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Original Article

Inhibition of IL17A promotes bufalin-induced apoptosis in colon cancer cells via miR-96/DDIT3

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Abstract: Bufalin is used clinically to treat patients with many solid malignant tumors. However, the mechanisms remain to be further elucidated. Our study focused on IL17A involved in bufacin inducing apoptosis of colon cancer cells. The data showed that bufalin could induce colon cancer cell apoptosis via inhibiting IL17A. Ectopic expression of IL17A promoted the proliferation and induced anti-apoptosis of colon cancer cells by MTT and flow cytometry analysis. Further study verified bufalin inhibiting IL17A induced apoptosis was through miR-96-DDIT3, our study demonstrated that bufalin may inhibit the proliferation and promote the apoptosis of colon cancer cells. Bufalin-associated IL-17A inhibition may indirectly be involved in cell proliferation and apoptosis by miR-96 targeting DDIT3, pointing to use as a potential molecular target of bufacin in colon cancer therapy.

Keywords: Bufalin, IL17A, DDIT3, colon cancer, miR-96

Introduction

Colon cancer is one of the common cancers in the world and has higher cancer mortality and morbidity in developed countries [1]. Many studies indicated that the malignant transformation and progression of cancer due to epigenetic changes and oncogenic signaling activation. In recent years, the epigenetic alterations, in particular, the aberrant expression of microRNAs (miRNAs), have been shown critical roles in cancer formation, metastasis, and response to cancer therapy [1-3]. However, the interaction between immune cells, inflammatory cytokines, and cancer evolution is still largely unknown. Recently, several inflammatory cytokines have been shown to promote colon cancer progression [4].

IL-17 (IL-17A), initially termed as cytotoxic T-lymphocyte-associated antigen (CTLA)-8, is the member of IL-17 cytokine family consisting of six homologous proteins (from IL-17A to IL-17F) [5, 6]. A large body of evidence suggests that IL-17 is an essential proinflammatory cytokine due to inducing a mass of cytokines and chemokines secretion by distinct cell types, such as mesenchymal cells and myeloid cells, which recruit monocytes and neutrophils into the site of inflammation [7]. IL-17 promotes the expression of antimicrobial peptides from epithelial cells and facilitates host defense against infections [6-8]. This evidence indicates that IL-17 is an important inflammatory cytokine which links innate and adaptive immunity. Recently, several studies have shown that IL-17 has either a protumor or antitumor role in different cancer models. However the majority of studies consider that IL-17 acts as a promoter in tumor initiation and progression in colon [9].

Bufalin, one of Chinese medicine, inhibits cell proliferation and induces apoptosis in various tumor cell lines [10, 11], including colon cancer [12]. However, the precise molecular mechanisms of the bufalin induced apoptosis of colon cancer cells are still unclear. At the present study, we will investigate mechanism of bufalin induced colon cancer cell apoptosis. We found that bufalin could promote cell apoptosis via inhibiting IL-17A-miR-96-DDIT3 in colon cancer.

Material and methods

Cell culture

Human colon cancer cell lines including HCT-116, HCT8, HT29, LS174T, LOVO, SW480 and

