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Original Article

Bufalin inhibits invasion and metastasis in colorectal cancer cells through miRNA-497 mediated IGF1R-PI3K-Akt signaling pathway

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Abstract: Objective: Bufalin, a major active ingredient of the Chinese traditional medicine Venenum Bufonis, exerts anti-tumor activity in multiple cancers. This study aimed to assess the role and mechanism of bufalin in invasion and metastasis inhibition in colorectal cancer. Methods: Human colorectal carcinoma Lovo cells were treated with bufalin at different concentrations, and cellular function and proliferation, as well as the expression of related molecules were evaluated. Results: Bufalin inhibited the proliferation of colorectal cancer cells in a time- and dose-dependent manner. Further mechanistic assessment showed that miR-497 down-regulation promoted invasion and metastasis in colorectal cancer cells by effecting the insulin-like growth factor 1 receptor (IGF1R)-Phosphoinositide 3 kinase (PI3K)-Protein Kinase B (Akt) signaling pathway. Meanwhile, bufalin could reverse these changes. Conclusion: the current findings suggested that bufalin could inhibit invasion and migration in colorectal cancer cells through miRNA-497 mediated IGF1R-PI3K-Akt signaling.

Keywords: Bufalin, colorectal cancer cell, miRNA-497, insulin-like growth factor 1 receptor, phosphoinositide 3 kinase, protein kinase B, IGF1R-PI3K-Akt signaling pathway

Introduction

Colorectal cancer is one of the most common clinical malignant tumors; its incidence ranks third in the United States, and fourth and fifth in major and remote cities of China, respectively [1, 2]. In China, the morbidity and mortality rates of colorectal cancer rank first worldwide [3]. About 50% of patients display local or distant metastases at the time of diagnosis [4]. Metastasis formation is a complex multi-step process, which involves initial malignant cell invasion, infiltration into the bloodstream, proliferation during migration, and extravasation into distant organs [5, 6]. Currently, the clinical treatment of colorectal cancer still largely depends on surgery, but the early surgical resection rate is only about 60% to 70%; meanwhile, middle and late stage cancers are prompt to metastasis and recurrence [7]. Therefore, new methods of diagnosis and treatment are urgently needed.

Bufalin is one of the main active ingredients of the important anticancer traditional Chinese medicine Venenum Bufonis. As a cardiotonic steroid isolated from Chansu, bufalin is a galenical preparation of the dried white venom [8, 9]. Several reports have shown that through Na^+/K^+-ATPase inhibition, bufalin blocks vasodilation, and increases vasoconstriction, vascular resistance, and blood pressure [10-12]. In addition, bufalin exhibits significant anti-tumor activity by inducing apoptosis in tumor cells, and inhibiting cell proliferation and metastasis [13]. Several signaling pathways play a role in the anti-tumor process of bufalin, including the mitochondrial [14], death receptor pathway [15] and endoplasmic reticulum stress [16] pathways. Bufalin exerts anti-tumor effects on many cancer types. It could also inhibit colon cancer cell growth, while inducing apoptosis, with Jak-stat3 signaling as proposed mechanism [17]. Other signaling pathways, including reactive oxygen species and JNK pathways, are also

involved in bufalin induced colon cancer cell death [18].

MicroRNAs (miRNAs) are involved in multiple cell activities, including differentiation, proliferation, apoptosis, and immunity [19-21]. Recently, multiple studies have shown that microRNA-497 (miR-497) is downregulated in tumors, functioning as a tumor suppressor in several types of human cancer [22-26]. In humans, bufalin could inhibit angiogenesis and metastasis in colorectal cancer cells, synergistically with miR-497 [27].

Insulin-like growth factor-1 receptor (IGF1R), a member of the transmembrane receptor, belongs to the tyrosine kinase family and is activated by insulin-like growth factor 1 (IGF-1) and IGF-2 [28]. IGF1R has a critical role in tumor cell transformation, survival, and invasion, and its high expression is implicated in several cancers [29-32]. IGF1R mRNA and protein expression levels are increased in tumor tissues, and significantly correlated with patient prognosis [33]. Reports also indicated that IGF1R overactivation allows the cytotoxic drug resistance property of malignant cells [34]. In addition, IGF1R activates the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway, which is curial to cell proliferation [35, 36].

To date, studies have demonstrated that miR-497 plays its cell inhibitory role by targeting IGF1R [22, 26]. Therefore, bufalin appears to inhibit proliferation and metastasis in tumor cells by altering the expression of miR-497, subsequently modulating the IGF1R signaling pathway. Whether miR-497 could target IGF1R directly in CRC cells as a tumor suppressor during bufalin treatment remains unclear. In the current study, after treatment with various concentrations of bufalin, we assessed invasion and metastasis in colorectal cancer cells as well as the expression levels of miR-497 and its downstream IGF1R-PI3K-Akt signaling pathway.

