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Curcumin inhibits proliferation and enhances apoptosis in A549 cells by downregulating lncRNA UCA1

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Objective: Curcumin has been reported to possess anti-tumor effects on multiple cancers, including lung cancer. However, the mechanisms of its anti-tumor effect on lung cancer have not been fully elucidated. Our study attempted to identify the effect of curcumin on A549 cells and further explore the potential mechanism. *Methods:* Different concentrations of curcumin were exposed to A549 cells for 24 h and cell viability was measured by CCK-8 assay. The expression of UCA1 was overexpressed in A549 cells by transfection with pEX-UCA1. Cell proliferation was determined by BrdU staining and assessing the expression of CyclinD1 using western blot and RT-PCR assay. Apoptotic cells were measured by flow cytometry assay. Western blot was performed to assess the expression of apoptosis-related, Wnt and mTOR pathways-related factors. *Results:* Curcumin incubation dramatically reduced viability of A549 cells in a dosage-dependent manner. Curcumin (0.6 μM) significantly reduced $BrdU^+$-positive cells, declined the expression of CyclinD1, and enhanced cell apoptosis. Interestingly, we found that curcumin inhibited the expression of UCA1 and UCA1 overexpression abolished the effect of curcumin on cell apoptosis. In addition, we also found that curcumin inhibited Wnt and mTOR pathways through down-regulation of UCA1. *Conclusion:* We demonstrated that curcumin inhibited the growth of A549 cells through downregulation of UCA1, which might provide new insight for the treatment of lung cancer.

1. Introduction

Lung cancer is one of the leading causes of cancer-related death (Wang et al. 2011). It is characterized by the uncontrolled growth of cells in lung tissues, which would spread to other lung tissues or other parts of the body through cell metastasis (Yano et al. 2005). About 75% of lung cancer is non-small cell lung carcinoma (NSCLC) (Yoo et al. 2012). Even though advantages have made in the clinical therapy for NSCLC patients, the efficiency still remains poor due to the severe side effects of some first-line chemotherapeutic agents (Kostova 2006). Thus, it's urgent to find and develop novel and potential drugs with more efficiency and less side effects including natural compounds.

Curcumin (diferuloylmethane) (Fig. 1A) is the active ingredient derived from the rhizome of turmeric (*Curcuma longa*), which is a widely used traditional Chinese herbal medicine (Hatcher et al. 2008; Liao et al. 2011). Curcumin possesses multiple pharmacological properties, including anti-inflammatory, anti-tumor, anti-oxidative stress activities (Arshad et al. 2017; Basnet and Skalko-Basnet 2011; Qian et al. 2015). For example, Subramaniam et al. (2012) revealed that curcumin promoted cell apoptosis and inhibited cell proliferation of esophageal cancer cells through targeting Notch signaling. Chendil et al. (2004) also demonstrated that curcumin enhanced the sensibility of a prostate cancer cell line (PC-3 cells) to radiation induced injury through altering the ratio of Bcl-2/Bax. Regarding lung cancer, previous studies have reported that curcumin could inhibit cell proliferation, migration and invasion of A549 cells (Li et al. 2013; Lin et al. 2009). However, the underlying mechanisms of the anti-tumor effects of curcumin in lung cancer have not been fully explored yet.

Our current study attempted to assess the effect of curcumin on cell growth of A549 cells and further explore the underlying