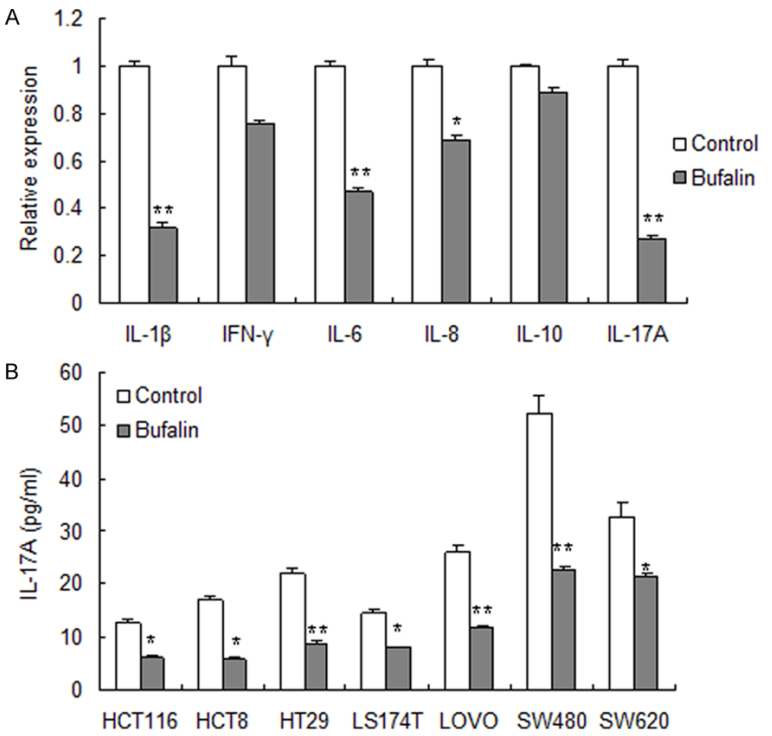

Figure 1. Bufalin inhibited IL-17A expression in colon cancer cells. A. Cytokines profile in HT-29 colon cancer cells with exposure to bufalin for 24 h. The conditioned medium was collected for cytokine ELISA assay. B. Colon cancer cells including HCT116, HCT8, HT29, LS174T, LOVO, SW480 and SW620 were treated with bufalin for 24 h, and the conditioned medium was assayed for IL-17A protein by ELISA.

SW620 were obtained primarily from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL of streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2.

MiRNA and siRNA transfection

The lentiviral vectors mediated miR-96, IL-17A and DDIT3 were constructed. Lentivirus was producted using 293T cells transfected with lentivirus vectors and packaging plasmids with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The expression of miR-96 was examined by qRT-PCR. IL-17A and DDIT3 siRNAs were ordered from Sigma (Sigma-Aldrich, Saint Louis, MO, USA).

ELISA assay

Cytokine levels were measured in the supernatant of colon cancer cells with bufalin treatment. ELISA assays were performed with Quantikine kits according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN, USA).

QRT-PCR

Total RNA from the cells transfected with miRNAs or siRNA was isolated from the cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). QRT-PCR was performed to measure mRNA expression. Primers were designed and ordered from Sangon Biotech (Shanghai, China). The relative expression levels were calculated by comparing Ct values of the samples with those of the reference, all data normalized to the internal control GAPDH or U6 snRNA.

MTT assay

The colon cancer cells were transfected with miRNAs or siRNA and then counted and re-seeded to 96-well plates with 5×10^3 cells per well. 10 μl MTT solution (5 mg/ml) was added to each well at day 1, 3 and 5, and then the cells were cultured for 4 h. After the incubation, the crystal was dissolved with 0.1% SDS and finally the ELISA reader was used to measure the absorbance at 570 nm.

Colony forming assay

Colon cancer cells were transfected the miRNAs, seeded in 6 well plates (200 cells in every well) and incubated at 37°C, 5% CO_2 for 24 h. Non-adherent cells were removed. Cell growth medium was changed every 3-5 days. The colonies with more than 50 cells were counted after 14 days.

Cell apoptosis

Colon cancer cells transfected with plasmids were seeded in 6-well plates and harvested after 48 h. The cells were stained with 5 μl of Annexin V and 5 μl of propidium iodide (BD

