Oncology Research, Vol. 22, pp. 267–273
Printed in the USA.

0965-0407/15 $90.00 + .00
DOI: http://dx.doi.org/10.3727/096504015X14410238486685
E-ISSN 1555-3906
www.cognizantcommunication.com

Ursolic Acid Inhibits the Proliferation of Gastric Cancer Cells by Targeting miR-133a

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Ursolic acid (UA), a potential chemotherapeutic agent, has the properties of inhibition of the growth of many human cancer cell lines. Whether UA can inhibit the growth and metastasis of human gastric cancer cells remains unknown. In this study, it was found that UA inhibited the growth and metastasis of human gastric cancer cells in vitro. Our results showed the increase of the percent of apoptotic cells and G_1 phase, the inhibition of cell migrations well as the decrease of the expression of Bax, caspase 3 and Bcl-2 in BGC-823 cells after the treatment with UA. Real-time quantitative PCR analysis showed that UA treatment upregulated the level of miR-133a in BGC-823 cells. Overexpression of miR-133a increased the G_1 phase of cell cycle and decreased Akt1 expression in BGC-823 cells. These outcomes might be secondary to the increased expression of miR-133a after the treatment with UA.

Key words: Ursolic acid (UA); Gastric cancer; miR-133a; Akt1

INTRODUCTION

UA is a pentacyclic triterpene acid, which is widely distributed in medical herbs and edible plants, such as the wax-like coating of apples and other fruits (1). Recent studies demonstrated that UA exhibited the growth inhibition properties against many human cancer cell lines, including breast, leukemia, prostate, liver, colon, and skin cancers (2–4). In addition, UA was reported to display a remarkable spectrum of biochemical activities that could influence the dysregulated processes in cancer development. These include the inhibition of tumorigenesis, tumor promotion, invasion, metastasis, angiogenesis, and the induction of tumor cell differentiation (5). It also interferes with the function of DNA synthesis enzymes, including DNA polymerase and DNA topoisomerase (6). Therefore, UA is considered a potential chemotherapeutic agent suitable for cancer treatment.

Gastric cancer, the fourth most common type of cancer, is prevalent in East Asia, Eastern Europe, and particularly China. The prognosis of gastric cancer is generally rather poor, with a 5-year relative survival below 30% in most countries (7). The development of chemoresistance, poor prognosis, and metastasis often renders the current treatments for gastric cancer ineffective. Whether UA could inhibit the growth and metastasis of human gastric cancer cells was still unknown.

In this study, we investigated whether UA was able to inhibit the growth and metastasis of human gastric cancer cells. UA was found to significantly inhibit the growth and metastasis of gastric cancer cells through the upregulation of miR-133a expression.

MATERIALS AND METHODS

Cell Lines

Human gastric cancer cell line BGC-823 was purchased from the Institute of Cell Biology (Shanghai, China) and cultured in RPMI-1640 medium supplemented with 10% FBS at 37°C in a humidified incubator containing 5% CO_2.

Preparation of Drug

UA was dissolved in dimethylformamide and filtrated through a 0.22-μm micropore filter and stored at 4°C. UA was diluted to the working concentration by RPMI-1640 medium before use. The concentration of dimethylformamide in the culture medium does not exceed 0.1%.

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Cell Proliferation

First, the cells (0.5×10^4/well) in logarithmic growth phase were seeded into a 96-well plate. After 24 h of incubation, UA was added to the culture at the final concentrations of 50 μM, 100 μM, 125 μM, 150 μM, 200 μM, and incubated for 48 h. After 48 h, the live cell population was determined using Cell Proliferation Reagent WST-1 according to manufacturer's instructions. The calculation formula of tumor cell growth inhibition rate and IC_{50} value was: [A (negative control group) − A (medication group)]/[A (negative control group) − A (blank control group)]. All assays were repeated at least three times.