Materials and methods

Chemicals and reagents

Bufalin, isolated from Bufotoxine with purity more than 99%, was purchased from Sigma-Aldrich Chemical Corp. (St. Louis, MO, USA). Bufalin dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C was diluted in the cell culture medium before use. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Primary antibodies to human Phospho-Akt (Thr308), Phospho-Akt (Ser473), Pan Akt, IGF-1 receptor beta, and beta-actin were purchased from Cell Signaling Technology Inc. (Beverley, MA, USA). LY294002 was purchased from Selleck Chemicals. Enhanced chemiluminescence (ECL) plus system was purchased from Amersham Pharmacia Biotech.

Cell culture and transfection

Human colorectal adenocarcinoma Lovo cells were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China), and cultured in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37°C in a humidified atmosphere with 5% CO_2. Lovo cells in logarithmic growth phase digested by Pancreatin were seeded in 6-well plates at a density of 2×10^5 cells/well with 2 mL of medium, and cultured for 12 hours at 37°C in 5% CO_2. At 70% confluency, the plasmid containing miR-497 no-load control (miR-497 NC) and miR-497 lentiviral vector (Hanbio, Biotechnology Co., Ltd.) were added to the cultures in 6-well plates, respectively, 20 μl; then, polybrene (at a final concentration of 5 μg/ml) was added and mixed gently. Lovo cells were cultured for 24 h at 37°C in 5% CO_2, followed by medium replacement. GFP-positive cells were selected by flow cytometry and sub-cultured, establishing a stable transfection line of miR-497 Lovo cells.

RNA extraction and real-time PCR

Total RNA was extracted with TRIzol Reagent (Invitrogen Corporation, CA, USA). MiR-497 expression was analyzed by the specific Bulge-Loop assay, which detects mature miRNAs. Reverse transcription and qPCR were performed with Bulge-Loop™ miRNA qRT-PCR Starter Kit (RiboBio Co., Ltd. Guangzhou, China) on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. MiRNA-497 levels were normalized to U6 RNA transcript levels. Relative expression levels between the samples were calculated using the comparative delta CT (threshold cycle number) method ($2^{-\Delta\Delta CT}$), with a control sample as reference.

Cell viability assay

Cell viability was evaluated using Cell Counting Kit-8 (CCK-8) assay. In brief, cells were seeded in 96-well plates at 2×10^4 cells/well and cultured overnight. After treatment for 24-72 h with bufalin at 12.5-1600 nmol/L (eight serial concentrations) (six replicates per dose), the CCK-8 solution (10 μl) was added to each well, followed by 3 h of incubation at 37°C, 5% CO_2. Absorbance at 450 nm was recorded for each well on a FlexStation 3 microplate reader (Molecular Devices, Sunnyvale, California, USA), and cell viability accessed based on the manufacturer's instructions.

Detection of apoptosis with Annexin V-FITC/PI staining

Cell apoptosis was determined by Annexin V-fluorescein isothiocyanate (FITC)/Propidium Iodide (PI) Apoptosis Detection Kit (EMD Biosciences, La Jolla, USA). Cells were cultured in 24-well plates at a density of 5×10^4 cells/ml, and treated with bufalin for 48 h. Cells were then digested and resuspended in binding buffer, and stained with 2.5 μl Annexin V-FITC and 5 μl PI for 15 min at room temperature in the dark. The stained cells were analyzed within 30 min on a BD FACS AriaII flow cytometer (BD biosciences, San Jose, California, USA). The lower right quadrant represented early apoptotic cells (Annexin V-FITC binding positive and PI negative), while the upper right one comprised late apoptotic cells (Annexin V-FITC and PI positive staining).

Cell cycle analysis

After 48 h of treatment with bufalin, cells were harvested for cell cycle phase distribution analysis. Stained cells were measured on an Accuri C6 flow cytometer according to the instructions of Cycle Test™ Plus DNA Reagent Kit (BD biosciences); data were analyzed with the Accuri C6 software package.

Wound healing assay

For cell migration measurements, 5×10^5 cells were seeded per well of 24-well plates, and grown for 24 h. A linear wound was generated by scraping a pipette tip across the confluent cell monolayer. Cells were rinsed twice with PBS and supplemented medium with or without bufalin at 25-400 nmol/L for additional 48 h of culture. Cell migration in terms of wound closure was measured by photographing at three random fields at the time of wounding (time 0) and at 48 h.

