

Liquiritigenin Inhibits Colorectal Cancer Proliferation, Invasion, and Epithelial-to-Mesenchymal Transition by Decreasing Expression of Runt-Related Transcription Factor 2

Fan-Chun Meng and Jun-Kai Lin

Department of Gastrointestinal Surgery, Shengli Oilfield Central Hospital, Dongying, Shandong, P.R. China

Inhibition of tumor metastasis is one of the most important purposes in colorectal cancer (CRC) treatment. This study aimed to explore the effects of liquiritigenin, a flavonoid extracted from the roots of *Glycyrrhiza uralensis* Fisch, on HCT116 cell proliferation, invasion, and epithelial-to-mesenchymal transition (EMT). We found that liquiritigenin significantly inhibited HCT116 cell proliferation, invasion, and the EMT process, but had no influence on cell apoptosis. Moreover, liquiritigenin remarkably reduced the expression of runt-related transcription factor 2 (Runx2) in HCT116 cells. Overexpression of Runx2 obviously reversed the liquiritigenin-induced invasion and EMT inhibition. Furthermore, liquiritigenin inactivated the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway in HCT116 cells. Upregulation of Runx2 reversed the liquiritigenin-induced PI3K/AKT pathway inactivation. In conclusion, our research verified that liquiritigenin exerted significant inhibitory effects on CRC invasion and EMT process by downregulating the expression of Runx2 and inactivating the PI3K/AKT signaling pathway. Liquiritigenin could be an effective therapeutic and preventative medicine for CRC treatment.

Key words: Colorectal cancer (CRC); Liquiritigenin; Epithelial-to-mesenchymal transition (EMT); Runt-related transcription factor 2; PI3K/AKT signaling pathway

INTRODUCTION

Colorectal cancer (CRC), characterized by uncontrolled cell proliferation and invasion in human colon and rectum, is the third most common malignant cancer worldwide¹. Many factors contribute to the occurrence of CRC, including inflammation in the colon and rectum, advanced age, low intake of fruits and vegetables, and family history of CRC². Surgical resection, chemotherapy, and radiotherapy remain the primary therapeutic methods for CRC³. However, these therapeutic methods cannot inhibit CRC tumor metastasis effectively, which is considered as the primary cause of mortality in CRC patients⁴. New therapeutic strategies and new therapeutic medicines for CRC are urgently needed.

Liquiritigenin, an aglycone of liquiritin, is one of the flavonoids extracted from the roots of *Glycyrrhiza uralensis* Fisch that has been used as a traditional medicine in Asia for centuries^{5,6}. Liquiritigenin has been known for its wide beneficial effects and bioactivities including anti-inflammation⁷, antioxidation⁸, antiallergy⁹, antitumor¹⁰, cytoprotection¹¹, and neuroprotection¹². Wang et al.

demonstrated that liquiritigenin inhibited the migration of human lung cancer A549 cells via downregulating the expression of pro-matrix metalloproteinase-2 (pro-MMP-2) and the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling pathway¹³. Shi et al. proved that liquiritigenin potentiated the inhibitory effects of cisplatin on melanoma cell invasion and metastasis¹⁴. In addition, Liu et al. presented that liquiritigenin reduced the tumor growth and angiogenesis in a mouse model of HeLa cells by downregulating the vascular endothelial growth factor (VEGF) expression¹⁵. These previous studies imply that liquiritigenin may also have inhibitory effects on CRC invasion and metastasis.

In the process of CRC metastasis, CRC cells spread from the primary tumor site to other locations in the colon and rectum or liver and lung tissues for formation of secondary tumors^{16,17}. Epithelial-to-mesenchymal transition (EMT), a phenotypic cellular process, induces loss of cell–cell junctions and apicobasolateral polarity in tumor tissue, thereby resulting in the invasion and metastasis of tumor cells^{18,19}. Runt-related transcription

Address correspondence to Jun-Kai Lin, Department of Gastrointestinal Surgery, Shengli Oilfield Central Hospital, No. 31 Jinan Road, Dongying District, Dongying 257000, Shandong, P.R. China. E-mail: foresighdo55t@gmail.com

factor 2 (Runx2) is a member of the Runx superfamily, which participates in the EMT process of numerous tumor cells²⁰. In this study, we explored the effects of liquiritigenin on CRC cell proliferation, invasion, and the EMT process. The regulatory roles of Runx2 in liquiritigenin-induced CRC cell invasion and EMT inhibition were also investigated. Our findings will be helpful for better understanding the inhibitory effects of liquiritigenin on CRC invasion and metastasis, and may provide an effective therapeutic strategy for CRC treatment.