Cell Migration Assay

The cells in logarithmic growth phase were adjusted to 6×10^6/well using complete culture medium and cultured in a dish. UA (90 μM) was added to the culture after 24 h of incubation. The migrated cells were evaluated by the cell migration Assay Kit (Corning) according to the manufacturer's instructions. Five 200× microscopic fields were randomly selected to calculate the total counts of the migrated cells.

Analysis of Cell Cycle

UA was added to the cultured cells (5×10^5/well) in a six-well plate after 24 h. The experiment was set with two groups, the negative control group (0 μM) and the drug group (IC_{50}). There were three wells in each group. Cells were pelleted by spinning for 5 min at 1,500 rpm and resuspended in 1 ml of cold PBS. After fixation by adding 1 ml of 70% absolute ethanol, the cells were centrifuged and resuspended in 1 ml of PBS overnight. The cells were stained with 300 μl propidium iodide and incubated for 30 min at room temperature before analysis.

Western Blot

Cells were washed with cold PBS three times after the addition of UA followed by incubation for 48 h. Then the cells were placed on ice for 20 min after the addition of 100 μl lysis buffer containing protease protein inhibitor. The supernatant was collected after centrifugation for 10 min at 3,000 rpm/min. Equal amounts of protein were loaded and separated discontinuously on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and subsequently transferred onto a PVDF membrane (Millipore). The membrane was then incubated in TBST blocking solution (Tris-buffered saline including 0.1% Tween-20) containing 5% fat-free milk for 1 h at room temperature, followed by separate incubation with primary antibodies (1:1,000 dilution) overnight at 4°C. After washing, the membrane was incubated with HRP-conjugated secondary antibody (1:1,000 dilution) for 2 h. After several washes, the immunoblots were visualized with enhanced chemiluminescent technology (Pierce Biotechnology) according to the manufacturer's instructions.

Quantitative Real-Time RT-PCR

The cells (6×10^6/well) were cultured in a dish for 24 h. For qRT-PCR analysis, miRNA was isolated using the miRcute miRNA isolation kit (TIANGEN Bio, Inc.), and reverse transcription was performed using the PrimeScript RT reagent kit (TIANGEN Bio, Inc.), according to the manufacturer's instructions. The expression of miRNA-133a was determined by real-time PCR (StepOne plus; Applied

