globally, with 135,430 new cases and 50,260 deaths in 2017 [1]. A large body of evidence indicates that multiple risk factors including lifestyle, genetics, environmental factors and several diseases are associated with the onset of colorectal cancer [2-7]. The major therapeutic strategy is surgery, which is effective for patients who are in the early stages while less effective for later-stage patients with multiple metastases. Hence, it is imperative to develop novel therapeutic strategies for colorectal cancer.

The phosphatidylinositol 3-kinase (PI3K) / protein kinase B (AKT) pathway plays indispensable roles in regulating cellular processes as diverse as reducing apoptosis, stimulating cell growth and increasing proliferation, furthermore, components of PI3K/AKT signaling pathway are frequently reported to be disrupted in colorectal cancer development and progression [8, 9]. Thus, several drugs targeting the pathway are under investigation and development [10].

Currently, the extensive use of herbal therapies and traditional Chinese drugs has been increasing globally due to their demonstrated effectiveness in treating cancers [11]. Isoliquiritigenin, a bioactive natural phenolic compound isolated from licorice. ISL has been reported to possess a number of biological activities, for instance, anti-inflammatory, anti-oxidative, immunomodulatory effects, and anticancer activities [12]. ISL has been shown to have inhibitory effects of various cancers, including breast, prostate, hepatoma and other types of cancers, and it has been indicated that regulation of the PI3K/AKT signaling pathway by ISL may be involved in its antitumor activity in breast cancer [13-15]. However, the effects of ISL on the regulation of PI3K/AKT signaling pathway and related anticancer effects in colorectal cancer have received relatively little attention to date.

In this study, we show that ISL suppressed human colorectal cancer cell proliferation, invasion and migration and induced apoptosis, which may involve regulation by the PI3K/AKT signaling pathway.
2 Materials and methods

2.1 Chemicals and antibodies

ISL was purchased from MedChemExpress Biotechnology (New Jersey, USA). Primary antibodies against the AKT, p-AKT, mTOR, p-mTOR were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Other antibodies against the Cyclin D1, p-P70S6K, Active-Caspase3, Tubulin and peroxidase-conjugated secondary antibodies were obtained from Proteintech Group, Inc (Wuhan, China). The enhanced chemiluminescence (ECL) detection system was from Proteintech Group, Inc (Wuhan, China).

2.2 Cell culture

HCT-116 cell lines (Shanghai Institute of Cell Biology, China) were cultured at 37°C and 5% CO₂ in RPMI-1640 (Hyclone Laboratories, Logan, UT, USA) supplemented with 10% FBS (Gibco Cell Culture, Carlsbad, CA, USA), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells in logarithmic phase of growth (0.5 × 10⁶ ~ 1 × 10⁶ cells/ml) were used in experiments. Experimental groups were treated with 20 µM ISL (10 mM ISL as stock solution was dissolved in DMSO and stored at -20°C. 20 µM ISL was prepared by diluting the stock solution with RPMI-1640) [16, 17], and the negative control group (NC) was cultured with 0.1% DMSO in culture media.

2.3 Cell proliferation assay

The effects of ISL on cell growth were determined by the Cell Counting Kit-8 (Beijing Solarbio Science & Technology Co., Ltd., China). HCT-116 cells were plated in triplicate wells of 96-well plates at a density of 10³ cells/well. Following overnight incubation, 20 µM ISL was added and incubation of plates was continued for 24 h, 48 h and 72 h. Briefly, 10 µl CCK8 solution was added to each well and the plates were incubated for another 1.5 h at 37°C. Subsequently, the Cell Counting Kit-8 system was used to spectrophotometrically measure the absorbancies of the wells in the plate at 450 nm.

2.4 Cell migration and invasion assay

Cells invasion and migration were analysed using 24-well trans-well chamber (Millipore, USA) with/without Matrigel matrix (BD Biosciences, USA), as per the protocol provided by the manufacturer. Briefly, 1 × 10⁶ cells were placed in each trans-well membrane filter insert, the lower chamber was filled with 500 µl of complete medium, and the samples were incubated overnight. Subsequently, residual cells were cleaned using cotton-tipped swabs, invasive cells were fixed with 4% paraformaldehyde for 30 min and then stained with 0.1% of crystal violet for 20 min and counted under a microscope in five fields random per membrane. For detecting cell migration, the steps were the same as for the detection of invasion, however, Matrigel was not used.