MATERIALS AND METHODS

Cell Culture and Treatment

Human CRC HCT116 cells were purchased from the American Type Culture Collection (ATCC; CCL-247™; Manassas, VA, USA). The cell line was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, Life Technologies), 100 U/ml penicillin–100 µg/ml streptomycin solutions (Gibco, Life Technologies), and 2.5 mg/ml sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified incubator (Thermo Fisher Scientific, Waltham, MA, USA) with 5% CO₂. Transforming growth factor-1 (TGF-1; 10 ng/ml; Sigma-Aldrich) was used to stimulate the EMT process²¹.

Preparation of Liquiritigenin Solution

Liquiritigenin was obtained from Sigma-Aldrich (Cat. No. 78825) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) to a storage concentration of 100 mg/ml and stored at –20°C until use. Liquiritigenin solution was freshly diluted with serum-free DMEM to the experimental concentration (0.1% final concentration of DMSO in medium).

Cell Counting Kit-8 (CCK-8) Assay

Cell viability was measured using CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan). Briefly, 1,000 HCT116 cells per well were seeded into a 96-well plate (BD Bioscience, Franklin Lakes, NJ, USA). After 0, 10, 20, 50, or 100 µg/ml liquiritigenin treatment for 24 h, CCK-8 solution (10 µl) was added into each well and incubated for another 1–4 h. The absorbance at 450 nm of each well was recorded using a Bio-Tek multiwell plate reader (ELx800; Winooski, VT, USA). Cell viability (%) was calculated as: average absorbance of treatment group/average absorbance of control group × 100%.

Cell Invasion Assay

Cell invasion was detected using a 24-well modified Boyden chamber with 8-mm polyethylene terephthalate

(PET) membranes (Costar, Corning Incorporated, New York, NY, USA). Briefly, after relevant treatment, 200 µl of HCT116 cells with serum-free DMEM was seeded into the upper chamber of a 24-well modified Boyden chamber, and 600 µl of complete medium was added into the lower chamber. Then the 24-well chambers were cultured in an incubator for 48 h and fixed with 4% paraformaldehyde solution (Sigma-Aldrich) according to the manufacturer's instruction. After that, the nontraversed cells in the upper chamber were carefully removed using a cotton swab. The traversed cells in the lower chamber were stained with 0.15% crystal violet solution (Beyotime Biotechnology, Shanghai, P.R. China). A microscope (Nikon, Tokyo, Japan) was used to count the number of traversed cells in the lower chamber. Relative invasion (%) was calculated as follows: number of traversed cells in treatment group/number of traversed cells in control group × 100%.

Cell Apoptosis Assay

Cell apoptosis was determined using fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) staining (Invitrogen, Carlsbad, CA, USA), which utilizes annexin V–PI to detect the phosphatidylserine on the external membrane of apoptotic cells²². Briefly, 3,000 HCT116 cells per well were seeded into 24-well plates (BD Bioscience). After 0, 10, 20, 50, or 100 µg/ml liquiritigenin treatment for 24 h, cells in each well were collected, respectively, and washed with phosphate-buffered saline (PBS, Sigma-Aldrich) twice. After that, 100 µl of cells from each group was incubated with 100 µl of FITC–annexin V–PI solution for 20 min at room temperature in the dark. The apoptotic cells (%) were quantified using flow cytometer (FACSCanto II; BD Bioscience).

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA in HCT116 cells after relevant treatment was isolated using MagJET RNA Purification Kit (Thermo Fisher Scientific) in line with the manufacturer's protocol. VexMAX™ Plus One-Step RT-PCR Kit (Applied Biosystems, Foster City, CA, USA) was used for testing the relative mRNA levels of Runx2. The conditions of the PCR program were set at 30 min at 60°C for reverse transcription, 2 min at 94°C for denaturation, and 30 cycles of amplification for 40 s at 94°C, 1 min at 60°C, and 1 min at 72°C. The sequences of the primers were as follows: Runx2, 5'-GGCAGGCACAGTCTTCC C-3' (forward) and 5'-GGCCAGT TCTGAAGCACC-3' (reverse); GAPDH, 5'-ACCAGGAAATGAGCT TGAC A-3' (forward) and 5'-GACCACAGTCCATGCCATC-3' (reverse). All reactions were performed three times, and the data were quantified using the 2^{–Ct} method²³.

