Ursolic Acid Inhibits Tumor Growth via Epithelial-to-Mesenchymal Transition in Colorectal Cancer Cells

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Ursolic acid (UA), a natural pentacyclic triterpenoid, is a promising compound for cancer prevention and therapy. However, its mechanisms of action have not been well elucidated in colorectal cancer cells. Here, using cultured human colon cancer cell lines SW620 and HCT116, this assay demonstrates that UA reduces cell viability, inhibits cell clone formation, and induces caspase-3 mediated apoptosis. Additional experiments show that UA inhibits cell migration and epithelial-mesenchymal transition (EMT), including E-cadherin, Vimentin, Integrin, Twist, and Zeb1 biomarkers. These results suggest that UA inhibits cell proliferation, invasion, and metastasis in colorectal cancer cells by affecting mechanisms that regulate EMT. Taken together, the results suggested that the anti-proliferation and anti-metastasis activities of UA was through EMT inhibition in colorectal cancer.

Key words colorectal cancer; epithelial–mesenchymal transition; metastasis; ursolic acid; invasion; metastasis

INTRODUCTION

Colorectal cancer (CRC), whose main treatments include surgery, chemotherapy and radiotherapy,1 is a common malignancy throughout the world and now ranking third among cancers in incidence and mortality in China.2,3 Medical treatments often fail because of metastasis and recurrence after surgery, as well as resistance to chemotherapy.4 In order to improve the quality of patients’ life, it is necessary to find a novel agent to effectively inhibit tumor recurrence and metastasis.

As a malignant disease, the mechanisms of CRC development are complicated and diversified, involve multiple pathways, one such is the epithelial–mesenchymal transition (EMT).5 EMT is a process in which tumor cells shift from epithelial phenotype to a mesenchymal phenotype, enabling migration and invasion.6 The symbol of EMT include the appearance of cell-surface proteins (E-cadherin, N-cadherin, and integrin), cytoskeletal proteins (α-smooth muscle actin, vimentin, and β-catenin), extracellular matrix proteins (collagens, fibronectin, and laminin), and transcription factors (Snail1, Snail2, TWIST, and LEF-1) in vitro and in vivo.7 Evidence suggests that EMT biomarkers such as N-cadherin, integrin, vimentin, TWIST, and Zeb1, play significant roles in CRC invasion and metastasis.

Several natural products have shown promising chemotherapeutic effects without harming normal tissues.8 Ursolic acid (UA; 3β-hydroxyurs-12-en-28-oic acid), a pentacyclic triterpenic acid, is widely existing in edible plants and medicinal materials.9–11 A lot of traditional Chinese medicinal herbs containing this compound, such as Hedyotic diffusa,12) Radix actinidiae,13) and Hippophae rhamnoides L INN,14) are frequently used to treat colorectal cancer in China, nonetheless the mechanisms underlying their effects are still unclear. UA shows a variety of biological effects that are likely to influence cancer development, including antiviral, anti-inflammatory, antioxidant, hepatoprotective, anti-tumor, cytotoxic, anti-metastatic activities, and anti-angiogenic.15–17) UA inhibits the metastasis of colorectal cancer by suppressing multiple biomarkers associated to invasion, angiogenesis, and metastasis.8 A recent study demonstrated that UA could inhibit EMT in non-small-cell lung cancer (NSCLC) through transforming growth factor-β (TGF-β)1 signaling pathway, and this might be the potential mechanism of resveratrol on the inhibition of invasion and metastases in NSCLC.18) However, no reports has showed on its role in EMT mediated metastasis in colorectal cancer. Therefore, we demonstrate that UA inhibits the growth and metastasis of colorectal cancer cells by affecting targets that promote EMT.