Transwell assay

Matrigel diluted in RPMI-1640 (50 μl) was added into the upper chamber of the transwell plate, followed by overnight incubation in 37°C. Cells in logarithmic phase were plated in the 24-well transwell chambers pre-treated with Matrigel, at a density of 7×10^4 cells/100 μl. RPMI-1640 (600 μl) containing 20% FBS was added to the lower chamber. Control and bufalin treatment groups were set in duplicate. Cells were cultured for another 48 h, at 37°C in a humidified atmosphere with 5% CO_2. Then, the filter membrane was fixed with 4% paraformaldehyde for 10 min, dyed with Giemsa for 5 min, and mounted with neutral balsam. Invasive cells were counted at ×200 under an optical microscope. For each filter membrane, five different views (upper, lower, left, right and middle) were assessed and averaged.

Immunoblotting analysis

Cells were cultured in 6-well plates at a density of 1×10^6 cells/well (2 ml of culture medium), and treated with PI3K inhibitors LY294002 at 10 μmol/L for 4 h. Then, bufalin was added to the cells, followed by 1 h of incubation at 37°C in 5% CO_2. Total cell lysates were prepared with cell lysis buffer containing 1% proteinase inhibitor cocktail and 1 mmol/L PMSF, both from Beyotime Institute of Biotechnology, China. Cell proteins (50 μg) were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA). The membranes were blocked with 1% BSA (Bovine Serum Albumin), incubated with anti-human Phospho-Akt (Thr-308), Phospho-Akt (Ser473), Pan Akt, IGF-1 receptor beta, and beta-actin primary antibodies, respectively, followed by treatment with secondary antibodies. Detection was performed with Bio-Rad ChemiDoc MP Imaging System (California, USA).

Statistical analysis

All analyses were performed with the SPSS software version 13.0 (SPSS Inc., Chicago, USA). In this study, the data were all measurement data expressed as means ± standard deviations (SD) and were normally distributed. The one-way analysis of variance (ANOVA) was adopted to assess whether there were any statistically significant differences between the

