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Knockdown of long noncoding RNA urothelial cancer-associated 1 enhances cisplatin chemosensitivity in tongue squamous cell carcinoma cells

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Cisplatin-based chemotherapy has been found to improve the prognosis of patients with tongue squamous cell carcinoma (TSCC), the most common oral cancer with a poor prognosis. Chemoresistance to cisplatin appears to be an important clinical problem for cisplatin-based TSCC chemotherapy. Long noncoding RNAs (lncRNAs) play important roles in regulating tumor cells' sensitivity to chemotherapeutic agents. A recent study has shown that the expression of lncRNA UCA1 is significantly enhanced in TSCCs, suggesting that UCA1 may play a role in TSCC progression. In the present study, we explored the effects and the underlying mechanisms of UCA1 on cisplatin chemosensitivity/chemoresistance and apoptosis in TSCC cells. Transient transfection of siRNA was used to knock down UCA1 in human TSCC cell lines CAL 27 and SCC-9, where UCA1 was highly overexpressed compared to normal human tongue tissues. Knockdown of UCA1 markedly increased cisplatin-induced caspase 3 activity and apoptosis in CAL 27 and SCC-9 cells. On the other hand, it decreased cisplatin-induced phosphatidylinositol 3-kinase (PI3K) activity and activation phosphorylation of Akt. UCA1 knockdown resulted in one magnitude decrease in the half maximal inhibitory concentration (IC_{50}) of cisplatin in CAL 27 and SCC-9 cells, from 8.9 mM and 20.7 mM down to 0.6 mM and 1.7 mM, respectively. In conclusion, this study has shown that UCA1 knockdown markedly increased cisplatin-induced apoptosis and chemosensitivity in TSCC cells, likely through inhibiting cisplatin-activated PI3K/Akt signaling. It provides new insights into the functional role of UCA1 in cancer cells and suggests that UCA1 knockdown could be a new strategy to increase cisplatin chemosensitivity and thereby improve the therapeutic outcomes of cisplatin-based chemotherapy for TSCC.

1. Introduction

Oral squamous cell carcinoma is the most common head and neck cancer, estimated to have a 275,000 incidence per year (Xing et al. 2013; Peng et al. 2015). As the most common oral squamous cell carcinoma with a five-year survival rate of 50%, tongue squamous cell carcinoma (TSCC) is associated with poorer local tumor control than other head and neck cancers (Xing et al. 2013; Peng et al. 2015). Chemotherapy is a major therapy for TSCC (Yu et al. 2010), reportedly leads to improved local disease control and organ preservation (Department of Veterans Affairs Laryngeal Cancer Study Group 1991). Cisplatin, one of the most potent platinum-based chemotherapeutic agents currently in use, has proven effective for the treatment of TSCC (Price KA and Cohen EE 2012). However, chemoresistance to cisplatin appears to be an important clinical problem for cisplatin-based TSCC chemotherapy (Gu et al. 2011). Identifying new targets to overcome cisplatin chemoresistance or to increase cisplatin chemosensitivity may improve TSCC therapeutic outcomes.

Studies on human genomes have revealed that transcription from <2% of the human genome yields many short or long noncoding RNAs (lncRNAs) with limited or no protein-coding capacity (Gibb et al. 2011; Kapranov et al. 2007; Mattick and Makunin 2006; Brosnan and Voinnet 2009). lncRNAs, defined as noncoding RNAs more than 200 nucleotides in length, is emerging as regulatory RNAs that play key roles in various critical cellular processes such as gene regulation and cell differentiation (Mysiore et al. 2009; Wang et al. 2014). Accumulating evidence has indicated that lncRNAs have a role in the initiation and progression of cancer (Fang and Fullwood 2016). Recent studies have shown that lncRNAs are closely involved in regulating tumor cells' sensitivity to chemotherapeutic agents, including cisplatin (Liu et al. 2015; Wu et al. 2016; Li et al. 2015). It has been reported that the expression level of urothelial cancer-associated 1 (UCA1), an oncogenic lncRNA believed to function in regulation of embryonic development and in bladder cancer invasion and progression (Wang et al. 2008; Yang et al. 2012), is significantly elevated in TSCC tissues and statistically correlated with TSCC lymph node metastasis (Fang et al. 2014), suggesting that UCA1 is associated with TSCC progression.