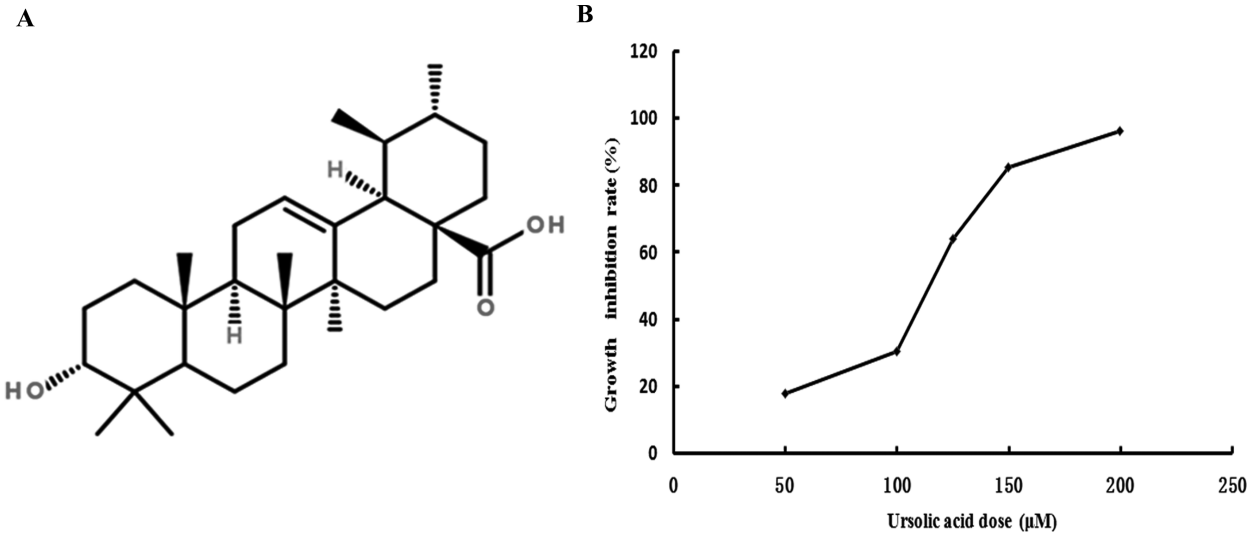

Figure 1. Determination of the growth of BGC-823 cells treated by UA. (A) The structure of UA, compound of pentacyclic triterpenoid, is shown on the left. (B) BGC-823 cells were treated with UA for 48 h at the indicated concentrations. The proliferation of cells was determined using Cell Proliferation Reagent WST-1.

Biosystems, USA) using a standard SYBR-Green PCR kit (TIANGEN Bio, Inc.). Reactions were conducted at 94°C for 2 min, followed by 45 cycles of 94°C for 20 s, and 60°C for 34 s. Total RNA was isolated from the cells cultured in dish for 48 h by using the TRIzol reagent kit (Takara). The method for measurement of mRNA of expression was similar to that for miRNA measurement. Reactions were conducted at at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 30 s. The relative expression of each target gene normalized with GAPDH was calculated using the $2^{-\Delta\Delta ct}$ method. Primers used were as follows. GAPDH, F: 5′-CCACTCCTCCACCTTTGAC-3′, R: 5′-ACCCTG TTGCTGTAGCCA-3′; Bax, F: 5′-CCCGAGAGGTCT TTTTCCGAG-3′, R: 5′-CCAGCCCATGATGGTTCTG AT-3′; Bcl-2, F: 5′-TACCTGAACCGGCACCTG-3′, R: 5′-GCCGTACAGTTCCACAAAGG-3′; Akt1, F: 5′-AGC GACGTGGCTATTGTGAAG-3′, R: 5′-GCCATCATTCT TGAGGAGGAAGT-3′.

Statistical Analysis

For all the analyses, measurements obtained from the groups were expressed as means ± SD for all data determined. Statistical analysis was performed using an unpaired Student's *t*-test followed by Tukey's test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

UA Inhibits the Proliferation of BGC-823 Cells

To determine whether UA inhibits the growth of BGC-823 cells, the cells were cultured in RPMI-1640 medium for 48 h at different concentrations of UA, and the numbers of viable cells were measured using