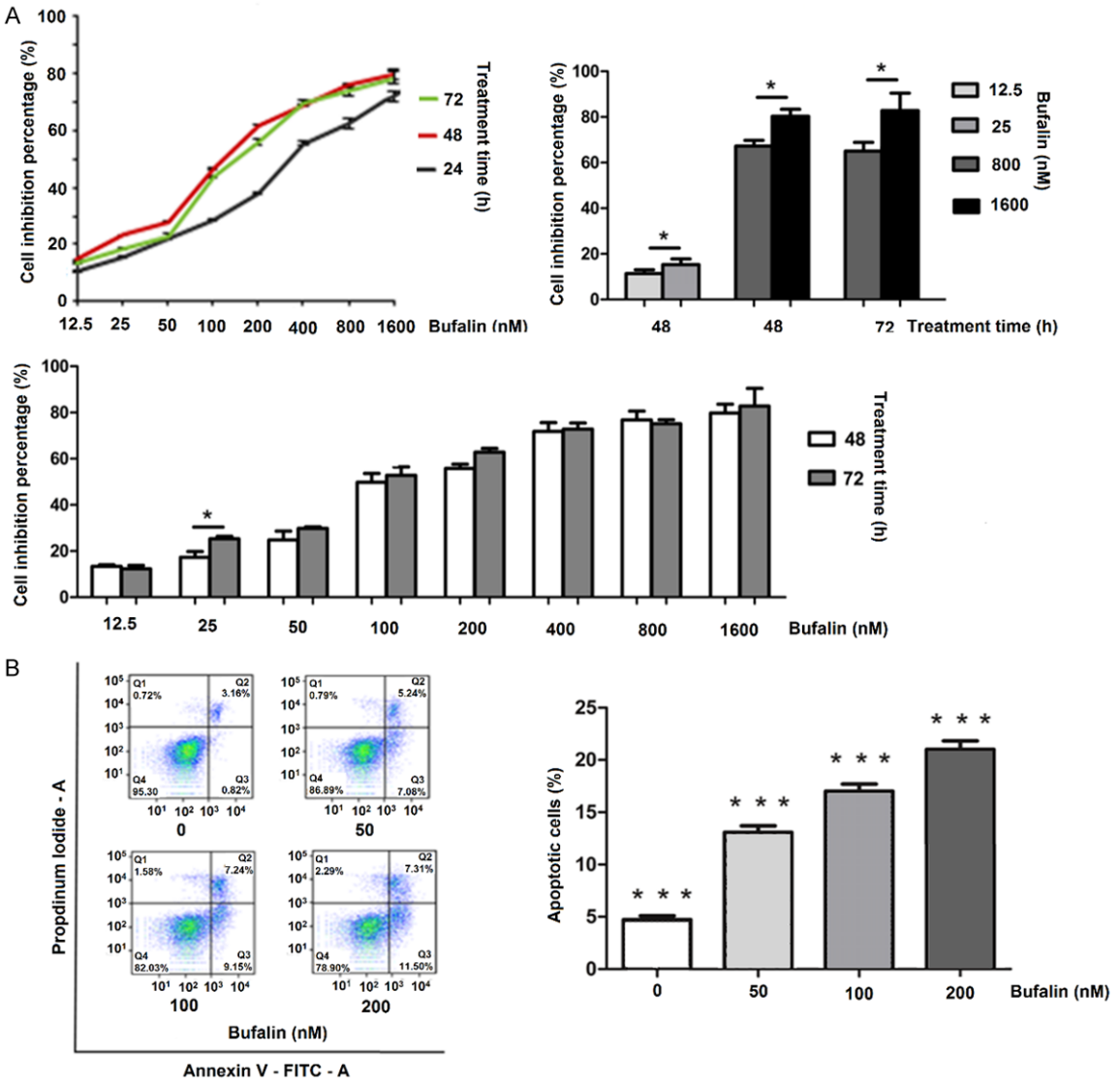

Figure 1. Bufalin inhibits viability and promotes apoptosis in Lovo cells. A: Bufalin inhibits cell viability. Lovo cells cultured in 96-well plates were treated with different concentrations of bufalin for 24-72 h. Cell viability was quantified using CCK-8 assay. Cell inhibition percent was calculated by the following equation: (A450 nm control - A450 nm experiment)/A450 nm control ×100%. Six replicates were assessed per group. The differences between various Bufalin concentrations showed statistical significance ($P < 0.01$). Between the 24 h and 48 h or 72 h groups, the differences were statistically significant for most Bufalin concentrations. Data for multiple comparisons among groups not shown in the column graph inferred the *P* values were all below 0.01. B: Bufalin induces cell apoptosis. Lovo cells cultured in 24-well plates were treated with different concentrations of bufalin for 48 h. Cell apoptosis was assayed with Annexin V-FITC and PI Detection Kit.

means of different experimental groups. P< 0.05 was considered statistically significant.

Results

Bufalin suppresses proliferation and promotes apoptosis in Lovo cells

Rapid proliferation is a critical property of tumor cells. Therefore, we aimed to explore the impact of bufalin on the proliferation of Lovo cells. To address this, Lovo cells were cultured in presence of bufalin at various concentrations. Cell viability was then determined by CCK-8 assay. As shown in **Figure 1A**, treatment with bufalin at different concentrations (12.5-1600 nmol/L) for 24-72 h resulted in significantly reduced proliferation of Lovo cells, in a dose- and time-dependent manner (24-48 h). In addition, a 10% DMSO/DMEM control group was set up