In the present study, we explored the effects and the underlying mechanisms of UCA1 on cisplatin chemosensitivity/chemoresistance and apoptosis in TSCC cells.

2. Investigations and results

2.1. UCA1 is highly overexpressed in human TSCC cells

As shown in Fig. 1, UCA1 was highly overexpressed in human TSCC cell lines compared with that in normal human tongue tissues. In CAL 27 and SCC-9 cells, the expression level of UCA1 was 18 fold and 25 fold of that in normal human tongue tissues, respectively (Fig. 1). Transfection of human UCA1 siRNA significantly decreased the expression of UCA1 by 75% in CAL 27 cells and by 68% in SCC-9 cells, while the negative control siRNA showed no significant effect (Fig. 1).

2.2. UCA1 knockdown increases cisplatin chemosensitivity in human TSCC cells

We next examined cisplatin IC_{50} in CAL 27 and SCC-9 cells with or without UCA1 knockdown. A lower IC_{50} value was considered to correspond to higher chemosensitivity or lower chemoresistance to cisplatin. As shown in Fig. 2, knockdown of UCA1 markedly decreased TSCC cell viability at nearly all time points

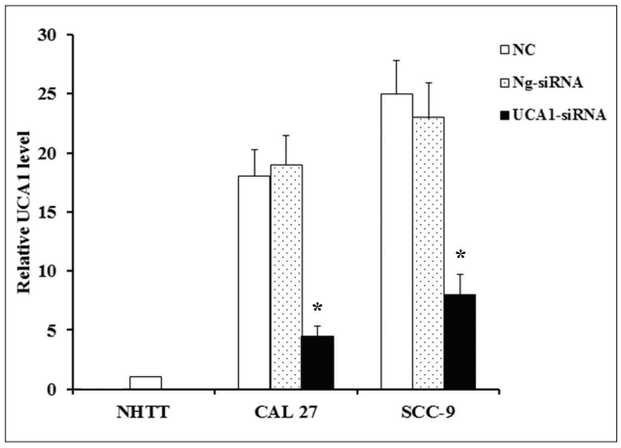

Fig. 1: Knockdown of UCA1 in tongue squamous cell carcinoma (TSCC) cells
Human CAL27 and SCC-9 TSCC cells were transfected with human UCA1 siRNA to knock down UCA1. Nontransfected cells (NC) and cells transfected with negative control (Ng) siRNA were used as controls. Twenty-four hours after transfection, the expression level of UCA1 was determined with real-time quantitative RT-PCR and expressed as fold changes to that of normal human tongue tissue (NHTT), which was used as a reference (designated as 1). $^{*}p<0.05$ vs. NC.

compared with the controls. According to the IC_{50} dose-response curves plotted with GraphPad Prism 5.0 (GraphPad Software), the cisplatin IC_{50} values of CAL 27 and SCC-9 cells were 8.9 mM and 20.7 mM, respectively; knockdown of UCA1 respectively decreased the values to 0.6 mM and 1.7 mM, resulting in an over one-magnitude decrease in cisplatin IC_{50}. The findings indicated that UCA1 knockdown effectively increased cisplatin chemosensitivity in human TSCC cells.

2.3. UCA1 knockdown increases cisplatin-induced apoptosis and caspase 3 activity in human TSCC cells

As shown in Fig. 3, cisplatin treatment significantly induced apoptosis without significantly altering the expression level of UCA1 in CAL 27 and SCC-9 cells. While UCA1 knockdown showed no significant effect on nontreated control cells, it markedly enhanced cisplatin-induced apoptosis in CAL 27 and SCC-9 cells by approximately 3.7 fold and 3.4 fold, respectively (Fig. 3*B*). As shown in Fig. 4, cisplatin treatment significantly induced in CAL 27 and SCC-9 cells the activity of caspase 3, a major activated caspase present in apoptotic cells (Woo et al. 1998). While UCA1 knockdown showed no significant effect on the caspase 3 activity in nontreated control cells, it markedly enhanced cisplatin-induced caspase 3 activity in CAL 27 and SCC-9 cells by approximately 3 folds (Fig. 4).

