

Original Article

Long non-coding RNA UCA1 enhances drug resistance of lung cancer cells via activating mTOR signaling

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Abstract: There is accumulating evidence suggesting that long non-coding RNA (lncRNA) UCA1 is involved in the resistance of the tumor to chemotherapeutic agents. Enhanced expression of UCA1 is frequently observed in patients with lung cancer. However, the role of UCA1 in regulating chemoresistance of lung cancer cells remains largely unknown. Here we reported that overexpression of UCA1 significantly enhanced the resistance of lung cancer cells to rapamycin. Overexpression of UCA1 enhanced mTOR phosphorylation and potentially reduced rapamycin-induced inhibition of mTOR signaling, resulting in enhanced viability of A549 cells upon rapamycin treatment. Consistently, knockdown of mTOR largely abolished UCA1-mediated resistance to rapamycin treatment. In addition, we showed that A549 sphere was characterized by enhanced expression of UCA1, high activity of mTOR signaling as well as elevated resistance to rapamycin treatment. Together, these results indicated that UCA1 could enhance chemoresistance of lung cancer cells via activating mTOR signaling, suggesting a role of UCA1 as a new effective therapeutic target for lung cancer treatment.

Keywords: UCA1, rapamycin, lung cancer, mTOR signaling

Introduction

Long non-coding RNAs (lncRNAs) are recently discovered molecules that regulate eukaryotic gene expression [1, 2]. There is now growing evidence suggesting that lncRNAs are playing an essential role in regulating signaling pathway crucial for development, differentiation, survival and homeostasis during normal development and disease [3-5]. Of note, deregulation of lncRNA has been frequently observed in various cancers, which is directly linked to essential growth-promoting activities and drug resistance. For example, previous results showed that HOTAIR lncRNA, which was known for its role in fine-tuning the expression of HOX gene clusters [6], was significantly up-regulated and was immediately linked to poor prognosis and high chance of tumor recurrence in lung, breast, and bladder cancer [7]. Elevated expression of another lncRNA, namely, MALAT-1, was also frequently observed in prostate and lung cancer. High MALAT-1 expression was correlated with metastasis development, tumor stage and resistance to chemotherapy [8]. Moreover, lncRNA GAS5 is highly expressed in hepatocellular carcinoma and is associated with a poor

prognosis as well as metastasis formation [9]. It has also been shown that lncRNA PANDA could interact NF- κ B to inhibit transcription of pro-apoptotic genes and reduce the apoptotic index in fibroblasts upon doxorubicin treatment [10].

Urothelial carcinoma associated 1 (UCA1) is a recently identified non-coding RNA that was first reported to be highly expressed in bladder cancer [11, 12]. Recent studies have shown that UCA1 is up-regulated generally observed in other tumor types such as breast cancer [13-15]. It could facilitate growth and proliferation of tumor cells and thereby promotes the development of cancer. Moreover, highly expressed UCA1 is immediately linked to reduced sensitivity of cancer cells to chemotherapeutic agents by affecting their apoptotic index during chemotherapy [11, 12]. Accordingly, deregulation of UCA1 is causally linked to pathogenesis of cancer and represents a potential target to overcome drug resistance [3]. Recently, it has been shown that expression level of UCA1 is significantly elevated in tumor tissues [16, 17]. However, whether UCA1 could likewise regulate chemoresistance of lung cancer cells remains to be determined.

UCA1 activates mTOR signaling

Here we investigated the role of the UCA1 lncRNA in rapamycin resistance during chemotherapy for lung cancer. To this end, we overexpressed UCA1 lncRNA in A549 lung cancer cell line. Our results provided clear evidence that forced expression of UCA1 strongly enhanced cell viability upon treatment with rapamycin by activating mTOR signaling. Consistent with this result, mTOR silencing by shRNA largely abolished the protective function of UCA1 upon rapamycin treatment. Moreover, we observed that UCA1 expression was significantly enhanced in fibroserve tumorspheres formed by A549 cells, which contains a high number of lung stem-like cancer cells and is characterized by enhanced resistance to rapamycin. Taken together, these results indicate that UCA1 plays an oncogenic role in lung cancer in part through activating mTOR pathway and enhancing drug resistance.