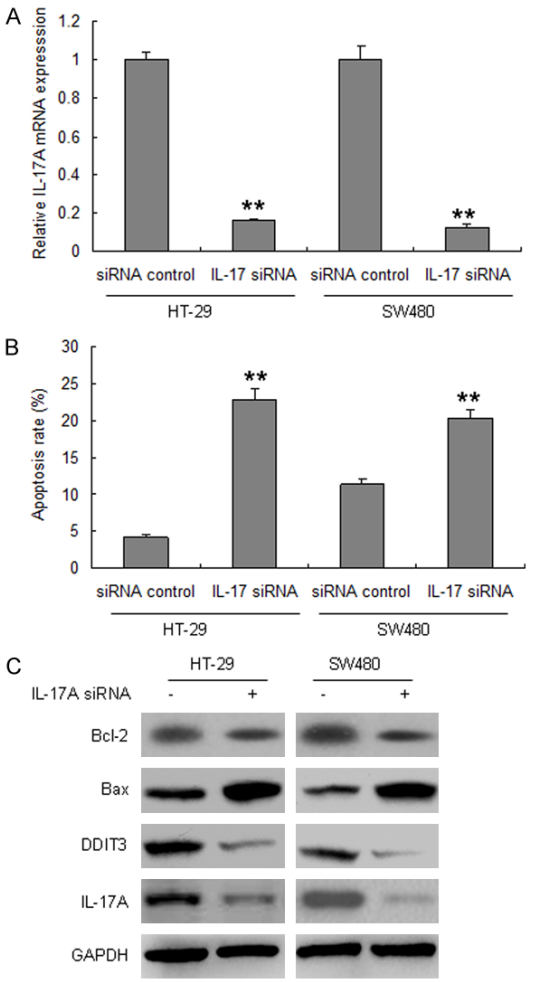

Figure 2. Inhibition of IL-17A promoted bufalin-induced apoptosis via DDIT3. A. Knocking-down of IL-17A in colon cancer cells. B. IL-17A deletion promoted apoptosis of HT-29 and SW480 colon cancer cells with bufalin treatment. HT-29 and SW480 colon cancer cells were transfected with IL-17A siRNA for 24 h, and then treated with bufalin (100 ng/ml) for another 24 h. Cells were collected for flow cytometry analysis. C. Apoptosis associated protein was detected by Western blotting in colon cancer cells with IL-17A down-regulation after bufalin treatment (100 ng/ml). HT29 and SW480 cells were transfected with IL-17A siRNA or its control for 24 h and then exposed to bufalin (100 ng/ml) for another 24 h. Protein was extracted for western blot.

Biosciences, San Jose, CA, USA) for 15 min at room temperature. The apoptosis rate (%) of the stained cells was analyzed using a flow cytometry (BD, USA).

Hoechst staining

Hoechst 33342 dye (Molecular Probes, Eugene, OR, USA) was used to stain the nuclei. Cells were transfected miRNAs and incubated in the Hoechst labeling solution for 30 mins at room temperature. Images were obtained using a Leica DM IRB inverted fluorescence microscope (Wetzlar, Germany) at 400× magnification.

Western blot analysis

Colon cancer cells were lysed in RIPA lysis buffer with protease and phosphatase inhibitors. The protein concentration was determined using Bradford assay (Bio-Rad, Philadelphia, PA). Equivalent protein was performed to SDS-PAGE and transferred to NC membranes (Millipore, Bedford, MA, USA). The NC membranes with protein blotting was blocked in 5% non-fat, incubated with primary antibodies, and then incubated with secondary antibodies conjugated with HRP. Blots were visualized on X-ray films using an ECL detection system (Pierce, IL).

Statistical analysis

Data were analyzed by SPSS 13.0 software and presented as mean ± SEM of at least three independent experiments. Two-tailed Student's t test was used for comparisons of two independent groups. Gene expression was analyzed by Mann-Whitney U test. *P* values of <0.05 were considered statistically significant.

Results

Bufalin inhibits IL-17A expression in colon cancer cells

Cytokines play important roles in cancer progression. To investigate whether bufalin influence cytokines in colon cancer, the colon cancer cells were treated with bufalin and conditioned medium was collected for ELISA analysis. The results showed that bufalin could induce changes of many cytokines' expression (**Figure 1A**). IL-17A decreased significantly in HT-29 cell. Cytokines such as IL-6 decreased, but it was research widely. In our research, we focused on IL-17A. To verify the result, HCT116, HCT8, HT29, LS174T, LOVO, SW480 and SW620 cells were treated with bufalin and IL-17A expression was evaluated by ELISA. We found IL-17A protein was reduced in most of the cell lines with bufalin treatment (**Figure 1B**). These gave us the evidence that IL-17A inhibition could have a suppressive effect on colon cancer.