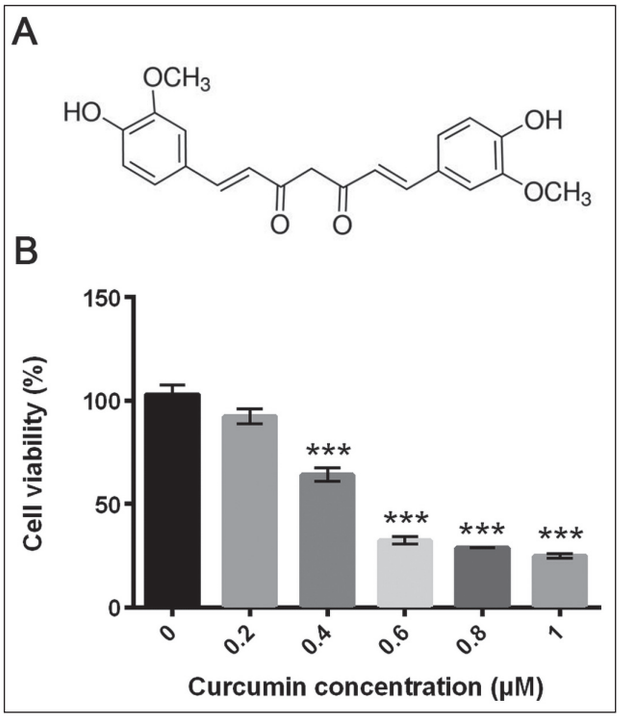

Fig. 1: Curcumin treatment reduced cell viability of A549 cells. (A) The chemical structure of curcumin. (B) Treatment of curcumin significantly reduced cell viability of A549 cells in a concentration-dependent manner. *** $P < 0.001$.