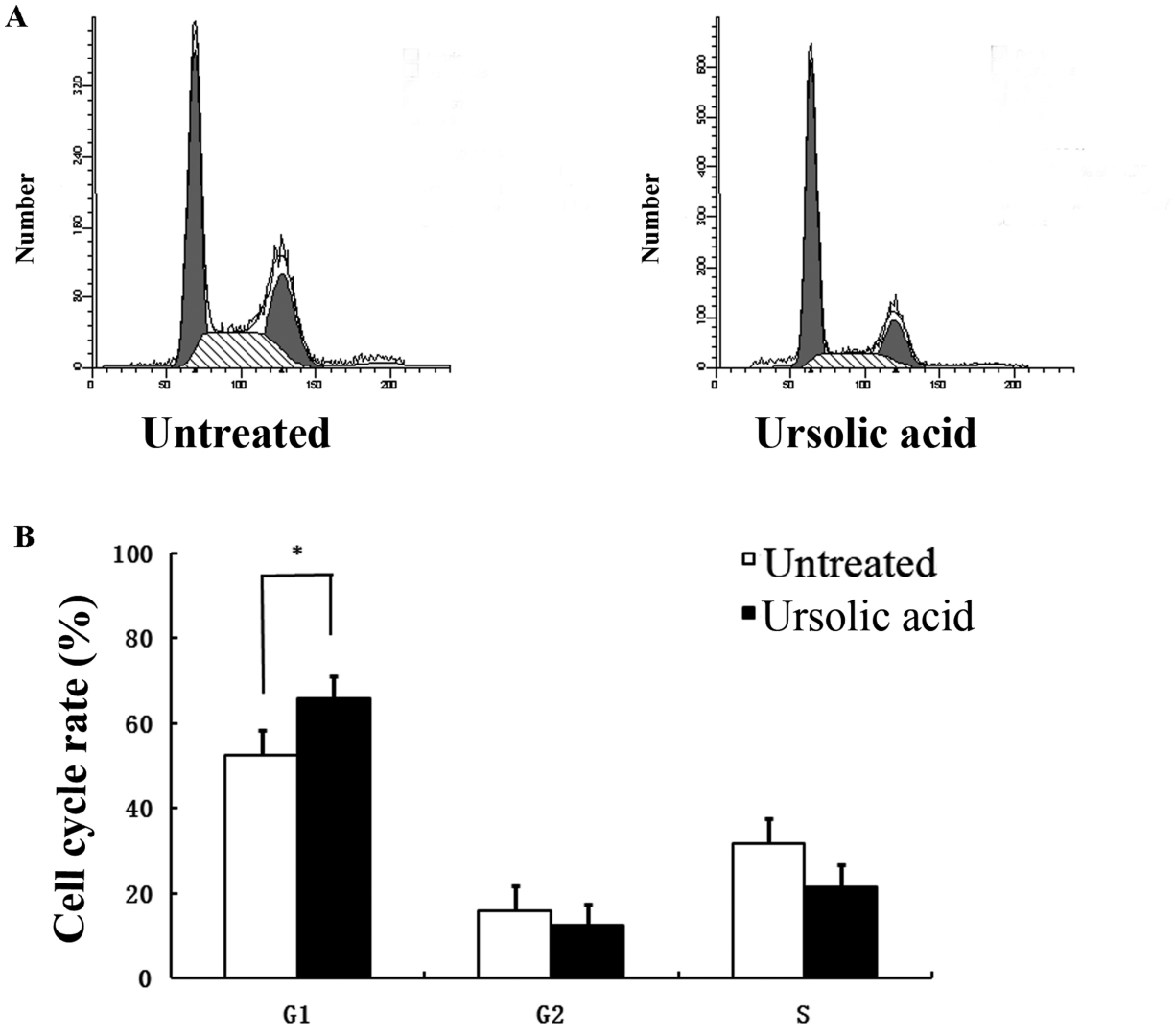

Figure 2. Cell cycle analysis of BGC-823 cells treated with UA. (A) Flow cytometric analysis of cell cycle of BGC-823 cells treated with UA. (B) The number of G_1, G_2, and S phase of BGC-823 cells treated with UA.

a WST-1 assay. The results showed that the treatment of BGC-823 cells with UA in a concentration range of 50–200 μM resulted in a dose-dependent suppression of cell proliferation (Fig. 1).

UA Induces Cell Cycle Arrest in BGC-823 Cell

To determine why UA suppresses the proliferation of BGC-823 cells, DNA flow cytometric analysis was performed to determine the effect of UA on the cell cycle of BGC-823 cells. The results showed that UA induced G_1 cell cycle arrest in gastric BGC-823 cells (Fig. 2).

Regulation of Cell Apoptosis by UA in BGC-823 Cells

To determine the effects of UA on cell apoptosis of BGC-823 cells and if UA affects mRNA levels of Akt, Bax, caspase 3, and Bcl-2, BGC-823 cells were treated with UA (90 μM) for 48 h. Then the apoptotic cells were quantificated with annexin V, and the total RNAs were isolated, and the Bax, caspase 3, Bcl-2, and Akt mRNA transcription levels were determined using quantitative RT-PCR. As shown in Figure 3, a significant increase in the percentage of apoptotic cells was observed, and the mean levels of Bax and caspase 3 mRNA expression were increased compared with the untreated group. However, Akt and Bcl-2 levels were decreased significantly.

UA Inhibits the Migration and Invasion of BGC-823 Cells

To investigate the effects of UA on cell migration and invasion of BGC-823 cells, a Transwell assay was used to determine whether UA is able to regulate the migration of BGC-823 cells. In migration assays, the number of migrated cells was significantly lower in BGC-823 cells treated with UA than that in their respective controls (Fig. 4). The relative number of migrated cells was 53.63%.

