Ursolic acid suppresses the invasive potential of colorectal cancer cells by regulating the TGF-β1/ZEB1/miR-200c signaling pathway

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Abstract. Ursolic acid (UA) is a biologically active compound, commonly used in traditional Chinese medicine (TCM). It has been reported to exhibit strong anticancer properties against a variety of cancers. Our previous studies showed that UA promoted apoptosis in colorectal cancer (CRC) cells and inhibited cellular proliferation and angiogenesis. However, the effect and underlying molecular mechanism of UA in CRC progression remain unclear. In the present study, the role of UA in suppressing the migration and invasion of human colon cancer HCT116 and HCT-8 cells was investigated, using transwell assays. In addition, to evaluate whether the anticancer properties of UA were mediated by the regulation of a double-negative feedback loop consisting of the transforming growth factor-β1 (TGF-β1)/zinc finger E-box-binding homeobox (ZEB1) pathway and microRNA (miR)-200a/b/c, reverse transcription-quantitative PCR and western blot analysis were performed. The results indicated that UA treatment significantly suppressed cellular growth, migration and invasion in HCT116 and HCT-8 cells in a dose-dependent manner. Furthermore, following UA treatment, several crucial mediators of the TGF-β1 signaling pathway, including TGF-β1, phosphorylated (p)-Smad2/3, p-focal adhesion kinase and ZEB1, were significantly downregulated in the HCT116 and HCT-8 cell lines compared with the control group. Furthermore, the ratio of N-cadherin/E-cadherin, two proteins directly downstream of the TGF-β1 signaling pathway, was found to be downregulated in UA treated CRC cells. Finally, UA significantly upregulated miR200a/b/c, with miR-200c exhibiting the highest increase in expression levels following UA treatment. Collectively, the present study suggested that inhibition of CRC cell invasion by UA occurred via regulation of the TGF-β1/ZEB1/miR-200c signaling network, which may be one of the mechanisms by which UA appears to be an effective therapeutic agent against colon cancer.

Introduction

Globally, colorectal cancer (CRC) is one of the most common types of cancer (1). At the early stage of CRC (i.e., stages I and II), CRC is amenable to surgery and curative treatment, with a 5-year survival rate >60% for patients with CRC. However, >50% of patients present with advanced disease (at or beyond stage III) and a high incidence of distant metastasis (2). In these patients, the 5-year survival rate drops to 10% (2). During metastatic progression, cancer cells can detach from the primary tumor site, passing through the circulation system to form metastatic tumors in distant organs (3). The epithelial-mesenchymal transition (EMT) is a process that contributes to the early stages of cancer metastasis, in which polarized epithelial cells lose their cell polarity and cell-cell adhesion to acquire a mesenchymal phenotype (4). The EMT process allows cancer cells to gain migratory and invasive properties that promote cancer metastasis (4). Since metastasis is considered as the leading cause of failure in cancer treatment, the development of novel pharmaceuticals for the prevention and treatment of cancer metastasis is critical.

The transforming growth factor (TGF)-β1 pathway is essential to the EMT in cancer cells. In this process, TGF-β1 initiates downstream signaling, by the dimerized TGF-β1 ligand binding to the type III TGF-β1 receptor, which then presents the ligand to the type II TGF-β1 receptor, which leads to the phosphorylation of the type I TGF-β1 receptor (TβR-I) (5,6). In canonical TGF-β1 signaling, activated TβR-I phosphorylates the intracellular proteins Smad2/3, which in turn bind to Smad4, before translocating into the nucleus where they initiate transcriptional changes of target genes, including of the zinc finger E-box-binding homeobox (ZEB)
family (7,8). ZEB members act as suppressors of these transcriptional factors, downregulating epithelial markers, such as E-cadherin, while upregulating mesenchymal markers, such as N-cadherin, which contributes to the development of cancer metastasis (9). In addition, TGF-β1 can also signal through the non-canonical pathway, which includes focal adhesion kinase (FAK). TGF-β1 has been shown to induce the Src-dependent phosphorylation of FAK, and its consequent activation is required for the upregulation of mesenchymal and invasiveness markers and the delocalization of E-cadherin, which promotes metastasis of cancer cells (10).