Materials and methods

Cells culture

A549 human lung carcinoma cells (ATCC) were grown in Dulbecco's modification of eagle's medium (DMEM, Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 units of penicillin/ml and 100 µg of streptomycin/ml (Lonza). Cells were incubated at 37°C and supplemented with 5% CO₂ in the humidified chamber. Suspended, stem-like sphere was established by diluting and seeding A549 cells in 6-well plates at 1×10³ cells/well with DMEM supplemented with 10% fetal FBS for three weeks. Thereafter, holoclones (50-200 cells) were isolated using cloning cylinders (Corning, USA) and cultured with serum-free stem cell medium containing DMEM/F12 (Gibco), B27 (1×, Gibco), recombinant human epidermal growth factor (rhEGF, 20 ng/ml; Sigma, USA), basic fibroblast growth factor (bFGF, 20 ng/ml; Upstate, USA), and insulin (4 U/l; Sigma) for two more weeks. When primary tumor spheres reached approximately 100-200 cells/sphere, the spheres were dissociated and single cells were cultured for another 1-2 weeks until secondary spheres were formed, which were used for the subsequent experiments.

Apoptosis assay

A549 cells were seeded in 6-well plates for 24 hours and then transfected with UCA1-1 or the

control expression vectors. After allowing protein expression for 24 h, cells were treated with rapamycin for 24 h, the cells were trypsinized, washed twice with PBS, and resuspended in Annexin V binding buffer. They were then stained with Annexin V/PI (Invitrogen, USA) for 15 min in the dark at room temperature, and the cell populations were analyzed by a BD FACSCalibur Flow Cytometer.

Proliferation assay

A549 cells or those dissociated from A549 secondary spheres were prepared into single cell suspension and seeded into 96-well plates at a density of 5000 cells/ml, 100 µl/well. Cell viability was determined using a Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher, USA). Briefly, after different treatment, 10 µl MTT stock solution (5 mg/ml) is added to each culture and incubated for 4 hr. The optical density of the solution in the wells was measured at 570 nm in an Enzyme-linked immunosorbent Monitor (U-Quat, USA). We have three replicates for each sample and the mean ± sd was shown. Differences in each group were determined by t-test and P<0.001 was considered significant.

Western blot

Cells were lysed using RIPA protein extraction reagent (Beyotime, Beijing, China) supplemented with phenylmethanesulfonyl fluoride (PMSF) [Riche, USA]. Approximately 50 µg of protein extracts were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes (Sigma), and incubated with specific antibodies. Antibodies used included anti-mTOR antibody (Cell Signaling Technology, USA), Phospho-mTOR Antibody (Cell Signaling Technology, USA) and anti-α-actin (Sigma-Aldrich, USA).

UCA1 clone and transfection

Full length of UCA1 was amplified using the primer pair: UCA1_f: 5'-CGGGATCCTGACATTC-TTCTGGACAATGAG-3'; UCA1_r: 5'-TTTTGTCCCC-ATTTCCATCATACG-3'.

The PCR product was cloned into TA vector using TOPO® TA Cloning kit (Invitrogen, USA),