Upregulation of miR-133a and Promotion of Cell Cycle Arrest at G_1 Phase in BGC-823 Cells After UA Treatment

Current results indicated that after BGC-823 treatment with UA (90 μM) for 48 h, mRNA expression of miRNA-133a increased 12.7-fold relative to the untreated cells, and this was found to be statistically significant ($p < 0.001$) (Fig. 5) (fold changes presented as mean ± SE). To determine the functional consequences of elevated miR-133a expression in gastric cancer, miR-133a was overexpressed in gastric cancer BGC-823 cells using miR-133a mimics. After the overexpression of miR-133a, the cell cycles of BGC-823 cells were assessed. As shown in Figure 5B, a significant increase in G_1 cell cycle arrest was observed

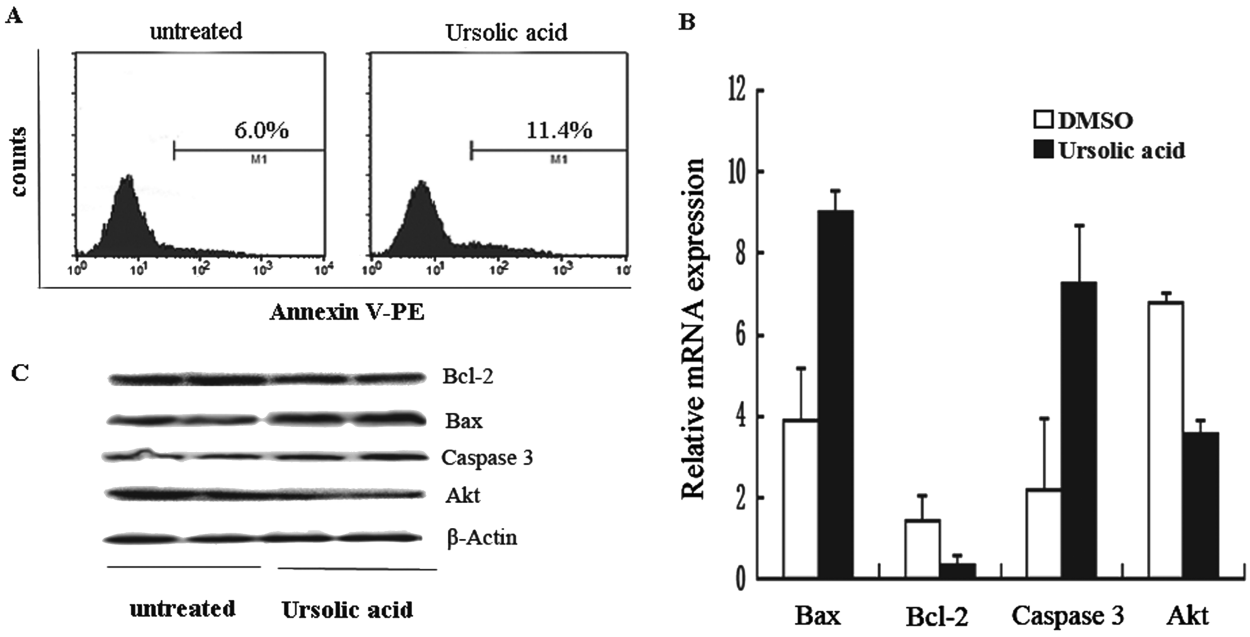

Figure 3. Analysis of cell apoptosis in BGC-823 cells after UA treatment. (A) The apoptosis of BGC-823 cells was analyzed by annexin V-PE staining. (B) Real-time PCR analysis of Bcl-2, Bax, caspase 3, and Akt mRNA expression. The results were normalized to the amount of β-actin as the internal control. Each value represents the average from three independent experiments. (C) Western blot analysis of Bcl-2, Bax, caspase 3, and Akt protein expression in each group. β-actin served as the loading control.