microRNAs (miRNAs) represent a class of non-coding RNAs, that are 21-24 nucleotides long, that suppress target gene expression in a sequence specific manner (11). The dysregulation of several miRNAs has been identified to induce EMT and promote colorectal metastasis associated with poorer survival (12). The miR-200 family consists of five members, including miR-200a/b/c, miR-141 and miR-429, and has been shown to be master regulators of EMT, promoting cell dissemination from the primary tumor and subsequent metastasis (13). Indeed, loss of miR-200a/b/c expression has been shown to promote cellular metastasis in several cancers by inhibiting the ZEB transcription factor family and is correlated with poorer survival in patients with CRC (14-16). Notably, at the miRNA level, DNA methylation of miR-200a/b/c is a key mechanism in the negative regulation of its expression, which has been reported to be mediated by TGF-β1 signaling (17). Thus, the TGF-β1/ZEB/miR-200a/b/c signaling network (a positive correlation between TGF-β1 and ZEB, negative correlations between TGF-β1 and miR-200a/b/c and between miR-200a/b/c and ZEB) supports the maintenance of the mesenchymal phenotype required for metastasis (18).

Worldwide, agents used in Traditional Chinese Medicine (TCM) have received increasing interest in recent years for the treatment of various cancers, due to relatively low toxicity and few side effects (19). There is an urgent need to identify naturally occurring agents for effective anticancer treatments. Ursolic acid (UA) is present in many herbs and plants used in TCM, including Hedychium diffusum, Scutellaria barbata, Spica prunellae and Patrinia scabiosaeofilia, and possesses excellent anticancer properties against various types of cancers, including CRC (20-23). Increasing evidence indicates that UA has several biological properties, such as anti-inflammatory, antiviral, antioxidant, cytotoxic, anticancer, and anti-diabetic (24-30). Our previous studies have shown that UA induced CRC cell apoptosis and suppressed cell proliferation and CRC angiogenesis via multiple signaling pathways (28,29). In the present study, the effect of UA on CRC cell migration and invasion in vitro was further evaluated and the potential molecular mechanisms of its action were elucidated.

Materials and methods

**Material and reagents.** UA was purchased from Sigma-Aldrich (Merck KGaA). RPMI-1640 medium, PBS and penicillin-streptomycin were purchased from Hyclone (GE Healthcare Life Sciences). Fetal bovine serum (FBS) and trypsin-EDTA were obtained from Gibco (Thermo Fisher Scientific, Inc.). 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Beijing Solarbio Science & Technology Co., Ltd. miR-200a/b/c and U6 primers were synthesized by Takara Biotechnology Co., Ltd. RNAiso for small RNA kit, Mir-X™ miRNA First-Strand Synthesis kit and TB Green™ Premix Ex Taq II kit were purchased from Takara Biotechnology Co., Ltd. N-cadherin (cat. no. ab18203) and E-cadherin (cat. no. ab1416) antibodies were purchased from Abcam. TGF-β1 (cat. no. 3711), phosphorylated (p-) Smad2/3 (cat. no. 8828), Smad2/3 (cat. no. 8685), p-FAK (cat. no. 3284), FAK (cat. no. 71433), ZEB1 (cat. no. 3396) and horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit IgG; cat. no. 7074; and anti-mouse IgG; cat. no. 7076) were purchased from Cell Signaling Technology, Inc. The β-actin antibody (cat. no. 66009-1-Ig) was purchased from ProteinTech Group, Inc. The Transwell chambers were obtained from Corning Life Sciences and the BD BioCoat Matrigel Invasion Chamber was purchased from BD Bioscience. All the other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (Merck KGaA).

