



# Overexpression of Long Non-Coding RNA Urothelial Carcinoma Associated 1 (LncRNA UCA1) Affects Paclitaxel (Taxol) Resistance in Colorectal Cancer Cells through the Promotion of Glycolysis

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## Abstract

**Purpose:** Emerging evidence demonstrates that long non-coding RNAs (lncRNAs) have critical roles in the regulation of cancer progression. Colorectal cancer is one of the most common malignancies of human. However, a significant fraction of colorectal cancer shows resistance to conventional chemotherapeutic agents such as Taxol. In this study, we investigate the roles of lncRNA urothelial carcinoma associated 1 (UCA1) in the modulation of Taxol resistance in human colorectal cancer cells.

**Methods:** UCA1 is significantly up-regulated in both colon cancer cell lines and tissues compared with normal colon cell lines or adjacent tissues. Through the construction of UCA1 overexpression vector, it is found that high UCA1 expression was better for Taxol resistance, and we also confirm that Taxol can induce UCA1 expression. Importantly, the rates of glucose consumption, lactate production, and extracellular acidification in Taxol resistant colorectal cancer cells are obviously higher than those in Taxol-sensitive cells. The glycolysis key enzymes Hexokinase 2 (HK2) and Lactate Dehydrogenase A (LDHA) are significantly up-regulated in Taxol-resistant cells. The survival rate of cancer cells is decreased when treated with HK2 and LDHA inhibitors.

**Results:** UCA1 can directly regulate glycolysis: overexpression of UCA1 promotes glycolysis whereas knockdown of UCA1 by siRNA suppresses glycolysis. We also demonstrate that the survival rate of colorectal cancer cells is increased by UCA1 knockdown by siRNA after the addition of HK2 and decreased by UCA1 overexpression after the addition of HK2 inhibitor.

**Conclusion:** Our study provides mechanisms for the UCA1-modulated chemo resistance and thus represents a potential long non-coding target to overcome chemo resistance in colorectal cancer, which provides a new target of chemo resistant drugs for colorectal cancer patients.

**Keywords:** Colorectal cancer; LncRNA UCA1; Paclitaxel resistance; Glycolysis

## Introduction

Long non-coding RNAs (lncRNAs) are conserved non-coding RNAs that are larger than 200 nucleotides in length [1]. It has been known that lncRNAs regulate gene expression at the post-transcriptional and transcriptional levels [2]. In addition, they act directly as structural, catalytic or regulatory RNAs [3]. Although they have no protein-coding capacity, lncRNAs have emerged as essential regulators in diverse biological processes, including embryonic development, cell growth and tumorigenesis [4-6]. So far, accumulating evidence suggests that lncRNAs play an important role in tumorigenesis of multiple tumor types [5,6]. The urothelial carcinoma associated 1 (UCA1) has been reported that it increases chemo resistance of bladder cancer cells by regulating Wnt signaling pathway, indicating UCA1 displays oncogenic roles in bladder cancer [7]. Moreover, another lncRNA, HOTAIR has been widely studied in cancers: up-regulated HOTAIR expression was associated with various cancers such as breast, hepatocellular, gastric, colorectal and pancreatic cancers [8]. Advanced colorectal cancer is one of the most common malignancies especially for the patients in China [9]. Although surgery is the effective method to this kind of patients, the survival

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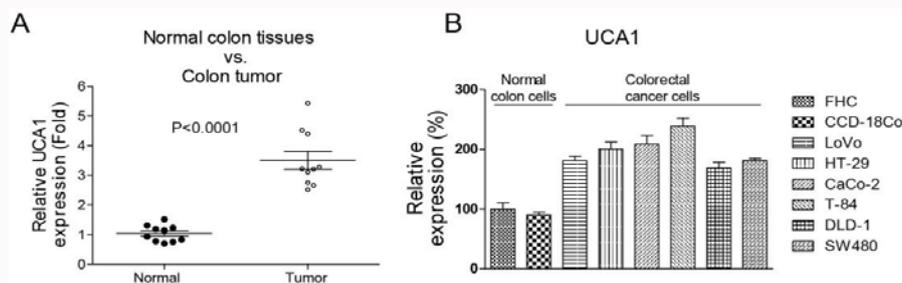
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**Figure 1:** UCA1 is upregulated in colon tumors and colon cancer cells: (A) The expressions of UCA1 were measured by real-time quantitative reverse transcription-PCR (qRT-PCR) in normal colon tissues and colon tumors. Each group contains 10 patient specimens. (B) The expressions of UCA1 were measured by qRT-PCR in two normal colon cell lines and six colon cancer cell lines respectively. Columns mean of three independent experiments; bars, SE.  $P < 0.05$  is considered statistical significance.