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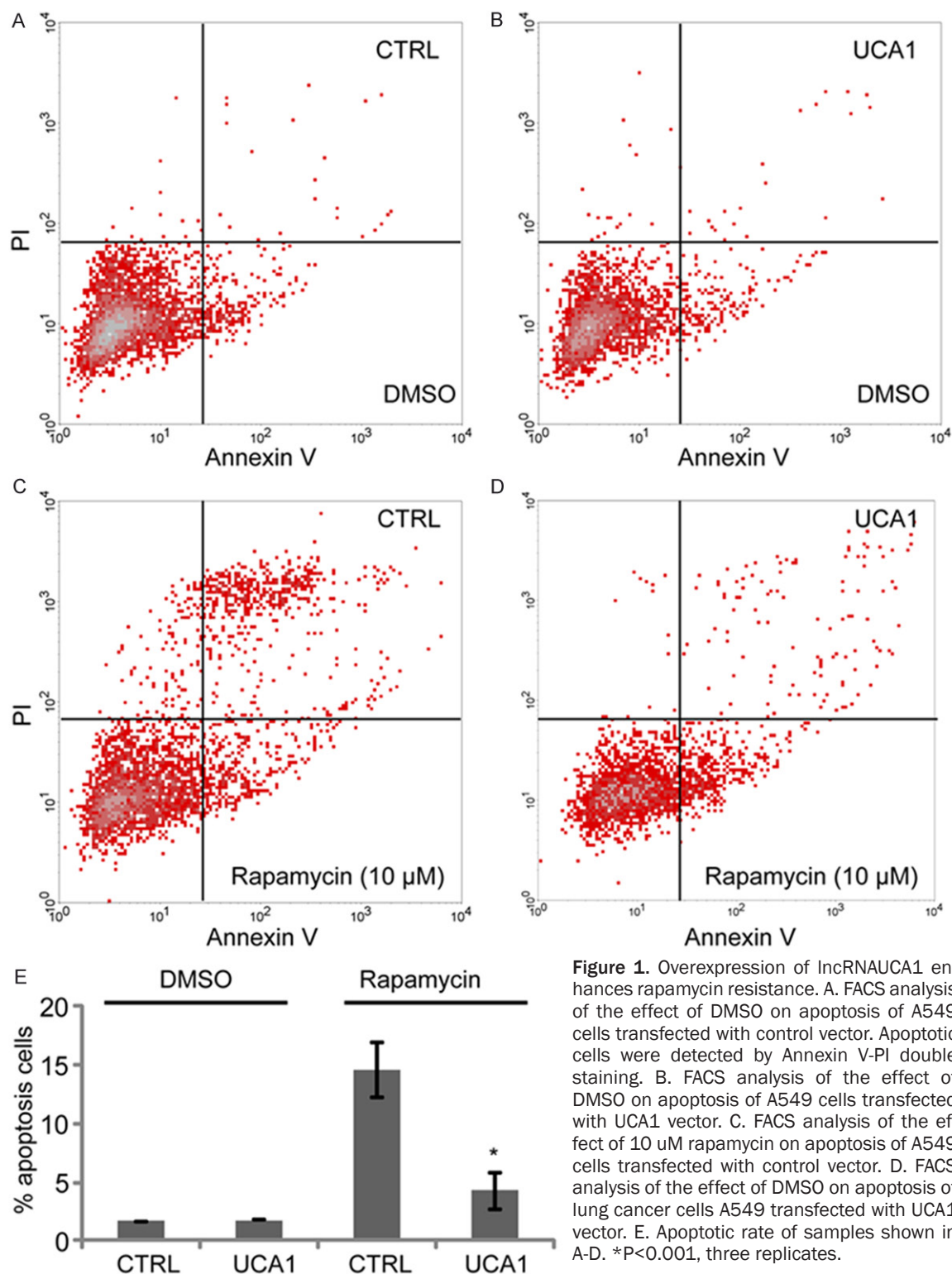


Figure 1. Overexpression of lncRNAUCA1 enhances rapamycin resistance. A. FACS analysis of the effect of DMSO on apoptosis of A549 cells transfected with control vector. Apoptotic cells were detected by Annexin V-PI double staining. B. FACS analysis of the effect of DMSO on apoptosis of A549 cells transfected with UCA1 vector. C. FACS analysis of the effect of 10 μM rapamycin on apoptosis of A549 cells transfected with control vector. D. FACS analysis of the effect of DMSO on apoptosis of lung cancer cells A549 transfected with UCA1 vector. E. Apoptotic rate of samples shown in A-D. *P<0.001, three replicates.

and subcloned into pcDNA 3.1 vector using BamH I and Xho I.

