Liquiritin represses proliferation , migration and invasion of colorectal cancer cells through inhibition of the miR-671/HOXB3 signaling pathway

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Abstract

Background: Colorectal cancer is a common malignant tumor and ranks third in cancer-related deaths. Considering that the side-effects of current therapies limit the clinical effectiveness, exploring new anti-colorectal cancer drugs from natural products is critical for the treatment of colorectal cancer. Among these drugs, liquiritin is an active component of the traditional Chinese herb *Glycyrrhiza Radix* and has been found to possess powerful anti-inflammatory and anti-tumor abilities. However, the direct molecular target of liquiritin remains unknown. Therefore, the aim of the present study was to identify potential molecular target of liquiritin to mediate its anti-colorectal cancer effect.

Methods: The function of liquiritin in cell proliferation, apoptosis, migration and invasion was estimated by CCK-8 assay, colony formation, EdU assay, flow cytometry analysis, TUNEL assay, wound healing assay and transwell assay, respectively. Animal experiment was carried out to further confirm the role of liquiritin *in vivo*. H&E staining analysis, TUNEL staining and immunohistochemistry (IHC) assay were adopted for histological analysis. The mechanism research was conducted with RT-qPCR, western blot and luciferase report assay.

Results: In this study, we found that liquiritin significantly inhibited the proliferation, migration, invasion and EMT processes of SW480 and HT-29 cells in a dose-dependent manner. MiRNAs have been extensively identified as drug targets in various studies. However, the miRNAs functioning as the direct targets of liquiritin remain unknown. In our study, we tested 8 potential pathogenic miRNAs screened from colorectal cancer patients, and we found that only the expression level of miR-671 was diminished due to liquiritin treatment. More importantly, overexpression of miR-671 partially abrogated the anti-tumor effects of liquiritin on colorectal cancer.

Conclusions: Collectively, our findings demonstrated that liquiritin exhibits great potential in the treatment of colorectal cancer through regulation of miR-671.

1. Background

Colorectal cancer is a common malignant tumor and is the third leading and second leading cause of cancer-related death among males and females, respectively [1]. Recently, the incidence of colorectal cancer worldwide increased to approximately 655,000 people annually [2]. Although the 5-year survival rate of early colorectal cancer is approximately 90%, the survival rate of metastatic colorectal cancer has decreased to 15% [3, 4]. Hence, invasive metastasis, especially extensive metastases to liver, lung, bone and abdominal cavity, are the leading causes of death. Colorectal cancer is a multistep, multistage and multigene cytogenetic disease, and it includes interactions between many factors, such as activation or inactivation of oncogenes and tumor suppressor genes, mismatch mutation of repair genes, gene overexpression and epigenetic modification of genome [5–7]. Current therapies, especially chemotherapy drugs, have achieved remarkable advances, but their side-effects restrict the clinical effectiveness. In this regard, many studies have focused on the development of drugs from natural products, which are

considered safe. Therefore, exploring new anti-colorectal cancer drugs from natural products is critical for the treatment of colorectal cancer.

Glycyrrhiza Radix, as a traditional Chinese medicine, is one of the most commonly used drugs in clinical practice, and it belongs to leguminous plants [19, 20]. Currently, Glycyrrhiza Radix is widely used for anti-cancer effects, liver protection, liver detoxification, anti-inflammation effects, anti-allergy effects, immune regulation and other aspects [21, 22]. Liquiritin (Fig. 1A), an active component of Glycyrrhiza Radix, possesses multiple pharmacological activities including anti-inflammation, neuroprotection, anti-cancer activities [23, 24]. For example, liquiritin induces apoptosis and autophagy in cisplatin (DDP)-resistant gastric cancer [25]. In addition, liquiritin exhibits significant inhibitory effects on the growth of cervical cancer cells via activation of caspase-3 [26]. Furthermore, in combination with isoliquiritin and isoliquirigenin, liquiritin significantly aggravates apoptosis in non-small cell lung cancer cells via regulation of p53 and p21 [27]. However, the specific role and the direct molecular target of liquiritin remain unknown, which restricts its clinic application and potential structure modification to improve its beneficial functions. Thus, this study was performed to explore the precise effects of liquiritin on colorectal cancer in vitro and in vivo, as well as its underlying mechanism.