rate following excision of the primary tumor is still high, systemic treatments such as chemotherapy, radiotherapy and immunotherapy are the necessary procedures when colorectal cancer cells spread to distant sites [10]. However, a significant fraction of patients show resistance to conventional chemotherapeutic agents and chemo resistance to anti-tumor agents become a major obstacle to improve the survival of patients [11]. Paclitaxel (taxol), a member of the taxane class of agents, is currently used for the treatment of a wide range of carcinomas through targeting microtubules [12,13]. However, similar to other anti-cancer drugs, acquired chemo resistance remains a significant clinical problem and a major limitation to the clinical application of taxol [14]. Therefore, the exploration of molecular mechanisms is needed for the development of novel therapeutic strategies responsible for drug resistance. In recent years, studies on the energy metabolism of tumor cells have received extensive attention. Because of rapid proliferation of tumor cells, the increase in the intake of glucose and other nutrients and glycolysis often occurs. It has been reported that glycolysis is closely associated with HK2 and LDHA which catalyze glycolysis in tumor disease. The aim of this study is to investigate the roles of lncRNA UCA1 in taxol treatment of colorectal cancer cells. Moreover, there are a few UCA1 glycolysis mechanism-related research reports, so we will characterize the glucose metabolic mechanism of taxol-resistant cells and assess whether UCA1 can regulate glycolysis of colorectal cancer *in vitro* for the discovery of novel therapeutic targets of anti-chemo resistant treatment.

## Materials and Methods

### Cell lines and patients specimen

The human normal colon cell lines, FHC and CCD-18Co were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in DMEM medium Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and penicillin (100 U/ml) at 37°C in a humidified incubator containing 5% CO<sub>2</sub>. Six human colon carcinoma cell lines, LoVo, HT-29, CaCo-2, T-84, SW480 and DLD-1 were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in RPMI Roswell Park Memorial Institute 1640 medium (Gibco, 11875093) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and penicillin (100 U/ml) at 37°C in a humidified incubator containing 5% CO<sub>2</sub>. All human colon cancer patient specimens were obtained from patients undergoing surgery for original colorectal tumor during 2016 to 2017 at The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China and stored in liquid nitrogen until analysis. Tumors were obtained

under institutional review board approved protocol by the Ethics Committee of The First Affiliated Hospital, Sun Yat-sen University, and Guangzhou, China.

### Antibodies and reagents

The rabbit monoclonal antibody against Hexokinase 2 was purchased from Cell Signaling Technology (#2867, Beverly, MA, USA). The rabbit monoclonal antibody against LDHA was purchased from Cell Signaling Technology (#3582, Beverly, MA, USA). The mouse monoclonal antibody against  $\beta$ -Actin was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The 3-BrPA and Oxamate were purchased from Sigma-Aldrich (Shanghai, China). Paclitaxel was purchased from Sigma-Aldrich (Shanghai, China) and stored as a 20 mM solution in dimethyl sulfoxide (DMSO) with a final concentration of 0.1% (v/v) at 80°C and diluted with DMEM medium prior to use.