Cell Transfection

The full-length Runx2 sequence was constructed into a pEX-2 plasmid (GenePharma Corp., Shanghai, P.R. China) in line with the manufacturer's protocol and referred to as pEX-Runx2. Cell transfection with an empty pEX-2 plasmid was used as a negative control and referred to as pEX. Transfection efficiency was detected using RT-qPCR and Western blotting.

Western Blotting

Total proteins in HCT116 cells after relevant treatment were isolated using RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) according to the manufacturer's instruction. The concentrations of proteins were calculated using BCA™ Protein Assay Kit (Thermo Fisher Scientific). Equal concentrations of protein samples were electrophoresed using a sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) system (Bio-Rad Laboratories, Hercules, CA, USA) and transferred onto polyvinylidene difluoride (PDVF) membranes (0.22 µm; Millipore, Bedford, MA, USA), which were incubated with primary antibodies. The following primary antibodies were used in this experiment: anti-B-cell lymphoma-2 (Bcl-2) antibody (ab59348), anti-Bcl2-associated X (Bax) antibody (ab32503), anti-caspase 3 antibody (ab13585), anti-caspase 9 antibody (ab32068), anti-proliferating cell nuclear antigen (PCNA) antibody (ab92552), anti-cyclin A antibody (ab185619), anti-cyclin E1 antibody (ab33911), anti-cyclin D1 antibody (ab134175), anti-cyclin-dependent kinase 2 (CDK2) antibody (ab32147), anti-CDK4 antibody (ab199728), anti-E-cadherin (E-cad) antibody (ab1416), anti-N-cadherin (N-cad) antibody (ab76057), anti-vimentin (Vim) antibody (ab137321), anti-zinc finger E-box-binding protein 1