Cells were transfected using Lipo2000 reagent (Thermo, USA) following the manufacturer's protocol.

Quantitative real-time PCR

Total RNA was isolated using Trizol reagent (Thermo, USA). 200 ng of RNA was reverse-transcribed to cDNA using SuperScript First Strand cDNA System (Thermo, USA) and gene

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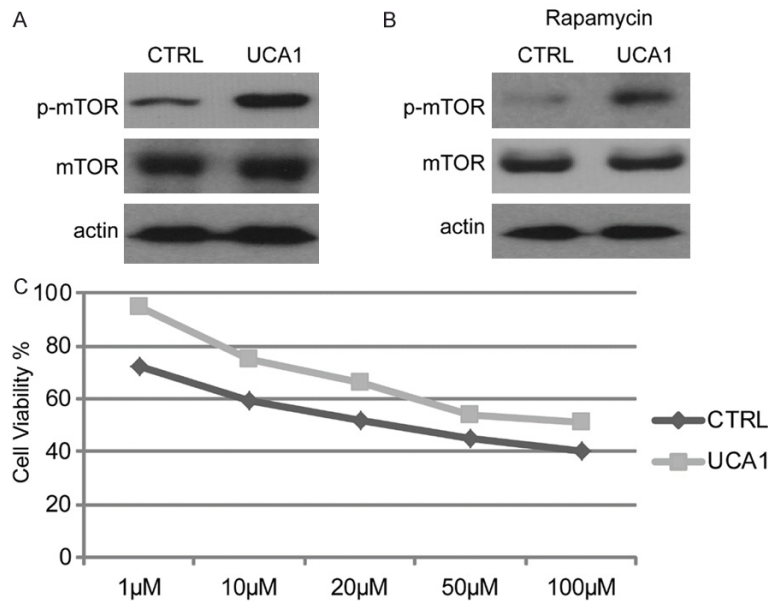


Figure 2. Overexpression of lncRNAUCA1 activates mTOR signaling. A. Western-blot analysis of phosphorylated mTOR and total mTOR in cells transfected with control or UCA1 vector. Actin was used as a loading control. B. Western-blot analysis of cells transfected with control or UCA1 vector following treatment with 10 μM rapamycin. C. Cells transfected with UCA1 or control were treated with 1, 10, 20, 50 or 100 μM of rapamycin for 24 h. Cell viability was determined by MTT approach.

specific primers. Quantitative real-time PCR was performed using SYBR Green Supermixes (BIO-RAD Laboratories, USA) and a LightCycler system (Roche Diagnostics). A dissociation curve was produced and relative gene expression was calculated by using the $2^{-\Delta\Delta CT}$ method. Experiments were performed in triplicates with the following PCR primers: UCA1: F 5'-CTTC-TGCATAGGATCTGCAATCAG-3'; R 5'-TTTTGTCC-CCATTTCCATCATACG-3'; GAPDH: F 5'-AGGTCG-GAGTCAACGGATTTG-3'; R 5'-GTGATGGCATGG-ACTGTGGT-3'.

Results

Overexpression of UCA1 enhances A549 resistance to rapamycin

Previous results suggested that UCA1 plays an important role in cisplatin resistance during chemotherapy for bladder cancer [15]. Recently, it has been shown that UCA1 is frequently upregulated and contributes to progression of lung cancer [16, 17]. However, the role of UCA1 in drug resistance of lung cancer cells remains unknown. To this end, we have cloned the full length spliced RNA of UCA1 transcript, transfected it into lung cancer cell line A549 and investigated the molecular mechanisms of ac-

quired drug resistance in these cells. Overexpression of UCA1 had no significant influence on the number of apoptotic cells under normal conditions (Figure 1A and 1B). However, strikingly, overexpression of UCA1 significantly increased the cell viability upon rapamycin treatment (Figure 1C-E, $P < 0.01$, t-test). These results provided clear evidence suggesting that in addition to its role in facilitating proliferation, UCA1 could also enhance drug resistance in lung cancer cells.