### Transfection of plasmid DNA and siRNA

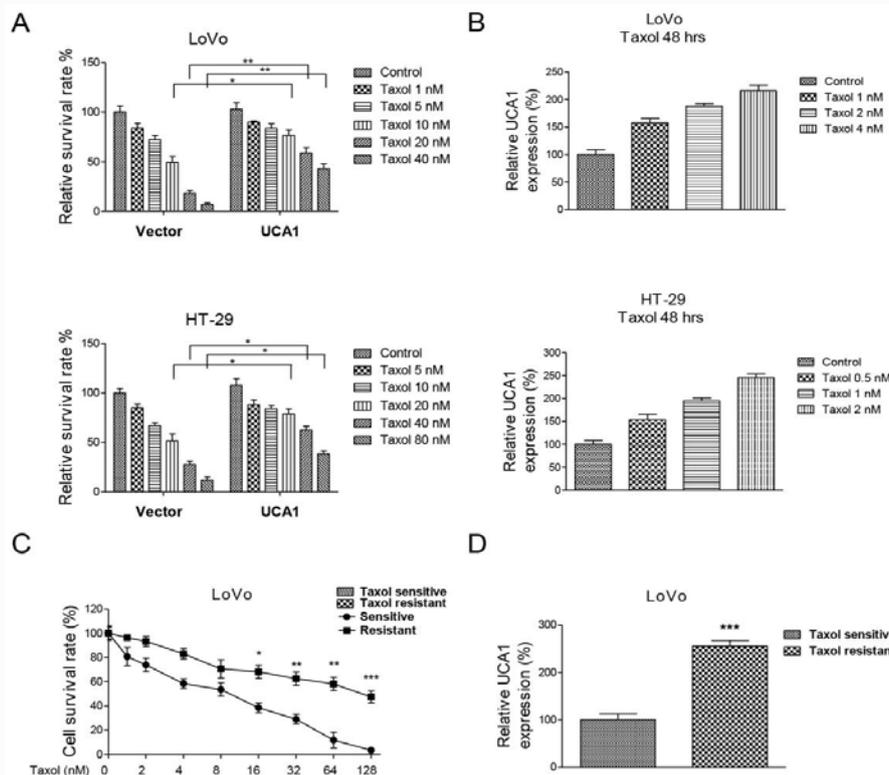
We constructed two recombinant overexpression vector pcDNA3.1-UCA1 by introducing a BamHI-EcoRI fragment containing the UCA1 precursor into pcDNA3.1 (Invitrogen, Carlsberg, CA, USA). The UCA1-siRNAs (A01035) and negative control siRNA were purchased from Genepharma (Shanghai, China). Cells were transfected with appropriate siRNA or plasmid using Lipofectamine RNAiMAX Reagent or Lipofectamine 2000 (Invitrogen, Carlsberg, CA, USA) according to the manufacturer's instructions. Forty-eight hrs after transfection, cells were collected for the following experiments.

### Real-time PCR

The total RNA was isolated using RN easy Protect Mini Kit (Qiagen, Valencia, CA, USA). The RNA was reverse transcribed into cDNA with a Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Real-time quantitative PCR assay was carried out with SYBR Premix Ex Taq II (TaKaRa, China) and monitored with the CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Comparative cycle threshold method was used to calculate fold change in gene expression. The mRNA expression was normalized with  $\beta$ -Actin. Primers for  $\beta$ -Actin: sense, TCA GGT CAT CAC TAT CGG CAA T; reverse, AAA GAA AGG GTG TAA AAC GCA All experiments were carried out in triplicate.

### Cell survival rate assay

Cells were plated at 10<sup>4</sup> cells per well in a 96-well plate and incubated in medium supplemented with 10% fetal bovine serum for overnight. The medium was removed, and 100  $\mu$ L of fresh medium containing the variant concentrations (1 nM, 5 nM, 10



**Figure 2:** UCA1 contributes to Taxol resistance in colon cancer cells: (A) LoVo cells (upper) and HT-29 cells (lower) were transfected with control vector or UCA1 overexpression vector for 48 hrs. LoVo cells were treated with Taxol at 0, 1, 5, 10, 20 and 40 nM and HT-29 cells were treated with Taxol at 0, 5, 10, 20, 40 and 80 nM for 48 hours, followed by the measurements of cell survival rate using MTT assay. (B) LoVo cells (upper) and HT-29 cells (lower) were treated with Taxol at 0, 0.5, 1 and 2 nM for 48 hours, and then the UCA1 expressions were measured. (C) Establishment of Taxol resistant cell line from LoVo parental cells through treatments with stepwise dose increase of Taxol (up to 100 nM) in regular cell culture conditions for selection of resistant cells. After successive treatments in continuous culture for 3 months, several resistant cell clones were developed from the parental cell line. Taxol -sensitive and -resistant cells were treated with Taxol at the indicated concentrations for 48 hours, and then the cell survival rates were analyzed by MTT assay. (D) The expressions of UCA1 were measured by qRT-PCR in Taxol -sensitive and -resistant LoVo cells. Columns, mean of three independent experiments; bars, SE. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

nM, 15 nM) of Taxol was added. After an additional 48 hr, the cell survival rate was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described.