MATERIALS AND METHODS

Cell Culture and Reagents Human colorectal adenocarcinoma HCT116 and SW620 cells were purchased from the Shanghai Cell Collection (Shanghai, China). HCT116 and SW620 cells were cultured in RPMI 1640 and L-15 medium, respectively, which were all supplemented with 10% fetal bovine serum (Gibco, NY, U.S.A.), 2 mM glutamine, 100 units/mL streptomycin and penicillin (Invitrogen, Carlsbad, CA, U.S.A.). The cells were grown at 37°C in a humidified 5% CO2 atmosphere. Monoclonal antibodies specific for E-cadherin, Vimentin, Integrin, Twist, Zeb1 and glyceraldehyde-
3-phosphate dehydrogenase (GAPDH) were obtained by Cell Signaling Technology (Beverly, MA, U.S.A.). UA (>90%) was acquired to Sigma-Aldrich (St. Louis, MO, U.S.A.).

Cell Viability Assays  HCT116 and SW620 cells (ca. 6000/well in 96-well plates) were incubated with UA (0, 10, 20, 40, 60, 100, 200, and 400 μM) in triplicate at 37°C for 24 and 48 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assays were performed, and absorbance was measured at 450nm using a microplate microplate reader (Labsystems Dragon, Waltham, MA, U.S.A.). Both cell lines were performed using 5 wells per condition. Calculate the relative inhibition of cell proliferation according to the following formula: 

\[R = \left(\frac{A_{2} - A_{1}}{A_{2}}\right) \times 100\%\],

in which \(R\) is the relative inhibitory rate of cell proliferation, \(A_{1}\) is the mean absorbance value of untreated control cells, and \(A_{2}\) is the mean absorbance value of treated cells. The experiment was carried out at least 3 times.

Clonogenic Assays  Inoculated SW620 and HCT116 cells in a six-well plate (approximately 100 cells/well) and cultured in the complete medium with different concentrations of UA for 2 d. Cells were then cleaned twice by phosphate buffered saline (PBS), then the cells were cultured in medium UA and allowed to form colonies for 14 d. Medium was replaced every 4 to 5 d. Colonies were washed three times in PBS, The fixation of colonies was conducted with 95% ethanol for twenty minutes then stained with 1% crystal violet.

Scratch Wound Assays  Briefly, SW620 and HCT116 cells were seeded into 6-well-plate at a density of \(5 \times 10^5\) cells. A linear scratch wound was generated mechanically using a 20μL plastic pipette tip, after UA-pretreated. The cells were exposed to 10, 30 and 60μM of UA for 48 h. Images were captured under a microscope at 0, 6 and 12 h and the scratch width were counted.

Western Blot Analysis  SW620 cells and HCT116 cells were treated with different UA at indicated concentrations for 48 h. Total lysates from treated cells were prepared with RIPA buffer (50 mM Tris, pH 7.2; 150 mM NaCl; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate; 1% Nonidet P-40; 10 mM NaF; 1 mM Na3VO4; protease inhibitor cocktail). Lysates were sonicated for 10s and centrifuged at 14000 rpm for 10 min at 4°C. Protein concentration was determined by bicinchoninic acid assay with bovine serum albumin (BSA) as a standard (Pierce, Rockford, IL, U.S.A.). Equivalent amounts of protein (50 µg/lane) were separated on 7.5–12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, U.S.A.). Membranes were incubated with PBS containing 0.05% Tween 20 and 5% nonfat dry milk to block nonspecific binding and were incubated with primary antibodies, then with appropriate secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were visualized by using Renaissance chemiluminescence reagent (Perkin-Elmer Life Science, Boston, MA, U.S.A.). GAPDH was used as an invariant control for equal loading of total proteins. The levels of target protein bands were densitometrically determined using Image Lab Software 3.0. The variation in the density of bands was expressed as fold changes compared to the control in the blot after normalization to GAPDH.

Immunofluorescence Analysis  Immunofluorescence analysis of E-cadherin and Caspase-3 in colorectal cancer cells was conducted as previously above.\(^{19}\) In short, SW620 cells and HCT116 cells were seeded in 6-well plates and cultured in RPMI 1640 and L-15 medium supplemented with 10% FBS for 24 h, and then treated with different reagents at indicated concentrations for 48 h. Cells were incubated with anti-E-cadherin (primary antibody), anti-Caspase-3 (primary antibody) cleaned with PBS, incubated with desmin antibody (1:400), washed again and incubated with antibody conjugated which has a fluorescent tag. The nucleus were stained with 4',6-diamidino-2-phenylindole (DAPI). Cells were stained with Hoechst 33342 (Invitrogen) for 5 min and mounted. Five random fields were assessed at 400× magnification.