**Cell culture.** Human colon cancer HCT116 and HCT-8 cell lines were obtained from the Nanjing KeyGen Biotech. Co. Ltd. Cells were cultured in RPMI-1640 medium, supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin in a 37°C humidified incubator supplemented with 5% CO₂.

**MTT assay.** UA was dissolved in DMSO and diluted with culture medium to the desired concentrations. The final concentration of DMSO in the culture medium was <0.1% throughout the study. HCT116 and HCT-8 cells were plated into 96-well plates at a 1x10⁴ cells/well and treated with 0, 10, 20, 40 µM UA for 24 h and 48 h. Treatment with DMSO was included as the vehicle control. Following treatment, 100 µl of 0.5 mg/ml MTT solution was added in each well at 37°C for 4 h. The MTT formazan precipitate was dissolved in 100 µl of DMSO. Subsequently, the resulting absorbance of the purple formazan product was determined at 570 nm with a ELX800 microplate reader (BioTek Instruments, Inc.). The cell viability was determined using the formula: Cell viability (%)=sample optical density (OD)/control OD x100.

**Transwell assay.** To evaluate the cell migration and invasion, Transwell assays were conducted using Transwell cell culture chambers with 8 µm pore filters (Corning Life Sciences). After treatment with 0, 10, 20, 40 µM of UA for 24 h, HCT116 and HCT-8 cells were harvested and resuspended in serum-free RPMI-1640 without UA. Then, ~5x10⁴ cells that survived the indicated concentrations of UA treatment for 24 h were seeded into the upper chambers. The lower chambers were filled with RPMI-1640 media containing 10% FBS as a chemoattractant. Cells were allowed to migrate towards the complete medium for 12 h in the migration assay, the non-migrating cells in the upper chamber were wiped and the migrated cells were stained with crystal violet for 15 min at room temperature. For quantification, the average number of migrated cells per field was assessed by counting three random fields under a phase contrast microscope (Leica Microsystems GmbH) at a magnification of x200. The cell invasion assay was similar to the migration assay, except that the upper chambers were coated with Matrigel matrix (BD Biosciences).
RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Following treatment with the indicated concentrations of UA for 24 h, total small RNA was extracted using RNAiso for small RNA kit. Total small RNA was reverse transcribed with an Mir-X™ miRNA First-Strand Synthesis kit following the manufacturer's protocol. The primers for miR-200a (cat. no. DHM0178), miR-200b (cat. no. DHM0179), miR-200c (cat. no. DHM0180) and U6 (cat. no. D356-03) were obtained from Takara Biotechnology Co., Ltd. The obtained cDNA was used to determine the levels of miR-200a, miR-200b and miR-200c using TB Green™ Premix Ex Taq II in an ABI 7500 Fast PCR system, according to the manufacturer's instructions. The PCR conditions were as follows: Pre-denaturation at 95°C for 2 min, then 45 cycles of 95°C for 3 sec and 60°C for 30 sec. U6 was used as an internal control. Relative quantification was performed using the 2−ΔΔCq method (31). Each PCR amplification was carried out in triplicate.

Western blot analysis. After treatment with the indicated concentrations of UA for 24 h, total protein was extracted with cell lysis buffer (Pierce; Thermo Fisher Scientific, Inc.) containing protease and phosphatase inhibitors. The concentration of total protein was detected using a bicinchoninic acid assay. A total of 50 µg protein was separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes (EMD Millipore Corporation). The membranes were blocked with 0.5% BSA for 2 h at room temperature and then probed with primary antibodies against TGF-β1 (1:1,000), p-Smad2/3 (1:1,000), Smad2/3 (1:1,000), p-FAK (1:1,000), FAK (1:1,000), N-cadherin (1:1,000), E-cadherin (1:2,000, ZEB1 (1:1,000) and β-actin (1:5,000) overnight at 4°C, followed by 1 h incubation with the anti-rabbit IgG HRP-conjugated secondary antibodies (1:2,000) or anti-mouse IgG HRP-conjugated secondary antibodies (1:2,000) at room temperature. Subsequently, using TBS/Tween-20 to wash the membranes, the immunoreactive bands were visualized via Image Lab software (version 3.0; Bio-Rad Laboratories, Inc.) using enhanced chemiluminescence (Yuheng Biotechnology Co., Ltd.).