#### Glucose consumption and lactate production assays

The glucose consumption and lactate production assays were performed as previous described [15]. Briefly, cells were plated at  $5 \times 10^4$  cells per well in a 48-well plate and incubated in medium supplemented with 10% fetal bovine serum for overnight. Then the supernatants of cell culture media were collected and detected using a glucose uptake kit (#K676-100, BioVision, Milpitas, CA, USA) and lactate assay kit (#K627-100 BioVision, Milpitas, CA, USA) according to the manufacturer's instructions. Glucose consumption and lactate production were calculated based on the standard curve and normalized to the cell number. All experiments were carried out in triplicate.

#### Extracellular acidification rate assay

The extracellular acidification rate assays were detected using an Extracellular Acidification kit (#ab197244, Abcam, Cambridge, Massachusetts, USA) according to the manufacturer's instructions. Rates of extracellular acidification are calculated from changes in fluorescence signal over time and as the measurement is non-destructive and fully reversible (pH-sensitive reagent is not consumed), measurement of time-courses and multiple drug treatments are possible. The results were normalized to the cell number. All experiments were carried out in triplicate.

#### Western blot

Whole cell lysates were extracted from the cultured cells using  $1 \times$  SDS sample buffer (Pierce Biotechnology, Rockford, IL, USA). The protein concentration was determined with a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). The whole-cell extract (50  $\mu$ g) was separated by electrophoresis on SDS-polyacrylamide gel and transferred to nitrocellulose membranes followed by incubation in blocking buffer for 1 hr at room temperature. The blots were then washed by PBST and incubated with primary antibodies overnight at 4°C. All the antibodies were purchased from Bio-Rad. Membranes were washed by PBST and incubated in horseradish peroxidase (HRP)-linked secondary antibody (Amersham Biosciences, Piscataway, NJ, USA) at room temperature for 1 hr. Immunocomplexes were detected with Electro Chemiluminescence (ECL) plus Western Blotting Detection System (Amersham Biosciences) and visualized with a Molecular Imager FX (Bio-Rad).

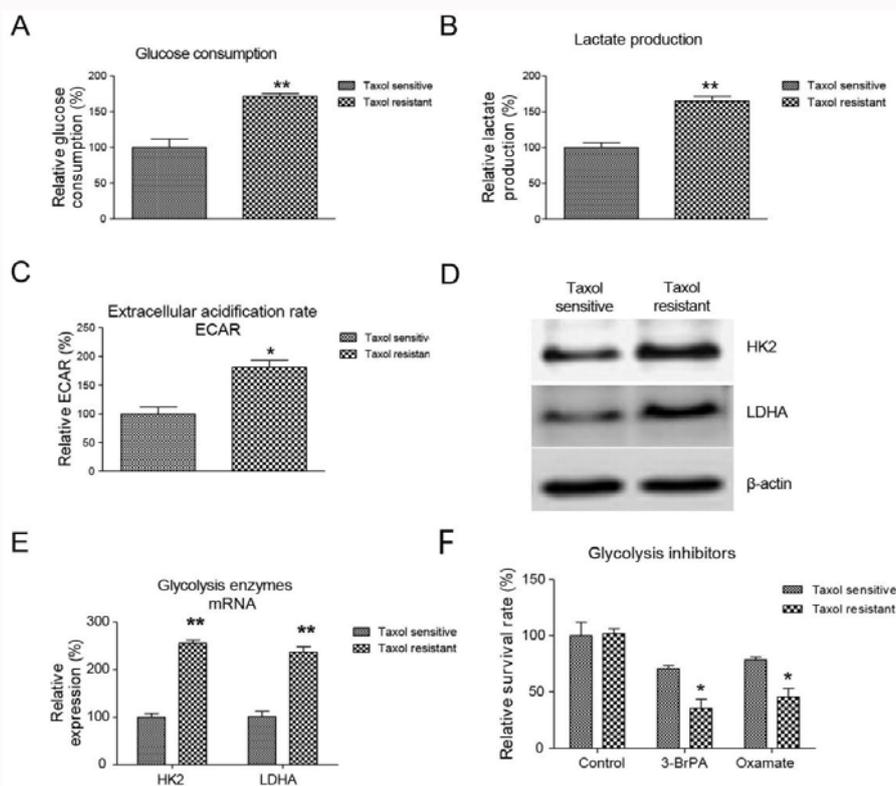
#### Statistical analysis

The Student t-test was used for all statistical analyses. Data were expressed as means  $\pm$  SD (standard deviation).  $P < 0.05$  was considered statistically significant.