Statistical Analysis  Data of experiments were presented as mean ± standard deviation (S.D.), repeat at least three times of experimental data before deciding to be used. Results were compared using Student’s t-test or covariance analysis. Differences were thought distinctiveness when p-values were less than 0.05. All analyses were calculated via SPSS13.0.

RESULTS

Effect of UA on Cell Proliferation  To explore the role of UA in CRC cell proliferation, we measured the cytotoxicity of UA on SW620 and HCT116 cells, which were treated with UA at various concentrations for 24 and 48 h. The data confirm that UA inhibited the growth of both cell lines, with effects proportional to incubation time and dosage. The doses of IC\(_{50}\) of UA for 24 h were 36.56 and 51.68 μM in SW620 and HCT116 cells and HCT116 cells, respectively. After 48 h of UA treatment, the viability of the SW620 cells was approximately 61% (IC\(_{50}\) = 39.15 μM), while the viability of the HCT116 cells was approximately 31% (IC\(_{50}\) = 68.62 μM) (Figs. 1A, B). Because cell proliferation is significantly suppressed by UA at concentrations less than 60 μM, this dosage range was used in subsequent experiments.

Effect of UA on Colony Formation and Tumorigenicity
HCT116 and SW620 cells were cultured with 0.1% dimethyl sulfoxide (DMSO) (as control) or UA at 10, 30, and 60 μM for 48 h, and examined for signs that they had been released from quiescence. The cells were then cultured an additional 14 d in the absence of UA at a density appropriate for formation of discernible colonies. Data show that cells in the 60 μM UA treatment groups formed fewer colonies compared with other groups (Fig. 1C). In both cell lines, these findings suggest that UA treatment reduced a dose-dependent decrease in the number of colonies formed.

**Effect of UA on Caspase 3-Mediated Apoptosis**

We next measured the apoptosis of HCT116 and SW620 cells through the intensity of fluorescence in caspase 3. Because the level of caspase 3 is usually considered to be an indication of apoptosis. With the treatment (Figs. 2A, B), the level of caspase 3 increased in a dose-dependent manner after treatment with UA. However, the rate of apoptosis seems to be higher in SW620 cells than that in HCT116 cells, similarity with the results of cell proliferation. Both of them showed the remarkable apoptosis inhibition of UA in colorectal cancer.

**UA Modulates Cell Invasion/Migration**

To explore the role of UA on the invasive and migratory capabilities of HCT116 and SW620 cells, we examined cellular mobility in a wound-healing assay (Figs. 3A, B). As expected, the ability of both cell lines to invade and migrate was significantly reduced by UA in a time- and dose-dependent manner.

**UA Affects the Epithelial Phenotype of Colorectal Cancer Cells**

The vast majority of studies support that EMT is a metastable cellular process, which closely related to cancer invasion and metastasis. Since UA inhibits CRC cell mobility and invasiveness, we speculated that UA might inhibit EMT-mediated cancer invasion and metastasis in HCT116 and SW620 cells. Cells were cultured with a range of UA concentrations for 48 h and then examined using fluorescence microscopy. As expected, HCT116 and SW620 cells are becoming round from spindle or matrix, compared with CRC cells without UA treatment. In brief, it showed that the transition to a mesenchymal phenotype was inhibited as UA concentration increases (Figs. 4A, B).

**UA Regulates the Expression of Proteins Associated with EMT**

In order to further understand the mechanisms of UA on EMT, five key proteins involved in CRC-related signal transduction cascades were examined using Western blots. From the analysis of grayscale value, compared with the SW620 and HCT116 cells without UA treatment, the E-cadherin expression elevated obviously, while the Vimentin, Integrin, Twist, and Zeb1 expression decreased remarkably in a dose-dependent manner (Figs. 5A, B). Among these proteins, the most important of which is E-cadherin, a key component of the adherens junctions that are integral in cell adhesion and
maintaining epithelial phenotype of cells. Therefore, the results accord with the experiments of invasion and migration, UA increased expression of E-cadherin and reduced expression of integrin (Figs. 5, 6). Simultaneously, we also used immunofluorescence techniques to investigate the E-cadherin expression in both colorectal cancer cells, and got the results which were consistent with Western blot. After UA treatment for 48h, the E-cadherin expression elevated remarkably.