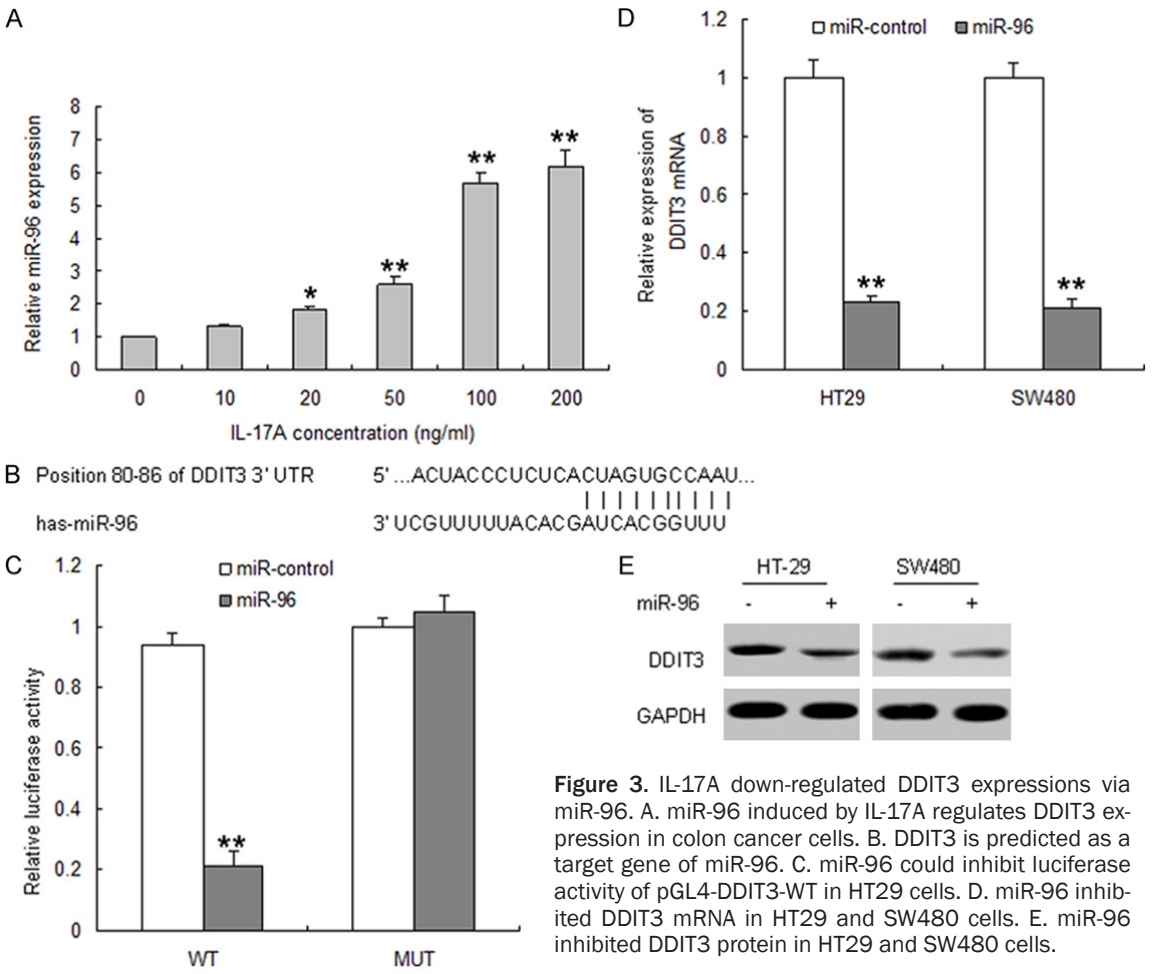

Figure 3. IL-17A down-regulated DDIT3 expressions via miR-96. A. miR-96 induced by IL-17A regulates DDIT3 expression in colon cancer cells. B. DDIT3 is predicted as a target gene of miR-96. C. miR-96 could inhibit luciferase activity of pGL4-DDIT3-WT in HT29 cells. D. miR-96 inhibited DDIT3 mRNA in HT29 and SW480 cells. E. miR-96 inhibited DDIT3 protein in HT29 and SW480 cells.

Inhibition of IL-17A promotes bufalin-induced apoptosis

Bufalin could induce apoptosis in various cancer cells. To answer whether IL-17A deletion induces colon cancer cell apoptosis, HT-29 and SW480 cells were transfected with IL-17A siRNAs or siRNA control, IL-17A mRNA and protein levels was decreased (**Figure 2A**). Cell apoptosis was examined by flow cytometry and it was shown that in the cells with IL-17A down-regulation, apoptosis rate increased compared with the control in HT-29 and SW480 cells (**Figure 2B**). Apoptosis associated protein was analyzed by western blot, and it was found that DDIT3 protein level was higher in HT-29 and SW480 cells with IL-17A inhibition (**Figure 2C**). Bcl-2 decreased and Bax increased. These data indicated that inhibition of IL-17A could promote cell apoptosis in HT-29 and SW480 cells. It is interesting that DDIT3 increases in the colon cells with IL-17A inhibition.