Overexpression of UCA1 activates mTOR signaling

As rapamycin is an allosteric inhibitor of mTOR [18], we thus asked whether UCA1-induced rapamycin chemoresistance was linked to hyperactivation of mTOR signaling in cells overexpressing UCA1. Western-blot results showed that phosphorylation of mTOR was significantly enhanced in A549 cells transfected with UCA1 as compared as control, while the expression level of total mTOR remained largely unchanged (Figure 2A). As expected, treatment with 10 μM rapamycin resulted in potent inhibition of mTOR phosphorylation (Figure 2B), which, however, was significantly counterbalanced by overexpression of UCA1 (Figure 2B). Interestingly, the protective effect of UCA1 was more pronounced when A549 cells were treated with lower concentration of rapamycin. As shown in Figure 2C, UCA1 reduced cell death by about 23% and 16% upon treatment with 1 μM and 10 μM rapamycin, respectively. In contrast, however, the effect was reduced to only 9% when treated with high dose (100 μM) of rapamycin. These results suggested that UCA1 activates mTOR signaling to enhance drug resistance to rapamycin in A549 cells.

Knockdown of mTOR abolished the protective effect of UCA1 in response to low dose of rapamycin

To further investigate whether UCA1 enhanced chemoresistance to rapamycin mainly via acti-

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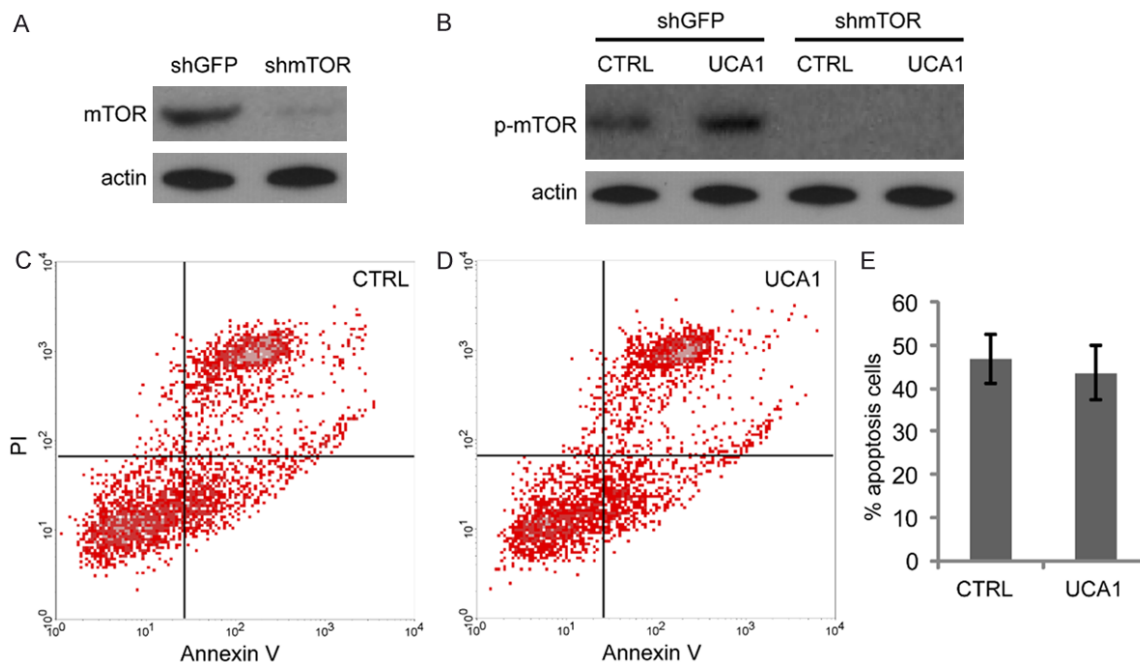