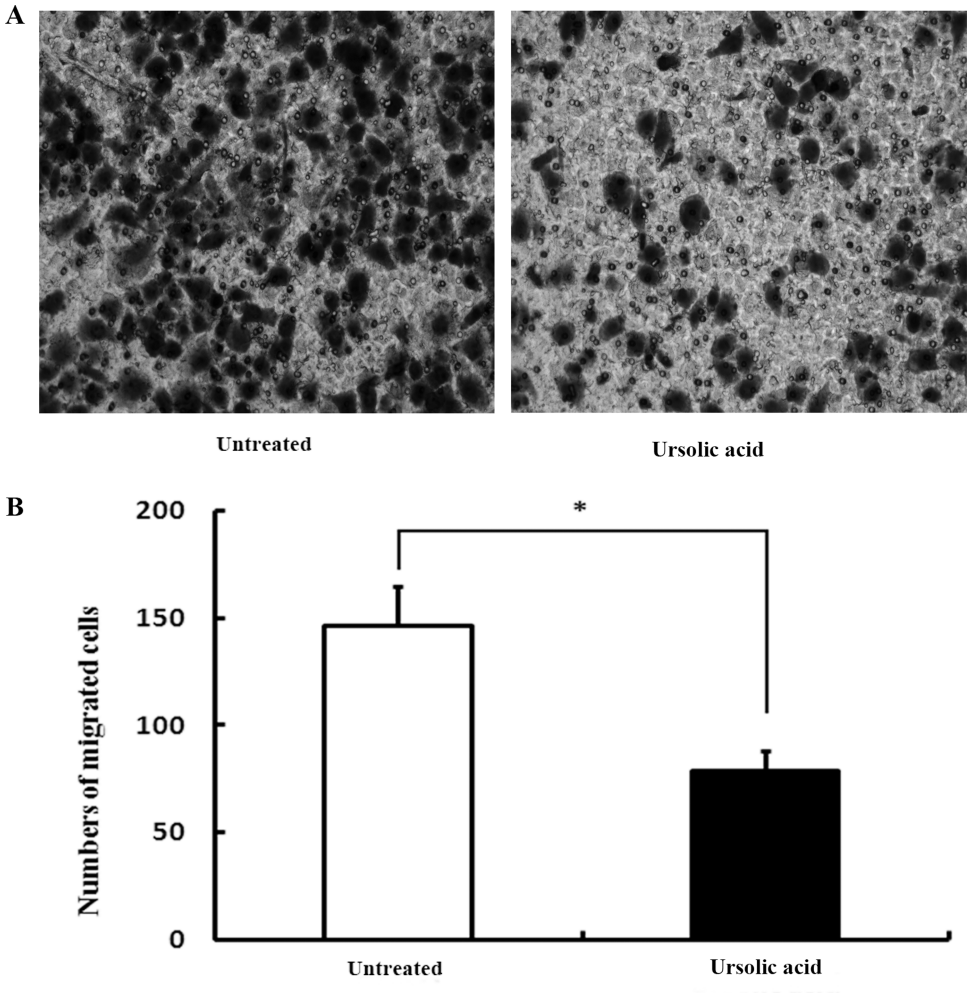

Figure 4. Migration of BGC-823 cells treated with UA. (A) Transwell assay to investigate the cell migration in BGC-823 cells treated with ursolic acid. (B) The migrated cells were visualized by staining with hematoxylin and quantitated by cell counter. Data are presented as mean ± SD from three independent experiments. *$p < 0.05$.

in the gastric BGC-823 cells that overexpressed miR-133a. Levels of Akt1 were also detected. As shown in Figure 5C, the mean levels of Akt1 mRNA expression were markedly lower than that of the control.