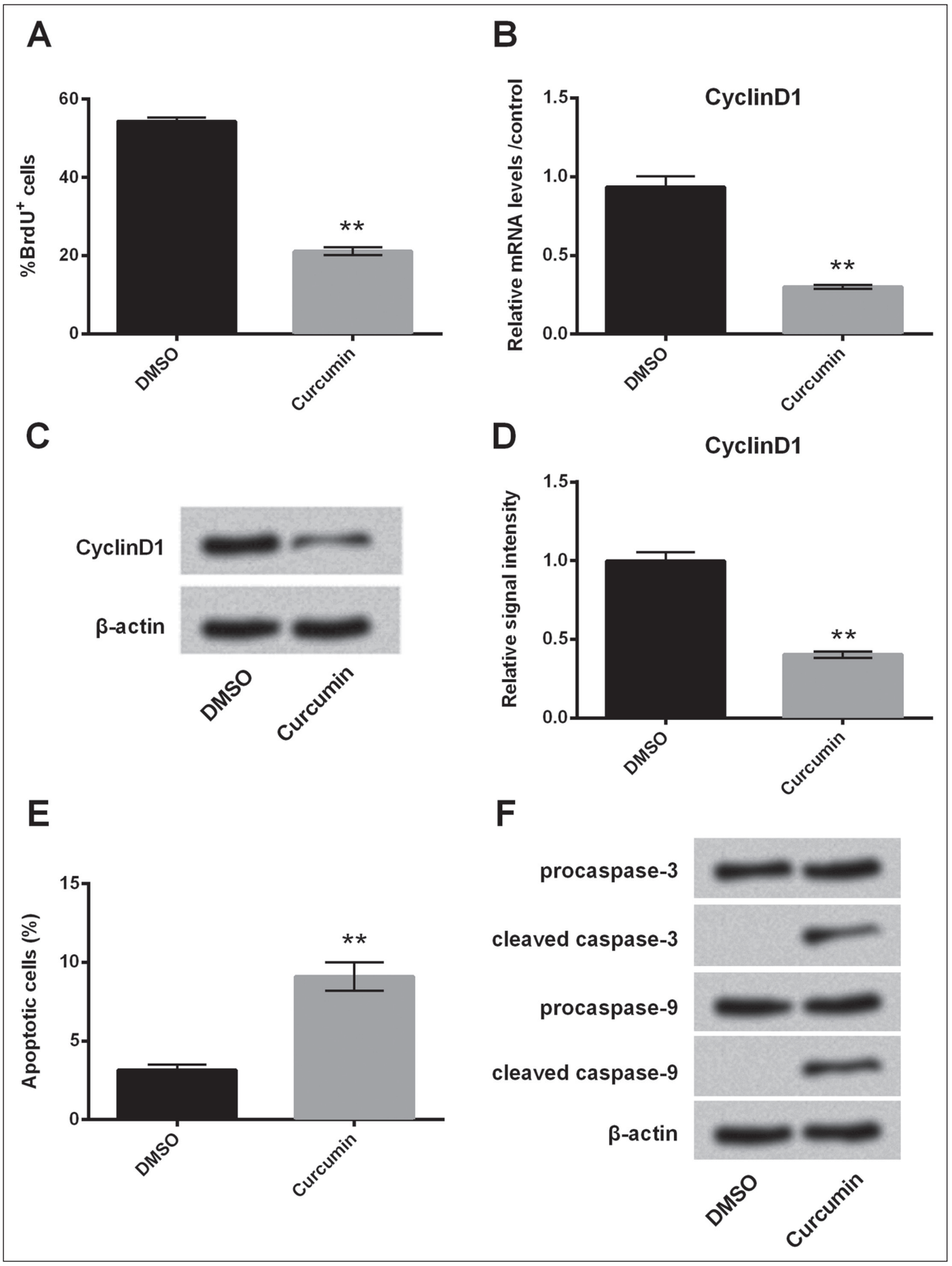

Fig. 2: Curcumin treatment inhibited cell proliferation but promoted cell apoptosis of A549 cells. Curcumin treatment significantly reduced (A) $BrdU^+$-positive cells, declined the (B) mRNA and (C and D) protein expression of cyclinD1, increased (E) apoptotic cell numbers, and (F) promoted the cleavage of caspase-3 and caspase-9. ** $P < 0.01$.

mechanism. We found that curcumin significantly reduced cell viability, inhibited cell proliferation and induced cell apoptosis in A549 cells. Interestingly, we also found that curcumin dramatically inhibited the expression of long non-coding RNA (lncRNA)

human urothelial carcinoma associated 1 (UCA1), which mediated the pro-apoptotic effect of curcumin on A549 cells. Furthermore, we found that curcumin inhibited the activation of Wnt and mTOR signaling pathways through down-regulation of UCA1. Our results further identified the anti-proliferation and pro-apoptotic effect of curcumin on lung cancer, and provided a novel mechanism of its function.

2. Investigations and results

2.1. Curcumin inhibited cell viability of A549 cells in a concentration-dependent manner

To assess the effect of curcumin on the growth of A549 cells, we firstly treated A549 cells with a series concentrations of curcumin (0 - 1 μM) for 24 h and then measured the cell viability of A549 cells. As shown in Fig. 1B, curcumin significantly reduced the viability with an increasing dosage (P <0.001). When the concentration of curcumin reached 0.6 μM, the cell viability was significantly reduced to less than 50%, thus 0.6 μM was selected as the treatment dose in the subsequent experiments.

2.2. Curcumin significantly inhibited cell proliferation but promoted cell apoptosis of A549 cells

We then investigated the effect of curcumin on cell proliferation and apoptosis in A549 cells. As shown in Fig. 2A, cell proliferation was significantly decreased after the addition of curcumin ($P < 0.01$). Consistently, the mRNA and protein expression of cyclin D1, a specific marker of cell proliferation, was dramatically downregulated in Curcumintreated cells (Fig. 2B-2D, $P < 0.01$). Similarly, curcumin treatment enhanced cell apoptosis of A549 cells, as significantly increased the number of apoptotic cells (Fig. 2E, $P < 0.01$), and upregulated the protein expression of cleaved caspase-3 and cleaved caspase-9 (Fig. 2F). Thus, curcumin could inhibit cell proliferation but promote cell apoptosis of A549 cells.

2.3. Curcumin enhanced cell apoptosis of A549 cells through downregulation of UCA1

Interestingly, we found that UCA1 expression was notably reduced by curcumin treatment in A549 cells (Fig. 3A, $P < 0.05$). We then investigated whether UCA1 was involved with the effect