2.5 Detection of apoptosis

Apoptosis in HCT-116 cells was determined using an annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (Beijing 4A Biotech Co. Ltd., Beijing, China) according to the manufacturer’s protocol. HCT-116 cells were plated at a density of 1 ~ 5 × 10⁶ cells/ml medium. After 24 h of incubation the cells were treated with ISL and were harvested from the culture dishes 24 h later by trypsinization (without EDTA), washed twice with PBS, resuspended in 500 µl 1 × binding buffer. Then, 5 µl FITC-labeled Annexin V (Beijing 4A Biotech Co. Ltd., Beijing, China) and 10 µl PI were added. Upon incubation in the dark for 5 min at room temperature, the cells were analyzed with a FACScan instrument (FACS Calibur, BD Biosciences, CA).

2.6 Western blotting analysis

HCT-116 cells were harvested in RIPA lysis buffer (CWBio, Beijing, China). The protein concentration of the supernatant was measured by BCA assay (CWBio, Beijing, China). Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) with loading equal amount of proteins per lane was undertaken. Then, gels were transferred to polyvinylidene difluoride membranes and blocked with 5% nonfat milk in TBST buffer (pH 7.4 tris-buffered saline buffer containing 0.1% Tween-20) for 1 h. Subsequently, the membranes were incubated with primary antibodies (1 : 1000 dilution) against Cyclin D1, p-P70S6K, Bcl-2, Bax, Active-Caspase3, AKT, p-AKT, mTOR, p-mTOR and (1 : 5000 dilution) against tubulin at 4°C overnight. Immunoreactive bands were detected using anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1 : 5000 dilution) and visualized using ECL Reagent.
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2.7 Statistical analysis

All values in this study are expressed as the mean ± standard deviation (SD) and analyzed by the SPSS (International Business Machines Corporation, State of New York) 17.0 software and GraphPad Prism 5 (San Diego, CA, USA). Treatment effects were compared between ISL groups and NC groups by the Student’s t-test and p < 0.05 was considered to indicate a statistically significant result.

Ethical approval: The conducted research is not related to either human or animals use.

3 Results

3.1 Inhibitory effects of ISL on the proliferation, migration and invasion of HCT-116 colorectal cancer cells

The in vitro anti-proliferative effect of ISL on the HCT-116 colorectal cancer cells were determined using CCK8 proliferation assays. The results revealed that ISL significantly reduced HCT-116 cell proliferation after 48 h of treatment (Fig. 1, p < 0.05).

Next, we tested whether ISL had the ability to impede the cell migration and invasion of HCT-116 cells. Transwell assays were used to investigate the impacts of ISL on these two behaviours of HCT-116 colorectal cancer cells. As shown in Fig. 2A, after treatment with ISL, the numbers of migrated cells dramatically decreased to 115 ± 5 compared to the control (185 ± 8) (Fig. 2A, p < 0.05). Trans-well invasion assays assessed the effects of ISL on HCT-116 cell invasion. Similarly, ISL treatment markedly reduced invasive cell numbers (24 ± 2), compared with NC groups (73 ± 4) (Fig. 2B).

3.2 ISL induces HCT-116 colorectal cancer cell apoptosis

Next, we investigated whether the observed anti-proliferative effects of ISL against human colorectal cancer cells are mediated via apoptosis. We determined the effect of ISL on apoptotic cells with flow cytometry using annexin V-FITC/PI staining, and found that treatment of HCT-116 cells with ISL led to a conspicuous increase in the apoptotic ratio of treated groups (17.26%) compared with NC groups (7.37%) (Fig. 3A). Furthermore, to investigate the potential mechanism of ISL-induced HCT-116 cells apoptosis, the impact of ISL on the expression of Bcl-2, Bax, Caspase-3 were examined. The results of western blot analysis indicated that, following treatment with ISL, the expression of the anti-apoptotic protein, Bcl-2, was decreased and the pro-apoptotic protein Bax was increased (Fig. 3B-D, *p < 0.05). Additionally, the expression level of cleaved caspase-3 was upregulated (Fig. 3B and E, *p < 0.05).

Taken together, these results suggested that ISL activates apoptotic signals in HCT-116 cells.