## Results

### UCA1 is aberrantly up-regulated in colon cancer cells and colon tumors

Since UCA1 has been reported to possess critical roles in



**Figure 3:** Taxol resistant colorectal cancer cells show elevated glycolytic rate: (A) The glucose consumption, (B) lactate production and (C) ECAR were measured in Taxol-sensitive and -resistant cells. (D) Western blot and (E) real-time PCR analysis of the expressions of HK2 and LDHA in Taxol-sensitive and -resistant LoVo cells.  $\beta$ -Actin is a loading control. (F) Taxol-sensitive and -resistant cells were treated with 3-BrPA at 50  $\mu$ M for 24 hrs and with Oxamate at 50 mM for 48 hrs, then cell survival rates were measured by MTT assay. Columns, mean of three independent experiments; bars, SE. \* $P$ <0.05; \*\* $P$ <0.01.

progression of multiple carcinogenesis we first investigated the expression levels of UCA1 in colon tumor patient specimens [16-20]. Interestingly, we observed the UCA1 was significantly up-regulated in colon tumor samples compared with their adjacent normal tissues (Figure 1A). Consistently, the expressions of UCA1 were higher in six colorectal cancer cell lines compared with two normal colon cell lines, FHC and CCD-18Co (Figure 1B). Taken together, our results demonstrated UCA1 is up-regulated in both clinical colon tumor specimens and cancer cells, suggesting that UCA1 overexpression may be related to the occurrence of colon cancer, which can be used as a target for the development of drugs for the treatment of colon cancer.

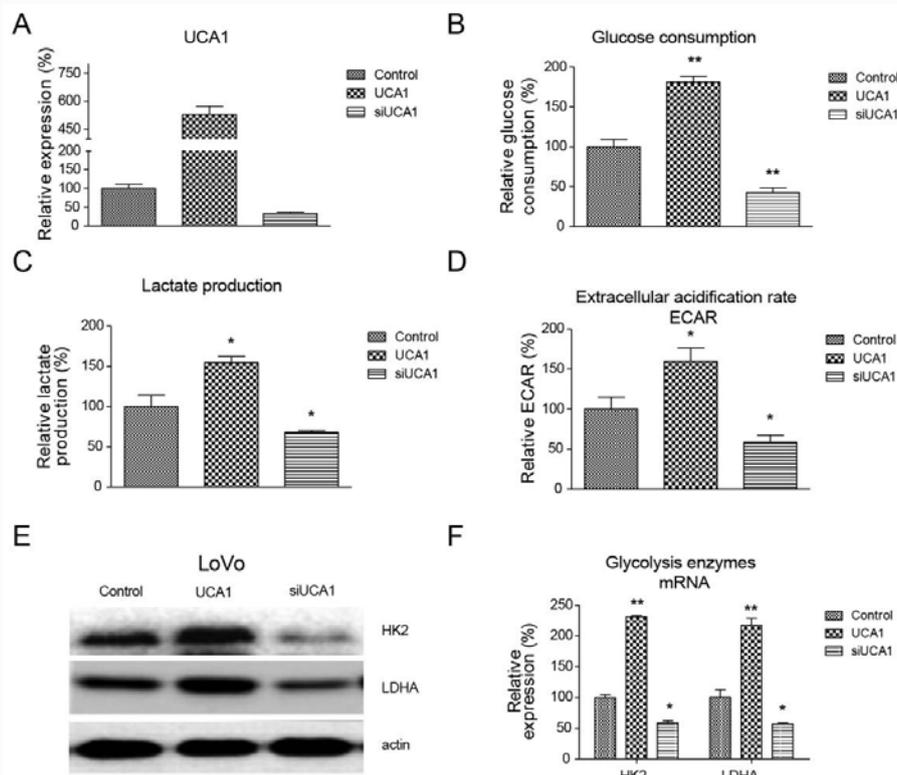
### UCA1 modulates Taxol sensitivity of colorectal cancer cells