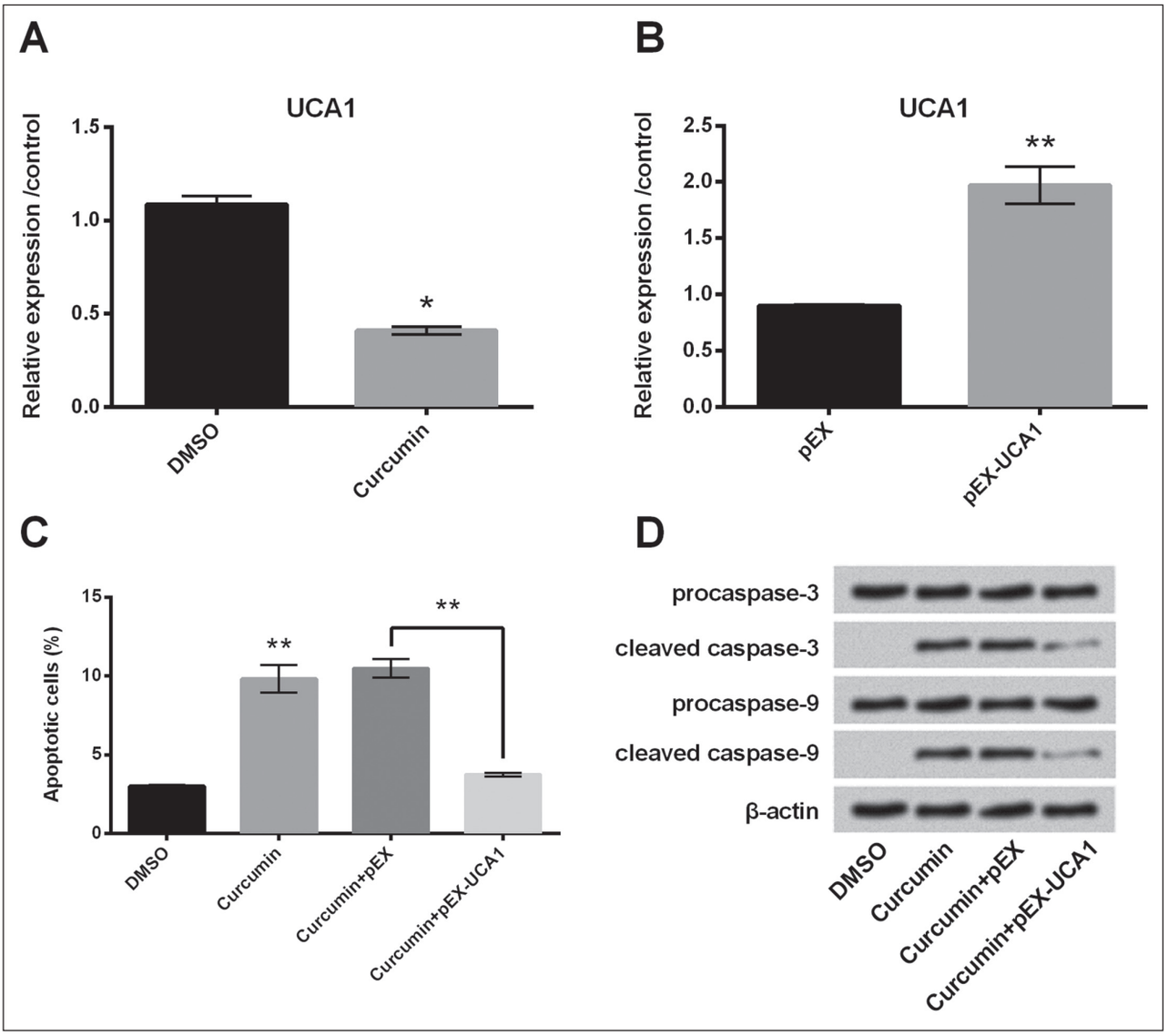

Fig. 3: Curcumin treatment promoted cell apoptosis through down-regulating UCA1. (A) Curcumin treatment significantly down-regulated the expression of UCA1. (B) UCA1 expression was enforced expressed by transfection with pEX-UCA1. pEX-UCA1 transfection reversed the promotive effect of curcumin on (C) apoptotic cell numbers and (D) the activation of caspase-3 and caspase-9. * $P < 0.05$, ** $P < 0.01$.