MicroRNAs (miRNAs) are a type of noncoding RNAs present in various organs and implicated in the regulation of multiple biological processes. Mature miRNAs are typically 19 to 25 nucleotides in length. It should be noted that abnormal expression patterns of miRNAs exist in human tumors, including the amplification, deletion, aberrant post-transcriptional control, epigenetic alterations or defects of miRNAs [8–10]. Hence, under certain conditions, these dysregulated miRNAs activate the proliferation of cancer cells, resist apoptosis, trigger invasion, promote metastasis signals, and induce angiogenesis [11, 12]. For the colorectal cancers, various miRNAs are also involved in the regulation of cell growth and metastasis of colorectal cancer. For instance, miR-141-3p is decreased in colorectal cancer, while overexpression of miR-141-3p significantly inhibits colorectal cancer cell proliferation, migration and invasion by targeting TRAF5 [13]. MiRNA-766 aggravates cell apoptosis through regulation of p53/Bax signaling via targeting MDM4 in human colorectal cancer [14]. Furthermore, miR-214 enhances the chemo-sensitivity of 5-FU by targeting HSP27 in colorectal cancer [15], indicating that miRNAs also function as drug targets. However, the miRNAs that function as the direct targets of liquiritin remain unknown. In our study, we tested 8 potential pathogenic miRNAs screened from colorectal cancer patients. Of these miRNAs, only miR-671 was sensitive to liquiritin treatment. MiR-671, as a member of the miR-671 family, is closely associated with the development of malignant tumors. For example, overexpression of miR-671 induces growth and metastasis of glioblastoma multiforme via targeting CDR1-AS, CDR1, and VSNL1 [16]. In addition, miR-671 is induced in clinical prostate cancer tissues, and repression of miR-671 remarkably inhibits prostate cancer cell proliferation by targeting SOX6 [17]. Moreover, miR-671 suppresses gastric cancer cell proliferation and enhances cell apoptosis by targeting URGCP [18]. Nevertheless, the specific effects and mechanisms of miR-671 on colorectal cancer have not been elucidated.

Herein, we found that liquiritin inhibited cell proliferation, promoted cell apoptosis, reduced cell migration, reduced invasion and suppressed EMT in a dose-dependent manner in colorectal cancer. Mechanistically,

liquiritin decreased the expression of oncogenic miR-671 but increased HOXB3 levels. More importantly, overexpression of miR-671 partially abrogated the beneficial effects of liquiritin on colorectal cancer progression, indicating the regulatory role of miR-671 in relaying liquiritin signals. In conclusion, our study indicated that liquiritin impedes the progression of colorectal cancer via regulation of miR-671/HOXB3.

2. Materials And Methods

2.1 Cell culture

Colorectal cancer cell lines, including SW480 and HT-29, as well as the NCM460 normal colonic epithelial cell line were obtained from the American Type Tissue Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) in a 5% CO₂ atmosphere at 37°C.

2.2 Cell viability assay

Cell viability of colorectal cancer cells treated with liquiritin was determined by the Cell Counting Kit 8 assay (CCK-8, Dojindo Molecular Technology, Rockville, MD, USA). In brief, HT-29 and SW480 cells ($1 \cdot 10^4$ cells/well) were cultured in 96-well plates. The medium was replaced with medium containing indicated concentrations of liquiritin (10, 30 and 90 µM) and incubated in a 5% CO₂ atmosphere at 37°C for 24, 48 and 72 h. Subsequently, 10 µL of CCK-8 solution was added to each well for an additional 3 h. Finally, a microplate reader was used to measure the absorbance at 450 nm.

2.3 Cell proliferation assay

Colony formation and EdU analyses were performed to determine the effects of liquiritin on the proliferation of HT-29 and SW480 cells. For the colony formation assay, HT-29 and SW480 cells (2×10^4 cells/well) were seeded into 24-well plates, and the medium was replaced with medium containing indicated concentrations of liquiritin (10, 30 and 90 µM) every 2–3 days. After incubation for 12 days, these cells were fixed with paraformaldehyde for 30 min and stained with 10% crystal violet for 30 min. For the EdU assay, cells were incubated with medium containing liquiritin (10, 30 and 90 µM) for 48 h and 100 µL of EdU (50 µM) was added into each well and cultured for an additional 8 h. SW480 and HT-29 cells were fixed and stained according to the manufacturer's protocol (Life Technologies, NY, USA).

2.4 Flow cytometry analysis

The role of liquiritin in apoptosis of HT-29 and SW480 cells was assessed by flow cytometry analysis. In brief, HT-29 and SW480 cells ($1 \cdot 10^4$ cells per well) were seeded in 24-well plates, and treated with indicated concentrations of liquiritin (10, 30 and 90 µM) for 48 h. Subsequently, 300 µL of $1 \times$ binding buffer was added to each well, and the treated cells were washed twice with PBS. Then, 5 µL of Annexin V-PE was added for 15 min, and 5 µL of 7-AAD solution was added for 5 min. Finally, 200 µL of $1 \times$ Binding Buffer was added for flow cytometry analysis.

2.5 TUNEL assay

For in vitro experiments, the TUNEL assay was performed to explore the effects of liquiritin on the apoptosis of HT-29 and SW480 cells according to the manufacturer's protocol (Roche, USA). In brief, HT-29 and SW480 cells were treated with indicated doses of liquiritin (10, 30 and 90 μM) for 48 h. Cells were then fixed with methanol-free formaldehyde solution for 30 min, and cells were permeabilized with permeabilization solution for an additional 2 min. The TUNEL reaction mixture was subsequently added to slides and incubated for 1 h at 37°C in dark. Representative images were acquired with a microscope. For in vivo experiments, the apoptosis of paraffin-embedded tumor sections was detected by the TUNEL assay. Briefly, sections were dewaxed, and permeabilized with proteinase K for 15 min at room temperature. Sections were then treated with 3% H_2O_2 to block endogenous peroxidases, and incubated with equilibration buffer and terminal deoxynucleotidyl transferase enzyme. Finally, sections were incubated with antidigoxigenin-peroxidase conjugate. Finally, DAB was used for visualization. Sections were examined under a light microscope.