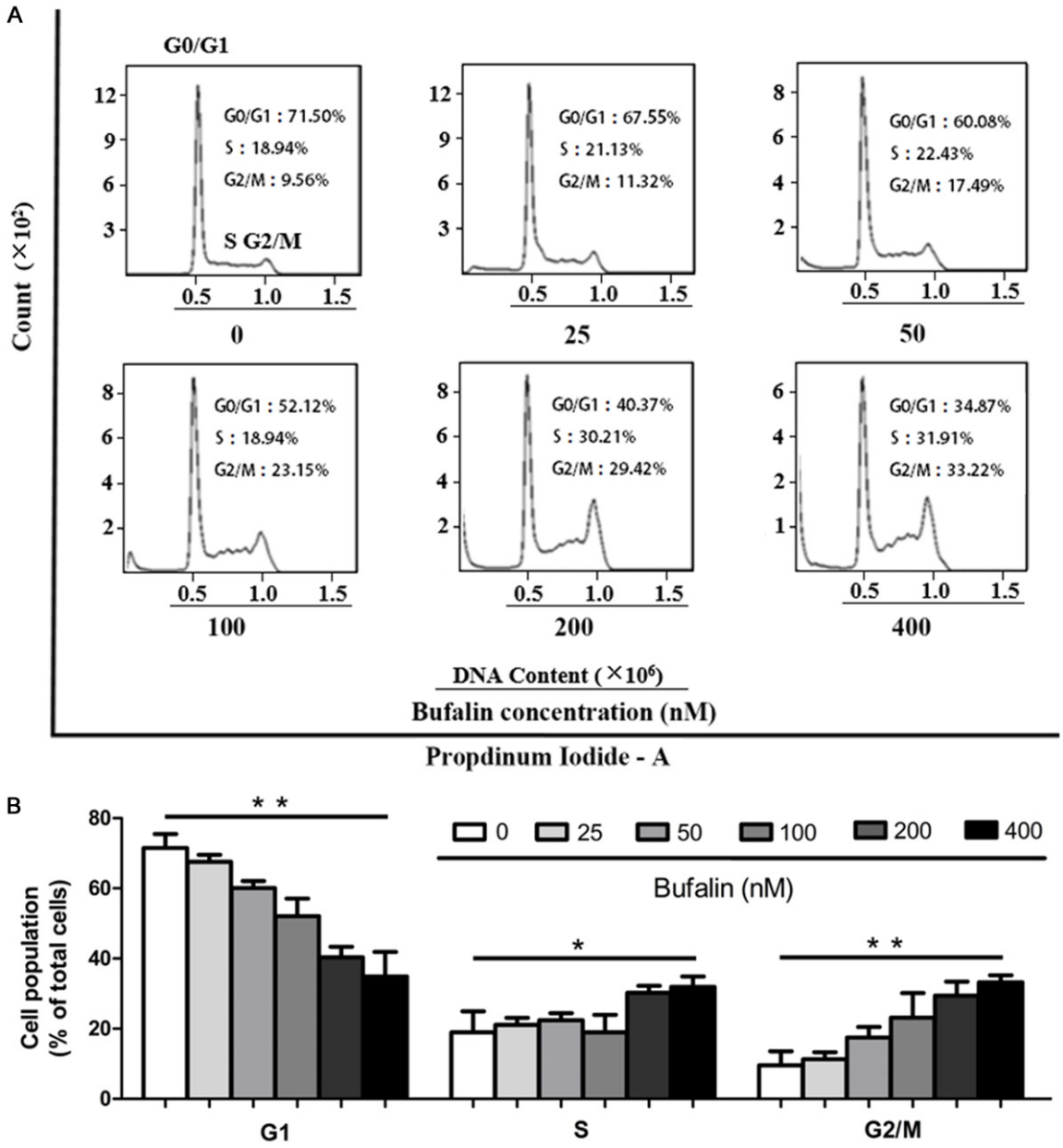

Figure 2. Cell cycle analysis: Lovo cells were stained with BD Cycle Test Plus DNA Reagent Kit and analyzed on a BD Accuri C6. One-dimensional PI histograms showed Lovo cells after 48 h treatment with different concentrations of Bufalin could be distinguished as G0/G1, S, and G2/M phase entities. The y-axis showed the number of cells while the x-axis presented increasing amounts of PI incorporation from left to right. The experiments performed in triplicate yielded similar results, and the graph is representative of three independent experiments. The peak to the far-left appearing after treatment with high bufalin concentration represented fragmented DNA from apoptotic cells.

since bufalin was dissolved in DMSO. Interestingly, cells treated with 10% DMSO/DMEM showed absorbance values similar to control cells. This finding indicated that cell death was caused by bufalin at a certain high concentration and not DMSO, because DMSO in the working solution had a concentration below 10% (data not shown). Meanwhile, resistance to apoptosis plays a critical role in cancer development, and whether a medicine induces a high degree of tumor cell apoptosis is an important criterion for evaluating its anti-tumor effects. Therefore, Lovo cell apoptosis was assessed by flow cytometry after treatment with bufalin at concentrations of 25-400 nmol/L for 48 h. Annexin V-FITC and PI staining revealed