of curcumin on A549 cells. A549 cells were transfected with pEX-UCA1 vector to overexpress UCA1, and the transfected efficiency was identified by qRT-PCR. As expected, the expression of UCA1 was efficiently upregulated by pEX-UCA1 transfection in A549 cells (Fig. 3B, P <0.01). Overexpression of UCA1 significantly reduced apoptotic cells of A549 cells, even though with the addition of curcumin (P < 0.01, Fig. 3C). Consistently, the expression of cleaved caspase-3 and cleaved caspase-9 was downregulated by UCA1 overexpression in curcumin-treated cells (Fig. 3D). Thus, curcumin enhanced cell apoptosis of A549 cells through down-regulating UCA1.

2.4. Curcumin inhibited the activation of Wnt and mTOR pathways through downregulation of UCA1

For the further mechanism exploration, the Wnt and mTOR pathways were investigated. As shown in Fig. 4A, curcumin treatment significantly declined the protein expression of Wnt 3a, Wnt 5a and β-catenin (P < 0.001). However, the effect of curcumin on activity of the Wnt pathway was reversed by UCA1 overexpression. The expression of Wnt 3a, Wnt 5a and β-catenin was dramatically upregulated in the curcumin-treated cells that were transfected with pEX-UCA1 (P < 0.001). In addition, similar results were observed in the mTOR pathway. Overexpression of UCA1 significantly reversed the inhibitory effect of curcumin on the activation of mTOR pathway, as enhanced the phosphorylation of mTOR and p70S6K (Fig. 4B, P < 0.001). Overall, curcumin inhibited the Wnt and mTOR signaling cascades through down-regulation of UCA1.

3. Discussion

Study results have indicated that curcumin exerts anti-tumor effects on multiple human cancers, such as melanoma (Odot et al. 2004), ovarian cancer (Weir et al. 2007), breast cancer and so on (Choud-