2.6 Wound-healing assay

The migration abilities of HT-29 and SW480 cells treated with liquiritin were evaluated by a wound-healing assay. Briefly, HT-29 and SW480 cells ($5 \cdot 10^6$ cells/well) were seeded into 6-well plates and grew to confluence. A standard wound (< 3 mm) was created on the cell monolayer (time set as 0 h), and then cells were subjected to liquiritin treatment at indicated doses in serum-free medium for 48 h. The width of the scratch was imaged and recorded under a microscope ($\times 200$).

2.7 Transwell migration and invasion assays

The migration and invasion abilities of SW480 and HT-29 cells in response to liquiritin treatment were determined using a transwell chamber assay. For the migration assay, $5 \cdot 10^4$ SW480 or HT-29 cells were seeded into the transwell chambers. For the invasion assay, Matrigel was diluted with medium (1:3), and 35 to 45 μL of the solution was added to transwell chambers. For transwell migration and invasion assays, the lower wells of the chamber were filled with 600 μL of medium containing 20% FBS. SW480 or HT-29 cells ($5 \cdot 10^4$ cells) were treated with indicated doses of liquiritin (0, 10, 30 and 90 μM) and allowed to migrate or invade for 24 h. Subsequently, non-migrated cells were removed from the upper side of the membrane with cotton swabs. Cells on the lower side of the membrane were stained with 350 μL of Wright-Gimsa dye for 5 min. Finally, four fields of view were imaged under a light microscope ($\times 400$).

2.8 RNA extraction and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from tumor tissues or cells using TRIzol reagent following the manufacturer's protocol. MiR-671, Bcl-2, Bax, cleaved-caspase-3, cleaved-caspase-9, E-cadherin, N-cadherin, vimentin and HOXB3 RNA levels were amplified by qPCR using the SYBR Green reagent (Takara, Dalian, China) on a Stratagene 3005p system (Agilent Technologies, Mississauga, Canada). GAPDH and U6 housekeeping

genes were used as internal standards. A $2^{-\Delta\Delta CT}$ method was used to measure the relative expression level. Primer sequences were used: miR-671 forward, 5'-GCCCCGAGGAAGCCCUGGAGGGGC-3'; miR-671 reverse, 5'-GTGCAGGGTCCGAGGT-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3'; U6 reverse, 5'-AACGCTTCACGAATTTGCGT-3'; Bcl-2 forward, 5'-AGATGTCCAGCCAGCTGCACC-3'; Bcl-2 reverse, 5'-TGACCCACCGAACTCAAAGA-3'; Bax forward, 5'-CCCCCGAGAGGTCATCGTCCG-3'; Bax-2 reverse, 5'-GGGCCTTGCGCACCAAGTTTGC-3'; cleaved-caspase-3 forward, 5'-ACATCTCCCGCGCGGGCCCGGA-3'; cleaved-caspase-3 reverse, 5'-TCCGCGGCCCGCCGCGGGAGATGT-3'; cleaved-caspase-9 forward, 5'-CGAACTAACAGGCAAGCA-3'; cleaved-caspase-9 reverse, 5'-CAAATCCTCCAGAACCAAT-3'; E-cadherin forward, 5'-GAAGCACAGAATCCCAAGTG-3'; E-cadherin reverse, 5'-GGCGTGTTTGTCTTCCATTTC-3'; N-cadherin forward, 5'-GTGCCATTAGCCAAGGGAATTCAGC-3'; N-cadherin reverse, 5'-GCGTTCTGTTCCTACTCATAGGAGG-3'; vimentin forward, 5'-CCGACACTCCTACAAGATTTAGA-3'; vimentin reverse, 5'-CCGACACTCCTACAAGATTTAGA-3'; HOXB3 forward, 5'-CCAAGAGCAA GGAGCTCAAC-3'; HOXB3 reverse, 5'-CTGTTTGGTGAGGGTGGAGT-3'; GAPDH forward, 5'-GGACCTGACCTGCCGTCTAG-3'; and GAPDH reverse, 5'-GTAGCCCAGGATGCCCTTGA-3'.

2.9 Western blot assay

Proteins from tumor tissues or cells were extracted according to the manufacturer's protocol, and the protein concentrations were determined using the BCA Protein Assay Kit (Beyotime, Beijing, China). Equal amount of protein was loaded on 12% SDS-PAGE for electrophoresis separation. The protein bands were transferred to PVDF membranes and then incubated at 4 °C overnight with primary antibodies purchased from Abcam, including Bcl-2 (#2872; 1:1000), Bax (#2774; 1:1000), Cleaved-caspase-3 (#9661; 1:1000), Cleaved-caspase-9 (#20750; 1:1000), MMP-2 (#4022; 1:1000), MMP-9 (#3852; 1:1000), E-cadherin (#17742; 1:1000), N-cadherin (#13116; 1:1000), vimentin (#5741; 1: 1000), HOXB3 (ab83404; 1: 1000) and GAPDH (#7076; 1: 2000). The protein bands were incubated with HRP- conjugated secondary antibody at room temperature for 1 h. The membrane was imaged using the ChemiDocTMMP imaging system, and ImageJ software was used to analyze the gray value. GAPDH was selected as the internal reference.