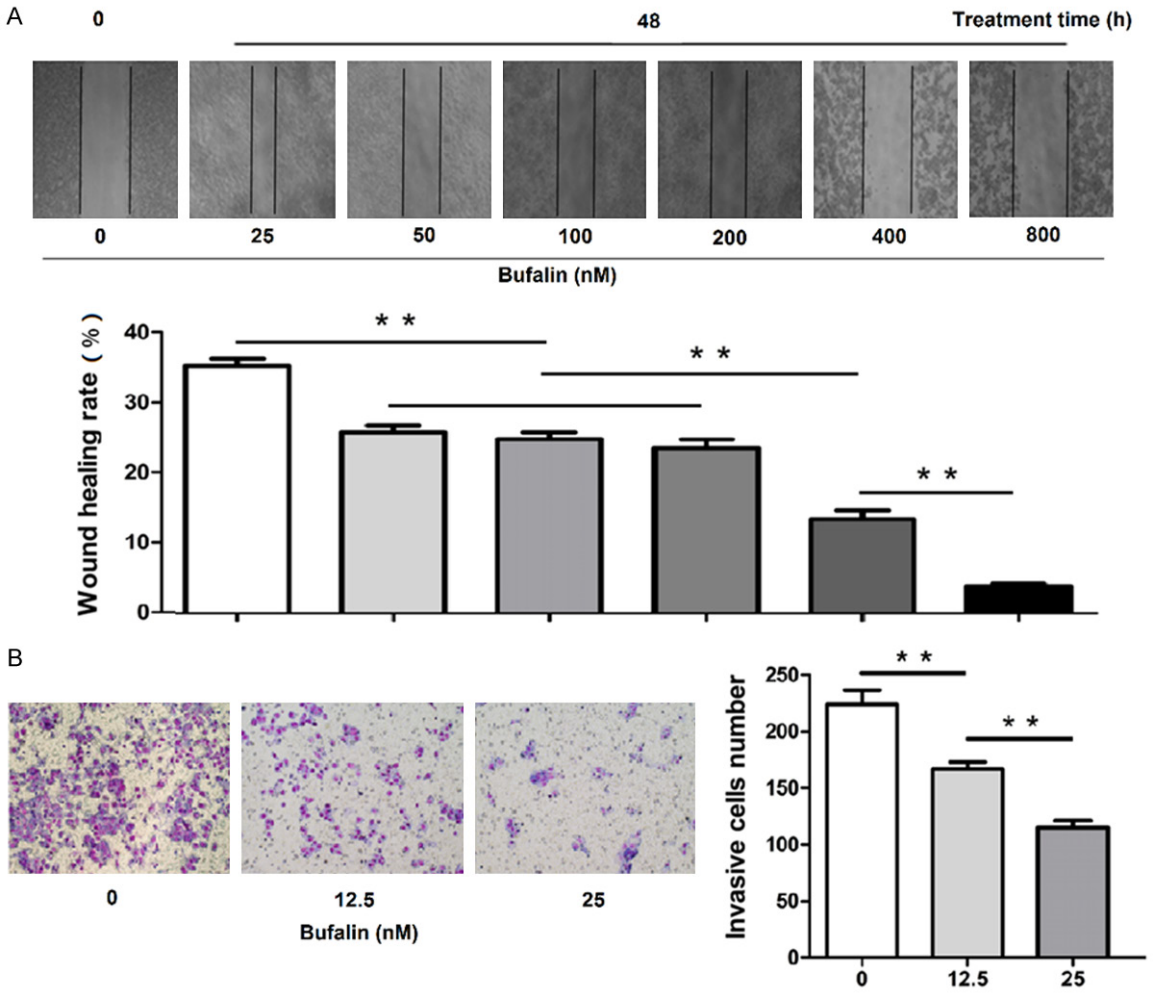

Figure 3. Bufalin inhibits cell migration and invasion. A: Lovo cells cultured in 1% FBS medium were treated with various concentrations of bufalin for 48 h. Migration assay was performed as described in Methods section, and microscopic analysis was carried out at the time of scratch (0 h) and after 48 h (48 h). Data were expressed as wound healing rate (width 0 h - width 12 h/width 0 h ×100%). B: The numbers of invasive cells were compared between different groups. Because the cells barely passed through the membrane after treatment with ≥ 50 nmol/L bufalin, 25 nmol/L was selected as maximum concentration in this assay. The original cell number in each well of the 24-well plate was 7×10^4. Data are mean ± SD from six independent experiments. $^{**}P < 0.01$.

that bufalin induced apoptosis in Lovo cells from low to high concentrations (**Figure 1B**).

Bufalin blocks cell cycle in the G2/M phase in Lovo cells

Normal cell cycle progression is necessary for tumor cell proliferation. Thus, the cell cycle status of Lovo cells was measured after bufalin treatment at 25-400 nmol/L for 48 h. As shown in **Figure 2**, after treatment with different concentrations of bufalin, the G0/G1 sub-population of Lovo cells was significantly increased from 71.50% ± 4.2% (control) to 34.87% ± 7.3% (400 nmol/L, $P < 0.01$). G2/M phase cells were significantly increased from 9.56 ± 4.70% (control) to 33.22 ± 2.23% (400 nmol/L, $P < 0.01$), in a concentration dependent manner. S phase cells were increased at high bufalin concentrations. These findings revealed that bufalin could regulate the cell cycle of Lovo cells, blocking the cancer cells in the G2/M phase, to inhibit proliferation (**Figure 2**).

