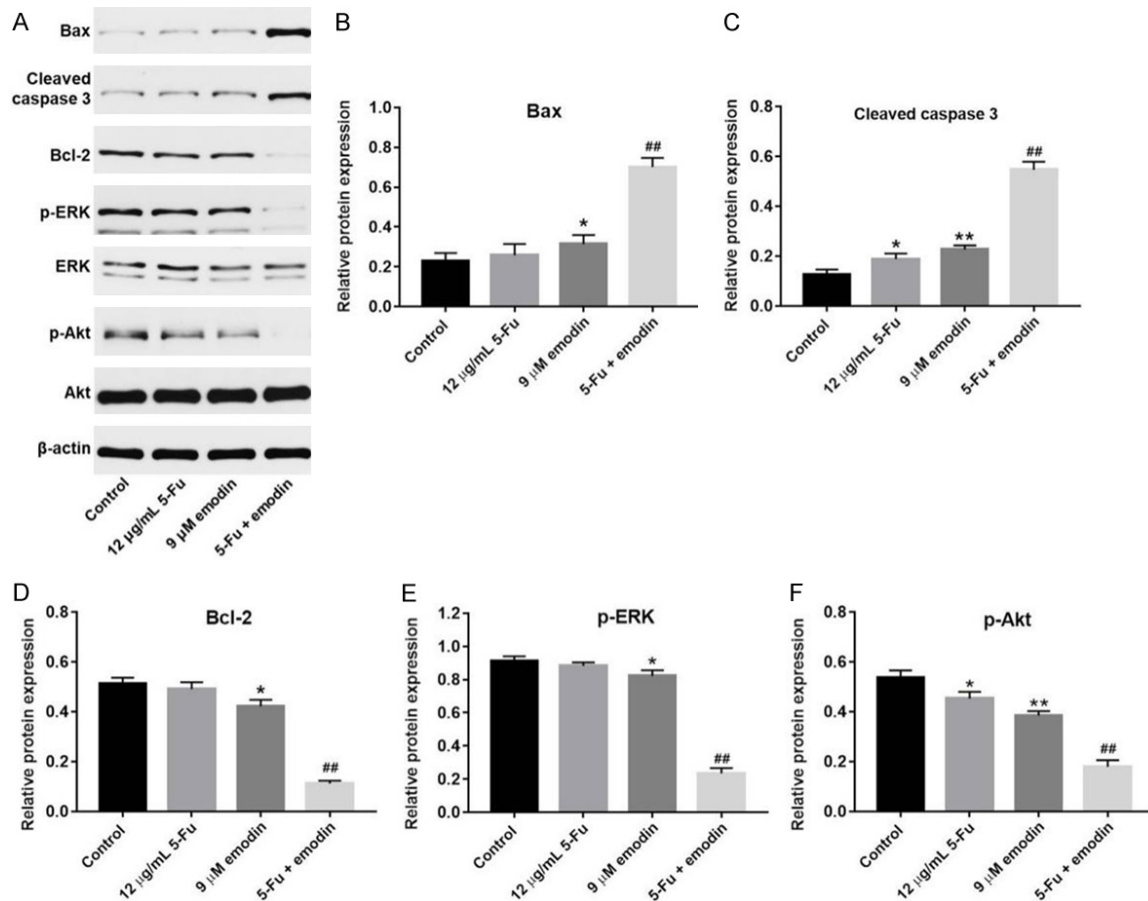


Figure 4. Emodin reversed 5-Fu chemoresistance in CRC via downregulation of PI3K/Akt signaling pathway. (A) SW480/5-Fu cells were treated with 5-Fu or/and emodin for 72 h. Then, the protein expressions of Bax, Bcl-2, cleaved caspase3, ERK, Akt, p-ERK and p-Akt in SW480/5-Fu cells were investigated by Western-blot. β -actin was used as an internal control. The relative protein expressions of (B) Bax, (C) cleaved caspase3, (D) Bcl-2, (E) p-ERK and (F) p-Akt were quantified by normalizing to β -actin. * P <0.05, ** P <0.01 compared to control group; ## P <0.01 compared to 5-Fu alone group.

combination of emodin with 5-Fu exhibited better inhibitory effect on tumor growth compared with emodin or 5-Fu alone (**Figure 5C**). Additionally, the tumor sizes in combination treatment group were much smaller than that in emodin or 5-Fu group. Besides, emodin or 5-Fu alone also exhibited inhibitory effect on tumor growth (**Figure 5C**). In addition, emodin, 5-Fu or combination treatment did not affect body weight of mice indicating the treatments were tolerable (**Figure 5D**). As revealed in **Figure 5E** and **5F**, the positive rate of TUNEL staining in tumor tissues was markedly increased by 5-Fu treatment, which was further enhanced in presence of emodin. Furthermore, the expression of p-Akt and p-ERK in tumor tissues of mice were obviously suppressed by 5-Fu treatment, which was further inhibited in presence of emodin (**Figure 6A** and **6B**). All

these results indicated emodin significantly resensitized CRC to 5-Fu treatment *in vivo*.