IL-17A down-regulates DDIT3 expressions via miR-96

It is interesting that DDIT3 increases in the colon cells with IL-17A inhibition. To investigate the potential molecule that regulates DDIT3 up-regulation in colon cancers, we next checked whether IL-17A down-regulates DDIT3 expression in colon cancer cells through miRNAs. DDIT3 may be regulated by many miRNAs including miR-96. HT-29 cells were treated with IL-17A, and the predicted miRNAs were analyzed and the data showed that miR-96 increased (**Figure 3A**). Bioinformatic analysis showed that DDIT3 was directly suppressed by miR-96 (**Figure 3B**). As shown in **Figure 3C**, the luciferase activity of wide type of DDIT3 3'UTR in HT29 cells was much lower than in control cells. The luciferase activity of mutation of DDIT3 3'UTR was rescued in the cells. Compared with control, endogenous DDIT3 mRNA levels (**Figure 3D**) were down-regulated

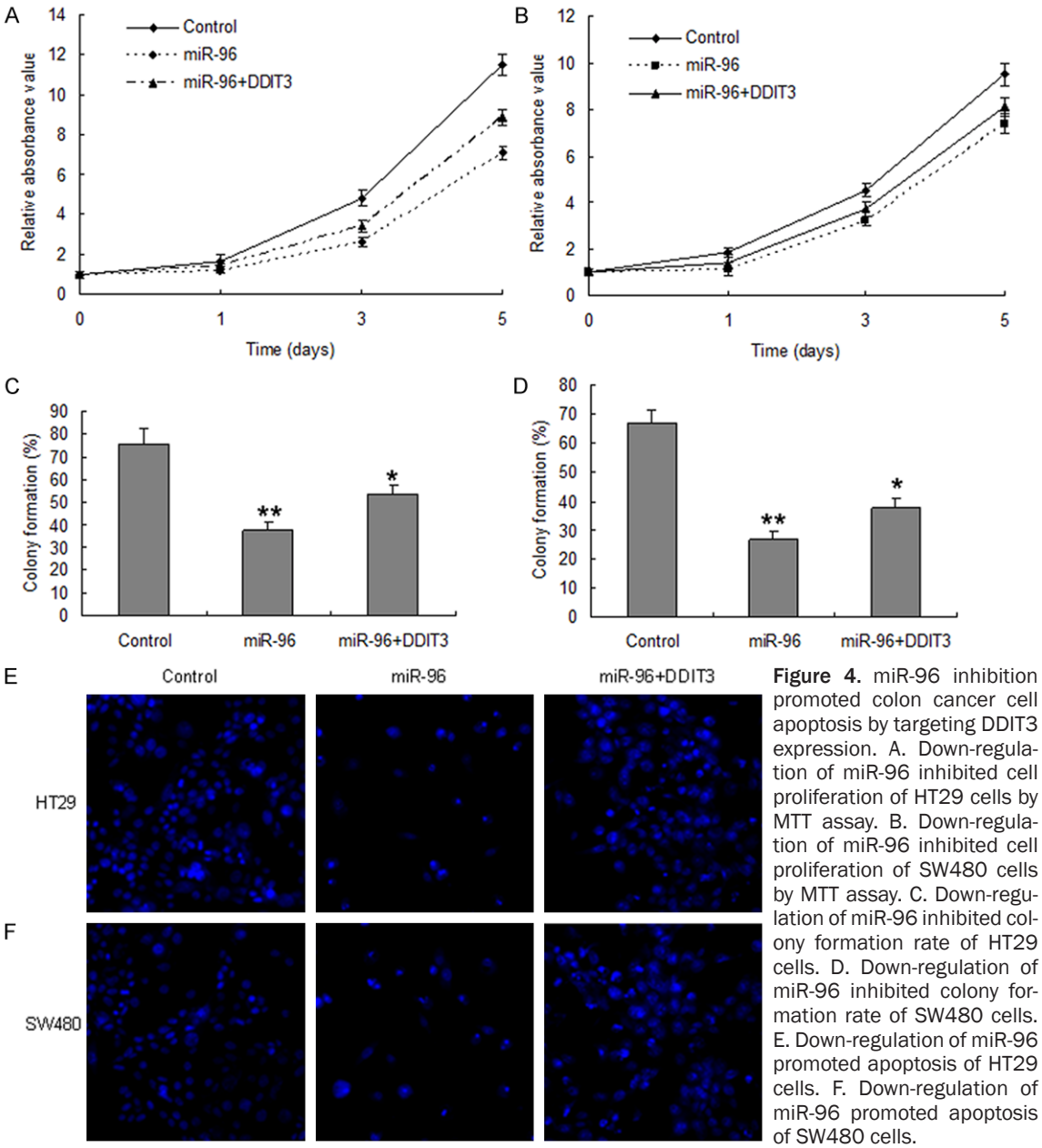

Figure 4. miR-96 inhibition promoted colon cancer cell apoptosis by targeting DDIT3 expression. A. Down-regulation of miR-96 inhibited cell proliferation of HT29 cells by MTT assay. B. Down-regulation of miR-96 inhibited cell proliferation of SW480 cells by MTT assay. C. Down-regulation of miR-96 inhibited colony formation rate of HT29 