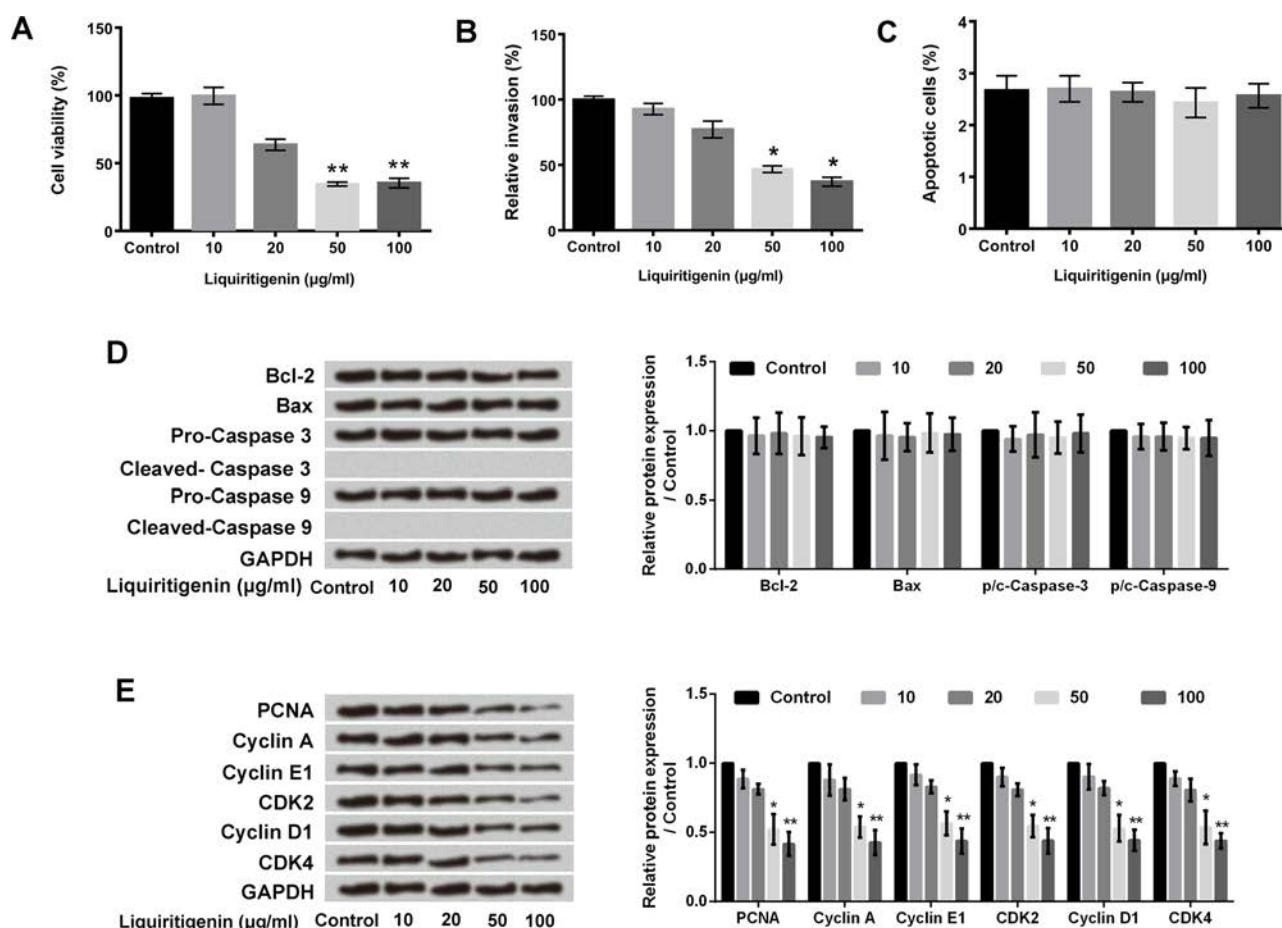


Figure 1. Liquiritigenin inhibited the proliferation and invasion of HCT116 cells. (A) HCT116 cell viability, (B) invasion, and (C) apoptosis after 0, 10, 20, 50, or 100 µg/ml liquiritigenin treatment were measured using cell counting kit-8 (CCK-8) assay, cell invasion assay, and fluorescein isothiocyanate (FITC)-annexin V-propidium iodide (PI) staining, respectively. (D) The expression levels of Bcl-2, Bax, caspase 3, and caspase 9, and (E) PCNA, cyclin A, cyclin E1, cyclin D1, CDK2, and CDK4 in HCT116 cells after 0, 10, 20, 50, or 100 µg/ml liquiritigenin treatment were analyzed using Western blotting. Data are expressed as mean ± standard deviation (SD). Bcl-2, B-cell lymphoma-2; Bax, Bcl2-associated X; PCNA, proliferating cell nuclear antigen; CDK2, cyclin dependent kinase 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. * $p < 0.05$ or ** $p < 0.01$ versus control group.

(ZEB1) antibody (ab181451), anti-Snail 1 antibody (ab82846), anti-Runx2 antibody (ab76956), anti-PI3K antibody (ab189403), anti-p-PI3K antibody (ab182651), anti-AKT antibody (ab18785), anti-p-AKT antibody (ab8933), and anti-GAPDH antibody (ab128915) (all were obtained from Abcam Biotechnology, Cambridge, MA, USA). After blocking with 5% milk-Tris-buffered saline-Tween (TBST; Sigma-Aldrich), PVDF membranes were incubated with goat anti-rabbit (or anti-mouse) IgG H&L (HRP) secondary antibodies (ab205718 and ab205719; Abcam Biotechnology) for 1 h at room temperature and transferred into the ChemiDoc™ XRS System (Bio-Rad Laboratories), supplemented with 200 μ l of Immobilon Western Chemiluminescent HRP Substrate (Millipore) on the surface of the membranes. Protein signals were recorded, and the intensities of the bands were quantified using the Bio-Rad Image Lab™ 3.0 version software (Bio-Rad Laboratories)²⁴.

Statistical Analysis

All experiments were performed in triplicate in this research. Data were expressed as mean \pm standard deviation (SD). GraphPad 6.0 software (GraphPad, San Diego, CA, USA) was used for statistical analysis. One-way analysis of variance (ANOVA) was performed to calculate the p values. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Liquiritigenin Inhibited Proliferation and Invasion of HCT116 Cells

Viability, invasion, apoptosis, and proliferation of HCT116 cells after liquiritigenin treatment were detected using CCK-8 assay, cell invasion assay, FITC-annexin

V-PI staining, and Western blotting, respectively. As presented in Figure 1A and B, liquiritigenin significantly inhibited HCT116 cell viability and invasion in a dose-dependent manner ($p < 0.05$ or $p < 0.01$). However, liquiritigenin had no influence on HCT116 cell apoptosis (Fig. 1C). The expression levels of Bcl-2, Bax, caspase 3, and caspase 9 were not changed after liquiritigenin treatment (Fig. 1D). In addition, Figure 1E shows that the expressions of PCNA, cyclin A, cyclin E1, CDK2, cyclin D1, and CDK4 in HCT116 cells were all downregulated after liquiritigenin treatment ($p < 0.05$ or $p < 0.01$), which suggested that liquiritigenin inhibited the proliferation of HCT116 cells. The above results indicated that liquiritigenin inhibited HCT116 cell viability, proliferation, and invasion but had no influence on cell apoptosis.