Our above results revealed an oncogenic role of UCA1 overexpression in colon cancer, we next explore whether UCA1 could modulate the chemo sensitivity of cancer cells. Taxol is one of the most effective chemotherapeutics against multiple cancers [11]. However, the mechanisms for the acquired resistance to Taxol remain unclear. We first examined the effect of UCA1 on Taxol resistance in colon cancer cells, and then we overexpressed UCA1 in LoVo cells and HT-29 cells by transfection of a vector containing UCA1 sequence. Cells with or without exogenous UCA1 were treated by Taxol. As expected, with the increase of Taxol concentration, the survival differences of LoVo cells and HT-29 cells with UCA1 overexpression were gradually obvious after 10 nm and 20 nm Taxol treatment respectively compared with the control group (Figure 2A upper, Figure 2A lower). The results showed that UCA1 overexpression

enhance Taxol resistance in colon cancer cells. In order to further detect the effect of Taxol on the expression level of UCA1, we first treated of colon cancer cells LoVo and HT-29 with Taxol for 48 hrs, and then detected the expression level of UCA1. It was found that the expression level of UCA1 in the LoVo cells (Figure 2B upper) and HT-29 cells (Figure 2B lower) was increased with Taxol concentration, leading us hypothesized that Taxol could promote UCA1 expression. To further demonstrate the above views, we next established a Taxol resistant colon cancer cell line (LoVo TR) by treatments of parental LoVo cells with gradually increased concentration of Taxol. Using the parental cells as the sensitive cell line and acquired drug-resistant cells as the resistant cell line, we found that the survival rate of resistant cells was significantly higher than that of sensitive cells. When the Taxol concentration was increased to 16 nM, the difference of the cell survival rate between resistant cells and sensitive cells was much more obvious (Figure 2C). As shown in Figure 2D, it was found that the expression level of UCA1 was significantly higher in Taxol resistant cells than sensitive cells. These results indicated that the survival rate and the expression level of resistant cells were obviously higher than those of sensitive cells. All these results suggested that inhibition of UCA1 might contribute to the development of anti-Taxol resistance in colon cancers.

### Glycolytic rates are elevated in Taxol resistant colorectal cancer cells

It has been widely studied that dysregulated glucose metabolism emerges as a new hallmarked of cancers [21]. Moreover, Taxol resistant cancer cells showed up-regulated nutrition demand for evading chemotherapy [22]. We hypothesized that UCA1 might



**Figure 4:** UCA1 promotes glycolysis of colorectal cancer cells: (A) LoVo cells were transfected by control vector, UCA1 overexpression vector or siUCA1 for 48 hrs. Cells were collected and the (B) glucose consumption, (C) lactate production, (D) ECAR, (E) protein levels of HK2 and LDHA and (F) mRNA levels of HK2 and LDHA were measured. Columns, mean of three independent experiments; bars, SE. \* $P < 0.05$ ; \*\* $P < 0.01$ .

regulate Taxol resistance in colon cancer cells through affecting glucose metabolism. To explore the putative mechanisms for the UCA1-modulated Taxol resistance of colorectal cancer cells, we focused on the metabolic characteristics between Taxol sensitive and resistant colon cancer cells. Our analysis of the glucose metabolism demonstrated that the glucose consumption (Figure 3A), lactate production (Figure 3B) and extracellular acidification rates (ECAR) (Figure 3C) of Taxol resistant cells were significantly higher than those of the Taxol sensitive cells and the glucose metabolism level of resistant cells was obviously higher than that of sensitive one. In addition, the protein and mRNA levels of glycolysis key enzymes HK2 and LDHA were up-regulated in Taxol resistant cells (Figure 3D and 3E), suggesting the HK2 or LDHA could be the selective targets to overcome Taxol resistance. To further support the above results that glycolysis is correlated with Taxol resistance of colorectal cancer cells, we treated LoVo Taxol sensitive and resistant cells with glycolysis inhibitors, 3-BrPA (3-bromopyruvate's) or Oxamate which targets on HK2 or LDHA, respectively. As we expected the relative survival rates of Taxol resistant cells and sensitive cells were decreased, meanwhile the Taxol resistant cells' survival rate was significantly decreased after 3-BrPA and Oxamate treatment. The results showed the Taxol resistant cells were more sensitive to glycolysis (Figure 3F). Taken together, our results illustrated the correlation between Taxol resistance and glycolysis in colon cancer cells.