Statistical analysis. All data were expressed as mean ± standard deviation. Data were analyzed using SPSS software (version 16.0; SPSS Inc.). Statistical analysis was performed using one-way analysis of variance and least significant difference post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

UA inhibits the growth of HCT116 and HCT-8 cells. To evaluate the inhibitory effect of UA on the growth of HCT116 and HCT-8 cells, MTT assays were performed. UA treatment significantly inhibited cell viability in both a dose- and time-dependent manner (Fig. 1A and B). After 24 h of treatment, the half maximal inhibitory concentration (IC50) values of UA for HCT116 and HCT-8 cells were calculated to be 37.2 and 25.2 µM, respectively. Similarly, after 48 h of treatment,
Figure 2. Effect of UA on the migration of HCT116 and HCT-8 cells. (A) HCT116 and (B) HCT-8 cell migration was determined using Transwell assays, following treatment with 0, 10, 20, 40 µM UA for 24 h. Cells were stained with crystal violet; the images were captured at a magnification of x200. The average numbers of migrated cells were counted in three random fields on the lower surface of the membrane. Data were normalized to the untreated control cells. Data are expressed as the mean ± standard deviation from three independent experiments. *P<0.01 vs. untreated control cells. UA, ursolic acid.

Figure 3. Effect of UA on the invasion of HCT116 and HCT-8 cells. (A) HCT116 and (B) HCT-8 cell invasion was determined using Matrigel-coated Transwell assays, following treatment with 0, 10, 20, 40 µM UA for 24 h. Cells were stained with crystal violet; the images were captured at a magnification of x200. The average numbers of invasive cells were counted in three random fields on the lower surface of the membrane. Data were normalized to the untreated control cells. Data are expressed as the mean ± standard deviation from three independent experiments. *P<0.01 vs. untreated control cells. UA, ursolic acid.
the IC50 values of UA were determined to be 28.0 and 19.4 µM for HCT116 and HCT-8 cells, respectively. Cells exhibiting condensed and fragmented nuclei are considered as growth inhibited. Phase-contrast microscopy was used to examine the effect of UA on HCT116 and HCT-8 cell morphology. Untreated control cells were observed in a confluent monolayer, healthy and attached to the culture plate, whereas UA treatment significantly decreased the confluence of these two cell lines and resulted in condensed, fragmented and detached cells, in a dose-dependent manner (Fig. 1C). Taken together, these results indicated that UA significantly inhibited the growth of HCT116 and HCT-8 cells.

UA inhibits migration and invasion in HCT116 and HCT-8 cells. Transwell assays were performed to evaluate the effect of UA on the migration of HCT116 and HCT-8 cells. As shown in Fig. 2, the results demonstrated that treatment with 10-40 µM UA significantly decreased the migratory rate of HCT116 and HCT-8 cells by 23.3±4.2-91.3±1.2% and 25.5±2.4-98.8±0.2%, respectively, when compared with untreated cells. Furthermore, UA was demonstrated to inhibit the invasive abilities of HCT116 and HCT-8 cells. The results revealed that the invasion rate of HCT116 and HCT-8 cells following UA treatment was 36.2±1.8-67.9±1.6% and 33.8±3.7-98.2±0.2%, respectively, compared with that in the untreated cells (Fig. 3). Taken together, these results suggest that UA exhibited an inhibitory effect on the migration and invasion properties of HCT116 and HCT-8 cells in a dose-dependent manner.