Liquiritigenin Suppressed the Process of EMT

TGF- β 1 was used as an EMT inducer in our experiment. Figure 2 shows that TGF- β 1 treatment obviously enhanced the expressions of N-cad, Vim, ZEB1, and Snail1 but remarkably reduced the level of E-cad in HCT116 cells ($p < 0.01$). However, the increase in TGF- β 1-induced expression of N-cad, Vim, ZEB1, and Snail1 was significantly reversed by different doses of liquiritigenin treatment ($p < 0.05$ or $p < 0.01$). Compared to the TGF- β 1 treatment group, the expression of E-cad in TGF- β 1 + liquiritigenin was markedly increased ($p < 0.05$ or $p < 0.01$). These results indicated that liquiritigenin suppressed the process of EMT.

Liquiritigenin Reduced the Expression of Runx2 in HCT116 Cells

A previous study demonstrated that Runx2 played critical regulatory roles in the EMT process by modulating

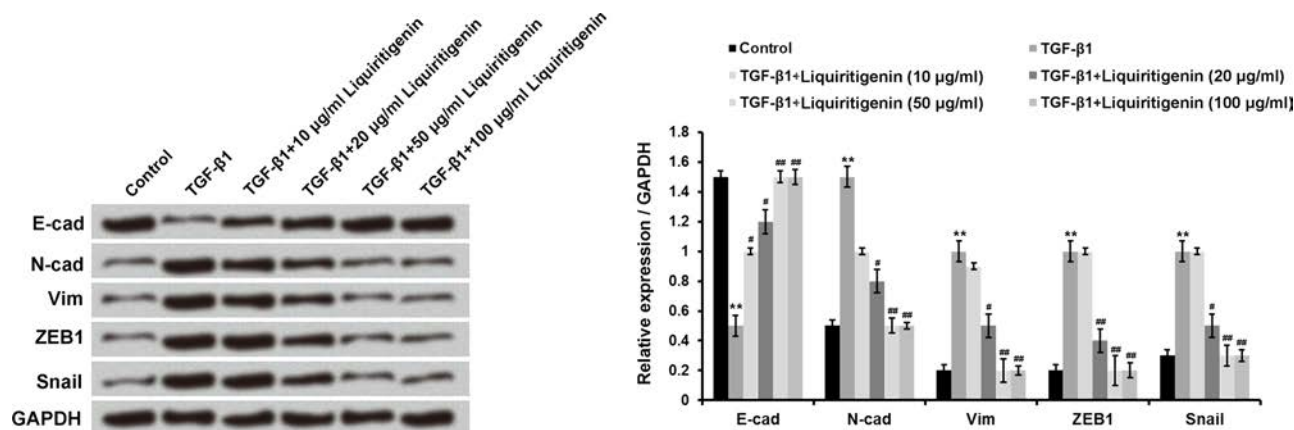


Figure 2. Liquiritigenin suppressed the process of EMT. The expressions of E-cad, N-cad, Vim, ZEB1, and Snail1 in HCT116 cells after TGF- β 1 (10 ng/ml) with or without liquiritigenin (0, 10, 20, 50, or 100 μ g/ml) treatment were detected using Western blotting. Data are expressed as mean \pm SD. EMT, epithelial-to-mesenchymal transition; TGF- β 1, transforming growth factor- β 1; E-cad, E-cadherin; N-cad, N-cadherin; Vim, vimentin; ZEB1, zinc finger E-box-binding protein 1. ** $p < 0.01$ versus control group; # $p < 0.05$ or ## $p < 0.01$ versus TGF- β 1 group.

the expression of proteins, which participated in the EMT process¹⁹. So we measured the effects of liquiritigenin on Runx2 expression in our research. The mRNA and protein levels of Runx2 in HCT116 cells after liquiritigenin treatment were determined using RT-qPCR and Western blotting, respectively. As shown in Figure 3A, liquiritigenin treatment significantly downregulated the relative mRNA expression of Runx2 in HCT116 cells in a dose-dependent manner ($p < 0.05$). After 100 $\mu\text{g/ml}$ liquiritigenin treatment, the relative mRNA expression of Runx2 was reduced to 27.33%. In addition, liquiritigenin treatment also obviously downregulated the protein level of Runx2 in HCT116 cells ($p < 0.05$ or $p < 0.01$) (Fig. 3B). These findings suggested that liquiritigenin negatively regulated the expression of Runx2 in HCT116 cells.