2.10 Luciferase reporter assay

The putative binding site in the 3'-untranslated regions (3'-UTR) of HOXB3 was mutated using mutagenesis kit. Wild type and mutant sequences were amplified and inserted into the vector to construct luciferase reporter plasmids according to the manufacturer's recommendations (Promega, USA). The luciferase activities were detected with the Dual Luciferase Reporter Kit (Promega, USA).

The 3' UTR region of human HOXB3 cDNA containing one putative target site for miR-671-3p was amplified and inserted into the vector to construct luciferase reporter plasmids according to the manufacturer's recommendations (Promega, Madison, WI, USA). A mutant version with a mutation of 7 bp (GAACCGG mutated to GACAAGC) from the sites of perfect complementarity was also generated. Wild-type (WT) and mutant (Mut) inserts were confirmed by sequencing. For transfection, 200 ng of WT or Mut reporter vector was transfected into either HT-29 or SW480 cells together with miR-671-3p mimics.

Relative luciferase activities (ratios of Renilla luciferase signal normalized to firefly luciferase) were determined 48 h after transfection. Equal amounts of NC mimic were used for all the transfections.

2.11 Xenograft tumors in nude mice

To detect the effect of tumor growth in vivo, SW480 cells (1×10^6 cells per mouse) were injected subcutaneously into the right axilla of the nude mice (Model Animal Research Center of Nanjing University, Nanjing, Jiangsu, China). Tumors were allowed to grow until their average diameter reached over 10 mm. Then, the experimental nude mice were treated with indicated doses of liquiritin (10, 20, 40 mg/kg i.p.) every 3 days for 30 days. Following a 30-day period, nude mice were euthanized by a high dose of anesthesia (150 mg/kg pentobarbital, intraperitoneal injection) as per protocols set by the Ethical Committee of Jiangsu Cancer Hospital. After the nude mice were sacrificed, neoplasms were isolated for further analyses. Tumor volumes were recorded by slide calipers every week and calculated with the formula: volume = $0.5 \times \text{length} \times \text{width} \times \text{depth}$.

2.12 Hematoxylin and eosin (H&E) staining

Tumor slices were stained with hematoxylin for 5 min, rinsed for 1 min, and incubated with 1% ammonia (30 s). Slices were then rinsed with running water (1 min). Slices were stained with by 0.5% H&E (for 1 min), rinsed (for 30 s), made transparent, and mounted with neutral gum.

2.13 Immunohistochemistry (IHC) assay

Tumor sample slides were deparaffinized (xylene), rehydrated (ethanol), and incubated with H_2O_2 at 37 °C for 10 min. Tumor sections were incubated with antibodies against Ki67 (ab15580; 1: 1000; Abcam, USA) and HOXB3 (ab83404; 1: 1000; Abcam, USA) at 4 °C overnight., followed by incubation with the biotin-conjugated goat anti-rabbit immunoglobulin G secondary antibody (ab6721; 1: 1000; Abcam, USA) at 37 °C for an additional 30 min. DAB was used as chromogenic agent. Images were obtained using a microscope ($\times 400$).

2.14 Statistical analysis

Graph Pad Prism 5.0 statistical software was used to perform all statistical analyses. Measurement data were represented as the mean $\pm$ SD. Statistical differences among multiple groups were analyzed by one-way ANOVA followed by a Bonferroni post hoc analysis. A value of $P < 0.05$ was considered as statistically significant.

3. Results

3.1 Liquiritin represses the proliferation of colorectal cancer cells

As shown in Fig. 1B, the CCK-8 assay revealed that liquiritin inhibited HT-29 and SW480 cell viability in a dose-dependent manner. To further assess the effects of liquiritin on the proliferation of colorectal cancer cells, we performed the EdU assay and found that liquiritin dose-dependently repressed the proliferation of HT-29 (inhibition by 61%) and SW480 cells (inhibition by 68%) in a dose-dependent manner (Fig. 1C). These results were confirmed by the colony formation analysis (Fig. 1D). These data suggested that liquiritin executes inhibitory effects on colorectal cancer cell proliferation.

3.2 Liquiritin aggravates apoptosis of colorectal cancer cells

To determine the effect of liquiritin on the cell apoptosis of HT-29 and SW480 cells, flow cytometry analysis was performed. As expected, liquiritin (10, 30 and 90 μM) significantly induced SW480 and HT-29 cell apoptosis compared to the untreated group (Fig. 2A). In addition, the TUNEL assay revealed that the apoptosis of HT-29 and SW480 cells was gradually augmented with increasing concentrations of liquiritin (Fig. 2B). In HT-29 and SW480 cells treated with liquiritin at the indicated doses, the mRNA and protein expression levels of cleaved-caspase-3, cleaved-caspase-9, and BAX were decreased, whereas the mRNA and protein expression levels BCL2 were increased (Fig. 2C and 2D). All of the effects of liquiritin occurred in a dose-dependent manner.