DISCUSSION

UA is a pentacyclic triterpenoid (a member of the cyclosqualenoid family) derived from berries, leaves, flowers, and fruits of medicinal plants, such as *Rosemarinus officinalis*, *Eriobotrya japonica*, *Calluna vulgaris*, *Ocimum sanctum*, and *Eugenia jumbolana* (8). UA has been shown to suppress tumorigenesis (9), inhibit tumor promotion (10–12), and suppress angiogenesis (13). Some of the effects of UA are mediated through the suppression of the expression of lipoxygenase, COX-2, MMP-9, and iNO (1,14). However, whether UA inhibits the growth and metastasis of human gastric cancer remains unknown.

In this article, the proliferation of BGC-823 cells was decreased after treatment with UA for 48 h, and the effects of UA on BGC-823 were found to be dose dependent (Fig. 1). Furthermore, the cell cycle of treated cells was assessed. The results showed that significantly more BGC-823 cells that received UA treatment were arrested at G_1/S phase transition than control cells. Also, after the treatment with UA for 48 h, the level of apoptosis of BGC-823 cells increased, and their migratory capability decreased significantly. All these results suggest that the UA may be effective in the treatment of gastric cancer.

It is widely accepted that miRNA dysregulation occurs in gastric and other cancers and contributes to carcinogenesis and tumor progression. The dysregulation of the miRNAs investigated in the current study was similar to that reported in other studies. It was reported that miR-133a can inhibit cell proliferation, migration, and