DISCUSSION

In this research, we analyzed the effects of UA treatment on 2 human colorectal cancer cell lines. Accumulating evidence suggests EMT triggers the metastasis potential of cancer cells. Moreover, much more clinical evidence suggests that the key markers of EMT in solid tumors are E-cadherin and its upstream targets. UA inhibited cell proliferation, migration, and EMT in a dose-dependent manner. We found that UA induces caspase-3 mediated apoptosis, and regulates expression of E-cadherin, integrin, Vimentin, Twist and Zeb1, thus blocking or reducing EMT. As far as we know, this is the first report of UA inhibiting cell proliferation and migration by regulating EMT in colorectal cancer cells.

The epithelial-to-mesenchymal transition is a process that converts tumor cells from an epithelial to a mesenchymal phenotype, enabling invasion and metastasis. The hallmark of EMT is to reduce expression of the epithelial cell marker E-cadherin, and increase that of mesenchymal cell markers such as vimentin, N-cadherin, and members of the Snail transcriptional factor family. EMT promotes migration by down-regulating E-cadherin and up-regulating transcription factors such as TWIST, Snail and Integrin. Liu et al. found that the Chinese medicinal herbal preparation Jianpijiedu (JPJD) inhibits TGF-β/Smad activity, stabilizes Snail, and up-regulates E-cadherin, thereby inhibiting EMT, tumor invasion, and metastasis. Curcumin, which is derived from the rhizome of turmeric (Curcuma longa), prevents EMT progression in
intestinal fibrosis. Apigenin, another plant compound, reverses EMT by affecting a nuclear factor-kappaB (NF-κB)/Snail pathway in human liver hepatoma. α-Hederin inhibits EMT by disruption of Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signaling in colon cancer cells.

UA is a compound proven to target multiple signaling pathways. For example, UA can inhibit growth and metastasis of colorectal cancer cells by down-regulating the expression of β-catenin, STAT3, NF-κB, MMP-9, CD31, EGFR. Another article also found the efficacy of inhibiting STAT3 signaling, reducing cell viability and inducing cleavage of caspase-3 in human colon cancer-initiating cells by UA. UA also inhibits lung cancer, breast cancer, liver cancer, gastric...
nisms by which EMT-triggered migration can be targeted and inhibits EMT and the active expression of some canonical EMT markers. Our results provide insight into the mechanisms by which ursoic acid inhibits the growth of colon cancer cells, combining with existing studies and our previous findings, we found that UA inhibited the activation of JAK2/STAT3 pathway was associated with EMT. Therefore, we hypothesize that UA inhibits colon cancer cell migration by affecting EMT.

In our study, we indicated that UA treatment significantly increased the expression of E-cadherin in HCT116 and SW620 cells. E-cadherin mediates calcium-dependent cell–cell adhesion, which is suppressed by vimentin, integrin, TWIST, and ZEB1. We found that UA inhibited the expression of integrin, which may result in the increased expression of E-cadherin. Integrin is an integral component of adherent junctions that help cells maintain epithelial layers, and is a target of the AKT-dependent signaling pathway. UA treatment decreases the expression of integrin in HCT116 and SW620, suggesting that modulation of colorectal cancer invasion by UA may be manifested mechanistically via alterations in EMT. We conclude that UA reduces migration and invasion by affecting EMT.

In summary, we investigated a molecular mechanism by which UA used its anticancer effects in 2 human colorectal malignant cancer cell lines, HCT116 and SW620. UA primarily affects processes related to cell proliferation and migration, and inhibits EMT and the active expression of some canonical EMT markers. Our results provide insight into the mechanisms by which EMT-triggered migration can be targeted and inhibited, and suggest that UA is expected to be the agent for preventing and curing human colon cancer.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