Discussion

The main reason for chemotherapy failure is the resistance of cancer cells to chemotherapeutic agents [23]. Some drugs such as cyclosporine A and verapamil have been reported that they could act as reversal agents, while they have significant adverse events which largely restrain their clinical application [24, 25]. Our study firstly demonstrated that emodin markedly reduced 5-Fu chemoresistance in CRC *in vitro* and *in vivo*. Previous studies have reported that emodin increased sensitivity of chemotherapeutic agents [7, 9, 26]. Our present study was similar to these results, indicating that emodin can be regarded as a reversal agent for treatment of CRC.

Emodin reverses 5-Fu resistance via inhibiting PI3K/Akt signaling

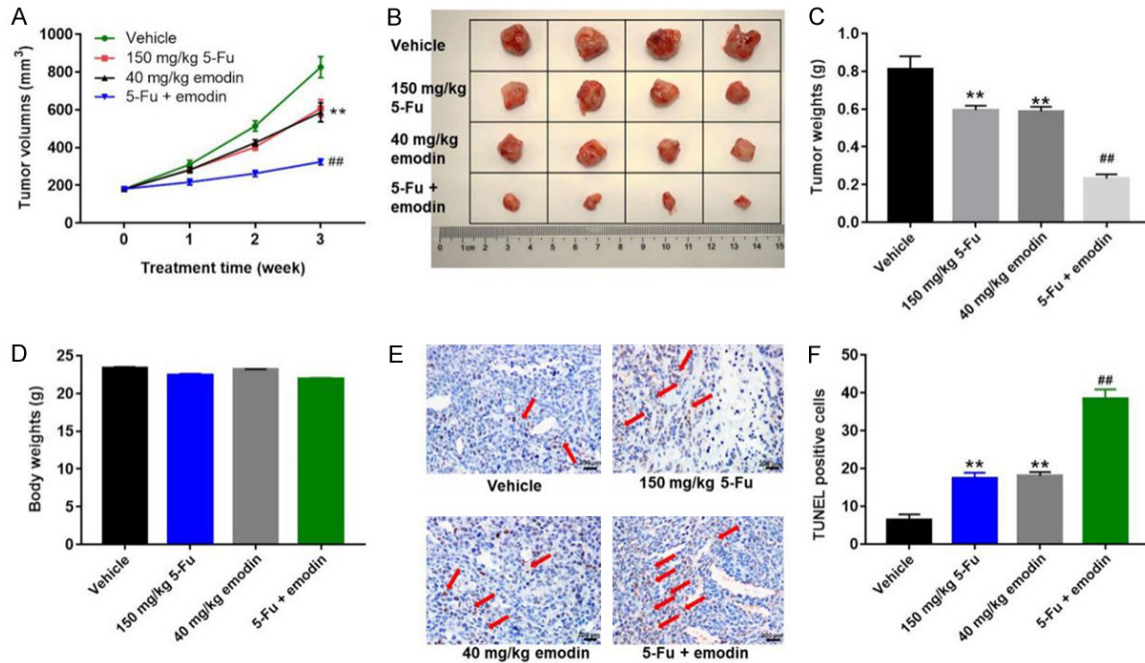


Figure 5. Emodin reduced 5-Fu chemoresistance in CRC *in vivo*. SW480/5-Fu cells were subcutaneously implanted into nude mice. Mice were divided into four groups: vehicle group, 5-Fu (150 mg/kg) group, emodin (40 mg/kg) group and 5-Fu + emodin group. A. Tumor volume of mice was measured weekly. B. The mice were sacrificed in 3 weeks and the tumors were collected. C. Tumor weights in each group were quantified. D. Body weight of mice was detected. E. Apoptosis in tumor tissues was detected by TUNEL staining. Red arrow indicated the positive cells. F. TUNEL positive cell rate in each group was calculated. ** $P < 0.01$ compared to vehicle group; ## $P < 0.01$ compared to 5-Fu (150 mg/kg) group.