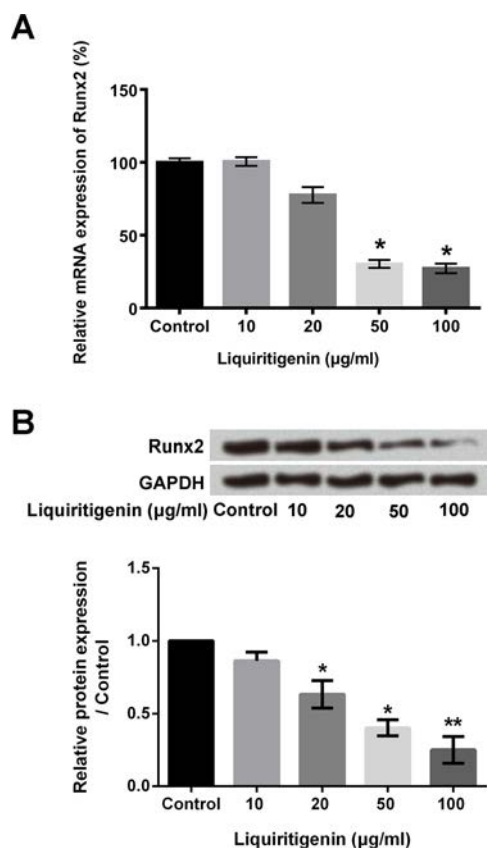


Figure 3. Liquiritigenin reduced the expression of Runx2 in HCT116 cells. (A) The relative mRNA expression of Runx2 in HCT116 cells after 0, 10, 20, 50, or 100 $\mu\text{g/ml}$ liquiritigenin treatment was determined using RT-qPCR. (B) The protein levels of Runx2 in HCT116 cells after 0, 10, 20, 50, or 100 $\mu\text{g/ml}$ liquiritigenin treatment were analyzed using Western blotting. Runx2, Runt-related transcription factor 2; RT-qPCR, reverse transcription quantitative polymerase chain reaction. Data are expressed as mean \pm SD. * $p < 0.05$ or ** $p < 0.01$ versus control group.

Overexpression of Runx2 Reversed the Liquiritigenin-Induced Invasion and EMT Inhibition

To investigate the roles of Runx2 in liquiritigenin-induced invasion and EMT inhibition, pEX-Runx2 was transfected into HCT116 cells. Considering that 50 $\mu\text{g/ml}$ liquiritigenin resulted in a significant inhibition of cell viability and invasion, 50 $\mu\text{g/ml}$ liquiritigenin treatment was selected for the following experiments. Figure 4A and B indicate that transfection with pEX-Runx2 notably increased the mRNA and protein levels of Runx2 in HCT116 cells ($p < 0.01$). Transfection with pEX-Runx2 significantly reversed the liquiritigenin-induced invasion inhibition, as evidenced by the enhancement of relative invasion ($p < 0.05$) (Fig. 4C). TGF- β 1 treatment significantly upregulated the expression of Runx2 in HCT116 cells ($p < 0.001$) (Fig. 4D), while liquiritigenin treatment remarkably reversed the TGF- β 1-induced increase of Runx2 expression ($p < 0.001$). Overexpression of Runx2 drastically alleviated the effects of liquiritigenin ($p < 0.001$). Similar results were found in N-cad, Vim, ZEB1, and Snail1 expressions ($p < 0.05$, $p < 0.01$, or $p < 0.001$). The above findings suggested that Runx2 was involved in the inhibitory effects of liquiritigenin on HCT116 cell invasion and EMT.

Overexpression of Runx2 Reversed the Liquiritigenin-Induced Inactivation of the PI3K/AKT Pathway in HCT116 Cells

The effects of liquiritigenin and Runx2 on the PI3K/AKT signaling pathway in HCT116 cells were analyzed using Western blotting. As displayed in Figure 5, single treatment with liquiritigenin remarkably downregulated the expression rates of p-PI3K/PI3K and p-AKT/AKT in HCT116 cells ($p < 0.05$). Transfection with pEX-Runx2 reversed the liquiritigenin-induced expression rate decreases of p-PI3K/PI3K and p-AKT/AKT in HCT116 cells ($p < 0.05$), which indicated that liquiritigenin inactivated the PI3K/AKT pathway by inhibiting Runx2 expression in HCT116 cells.

DISCUSSION

CRC is one of the most common malignant tumors with metastatic potential in the human gastrointestinal system²⁵. Thus, inhibition of tumor metastasis is one of the most important purposes in CRC treatment²⁶. This study revealed that liquiritigenin distinctly inhibited the proliferation, invasion, and EMT of HCT116 cells. Moreover, liquiritigenin obviously reduced the expression of Runx2 in HCT116 cells in a dose-dependent manner. Overexpression of Runx2 reversed the liquiritigenin-induced invasion and EMT inhibition as well as the inactivation of the PI3K/AKT signaling pathway.