cells. D. Down-regulation of miR-96 inhibited colony formation rate of SW480 cells. E. Down-regulation of miR-96 promoted apoptosis of HT29 cells. F. Down-regulation of miR-96 promoted apoptosis of SW480 cells.

when HT29 and SW480 cells were transfected with *miR-96*. DDIT3 protein was down-regulated in the cells with miR-96 (**Figure 3E**). So, DDIT3 is involved in bufalin induced colon cancer cell apoptosis by miR-96.

MiR-96 inhibition promotes colon cancer cell apoptosis by targeting DDIT3 expression

To further research the mechanism of DDIT3 regulation, colon cancer cells were transfected with miR-96 inhibitor for cellular function analysis. HT29 and SW480 cells were transfected with miR-96 inhibitor in or the control (miRNA-control), the results of MTT assay displayed that miR-96 inhibited cell proliferation in HT29 cells (**Figure 4A**) and SW480 cells (**Figure 4B**). The colony formation rate in the two cells with miR-96 overexpression increased compared with the controls (**Figure 4C** and **4D**). Cell apoptosis was increased in the HT29 and SW480 cells with miR-96 inhibition (**Figure 4E** and **4F**).

Correlation of miR-96 and DDIT3 in colon cancer tissues

Above data suggested that miR-96 inhibits colon cancer cell apoptosis by targeting DDIT3.