3.3 Liquiritin inhibits migration and invasion of colorectal cancer cells

To identify the potential role of liquiritin in the regulation of colorectal cancer cell migration and invasion, wound-healing, transwell migration and transwell invasion analyse were performed. As shown in Fig. 3A, the wounding healing rate in liquiritin (10, 30 and 90 μM) groups was significantly decreased compared to that of untreated group. Moreover, the transwell migration and invasion assays (Fig. 3B and 3C) showed that the migration and invasion of HT-29 and SW480 cells were notably repressed by liquiritin in a concentration-dependent manner (10, 30 and 90 μM). At the molecular level, the expression levels of migration- and invasion-associated proteins, including MMP-2 and MMP-9, were decreased in response to liquiritin treatment at the indicated doses (Fig. 3D). In addition, epithelial-mesenchymal transformation (EMT) plays an important role in the occurrence, development and metastasis of tumors [28, 29]. Key transcription factors, including E-cadherin, N-cadherin and vimentin, are extensively involved in the regulation of the EMT process [30]. Therefore, RT-qPCR and Western blot assays revealed that liquiritin significantly enhanced E-cadherin level and downregulated the expressions of N-cadherin and vimentin (Fig. 3E and 3F).

3.4 Liquiritin suppresses the growth of colorectal cancer xenografts in vivo

We next used SW480 tumor xenograft models to evaluate the suppressive effect of CLQ in vivo. As shown in Fig. 4A, the average tumor volume in the control group reached 2.5 mm^3 , whereas 10 mg/kg,

30 mg/kg and 90 mg/kg liquiritin treatments remarkably decreased tumor volume by 81.8%, 61.1% and 40.8%, respectively, compared to the control group. Consistently, the xenograft tumor weights were reduced by 73.7%, 56.6% and 31.6% in the 10 mg/kg, 30 mg/kg and 90 mg/kg liquiritin treatment groups, respectively (Fig. 4B and 4C). Histologically, H&E staining analysis showed that liquiritin treatment significantly decreased tumor cell density and blurred tumor cell borders but increased severe necrosis (Fig. 4D). TUNEL staining revealed that liquiritin increased tumor cell apoptosis in vivo in a dose-dependent manner (Fig. 4E). Immunohistochemistry (IHC) analysis indicated that the number of Ki-67-positive cells gradually reduced in response to liquiritin treatment (Fig. 4F), confirming the inhibitory effects of liquiritin on tumor growth. Additionally, liquiritin treatment led to enhanced expression of E-cadherin and reduced N-cadherin levels (Figure S1A and 1B). These data indicated that liquiritin exerts anti-colorectal cancer properties in vivo.

3.5 Liquiritin decreases the expression of miR-671 and promotes the expression of HOXB3 in colorectal cancer in vitro and in vivo

MiRNAs have been extensively identified as drug targets in various studies [31]. However, the miRNAs that function as direct targets of liquiritin remain unknown. Based on a previously performed microarray, eight potential miRNAs were screened from colorectal cancer patients. We hypothesized that these miRNAs may be the target of liquiritin. We tested these miRNAs in our settings and found that only the expression level of miR-671 was diminished due to liquiritin treatment (Fig. 5A). In contrast to normal colonic epithelial cells NCM460, miR-671 level was elevated, and the HOXB3 level was downregulated in colorectal cancer cells (Figure S1C and 1D). On the other hand, according to previous studies, miR-671 and HOXB3 levels are abnormally expressed and exhibit important effects on the growth and metastasis in the pathogenesis of cancer [16–18, 32–34]. Therefore, we further examined the dose-dependent effect of liquiritin on the expression levels of miR-671 and HOXB3 in vitro and in vivo. As expected, liquiritin reduced the expression of miR-671 in HT-29 and SW480 cells (Fig. 5B). Similar results were demonstrated in the in vivo system (Fig. 5C). Furthermore, RT-qPCR and Western blot analyses showed that liquiritin increased the expression of HOXB3 in HT-29 and SW480 cells in a dose-dependent manner (Fig. 5D and 5E). In agreement with these results, the mRNA and protein expression levels of HOXB3 in xenografts were enhanced correspondingly with liquiritin treatment (Fig. 5F). These data suggested that liquiritin may exhibit inhibitory effects on the growth of colorectal cancer via regulation of miR-671 and HOXB3.

3.6 HOXB3 is a direct target gene of miR-671

To validate if HOXB3 is a target gene of miR-671 that mediates its function in the development and progression of colorectal cancer, TargetScan analysis was employed, which indicated that HOXB3 may be a candidate target of miR-671 (Fig. 6A). To further dissect the relationship between HOXB3 and miR-671, HT-29 and SW480 cells were transfected with NC mimics or miR-671 mimics. The overexpression efficiency was presented in Fig. 6B. The luciferase reporter assay revealed that miR-671 inhibited the activity of the human HOXB3 3'UTR region. More importantly, the inhibitory effects of miR-671 were abolished when the miR-671-3p-binding sites in the HOXB3 3'UTR region were mutated in HT-29 and

SW480 cells (Fig. 6C). Moreover, overexpression of miR-671 significantly decreased the HOXB3 expression in HT-29 and SW480 cells at transcriptional and translational levels (Fig. 6D and 6E). Therefore, HOXB3 may be a direct target gene of miR-671.