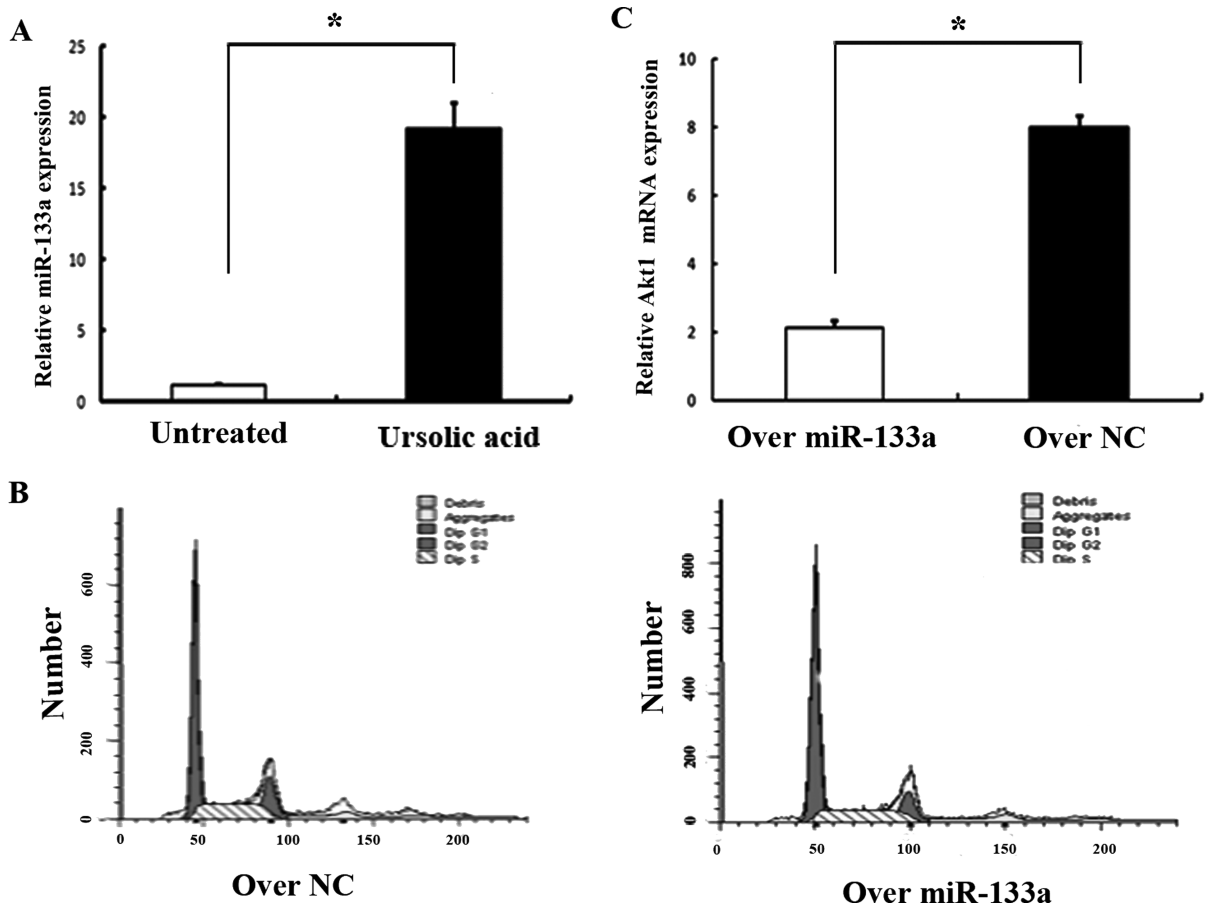

Figure 5. (A) Real-time PCR analysis of miR-133a expression in UA-treated BGC-823 cells. The results were normalized to the amount of U6 as the internal control. (B) Flow cytometric analysis of cell cycle of BGC-823 cells with overexpression of miR-133a. (C) Real-time PCR analysis of Akt1 mRNA expression in BGC-823 cells with miR-133a overexpression. Each value represents the average from three independent experiments.

invasion in bladder cancer (15) and prostate cancer (16) by targeting the epidermal growth factor receptor. A high-throughput screen showed that miR-133a could decrease lung cancer cell survival by activating caspase 3/7-dependent apoptotic pathways and inducing cell cycle arrest in S phase (17). The suppressive role of miR-133a could also be found in esophageal squamous cell carcinoma (18), ovarian cancer (19), colorectal cancer, and similar illnesses (20). miR-133b is a prognostic marker, and it can inhibit the progression of colorectal cancer by regulating CXCR4 (21).

Aberrant activation of the miR-133a signaling pathway is associated with neoplastic cell proliferation, migration, stromal invasion, angiogenesis, and resistance to apoptosis (22). In this way, agents that can control these multiple signaling pathways have the potential for use against human gastric cancer. The levels of expression of miR-133a in BGC-823 cells at 48 h after UA treatment were here determined. The results showed that the level of miR-133a was significantly higher than that in the control group. The current results showed that, after the overexpression of miR-133a, more cells were arrested in the G_1/S transition. The levels of Bax and caspase 3 mRNA were significantly higher in BGC-823 cells. However, the mRNA level of Bcl-2 was lower than that in the controls. It was concluded that the effects of UA on cell proliferation, apoptosis, and cell cycle arrest in BGC-823 cells were mediated by the regulation of the expression of miR-133a.

Our results showed that miR-133a did not regulate the levels of Bax and Bcl-2, suggesting that miR-133a regulates the apoptosis and proliferation of BGC-823 cells through other signals that targeted Bax, Bcl-2, or both. To further investigate the underlying mechanisms, the mRNA level of Akt1 was measured in BGC-823 cells after overexpression of miR-133a. The results showed that Akt1 silencing reduced the proliferation of gastric cancer cells and increased cell apoptosis both in vitro and in vivo, and this might be associated with the inactivation of the PI3K/Akt1 signaling pathway along with

the induced expression of proapoptotic protein Bax and a concomitant decrease in Bcl-2 expression (23). In this study, our results showed that the mRNA level of Akt1 was significantly lower than that in the control group. These results indicated that the decreased mRNA level of Akt1 may affect the expression of Bax and Bcl-2, which in turn reduced the proliferation but increased the apoptosis of BGC-823 cells. Akt1 may be inactivated by the increased expression of miR133a in response to UA.

Collectively, these data indicate that UA significantly suppresses the proliferation of BGC-823 cells, promotes cell cycle arrest, and inhibits the cell migration. These effects are attributable to the increased expression of miR-133a and the subsequent downregulation of Akt1by UA treatment.

ACKNOWLEDGMENT: This study was supported by grants from the Leading Academic Discipline Project of State Administration of Traditional Chinese Medicine of China (2013QK08).

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