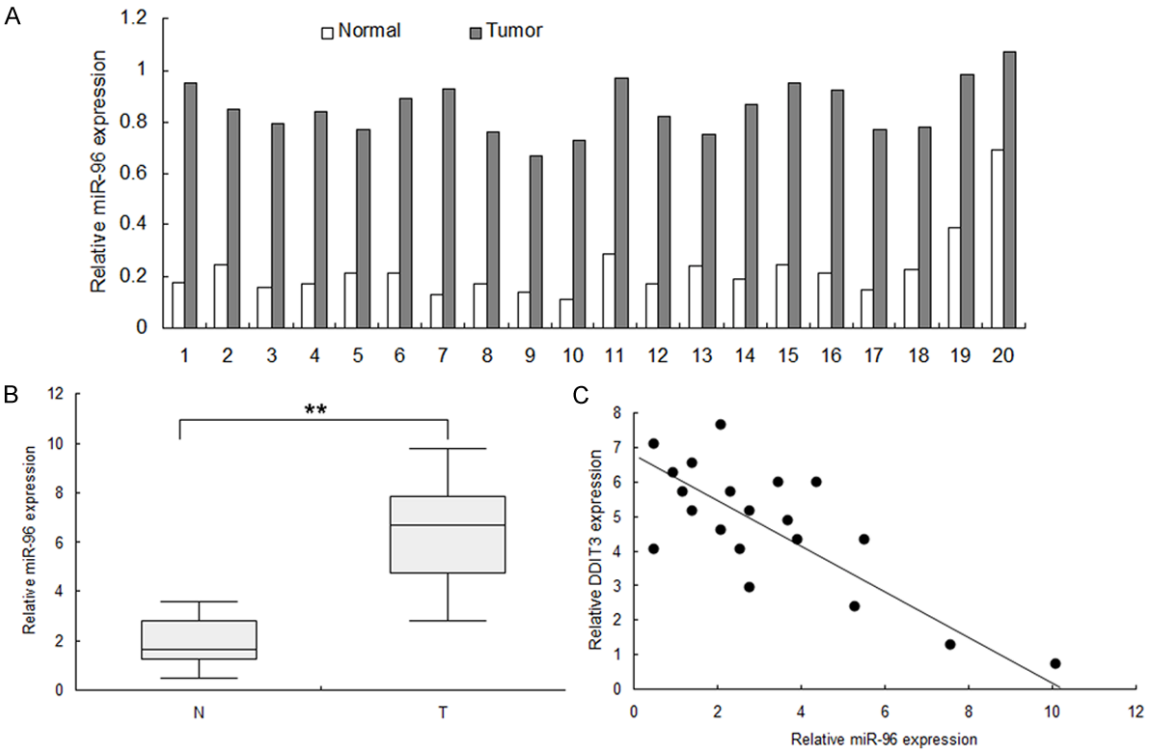

Figure 5. Correlation of miR-96 and DDIT3 in colon cancer tissues. (A) miR-96 expression in colon cancer tissues was up-regulated compared to their adjacent normal tissues by qRT-PCR assay. (B) Data analysis of (A). (C) miR-96 was negatively associated with DDIT3 protein in colon cancer tissues.

In order to investigate miR-96 expression in colon cancer tissues, the data from real time RT-PCR showed that miR-96 was up-regulated in colon cancer tissues than their normal tissues (**Figure 5A** and **5B**). The clinic data showed that miR-96 was negatively associated with DDIT3 protein in colon cancer tissues (**Figure 5C**).

Discussion

Bufalin, a class of toxic steroids purified from Chinese traditional medicine, has therapeutic effect on cancer, but its molecular mechanism on cancer is unclear. In the present study, we found that bufalin could suppress IL-17A expression in colon cancer cells. Further study showed that deletion of IL-17A could lead to cell apoptosis and DDIT3 expression. DDIT3 was identified as a target gene of miR-96 stimulated by IL-17A in colon cancer cells.

MiRNAs are a class of small noncoding RNAs that typically inhibit the translation and stability of messenger RNAs (mRNAs) by binding to the 3'-untranslated regions (3'-UTR) of their target mRNAs. MiRNAs have 19-22 nucleotides and are found in all multi-cellular eukaryotic cells. MiRNAs have important roles in various biological and pathological processes, such as development, cell proliferation, differentiation, apoptosis, inflammation, stress response and migration. Recent studies showed that the differential expression of miR-96 in many type of cancers, such as esophageal cancer, colorectal cancer, pancreatic cancer, glioma, breast cancer and etc. The target genes of miR-96 that identified such as RECK, FOXO1, FOXO3a, NUAK1, HERG1 and HBP [13-26]. Previous study suggested that miR-96 acted as a tumor suppressor miRNA. Our data show that miR-96 is up-regulated in advanced and metastatic colon cancer by targeting DDIT3. Taken together with previous studies, it suggests that roles of miR-96 can be complicated by regulating different downstream effectors in different cancer contexts and it functions mainly as an oncogen in colon cancer.

DNA-damage-inducible transcript 3 (DDIT3) is also known as CHOP, growth arrest- and DNA damage-inducible gene 153 (GADD153), and

C/EBPζ [27]. DDIT3 protein is composed of two known functional domains, an N-terminal transcriptional activation domain and a C-terminal basic-leucine zipper (bZIP) domain consisting of a basic amino-acid-rich DNA-binding region followed by a leucine zipper dimerization motif [27-29]. There are reports showed that bZIP domain is important for CHOP-induced apoptosis [27, 30, 31]. Re-expression of DDIT3 could promote cell apoptosis of colon cancer cells. However, in mouse model studies, re-expression of DDIT3 could significantly but not completely reverse the miR-96-imposed promotion on tumor growth, which suggests that other potential targets of miR-96 may have other genes.

Our present study has identified bufalin could inhibit IL-17A expression in colon cancer cells. IL-17A inhibits cell apoptosis via targeting DDIT3, an apoptosis inhibitor. DDIT3 is regulated by miR-96 which is negatively correlated in colon cancer tissues. Our finding suggested that IL-17A and miR-96 are therapeutic targets for colon cancer.

Disclosure of conflict of interest

None.

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