3.7 Liquiritin affects the proliferation of colorectal cancer cells through regulation of miR-671/HOXB3

To further demonstrate the role of miR-671 in mediating the inhibitory effect of liquiritin on cell proliferation, we transfected either miR-671 or NC mimics into HT-29 and SW480 cells for 48 h, followed by liquiritin treatment. The overexpression efficiency was presented in Fig. 6B.) CCK-8 and EdU analyses indicated that overexpression of miR-671 accelerated the proliferation of HT-29 and SW480 cells by 1.63-fold and 1.59-fold, respectively. More importantly, this overexpression abrogated the liquiritin-inhibited proliferation of HT-29 and SW480 cells (Fig. 7A and 7B), indicating that miR-671 mediates the beneficial effects of liquiritin on the proliferation of colorectal cancer cells.

3.8 Liquiritin affects the migration and invasion of colorectal cancer cells through regulation of miR-671/HOXB3

To further confirm if miR-671 is involved in the liquiritin-induced inhibition of colorectal cancer cell migration and invasion, we transfected HT-29 and SW480 cells with miR-671 mimics for 48 h followed by liquiritin treatment. As shown in Fig. 8A to 8C, overexpression of miR-671 alone promoted the migration and invasion of HT-29 and SW480 cells, while this overexpression partially antagonized the repression effect of liquiritin on cell migration and invasion. In addition, RT-qPCR and western blot assays indicated that overexpression of miR-671 repressed E-cadherin expression but promoted N-cadherin and vimentin expression at both mRNA and protein levels (Fig. 8D and 8E). Importantly, overexpression of miR-671 partially abrogated the beneficial effects of liquiritin on the EMT process. These results indicated that miR-671 partially mediates the function of liquiritin in the migration and invasion of colorectal cancer cells.

4. Discussion

Colorectal cancer is a common malignancy that has been ranked the third fatal cancer in recent years. In China, the morbidity and mortality of colorectal cancer have been increasing year by year [1, 2]. Surgical resection is the most effective treatment for colorectal cancer, but these patients have poor prognosis and are prone to recurrence after surgery [35]. Therefore, colorectal cancer treatment is largely dependent on medical treatment. However, although 5-fluorouracil is the most basic chemotherapy drug for colorectal cancer, its single drug efficiency is not high [36]. Hence, conventional chemotherapy is not functional for the treatment of colorectal cancer. In recent years, new chemotherapeutic drugs, such as capecitabine and irinotecan, as well as the molecular targeting drug cetuximab, have been developed and achieved

certain curative effects. However, shortcomings, including high price, large toxicity and side effects, are inevitable in such treatments [37–39]. As a result, developing highly effective, low cost anti-colorectal cancer drugs is still a top priority. In this study, we demonstrated liquiritin as a potential anti-colorectal cancer drug and identified the direct molecular target, namely, miR-671, which relays the liquiritin signals.

Traditional Chinese medicine has the advantages of multitarget, high efficiency, low toxicity and cost in the treatment of cancer. These herbs have unique superiorities in stabilizing the condition and alleviating pain, thus further improving life quality and extending the survival time of cancer patients. Recently, the anti-tumor effects of nature plant extracts have become one of the hotspots in anti-cancer drug development, and they have attracted increased attention from oncologists [38–42]. *Glycyrrhiza Radix* is a traditional Chinese medicine with the positive functions in clearing heat, clearing toxic material, relieving spasm, relieving pain, reinforcing spleen function, reconciling the stomach, expelling phlegm and arresting coughing [19, 20]. Liquiritin is one of active ingredients extracted from the root of *Glycyrrhiza uralensis*. Emerging studies have shown that liquiritin has multiple beneficial effects, such as anti-oxidation, anti-allergy, immunity-enhancing, anti-thrombosis and neuroprotection effects [23, 24]. More importantly, liquiritin has powerful inhibitory effects on the development of various cancers, such as gastric cancer, cervical cancer and lung cancer [25–27]. However, the effects of liquiritin on colorectal cancer remain unknown. In the present study, liquiritin repressed cell proliferation but remarkably induced apoptosis of HT-29 and SW480 cells. These inhibitory effects of liquiritin on cancer growth were further confirmed by an in vivo xenograft experiment. Hence, liquiritin may be a promising anti-colorectal cancer drug.

To enter the blood or lymphatic circulation, stationary epithelial-derived tumor cells migrate and invade, causing tumor metastasis, which is the main cause of death for cancer patients [43]. In the present study, we also found that liquiritin substantially diminished the migration and invasion of HT-29 and SW480 cells as evidenced by decreased migrated distances and reduced protein levels of MMP-2 and MMP-9. Moreover, the EMT process promotes the migration and invasion of stationary tumor cells [44]. The concept of EMT was first proposed by Hay and Greenburg in 1982, and it was mainly used to describe the physiological process of embryonic stage [45]. Further studies have broadened the EMT concept and found that this process is also involved in the chronic degeneration of mature organs, fibrosis of mature organs and tissue remodeling and that it is closely associated with the metastasis of tumors. As EMT is an early process of tumor metastasis, we examined the effect of liquiritin on the cancer cell EMT process. We found that liquiritin repressed the protein expression levels of EMT hallmarks, such as N-cadherin and vimentin, but increased E-cadherin levels. Given the importance of EMT in versatile biological processes, the pharmacological effects of liquiritin on diseases, such as chronic degeneration, remain to be further investigated.