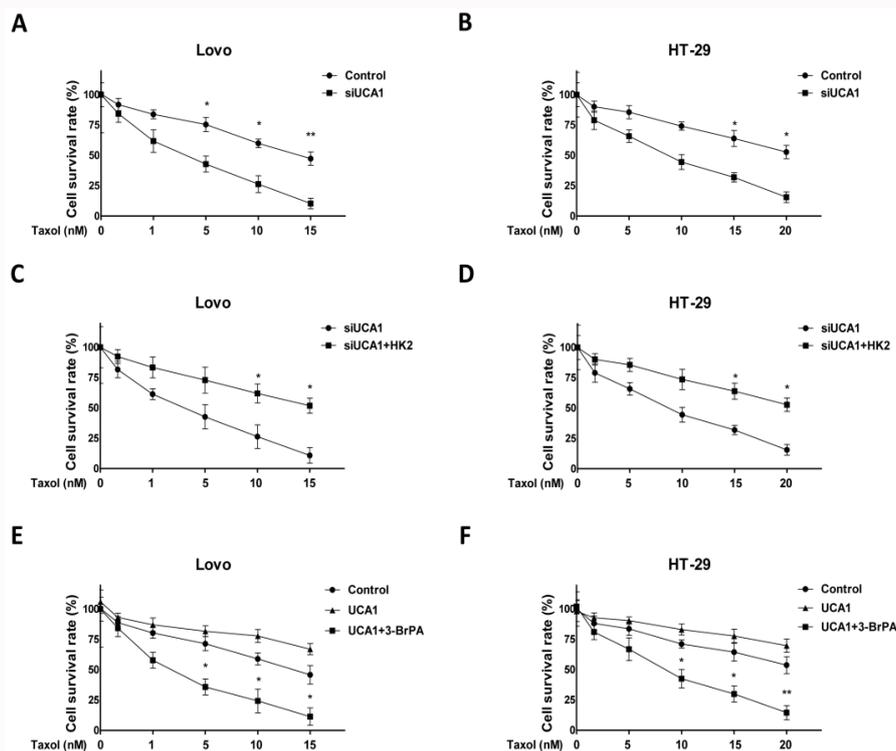
#### UCA1 up-regulates glycolysis enzymes and promotes glycolysis

We next investigated whether the UCA1-modulated Taxol resistance of colon cancer cells is associated with the elevated glycolysis in Taxol resistant cells. To study whether UCA1

could regulate glycolysis, we transfected LoVo cells with UCA1 overexpression vector or UCA1 specific siRNA followed by the measurements of glucose metabolism lactate production and ECAR (Figure 4A). Overexpression of UCA1 significantly up-regulated glucose consumption, lactate production and ECAR, in contrast, knockdown of UCA1 down-regulated the glucose metabolism (Figure 4B to 4D). Consistently, the glycolysis key enzymes, HK2 and LDHA were regulated by overexpression or knockdown of UCA1 at both protein (Figure 4E) and mRNA (Figure 4F) levels. In summary, our results demonstrated that UCA1 promoted glucose metabolism of colorectal cancer cells by upregulating HK2 and LDHA.

#### Inhibition of UCA1 sensitizes colorectal cancer cells to Taxol through suppression of glycolysis

The above results reveal the correlation between UCA1-modulated glycolysis and Taxol sensitivity, we assessed whether UCA1 has the potential to be an anti-chemo resistance agent with the combination of Taxol and inhibition of UCA1 in colon cancers. Knockdown of UCA1 significantly sensitized LoVo (Figure 5A) and HT-29 (Figure 5B) cells to Taxol treatments at 5, 10 and 15 nM and 15 and 20 nM, respectively. To figure out whether the sensitization of colon cancer cells to Taxol by knockdown UCA1 is through the suppression of glycolysis, we co-transfected overexpression vector of HK2 and siUCA1 to rescue the glycolysis. As we expected, restoration of glycolysis rate in UCA1-knockdown LoVo (Figure 5C) and HT-29 (Figure 5D) cells rendered cells resistant to Taxol treatments. To further support the above results, we treated glycolysis inhibitor, 3-BrPA which targets on HK2 in UCA1 overexpressing LoVo and HT-29 cells. Results in Figure 5E and 5F demonstrated although exogenous UCA1 increased the Taxol resistance of colon cancer cells,