Figure 3. Knockdown of mTOR abolishes the effects of UCA1 overexpression. (A) Western-blot analysis of mTOR abundance in mTOR knockdown cells or GFP knockdown cells. Actin was used as a loading control. (B) Western-blot analysis of mTOR phosphorylation in mTOR knockdown cells or GFP knockdown cells treated with DMSO or 10 μ M rapamycin. (C) FACS analysis of the effect of 10 μ M rapamycin on apoptosis of A549 cells with GFP knockdown or (D) with mTOR knockdown. Apoptotic cells were detected by Annexin V-PI double staining. (E) Percentage of apoptotic cells in (C and D). The values of three replicates are expressed as means \pm SD, * P <0.001.

vating mTOR signaling, we knockdown mTOR using a shRNA-based silencing approach. If mTOR signaling served as a main downstream target for UCA1, knockdown of mTOR should abolish the effects of UCA1 overexpression, leading to enhanced drug resistance to rapamycin in cells transfected with UCA1. This prediction we proved is true. As shown in **Figure 3A**, shRNA-based gene silencing resulted in significantly reduced expression of mTOR. As expected, knockdown of mTOR abolished activation of mTOR signaling induced by UCA1 (**Figure 3B**). Interestingly, mTOR knockdown resulted in significantly enhanced cellular sensitivity to rapamycin treatment (**Figure 3C**), and importantly, abolished the protective effect of UCA1 (**Figure 3D** and **3E**). This result provided independent evidence that UCA1 enhanced rapamycin resistance in A549 cells mainly by activating mTOR signaling.

A549 tumor sphere was characterized by enhanced expression of UCA1 and drug resistance

Cancer stem cells (CSCs) are playing critical roles in regulating tumor progression, metastasis and drug resistance [19]. Recent studies

showed that stem-like cells from tumor spheres derived from A549 expressed various stem cell markers such as CD44, CD133, Sox2 and Oct4 [20, 21]. Moreover, such A549 sphere showed dramatic changes in proliferation, cell-cycle progression as well as drug-resistant properties as compared to A549 adherent cells in monolayer culture [21, 22]. We thus asked whether expression of UCA1 was also altered in A549 sphere cells. To this end, using real-time PCR, we analyzed UCA1 expression in A549 sphere and monolayer cells. Interestingly, our result showed that UCA1 was enhanced by ~5 folds in A549 sphere (**Figure 4A**). In line with this result, we observed enhanced activity of mTOR signaling in A549 sphere, supporting the activity of UCA1 in regulating this crucial pathway (**Figure 4B**). Moreover, as expected, A549 sphere showed higher resistance to rapamycin as compared with monolayer A549 cells (**Figure 4C**). Together, these results support a model that sphere-derived cells enhanced expression of UCA1 to activate mTOR signaling pathway, leading to enhanced cell proliferation and resistance to drugs.