Recent studies have shown that chemotherapeutic drugs affect the expression of miRNAs in cancer cells, suggesting that miRNAs are extensively involved in the anticancer effects of chemotherapeutic drugs. For example, Kaempferol inhibits growth and metastasis of hepatocellular carcinoma HepG2 cells via decreasing miR-21 [46]. Ginkgetin induces G2-phase arrest in colorectal cancer cells by regulating b-Myb

and miRNA34a [47]. Dioscin exhibits protective effects against pancreatic cancer through suppression of Akt1 mediated by miR-149-3p [48]. These previous findings promoted us to hypothesize that targeting miRNAs may provide new strategies and ideas for the development of new anticancer drugs. From clinical sample sequences, eight miRNAs were identified to be potentially involved in the development of colorectal cancer. However, only miR-671 responded to liquiritin treatment, indicating its regulatory role in drug treatment. Indeed, miR-671 has been reported to be highly expressed in many tumors, such as breast cancer, glioblastoma multiforme, and gastric cancer, and down-regulation of miR-671 suppresses biological functions of tumor cells [16–18]. The role of miR-671 in cancer progression is still debated. For example, miR-671 inhibits the invasion and cell adhesion of breast cancer by binding to the 3'UTR of FOXM1 [49], whereas miR-671 promotes glioblastoma multiforme proliferation and migration by targeting CDR1-AS, CDR1, and VSNL1. Nevertheless, the specific effects and underlying mechanisms of miR-671 on colorectal cancer and liquiritin-mediated colorectal cancer have not been elucidated. In the present study, liquiritin decreased miR-671 expression, while overexpression of miR-671 promoted proliferation, suppressed apoptosis, enhanced migration, enhanced invasion and enhanced EMT in HT-29 and SW480 cells. These data suggested that miR-671 was an onco-miRNA in the colorectal cancers. Of note, overexpression of miR-671 partially abrogated liquiritin-induced inhibition of proliferation, migration, invasion and EMT as well as liquiritin-induced apoptosis in HT-29 and SW480 cells, suggesting that miR-671 is a potential liquiritin target. Moreover, it has been reported that miR-671 affects the expression of various genes to regulate cancer development [16–18]. Therefore, TargetScan was used to predict the target candidates of miR-671, and HOXB3 was found to be a potential candidate of miR-671. HOXB3 is a member of the HOX family, and it has been reported to be highly expressed in prostate cancer, pancreatic cancer and breast cancer, playing a crucial effect on the occurrence and progression of cancer [50–53]. Notably, HOXB3 is described as an oncogene in lung tumors due to its epigenetic silencing effects on the RASSF1A tumor suppressor. Consistently, overexpression of HOXB3 promotes cell growth, migration and invasion in endometrial cancer. HOXB3 also accelerates the progression of prostate cancer via transactivation of CDCA3. All of these findings indicate that HOXB3 functions as an oncogene in cancer progression. However, the role of HOXB3 in the colorectal cancer has not been identified. In our study, we demonstrated that HOXB3 was increased in response to the liquiritin treatment. In addition, the luciferase reporter assay indicated that HOXB3 was a target gene of miR-671 in HT-29 and SW480 cells. With the reduction of miR-671 induced by liquiritin, the expression level of HOXB3 was unexpectedly increased in vitro and in vivo. As HOXB3 is a molecular target of miR-671, this unexpected increase may be caused by the repressive effects of liquiritin on the miR-671. Hence, further studies are needed to investigate the direct role of HOXB3 by manipulating the expression of HOXB3 in the presence of liquiritin. Moreover, the beneficial effects of either liquiritin or suppressed miR-671 on colorectal tumor may not be simply due to one molecule. Although we found an undesirable increase of HOXB3, the net effects of liquiritin and the suppression of miR-671 caused by liquiritin impeded tumor progress. Broad bioinformatics and molecular biological analyses need to be performed to identify the direct molecular target of liquiritin/miR-671 in future studies.

5. Conclusion

To sum up, liquiritin, as a type of *Glycyrrhiza Radix*, retarded cell growth, migration and invasion of colorectal cancer *in vitro* and *in vivo* through regulation of the miR-671/HOXB3 signaling pathway. Therefore, liquiritin may be a powerful candidate compound for anti-colorectal cancer drug development.

Declarations

Ethics approval and consent to participate

This study was carried out based on the Ethics Committee of The Affiliated Cancer Hospital of Nanjing Medical University. The clinical analysis was performed according to the principles of Helsinki Declaration.

Consent for publication

All authors agree to publish.

Availability of data and material

Yes.

Competing interests

None.

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Authors' contributions

Liu Yang conceived and designed the study. Liu Yang, Yinan Zhang, Jun Bao, Ji-Feng Feng performed the literature search and data extraction. Liu Yang analyzed the data and the manuscript.

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Figures

Figure 1

Liquiritin represses the proliferation of colorectal cancer cells. (A) Chemical structure of liquiritin. (B) CCK-8 assay was performed to investigate the effect of liquiritin on the viability of HT-29 and SW480 cells. HT-29 and SW480 cells were treated with liquiritin at indicated doses. (C) EdU and (D) colony formation assays were used to determine the effects of liquiritin on cell proliferation. The results were expressed as the mean $\pm$ SD of three independent experiments, and each was experiment performed in triplicate. * $P < 0.05$, ** $P < 0.01$ vs. nontreated group.