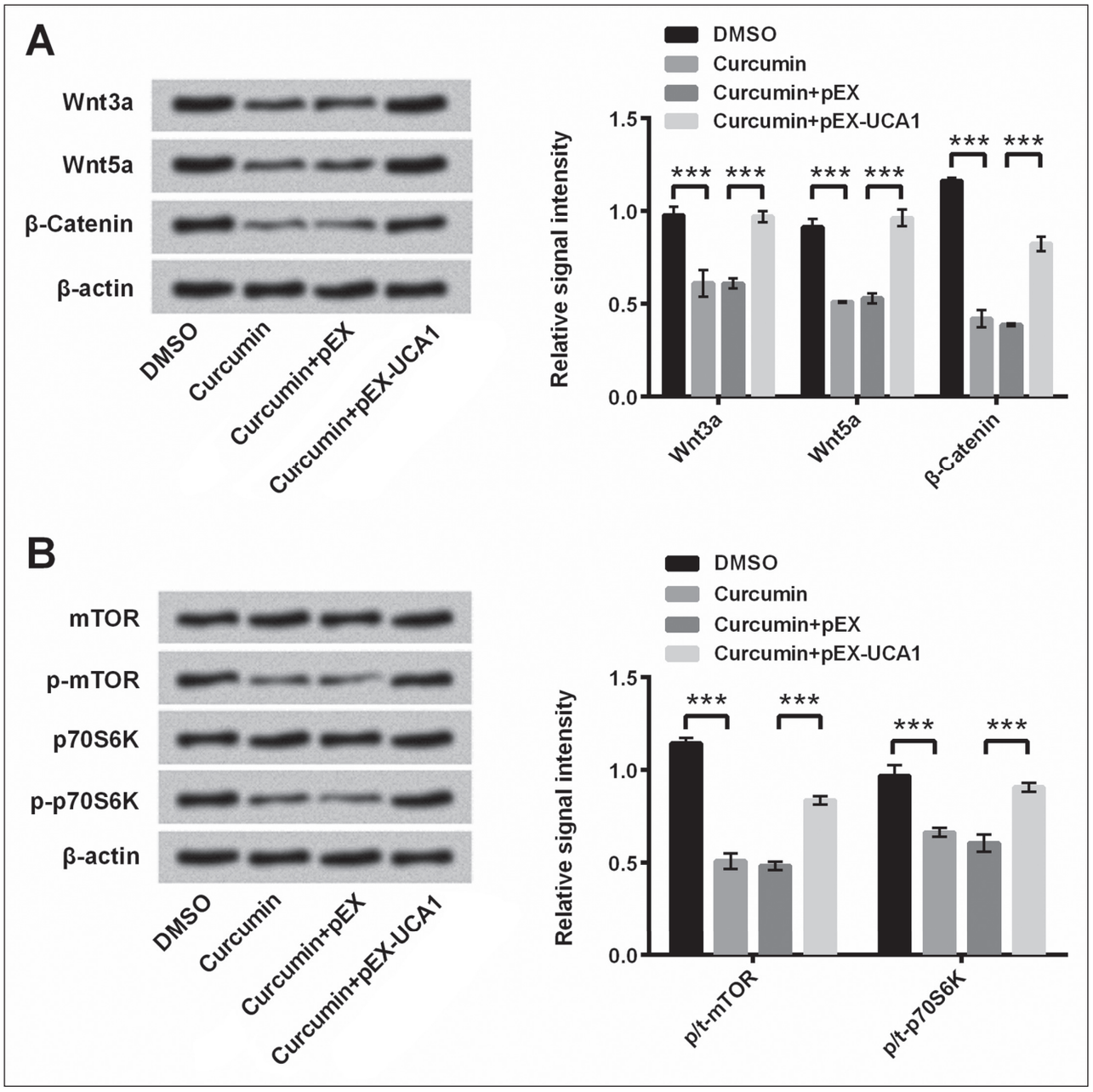

Fig. 4: Curcumin inhibited the activation of Wnt and mTOR pathways through down-regulating UCA1. (A) The expression of Wnt3a, Wnt5a and β-catenin was significantly inhibited by curcumin treatment and pEX-UCA1 transfection reversed the effect of curcumin. (B) The phosphorylation of mTOR and p70S6K was significantly reduced by curcumin treatment and pEX-UCA1 transfection reversed the effect of curcumin. *** P < 0.001.

huri et al. 2002; Dorai et al. 2001; Wilken et al. 2011). Previous reports have demonstrated that curcumin could significantly reduce cell viability, suppress the metastasis and proliferation, and induce cell apoptosis in lung cancer cells (Chen et al. 2008; Lin et al. 2009; Radhakrishna Pillai et al. 2004; Wu et al. 2010). Consistent with previous studies, our present study found that curcumin at a concentration from 0.2 μM to 1.0 μM decreased the viability of A549 cells with an increasing concentration. We also found that curcumin at a concentration of 0.6 μM significantly inhibited cell proliferation but enhanced cell apoptosis of A549 cells. Our results further confirmed the anti-proliferation and pro-apoptotic effects of curcumin on NSCLC.

LncRNAs possess multiple functions including modification of chromatin, determination of cell fate, transcriptional and post-transcriptional modulation (Gong and Maquat 2011; Kanduri 2011). LncRNAs have been indicated to play critical biological roles in various kinds of tumors through modulating cell proliferation, migration and apoptosis (Khaitan et al. 2011; Wang et al. 2015). UCA1 was initially found in human bladder cancer, with a role of promoting cell growth and metastasis in carcinoma (Fu et al. 2015; Wang et al. 2006; Xue et al. 2014). Our data showed that the expression of UCA1 was dramatically inhibited by curcumin treatment in A549 cells. In addition, overexpression of UCA1 by transfecting with pEX-UCA1 significantly reversed the pro-apoptotic effect of curcumin in A549 cells, as reduced the number of apoptotic cells, and suppressed the cleavage of caspase-3 and caspase-9, indicating that UCA1 mediated the regulatory effect of curcumin in lung cancer. The oncogenic role of UCA1 in lung cancer has been reported by previous studies. For instance, Nie et al. (2016) revealed that UCA1 was aberrant overexpressed in 112 pairs of NSCLC tissues and human NSCLC cell lines (A549, H1299, H446, H460, and NCI-H1650), and overexpression of UCA1 elevated cell viability and induced colony formation of A549 cells and H1299 cells. Wang et al. (2015) demonstrated that UCA1 expression was closely related to the clinical diagnosis of lung cancer, and upregulated expression of UCA1 expedited the development and progression of lung carcinoma. Our results firstly revealed that curcumin could function as an anti-tumor compound through modulation of UCA1 in A549 cells, which might enrich the potential mechanism of curcumin that it functions through.