3.3 ISL inhibits the activity of PI3K/AKT signaling pathway

Since we have observed that ISL could inhibit proliferation and induce apoptosis in HCT-116 colorectal cancer cells, we further investigated the possible mechanisms. The PI3K/AKT/mTOR signaling pathway is an intracellular signaling pathway which plays a vital role in cellular processes related to cell survival, growth, proliferation and death [18, 19]. We examined whether this pathway plays a central role in ISL-mediated effects on cell proliferation and apoptosis. As shown in Fig. 6A-E, ISL treatment triggers a significant decrease in the levels of phosphorylated AKT and mTOR, while there was no change observed in the total AKT and mTOR protein levels. Additionally, treatment of HCT-116 cells with ISL decreased the activation of PI3K/AKT downstream pathway intermediates such as p-P70S6K and Cyclin D1 (Fig. 6A, F and G). Overall, these data suggested that the ISL has an inhibitory action on the activation of PI3K/AKT/mTOR signaling pathway.
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Fig. 2. ISL decreases migration and invasion of colorectal cancer cells by trans-well membrane migration and invasion assays
A-B. ISL significantly decreased the number of cells that migrated to the chamber. C-D. ISL significantly decreased the number of invaded cells that passed through the Matrigel. * p < 0.05 versus NC groups (n=3).

4 Discussion

Our previous study has shown that ISL inhibits cellular proliferation, migration and invasiveness, and induces cellular apoptosis in human colorectal cancer cells. Moreover, the effects of ISL may result from the inhibition of PI3K/AKT/mTOR signaling pathway.

ISL has been reported to have anticarcinogenic functions in both in vivo and in vitro experimental models. Previous results from several clinical and animal studies discovered that ISL inhibits colitis-associated colorectal cancer [20], endometrial cancer growth [21] and pulmonary metastasis of murine renal carcinoma cells [22], and in vitro studies have suggested that ISL has antiproliferative activity in prostate, tongue squamous, melanoma, glioma, breast, non-small-cell lung [13, 16, 23-26]. In addition, some research has revealed that ISL exerts anti-invasive and anti-metastatic effects in breast and prostate cancer cells [17, 27]. Our findings also demonstrated that ISL inhibits cell proliferation, invasion and migration in human colorectal cancer cells. Our study, in combination with previous reports, provide powerful evidence suggesting that ISL may be an effective therapeutic drug for the treatment of colorectal cancer.

It has been well established that apoptosis plays a vital role in carcinogenesis and the progression of cancer. Studies on the growth-inhibitory effect of ISL are extensive, with observations showing it initiates...
which plays a pivotal role in cell growth via suppression of apoptosis in multiple human malignant tumors. AKT kinase is the core component of the PI3K/AKT signaling pathway, activation of AKT also promotes carcinoma metastasis and invasion, and phosphorylates mTOR protein kinase with critically involved in the regulation of cell proliferation and survival [31]. Cyclin D1, as one of PI3K/AKT signaling pathway downstream molecules, is correlated with abnormal proliferation, invasion, and poor prognosis of cancer cells [32]. In our study, ISL treatment inhibited Akt and mTOR activation through reduction of p-AKT and p-mTOR without changes in total Akt and mTOR expression. Furthermore, our results apoptosis [28]. Bax, Bcl-2, and Caspase proteins are the key molecular players that regulate cellular apoptosis [29, 30]. Our results showed that ISL possesses the capability to inhibit HCT-116 cells proliferation by inducing apoptosis. Western blot analysis showed that cleaved Caspase-3 and Bax, pro-apoptotic proteins, were activated, while anti-apoptotic protein Bcl-2 was downregulated. Thus, these results suggested that ISL suppresses cell proliferation through an apoptotic-associated mechanism.

To further investigate the underlying molecular pathways that impact on cell growth inhibition and death of HCT-116 cells induced by ISL, we evaluated the activity of ISL on the PI3K/AKT/mTOR signaling pathway.

Fig. 3. Induction of apoptosis in HCT-116 cells by ISL
A, The cells were stained with annexin V and propidium iodide (PI) and then analyzed by FACScan instrument. The percentage of apoptotic cells was significantly increased in ISL treated groups compared with the NC groups. B, Western blot assays detected apoptosis-related protein expression of Bax, Bcl-2, active caspase-3. C-E, protein expression levels were quantified and analyzed by Image-Pro Plus6.0 software. Data are expressed as the mean ± SD (n=3). * p < 0.05 indicates statistically significant differences between ISL groups and NC groups.
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In summary, our present findings suggest that ISL induces growth inhibition and apoptosis of human colorectal cancer cells, which may be regulated by the involvement of the PI3K/AKT signaling pathway. Based on these evidences, we demonstrate that ISL may be promising therapeutic for the treatment of human colorectal carcinogenesis.

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Abbreviations

ISL Isoliquiritigenin
AKT protein kinase B
p-AKT phospho-AKT
p-mTOR phospho-mTOR
p-P70s6k phospho-p70s6 Kinase
PI3K phosphatidylinositol 3-kinase
ECL enhanced chemiluminescence
CCK8 Cell Counting Kit-8
Annexin V-FITC annexin V-fluorescein isothiocyanate
SD standard deviation
SDS-PAGE Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

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