UA inhibits TGF-β1/Smad and TGF-β1/FAK signaling pathways and regulates EMT-related proteins in HCT116 and HCT-8 cells. EMT has been shown to be associated with the metastasis of tumor cells (32). TGF-β1 signaling pathways, including the canonical TGF-β1/Smad pathway and the non-canonical TGF-β1/FAK signaling pathway, can trigger EMT (10,33). To better understand whether UA inhibited TGF-β1 signaling pathways, the expression of several pivotal mediators of TGF-β1/Smad and TGF-β1/FAK signaling pathways was assessed in HCT116 and HCT-8 cells. As shown in Fig. 4, western blot analysis revealed that UA treatment (0, 10, 20, and 40 µM) dose-dependently decreased the expression

**Figure 4.** Effect of UA on the expression of TGF-β1 pathway-associated proteins in HCT116 and HCT-8 cells. (A) The protein expression levels of TGF-β1, p-Smad2/3, Smad2/3, p-FAK, FAK, ZEB1, N-cadherin and E-cadherin in HCT116 and (B) HCT-8 cells were determined using western blot analysis following treatment with 0, 10, 20, 40 µM UA for 24 h. β-actin was used as the internal control. Images are representative of three independent experiments. Relative densitometric analysis is shown. *P<0.05 and **P<0.01 vs. untreated control cells. UA, ursolic acid; TGF-β1, transforming growth factor β1; p-, phosphorylated; FAK, focal adhesion kinase; ZEB, zinc finger E-box-binding homeobox.
levels of TGF-β1, and the expression levels of the TGF-β1 target gene ZEB1. Activation of Smad2/3 and FAK is mediated by their phosphorylation, and UA treatment also significantly reduced the phosphorylation levels of both Smad2/3 and FAK (Fig. 4). Inhibition of the TGF-β1/Smad and TGF-β1/FAK signaling pathways by UA led to a decrease in the expression of the mesenchymal marker N-cadherin compared with that in the control cells (Fig. 4). However, no difference was observed between the control cells and UA-treated cells regarding the protein expression levels of the epithelial marker E-cadherin (Fig. 4). These results indicated that the antitumor properties of UA may be mediated by the inhibition of the TGF-β1 signaling pathways.

UA regulates the expression of miR-200a/b/c in HCT116 and HCT-8 cells. miR-200a/b/c maintains the epithelial phenotype and inhibits cell metastasis via downregulation of its target gene ZEB1 (34). In this process, TGF-β1 signaling increases the DNA methylation of miR-200a/b/c, thereby negatively regulating its expression (17). Thus, to further investigate whether UA inhibited colorectal cell invasion via regulation of the TGF-β1/ZEB1/miR-200a/b/c feedback loop, the expression levels of miR-200a/b/c were determined via RT-qPCR analysis. As shown in Fig. 5, the expression levels of miR-200a and miR-200c in HCT116 and HCT-8 cells were significantly increased following UA treatment compared with that in the untreated control cells. While the expression
levels of miR-200b did not significantly change after UA treatment in HCT116 cells compared with the untreated control, HCT-8 cells exhibited a significant increase in miR-200b levels following treatment with 40 µM UA (Fig. 5). These findings are consistent with the observation that UA inhibited the TGF-β1 pathway and the expression of ZEB1 (Fig. 4). As shown in Fig. 5, the expression levels of miR-200c exhibited the highest increase following UA treatment. Therefore, the present results suggested that UA may inhibit cell growth and invasion in HCT116 and HCT-8 cells via regulation of the TGF-β1/ZEB1/miR-200c feedback loop.