The Wnt pathway has been reported to be crucial in the progression of lung cancer through regulating cell proliferation and differentiation (Huang et al. 2015; Xia et al. 2015). Wnt3a, Wnt5a and β-catenin are key regulators of this pathway. One previous report has suggested that curcumin could suppress the growth of human breast cancer cells through regulating the activity of the Wnt signaling pathway (Prasad et al. 2009). Lu et al. (2014) demonstrated that the suppressive effect of curcumin on the proliferation and invasion in NSCLC cell lines was mediated by inhibition of the Wnt pathway. In addition, UCA1 has been reported to reduce sensibility of bladder cells to cisplatin and enhance epithelial-mesenchymal transition (EMT) process of breast cancer cells through regulation of the Wnt signaling cascade (Fan et al. 2014; Xiao 2016). Thus, we hypothesized that curcumin might be involved in the activation of Wnt signaling pathway through UCA1. We observed that curcumin inhibited the expression of Wnt3a, Wnt5a and β-catenin, while was reversed by overexpression of UCA1. These results suggested that curcumin inhibited the activation of Wnt pathway *via* suppression of UCA1, and which might be involved in the pro-apoptotic role of curcumin in A549 cells. Meanwhile, similar observations were found in the mTOR pathway. We found that curcumin inhibited the phosphorylation of mTOR and p70S6K, and UCA1 overexpression abolished the effect of curcumin. These results were consistent with previous study showing that curcumin could suppress the process of EMT in lung cancer cells through mTOR pathway (Jiao et al. 2016). Moreover, Cheng et al. (2105) showed that the mTOR pathway was activated by UCA1 in NSCLC cells, which was consistent with our results.

Taken together, our current study demonstrated that curcumin significantly reduced cell viability, inhibited cell proliferation but enhanced cell apoptosis in A549 cells. Moreover, curcumin dramatically suppressed the expression of UCA1, and UCA1 mediated the pro-apoptotic effects of curcumin in A549 cells and the inhibitory regulation on mTOR signaling pathways. Our study confirmed the anti-tumor properties of curcumin in lung cancer and provided novel regulatory mechanism, which might provide new clues for the treatment of lung cancer.

4. Experimental

4.1. Cell culture and treatments

A549 cells (provided by American Type Culture Collection, Rockville, MD, USA), the human lung cancer cell line, were cultured at 37 °C in a humidified condition of 5% CO2 and 95% air with the complete culture medium. The complete culture medium consisted of minimal Roswell Parker Memorial Institute (RPMI)-1640 medium (Cellgro, Herndon, VA, USA), 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), and 1% antibiotic-antimycotic mixture (Gibco).

A549 cells were treated with different concentrations of curcumin (Sigma-Aldrich, St Louis, MO, USA) for 24 h, which was dissolved in dimethyl sulfoxide (DMSO) and were diluted to the working concentration using RPMI-1640 culture medium. The cells in the control group were treated with corresponding concentration of DMSO.

4.2. Cell transfection

For the enforced expression of UCA1, full sequence of UCA1 was constructed in pEX plasmid (GenePharma, Shanghai, China) to form the UCA1-overexpressing vector, which was referred as pEX-UCA1, and the corresponding negative control was referred as pEX. A549 cells were transfected with pEX or pEX-UCA1 by using lipofectamine 3000 reagent (Life Technologies Corporation, Gaithersburg, MD, USA).