Tumor metastasis is a complex process, which is characterized by the increasing invasion ability of tumor

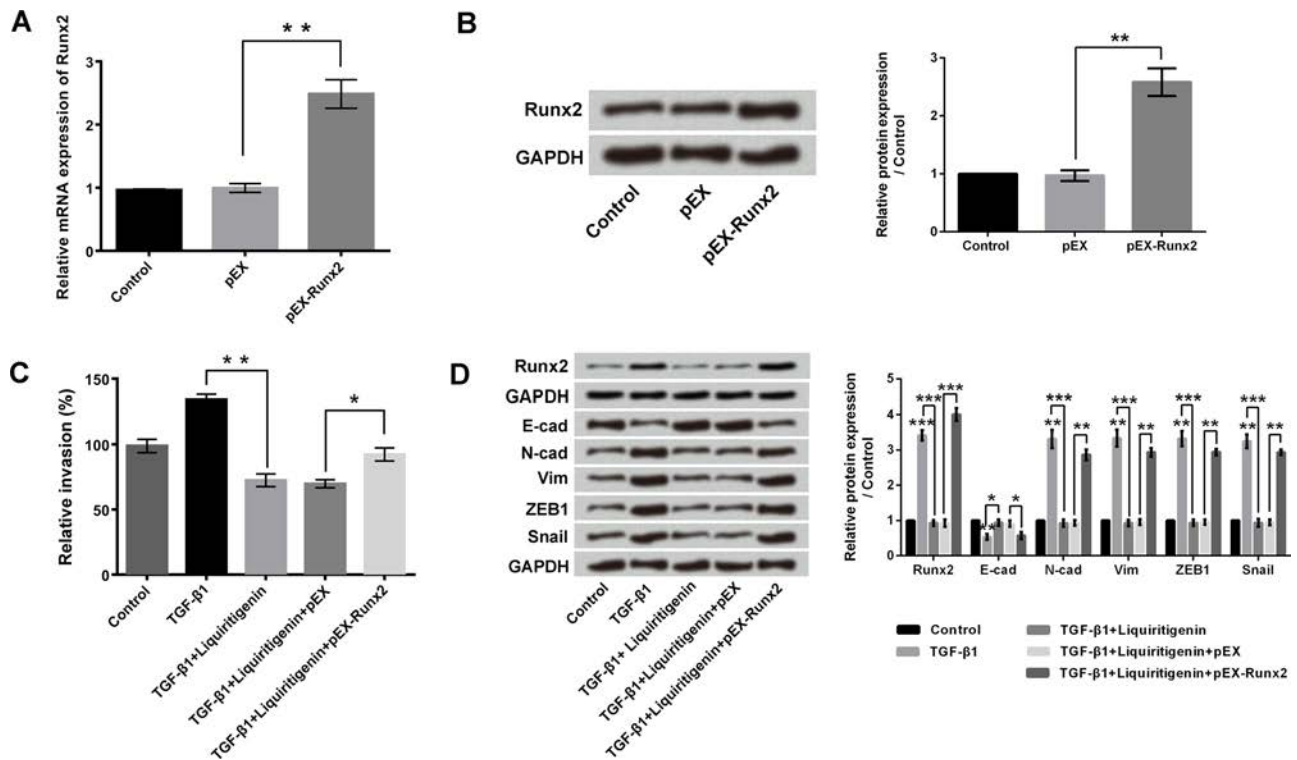


Figure 4. Overexpression of Runx2 reversed the liquiritigenin-induced invasion and EMT inhibition. After transfection with pEX or pEX-Runx2, the (A) mRNA and (B) protein expressions of Runx2 in HCT116 cells were measured using RT-qPCR and Western blotting, respectively. (C) Relative invasion of HCT116 cells after TGF- β 1 (10 ng/ml) with or without liquiritigenin (50 μ g/ml) treatment and pEX or pEX-Runx2 transfection were detected using cell invasion assay. (D) The expressions of Runx2, E-cad, N-cad, Vim, ZEB1, and Snail in HCT116 cells after TGF- β 1 (10 ng/ml) with or without liquiritigenin (50 μ g/ml) treatment and pEX or pEX-Runx2 transfection were analyzed using Western blotting. Data are expressed as mean \pm SD. * p <0.05, ** p <0.01, *** p <0.001.