Discussion

UA is a natural triterpene acid present in various plants, fruits, flowers and berries used in TCM (35). It mediates several pharmacological processes and can be used as a preventive and therapeutic medicine against multiple chronic diseases, including cancer, metabolic syndrome, cardiovascular diseases, brain disease, liver disease, and sarcopenia (36). In particular, several studies have revealed the antitumor effects of UA in brain disease, liver disease, and sarcopenia (42). It was observed that UA inhibited CRC cell growth while having no adverse effects on the weight of mice in vivo (29). In addition, UA significantly promotes CRC cell apoptosis and suppresses cell proliferation via the regulation of numerous CRC-related signaling pathways, including STAT3, ERK, JNK, and p38 (29). Of note, UA also inhibits CRC angiogenesis through the suppression of several pivotal mediators, such as vascular endothelial growth factor-A and basic fibroblast growth factor. The inhibition of multiple signaling pathways, including those related to hedgehog, STAT3, AKT and ribosomal protein S6 kinase β-1, may be the potential mechanisms by which UA may represent a promising compound against tumor angiogenesis (28). Furthermore, UA has anti-inflammatory effects on a dextran sodium sulfate-mediated colitis model, whereas it has no effect on normal intestinal epithelial cells (data not shown). In the present study, the effect of UA on the metastatic potential of CRC cells was investigated. The inhibitory effect of UA on cell viability was confirmed in the human colon cancer cell lines HCT116 and HCT-8 and these results are consistent with previous results in HT-29 cells (29). In addition, UA treatment significantly and dose-dependently inhibited the migration and invasion of HCT116 and HCT-8 cells in Transwell assays, suggesting that UA may strong suppressive effects on cancer progression.

Metastasis is considered the predominant cause of malignant cancer progression (43). Similar to angiogenesis, the process of metastasis is complex and involves complicated interactions between the tumor and the stroma (44). Indeed, multiple signaling pathways are involved in metastasis, including the integrin pathway, the TGF-β pathway, the chemokine pathway, and the dependence receptor pathway (45). These signaling pathways regulate multiple mesenchymal and invasiveness markers, as well as epithelial markers. A recent study has reported that UA inhibited the invasive phenotype of human gastric cancer cells by decreasing the expression of matrix metalloproteinase-2 (37). The synergism between UA and metformin has been shown to significantly inhibit the invasion and migration of breast cancer cells via modulation of the 5'-AMP activated protein kinase/TOR signaling pathways (46). Furthermore, UA attenuates EMT in non-small cell lung carcinoma by targeting integrin αvβ5/matrix metalloproteinase signaling (47). Aspirin combined with UA exhibits anti-metastatic ability via influencing both EMT and epidermal growth factor receptor-mediated pathways (48). In the present study, the focus was on the TGF-β1 pathways, including canonical TGF-β1/Smad and non-canonical TGF-β1/FAK. The results demonstrated that UA treatment significantly reduced the expression of several crucial mediators of these TGF-β1 signaling pathways, including TGF-β1, p-Smad2/3, p-FAK and ZEB1, leading to a decrease in N-cadherin protein expression. A recent study has shown that ZEB1 is the direct downstream target of miR-200a/b/c and is downregulated following miR-200a/b/c activation (49), miR-200a/b/c, ZEB1 and TGF-β1 are known to regulate tumor progression (50-53), and increased the expression of miR-200a/b/c or the decrease in ZEB1 or TGF-β1 or TGF-β1 could inhibit cancer cell EMT, which deactivates cellular mobility and subsequently suppresses tumor metastasis (54-57). Notably, the present study demonstrated that UA regulated the expression levels of miR-200a/b/c, with miR-200c exhibiting the highest upregulation in HCT116 and HCT-8 cells following UA treatment.

In summary, UA inhibited the viability, migration and invasion of CRC cells in vitro, by modulating the TGF-β1/ZEB1/miR-200c signaling network. The present findings elucidated the potential underlying mechanisms of UA, and suggested that it may be an effective and promising therapeutic agent in the treatment of CRC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contribution

LZ and JML conceived and designed the experiments, analyzed the data and drafted the manuscript. LZ, QYC and JXL performed the cell experiments. QYC, JXL and YQC performed the RT-qPCR and western blot experiments. TS and JP were involved in analyzing the data and revised the manuscript critically for important intellectual content. JML gave final approval of the version to be published. All authors read and approved the final manuscript.
Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interest
The authors declare that they have no competing interests.

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