4.3. CCK-8 assay

CCK-8 assay was performed to measure cell viability of A549 cells. In brief, A549 cells were seeded in 96-well plate with a density of 1×10^5 cells/well. After the curcumin treatment, the culture medium containing curcumin was removed, and cells were incubated with 10 μl CCK-8 solution (Beyotime Biotechnology, Shanghai, China) containing in 90 μl RPMI-1640 for another 1 h at 37 °C. The absorbance was read at 450 nm using a Vmax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

4.4. Proliferation assay

Cell proliferation was measured by bromodeoxyuridine (BrdU) cell proliferation kit (Sigma-Aldrich). Briefly, after the treatment of curcumin, cells were incubated with BrdU labeling solution for 1 h. Then, cells were fixed with 4% formaldehyde, permeabilized with 0.2 % Triton X100, and the DNA denatured in formamide. Thereafter, cells were incubated with anti-BrdU antibody, followed by the addition of horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich). Absorbance was quantified by a spectrophotometer (FLUOstar Optima, BMG Labtech, Offenburg, Germany) at 450 nm and 595 nm.

4.5. Apoptosis assay

The fluorescein isothiocynate (FITC)-conjugated Annexin V and propidium iodide (PI) kit provided by Beyotime Biotechnology was employed to assess apoptotic cells. Briefly, after the treatment of curcumin, cells were collected and washed twice with phosphate buffered saline (PBS), and then resuspended in binding buffer. Subsequently, cells were incubated with the mixture of 5 μl Annexin V-FITC and 5 μl PI for 1 h in the dark at room temperature. Flow cytometry analysis was immediately performed by using a FACS can (Beckman Coulter, Fullerton, CA, USA).

4.6. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from A549 cells with the use of Trizol reagent (Invitrogen, Carlsbad, CA, USA). To measure the mRNA expression of CyclinD1, RNA was synthesized into cDNA by employing RNA PCR Kit (AMV) Ver.3.0 (TaKaRa, Dalian, China) and RT-PCR was performed using SYBR® Premix Ex TaqTM II (TaKaRa). To detect the expression of UCA1, the One Step SYBR®PrimeScript®PLUS RT-RNA PCR Kit provided by TaKaRa was used. The relative expression of cyclinD1 was normalized to β-actin and the expression of UCA1 was normalized to U6, and the fold changes were calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

4.7. Western blot

Briefly, cells were harvested and lysed in RIPA lysis buffer (Beyotime Biotechnology) which containing protease inhibitors (Vazyme Biotech, Nanjing, China) to extract protein samples. Then, the concentration of protein samples were quantified by a BCA Protein Assay Kit (Beyotime Biotechnology). Protein samples (40 μg) were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto the polyvinylidene difluoride (PVDF) membranes at 0.45 μM pore size, which was provided by Millipore Corp. (Bedford, MA, USA). Subsequently, the membranes were blocked in 5% bovine serum albumin (BSA; Roche, Indianapolis, IN, USA) for 2 h at room temperature. Primary antibodies against cyclinD1 (#2978), caspase-3 (#9665), caspase-9 (#9502), Wnt3a

(#2721), Wnt5a (#2530), β-Catenin (#8480), mTOR (#2983), p-mTOR (#5536), p70S6K (#2708), p-p70S6K (#9204), β-actin (#4970) obtained from Cell Signaling Technology (Beverly, MA, USA) were respectively used to incubated membranes overnight at 4 °C. All antibodies were diluted in 5% BSA at a dilution of 1:1000. Then, the blots were washed with Tris-buffered saline plus Tween-20 (TBST) and incubated with secondary antibody marked by horseradish peroxidase for 2 h at room temperature. Signals were captured by enhanced chemiluminescence (ECL) method using Image Lab™ software (Bio-Rad, Hercules, CA, USA) and the relative intensity of bands was quantified.

4.8. Statistical analysis

Data were presented as mean±standard deviation (SD) and Graphpad Prism 6 software (GraphPad, San Diego, CA, USA) was used to analyze the statistical significance. The one-way analysis of variance (ANOVA) was used to calculate the *P* values and *P*-value of <0.05 was considered statistically significant.

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Conflicts of interest: None declared.

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