Figure 2

Liquiritin aggravates the apoptosis of colorectal cancer cells. HT-29 and SW480 cells were treated with liquiritin at indicated doses for 48 h. (A) Flow cytometry analysis and (B) TUNEL assay were performed to evaluate the effects of liquiritin on the apoptosis of HT-29 and SW480 cells. (C) RT-qPCR and (D) Western blot analyses of the expression levels of cleaved-caspase-3, cleaved-caspase-9, Bax and Bcl-2. The band intensity was quantified by Image software. The results were expressed as the mean $\pm$ SD of three independent experiments, and each experiment was performed in triplicate. * $P < 0.05$, ** $P < 0.01$ vs. nontreated group.

Figure 3

Liquiritin inhibits migration and invasion of colorectal cancer cells. HT-29 and SW480 cells were treated with liquiritin at indicated doses for 48 h. (A) Wound-healing and (B) Transwell migration analyses were performed to examine the effects of liquiritin on migration abilities of HT-29 and SW480 cells. (C) Transwell invasion assay was performed to examine the role of liquiritin in the invasion ability of HT-29 and SW480 cells. (D) Western blot assay was used to assess the effects of liquiritin on the protein expression levels of MMP-2 and MMP-9. (E) RT-qPCR and (F) Western blot analyses of the expression levels of E-cadherin, N-cadherin and vimentin. The band intensity was quantified by ImageJ software. The results were expressed as the mean $\pm$ SD of three independent experiments, and each experiment was performed in triplicate. * $P < 0.05$, ** $P < 0.01$ vs. nontreated group.

Figure 4

Liquiritin represses the growth of colorectal cancer xenografts in vivo. SW480 cells (1×10^6 cells per mouse) were injected subcutaneously into the right flank of nude mice. When tumors became palpable on day 7, mice were randomly divided into four groups and were treated with either vehicle (i.p., daily) or liquiritin (10 mg/kg, 30 mg/kg, 90 mg/kg, i.p., daily) for 30 days ($n=3$ for each group). (A) Mice and tumors in the different groups were shown. (B) Subcutaneous tumor volumes were measured at the

indicated days. (C) Tumor weight, (D) H&E staining, (E) TUNEL staining and (F) Ki-67 staining of tumor sections. All data were shown as mean $\pm$ SD. *P < 0.05, **P < 0.01 vs. nontreated group.

Figure 5

Liquiritin decreases the expression of miR-671 and promotes the expression of HOXB3 in colorectal cancer in vitro and in vivo. (A) RT-qPCR analysis of the RNA expression levels of eight miRNAs in cells treated with or without liquiritin at the 90 μ M dose. (B and C) RT-qPCR analysis of the expression levels of miR-671 in cells or xenograft tumors treated with liquiritin at indicated doses. (D) RT-qPCR and (E) Western blot analyses of the expression levels of HOXB3 in cells treated with liquiritin at indicated doses. The band intensity was quantified by ImageJ software. (F) RT-qPCR analyses of the expression levels of HOXB3 in xenograft tumors treated with liquiritin at indicated doses. The results were expressed as the mean $\pm$ SD of three independent experiments, and each experiment was performed in triplicate. *P < 0.05, **P < 0.01 vs. non-treated group.

Figure 6

HOXB3 is a direct target gene of miR-671. (A) TargetScan analysis for target genes of miR-671. (B) RT-qPCR analysis of the transfection efficiency of miR-671 mimics. (C) Luciferase reporter assay analysis of the effect of miR-671 on the 3'UTR promoter activities of HOXB3 in HT-29 and SW480 cells. (D) RT-qPCR and (E) Western blot assays for the expression of HOXB3 in HT-29 and SW480 cells transfected with either NC or miR-671 mimics. The band intensity was quantified by ImageJ software. The results were expressed as the mean $\pm$ SD of three independent experiments, and each experiment was performed in triplicate. * $P < 0.05$, ** $P < 0.01$ vs. non-treated group.

Figure 7

Liquiritin inhibits the proliferation of colorectal cancer cells through regulation of miR-671/HOXB3. HT-29 and SW480 cells were transfected with either miR-671 or NC mimic, followed by treatment with 90 μ M liquiritin. (A) CCK-8 assay. (B) EdU assay. The results were expressed as the mean $\pm$ SD of three independent experiments, and each experiment was performed in triplicate. *P < 0.05, **P < 0.01 vs. nontreated group.

Figure 8

Liquiritin inhibits the migration and invasion of colorectal cancer cells through regulation of miR-671/HOXB3. HT-29 and SW480 cells were treated as described in Figure 7. (A) Wound healing assay and (B) Transwell migration analysis were performed to examine the effects of miR-671 on migration abilities of HT-29 and SW480 cells. (C) Transwell invasion assay was performed to examine the role of miR-671 in the invasion ability of HT-29 and SW480 cells. (D) RT-qPCR and (E) Western blot analyses were utilized to determine the effects of miR-671 on the expression levels of E-cadherin, N-cadherin and vimentin. The band intensity was quantified by ImageJ software. The results were expressed as the mean $\pm$ SD of three independent experiments, and each experiment was performed in triplicate. * $P < 0.05$, ** $P < 0.01$ vs. nontreated group. # $P < 0.05$, ## $P < 0.01$ vs. miR-671 mimics control group.

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