**Figure 5:** Inhibition of UCA1 sensitizes colorectal cancer cells to Taxol. (A) LoVo cells and (B) HT-29 were transfected by control siRNA or siUCA1 for 48 hrs. Cells were treated with Taxol at the indicated concentrations respectively for 48 hrs followed by the measurements of cell survival rates. (C) LoVo cells and (D) HT-29 cells were transfected with siUCA1 or siUCA1+HK2 for 48 hrs. Cells were treated with Taxol at the indicated concentrations respectively for 48 hrs followed by the measurements of cell survival rates. (E) LoVo cells and (F) HT-29 cells were transfected with control vector or UCA1 overexpression vector for 48 hours. Cells were then treated with PBS control or 3-BrPA at 50  $\mu$ M for 24 hrs, followed by the measurements of cell survival rates by MTT assay. Columns, mean of three independent experiments; bars, SE. \* $P$ <0.05; \*\* $P$ <0.01.

treatments with glycolysis inhibitor overcome the resistance. Taken together, our data showed the UCA1-mediated Taxol sensitivity is through the regulation of glycolysis of colorectal cancer cells.

## Discussion

Currently, lncRNAs have been well characterized to be involved in a variety of biological processes including development, cell growth and tumorigenesis [4,5]. In addition, recent studies have revealed that lncRNAs are involved in regulating chemo resistance. lncRNA H19 has been described to induce P-glycoprotein expression and multi-drug resistance 1-associated drug resistance in liver cancer cells [23]. Consistently, our results demonstrate UCA1 is up-regulated in colon cancer patient samples compared with normal adjacent colon tissues. Previous study illustrated UCA1 is involved in cisplatin sensitivity of human bladder cancer cells intriguing us to explore the Taxol resistance mechanism of UCA1 in colon cancer. The results showed that Taxol promoted UCA1 expression in colorectal cancer cells. In turn, UCA1 also enhanced the Taxol resistance of colorectal cancer cells and UCA1 expression in Taxol resistant colorectal cancer cells was more significant suggesting inhibition of endogenous UCA1 could sensitize cancer cells to Taxol. Taxol were described as antibiotic agents that bind to  $\beta$ -tubulin to stabilize the microtubule polymer and protect it from disassembly, resulting in the cell cycle arrest at the  $G_2/M$  phase and triggering cell apoptosis [11,12]. Cancer cells prefer to rely on aerobic glycolysis for their energy and build materials supply, distinguishing them from normal differentiated cells which rely primarily on mitochondrial oxidative phosphorylation to generate the energy. This phenomenon termed “the Warburg effect”

[21]. It has been revealed that dysregulated glycolysis of cancer cells contributes to chemo resistance, such as cisplatin, 5-Fu and Taxol [24,25]. Moreover, a recent study described that UCA1 is a hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) target and HIF-1 $\alpha$  specifically bound to HREs in the lncRNA-UCA1 promoter [26]. Since glycolysis enzymes have been reported as downstream targets of HIF-1 $\alpha$ , suggesting UCA1 might be involved in the HIF-1 $\alpha$ -mediated glycolysis of cancer cells. In our study, by establishing Taxol resistant colorectal cancer cell line, we compared the glycolytic rate between Taxol-sensitive and -resistant cells. Interestingly, our results revealed Taxol resistant cells displayed higher glycolysis rate than sensitive cells. Importantly, we demonstrated a direct linkage between the UCA1-modulated Taxol resistance and the up-regulated glycolysis in Taxol resistant colon cancer cells: UCA1 could promote glycolysis of colon cancer cells through upregulating of glycolysis key enzymes, HK2 and LDHA. Our study first demonstrated the function of UCA1 in Taxol resistance of colon cancer cells by promotion of glycolysis enzymes indicating modulation of UCA-1 regulated glycolysis could be a novel anticancer strategy.

## Conclusion

In summary, this study reported a new role of lncRNA UCA1 in Taxol resistance through the regulation of glycolysis of colon cancer cells. Further studies will investigate detailed mechanism how UCA1 regulates HK2 and LDHA expression. A mouse model will be established to test the therapeutic efficiency by combination of Taxol, glycolysis inhibitor and inhibition of UCA1 *in vivo*.

## Compliance with Ethical Standards

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**Research involving human participants:** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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