Bufalin inhibits the ability of cell migration and invasion

Cell migration represents a critical event for tumor growth and metastasis. The effects of bufalin on the motility of Lovo cells were mea-

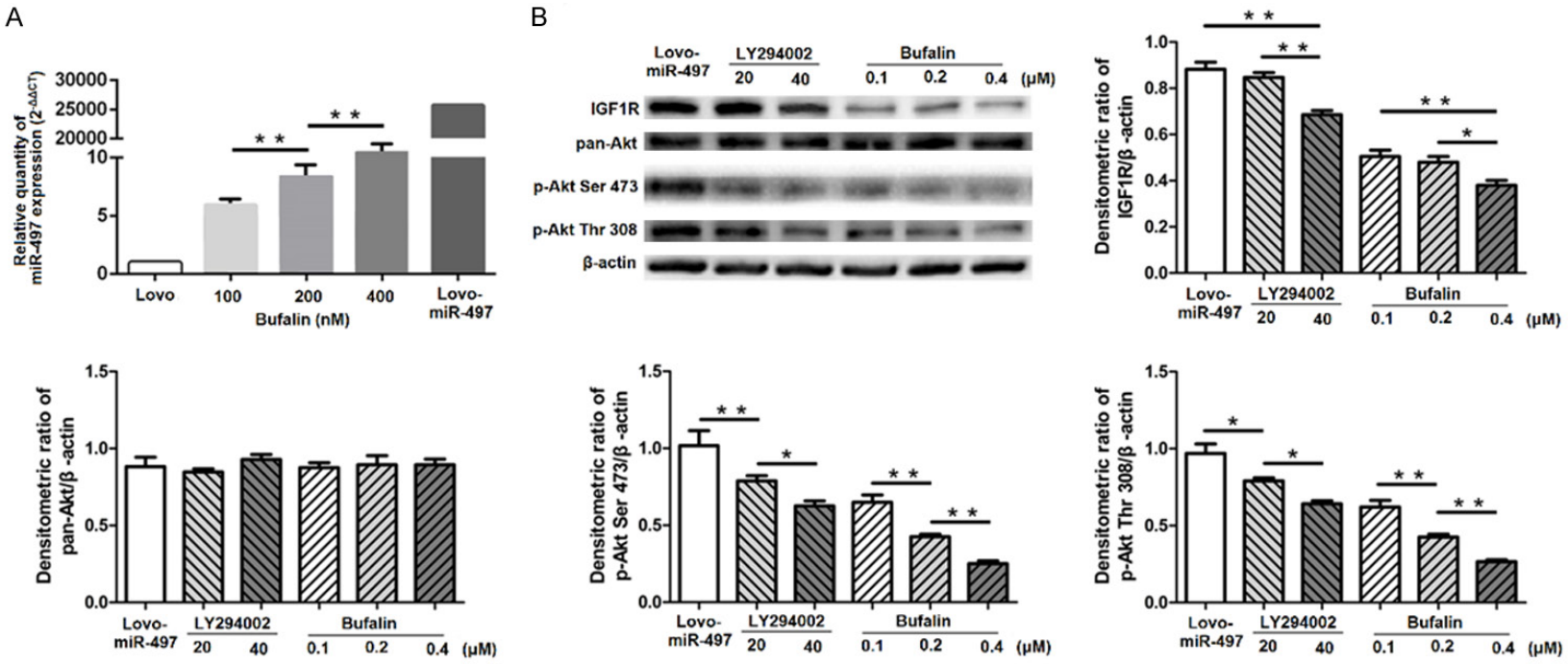

Figure 4. Expression levels of miR-497 and IGF1R related signaling effectors after treatment with bufalin. A. Bufalin upregulated miR-497 in Lovo cells after 24 h treatment; semi-quantitation of miRNA levels showed increasing trend with increasing bufalin concentrations. In addition, miR-497 transcripts in the cells was also analyzed. MiR-497 expression levels were determined by real time PCR, calculating the miR-497/U6 expression ratio. B. Cells were exposed to bufalin at different concentrations or transfected with mir-497 vector and incubated for 24 h; then the protein levels of IGF1R signaling pathway effectors were determined by Western blot.

sured by scratch assay. As shown in **Figure 3A**, treatment with bufalin at 25-400 nmol/L for 48 h resulted in decreased migration rates in Lovo cells in a dose-dependent manner, i.e. from 35.2% ± 2.1% (25 nmol/L) to 3.5% ± 1.6% (400 nmol/L); non-treated control cells showed no significant difference in migration ability. To assess the effects of bufalin on the invasion ability of Lovo cells, the transwell invasion assay was adopted. The amounts of Lovo cells after 48 h of treatment with bufalin at 0 nmol/L, 12.5 nmol/L and 25 nmol/L concentration were 225.3 ± 10.54, 167.7 ± 9.61, and 115.4 ± 10.52, respectively (200×, 5 fields). The differences between each treatment group and controls were statistically significant ($P < 0.01$). Lovo cells were also treated with bufalin at 50 nmol/L or more (data not shown), but no adherent cells were found at the surface of the transwell chamber. These results suggested that low bufalin concentrations could inhibit the invasive ability of cells (**Figure 3B**).