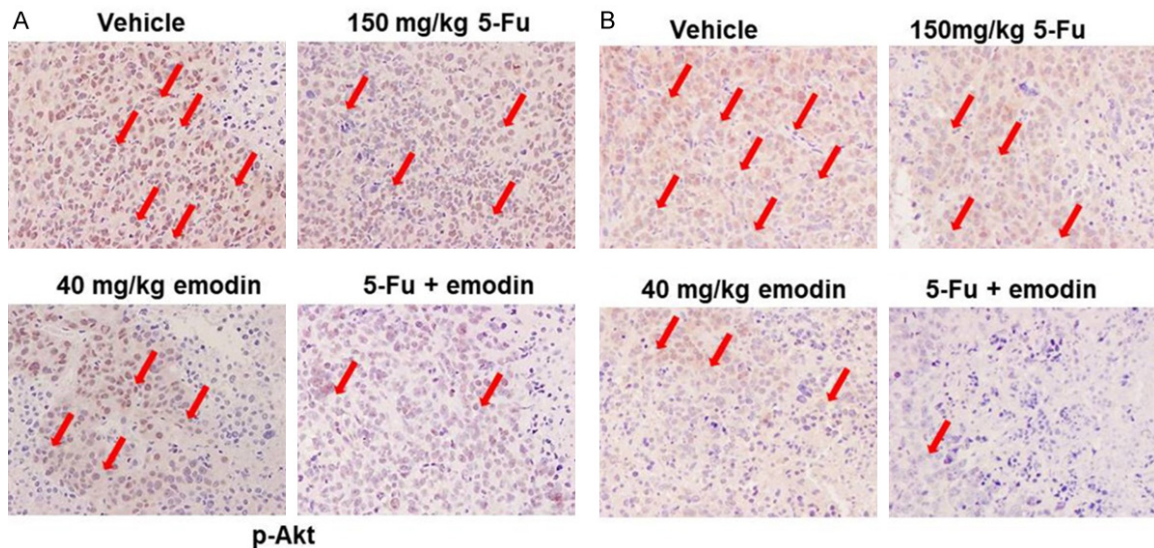


Figure 6. Emodin further enhanced the inhibitory effect of 5-Fu on CRC *in vivo*. A, B. The expression of p-Akt and p-ERK in tumor tissues of mice were detected by IHC staining. Red arrow indicated the positive cells.

Our current data indicated that emodin significantly resensitized CRC to 5-Fu treatment *in vitro* and *in vivo* without toxic effects. This finding was similar to the previous study that emo-

din in combination with cytarabine increased apoptosis in resistant cancers *in vitro* and *in vivo* without toxic and side effects [27]. Additionally, our results revealed that emodin,

5-Fu or combination treatment (emodin plus 5-Fu) had very limited effect on body weight of mice, which suggested that this strategy had no significant systemic toxicity.

In addition, emodin increased the apoptotic effect of 5-Fu *in vitro* via upregulating the expression of Bax and cleaved caspase3 and downregulating the expression of Bcl-2. Huang Q et al found that Bcl-2 played a critical role in modulating cell apoptosis [28]. On the other hand, lncRNA FAM99A induced trophoblast cell apoptosis via modulating expressions of Bax and cleaved caspase3 [29]. Consistently, combination of emodin with 5-Fu could induce apoptosis via suppression of Bcl-2 and increase of Bax and cleaved caspase3 in SW480/5-Fu cells. Meanwhile, Wu Y et al found that 5-Fu alone significantly induced apoptosis in gastric cancer cells [30]. However, 5-Fu exhibited limited apoptotic effect on SW480/5-Fu cells in our study. This discrepancy may probably due to the different cell line.

Next, our research revealed that emodin significantly decreased 5-Fu chemoresistance via downregulation of PI3K/Akt pathway. PI3K/Akt signaling was involved in tumorigenesis of cancer [31, 32]. Previous study has reported that PI3K/Akt signaling played a key role in cancer progression, drug resistance, and treatment [33]. Danielsen SA et al found that PI3K/Akt signaling lead to reduced apoptosis and increased proliferation in CRC [34]. This finding was similar to our current research. Moreover, our present study demonstrated that emodin further increased the inhibitory effect of 5-Fu on PI3K/Akt signaling pathway *in vitro*, indicating that emodin may act as a PI3K/Akt signaling inhibitor for treatment of CRC. Otherwise, NF- κ B and FoxM1/PHB1/RAF-MEK-ERK played important roles in drug resistance in cancer [35, 36]. Since this research focused only on the role of PI3K/Akt signaling in 5-Fu resistant CRC, our further study will investigate the roles of these two signaling pathways in drug resistance.

In conclusion, this study indicated that emodin significantly reversed 5-Fu resistance in CRC by inhibiting PI3K/Akt pathway. This finding provided a preclinical evidence for the treatment of 5-Fu resistant CRC.

Disclosure of conflict of interest

None.

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