cells²⁷. In our experiments, relative invasion of HCT116 cells was obviously decreased after liquiritigenin treatment, which proved that liquiritigenin inhibited the invasion ability of HCT116 cells. EMT is considered as a key process for promoting tumor cell dissociation, invasion, and metastasis^{18,28}. The increasing expression of N-cad

and the decreasing expression of E-cad in tumor cells are efficient biomarkers of EMT activation²⁹. In this study, TGF- β 1 treatment promoted the EMT process in HCT116 cells, which was evidenced by the increased E-cad expression and the decreased expression of N-cad. However, liquiritigenin remarkably suppressed the process of EMT

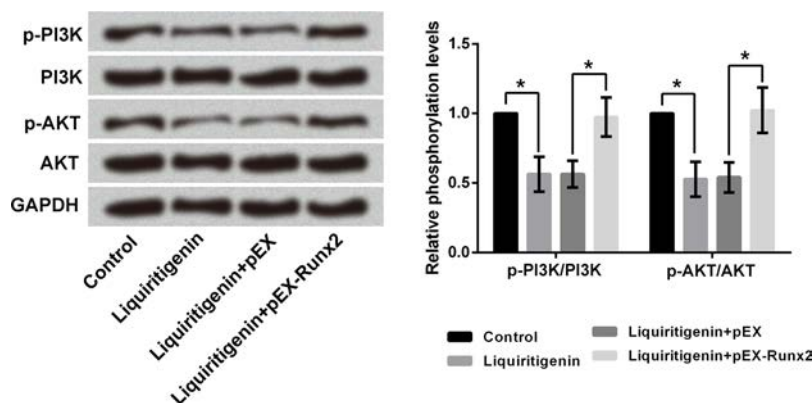


Figure 5. Overexpression of Runx2 reversed the liquiritigenin-induced inactivation of the PI3K/AKT pathway in HCT116 cells. The expressions of PI3K, p-PI3K, AKT, and p-AKT in HCT116 cells after 50 μ g/ml liquiritigenin treatment and transfection with pEX or pEX-Runx2 were analyzed using Western blotting. p-, phosphorylated; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B. * p <0.05.

by reversing the effect of TGF- β on E-cad and N-cad expressions. In addition, liquiritigenin also attenuated the effects of TGF- β on ZEB1, Snail1, and Vim, which all play important regulatory roles in the EMT process³⁰. The negative regulation of liquiritigenin on the EMT process further indicated the influences of liquiritigenin on CRC cell invasion and tumor metastasis.

Runx2 is highly expressed in various tumor cells and plays critical regulatory roles in the EMT process by regulating the expressions of matrix and adhesion proteins in tumor cells³¹. In terms of CRC, Georges et al. demonstrated that Runx2 was highly expressed in rat CRC metastasis tumor cells³². Wai et al. presented that Runx2 regulated the transcription of the metastatic gene osteopontin in CT26 cells³³. In our research, liquiritigenin downregulated both the mRNA and protein levels of Runx2 in HCT116 cells in a dose-dependent manner. Overexpression of Runx2 significantly reversed the liquiritigenin-induced invasion and EMT inhibition. These findings verified that Runx2 was involved in the inhibitory effects of liquiritigenin on CRC invasion and metastasis. Moreover, these results were consistent with the previous study, which pointed out that Runx2 was an intracellular molecular target for CRC treatment³⁴.

Previous studies had demonstrated that liquiritigenin inhibited the migration and invasion of lung cancer cells and melanoma cells by suppressing the PI3K/AKT signaling pathway^{13,14}. In our experiments, we also explored the effects of liquiritigenin on the PI3K/AKT pathway in HCT116 cells. The PI3K/AKT signaling pathway participates in the regulation of various cancer cell functions, such as proliferation, cell cycle transition, migration, invasion, and so on^{35–37}. Our results showed that liquiritigenin downregulated the expression rates of p-PI3K/PI3K and p-AKT/AKT in HCT116 cells. Overexpression of Runx2 reversed the liquiritigenin-induced inactivation of the PI3K/AKT signaling pathway, which further implied that liquiritigenin inactivated the PI3K/AKT pathway by inhibiting Runx2 in HCT116 cells.

In conclusion, our research verified that liquiritigenin exerted significant inhibitory effects on CRC cell invasion and EMT by negatively regulating the expression of Runx2 and the PI3K/AKT pathway. We propose that liquiritigenin could be an effective therapeutic and preventative medicine for CRC. However, further safety evaluation and in vivo studies are still needed.

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