UCA1 activates mTOR signaling

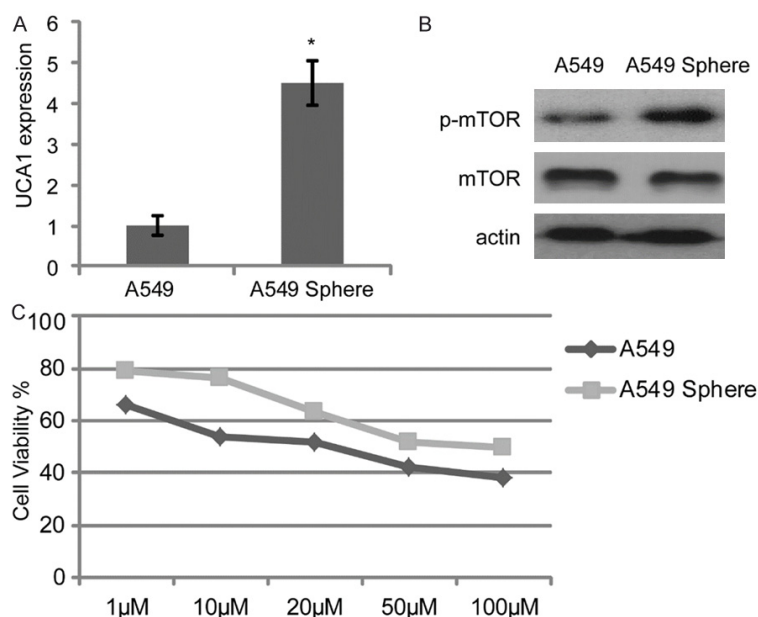


Figure 4. A549 sphere is characterized by high level of UCA1 expression. A. UCA1 expression was examined by quantitative real-time PCR in A549 monolayer cells or that from sphere. The values of three replicates are expressed as means \pm SD, * $P < 0.001$. B. Western-blot analysis of mTOR and phosphorylated mTOR abundance in A549 monolayer cells or that from sphere. C. A549 monolayer cells or that from sphere was treated with 1, 10, 20, 50 or 100 μ M of rapamycin for 24 h. Cell viability was determined by MTT approach.

Discussion

Effective systemic therapies for patients with lung cancer are urgently needed. Because of the heterogeneity and the aggressive nature of the disease, the therapeutic response and clinical outcome of patients with the same cancer type is highly variable [23]. Accordingly, understanding the mechanisms of tumorigenesis is critical to identify novel targets for therapeutic intervention of the disease [24].

Reducing cell proliferation and inducing cell death are the main principles for cancer chemotherapy [23]. Acquired drug resistance is a major problem in cancer treatment, which greatly limits the effectiveness of chemotherapeutic agents [24]. There are numerous factors may contribute to chemoresistance, such as induction of multiple drug resistance genes, somatic genetic or epigenetic alterations in the cancer cells, etc. More recently, deregulation of lncRNAs has been identified as a key player in resistance to multiple antitumor drugs in different tumors. Among them, UCA1 has emerged as an important player with oncogenic function

by inducing cell proliferation and survival [12, 14, 16, 17]. In line with this notion, here we demonstrated that overexpression of UCA1 was significantly correlated with activated mTOR signaling and enhanced drug resistance to rapamycin in lung cancer cells.

mTOR protein is a PI3 kinase (PI3K)-related kinase that plays a critical role in regulating protein synthesis, ribosomal protein translation, and cap-dependent translation [25, 26]. The PI3K-AKT-mTOR signaling pathway fine-tunes cell growth, proliferation, and survival [27]. Deregulations in mTOR signaling are frequently associated with tumorigenesis, angiogenesis, tumor growth and metastasis. It has been shown that activation of PI3 kinase (PI3K)-AKT-mTOR signaling pathway is observed in over 60% of all lung cancers

[28]. Rapamycin is a specific mTOR inhibitor that shows significant antitumor activity against a wide variety of solid tumors. Given its putative synergistic effect with other chemotherapeutic agents, rapamycin therefore has been incorporated in many treatment regimens. However, cancer cells frequently become resistant to rapamycin, how to circumvent this resistance to improve anticancer efficacy remains to be defined [29]. Accordingly, our results that overexpression of UCA1 enhances rapamycin resistance via activating mTOR signaling thus represents a significant advance towards a better understanding of the mechanisms that govern lung cellular growth and drug resistance. Moreover, we found that knockdown of mTOR largely abolished the function of UCA1, providing direct evidence that activation of mTOR signaling was causally linked to UCA1-induced chemoresistance.

From a clinical perspective, our results support the notion that UCA1 is a putative prognostic marker and potential therapeutic target for lung cancer. This is consistent with previous studies that high UCA1 expression was associ-

ated with a significant increase in metastatic recurrence rate and drug resistance in bladder cancer. Notably, using a tumor sphere model, we provided clear evidence that high expression of UCA1 is a robust marker of tumor stem cell and is immediately linked to enhanced drug resistance. Combinatorial targeting of UCA1 and mTOR signaling may thus represent a unique therapeutic regimen targeting tumor stem cell and may provide unique benefit for a subset of patients with elevated expression of UCA1 and advanced lung cancer.

Disclosure of conflict of interest

None.

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