Bufalin regulates miR-497 and the IGF1R-PI3K-Akt signaling pathway in Lovo cells

Recently, multiple studies demonstrated that MiR-497 plays a vital role in cancer development and relapse, with its downregulation closely associated with poor prognosis. Meanwhile, miR-497 causes a significant inhibition of tumor cell viability, proliferation, and metastasis [22]. To assess the role of miR-497 in human colorectal adenocarcinoma cells, a lentiviral system was used for the transfection of miR-497 in Lovo cells. Stable miR-497 expressing Lovo cells (miR-497-Lovo cells) or no-load Lovo cells (Lovo NC cells) were established via GFP-sorting by flow cytometry. Real-time PCR (RT-PCR) demonstrated that the mRNA levels of miR-497 were close to 30,000 times higher in miR-497-Lovo cells compared with those of Lovo NC cells ($P < 0.01$, **Figure 4A**). These findings indicated that lentivirus over-expression vector increased miR-497 mRNA levels in Lovo cells. In addition, to assess whether bufalin affects miR-497 expression, Lovo cells were treated with different concentrations of bufalin for 24 h, after which miR-497 expression levels were determined by RT-PCR. In this assay, a lentiviral system was used for the transfection of miR-497 in Lovo cells as positive control. Interestingly, miR-497 expression was increased by bufalin, in a concentration-dependent manner (**Figure 4A**). Since one of the predicted targets of miR-497 is IGF1R, (http://www.targetscan.org; http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5), which plays a critical role in colon cancer proliferation and survival [34, 37, 38], we also assessed the expression levels of IGF1R and the downstream PI3K-Akt signaling effectors in Lovo cells. As shown in **Figure 4B**, Lovo cells treated with bufalin showed a decreasing trend of IGF1R expression. Meanwhile, the protein expressions levels of p-Akt Ser473 and p-Akt Thr308 were markedly reduced after treatment of Lovo cells with bufalin or LY294002 (PI3K/Akt signaling pathway inhibitor), while no change was observed in pan-Akt and IGF1R amounts (**Figure 4B**), denoting impaired PI3K/Akt signaling. These findings indicated that bufalin played a role in the regulation of IGF1R-PI3K-Akt signaling mediated by miR-497.

Discussion

Bufalin is a compound extracted from Venenum Bufonis, and widely used due to its broad-spectrum anti-tumor activities and the advantages of natural drugs. It is considered a potential anticancer agent in a variety of cancer models [39-42]. MiRNAs are highly conserved small non-coding regulatory RNAs with sizes of 17-25 nucleotides. As posttranscriptional regulators, miRNAs can negatively regulate gene expression by binding directly to 3' untranslated region (3'UTR) of corresponding target messenger RNAs (mRNAs) in a sequence-specific manner; this induces mRNA degradation or protein translation repression [19, 43]. MiRNAs are involved in a number of important processes, including tumor occurrence, development and metastasis [43-46]. Among miRNAs, miR-497 has gained a lot of attention in recent years, due to its decreased level in tumors. One of the predicted targets of miR-497 is IGF1R (http://www.targetscan.org; http://www.ebi. ac.uk/enright-srv/microcosm/htdocs/targets/v5), which is an epidermal growth factor receptor that regulates the downstream PI3K-Akt signaling pathway and the malignant transformation. Several reports demonstrated the role of IGF1R in colon cancer cell survival, proliferation, and resistance to treatment [35, 37, 38, 47]. Previous findings showed bufalin could act in synergy with miR-497 in affecting the invasion and metastasis of colorectal cancer cells. However,

the detailed mechanism remains elusive. In addition, it remains unclear whether IGF1R and its downstream PI3K-Akt pathway are involved in this regulation process. In this study, the human colorectal cancer Lovo cell line was assessed.

Our results showed that upon bufalin administration, cell proliferation was significantly suppressed in a time- and dose-dependent manner. Flow cytometry and Annexin V-FITC/PI staining showed that rates of apoptosis were increased in a dose-dependent manner after bufalin treatment. In addition, treatment with bufalin increased the rates of Lovo cells in the G0/G1 phase. Besides, through transwell and scratch assays, we demonstrated that migration and invasion in Lovo cells were impaired after treatment with bufalin. For mechanistic assessment, we focused on IGF1R since it is a miR-497 target, and has been reported to regulate colon cancer. After treatment with the PI3K/Akt signaling pathway inhibitor LY294002 or bufalin, the protein expression levels of p-Akt Ser473 and p-Akt Thr308 were markedly reduced, indicating impaired PI3K/Akt signaling. Therefore, we speculate that IGF1R-PI3K-Akt signaling, which is mediated by miR-497, is responsible for the ef fects of bufalin in regulating invasion and metastasis in colorectal cancer cells.

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Disclosure of conflict of interest

None.

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