2.4. UCA1 knockdown inhibits cisplatin-induced PI3K activity and activation phosphorylation of Akt in human TSCC cells

Cisplatin treatment reportedly activates the PI3K/Akt pathway that protects cancer cells from apoptosis (Belyanskaya et al. 2005). To explore the potential effect of UCA1 on the PI3K activity in cisplatin-treated TSCC cells, we next examined the PI3K activity in cisplatin-treated CAL 27 and SCC-9 cells with or without UCA1 knockdown. As shown in Fig. 5, cisplatin significantly induced the PI3K activity in CAL 27 and SCC-9 cells by 2.7 fold and 2.9 fold, respectively, which was abolished by knocking down UCA1. PI3K activation reportedly leads to activation phosphorylation of Akt at serine 473 (ser473) (Liao and Hung 2010; Wu et al. 2013). As shown in Fig. 6, cisplatin significantly induced the ser473 activation phosphorylation of Akt in CAL 27 and SCC-9 cells by approximately 2.4 fold and 2.8 fold, respectively, which was abolished by knocking down UCA1.

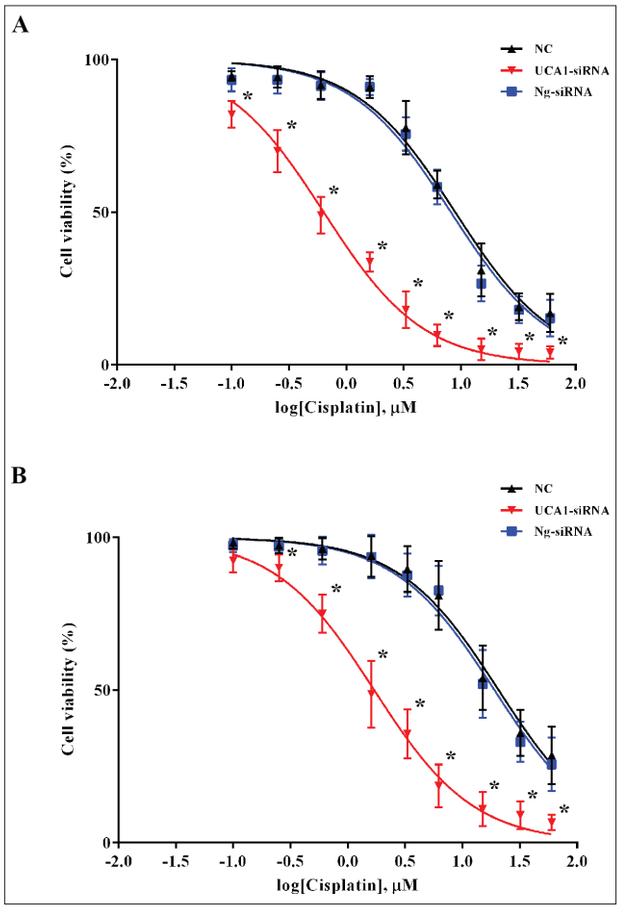

Fig. 2: Knockdown of UCA1 in tongue squamous cell carcinoma (TSCC) cells
(*A*) CAL 27 and (*B*) SCC-9 cells were transfected with human UCA1 siRNA to knock down UCA1. Nontransfected cells (NC) and cells transfected with negative control (Ng) siRNA were used as controls. Twenty-four hours after transfection, the cells were treated with or without various concentrations of cisplatin for 24 h. The half maximal inhibitory concentration (IC_{50}) dose-response curves were plotted with GraphPad Prism 5.0 (GraphPad Software). $^{*}p<0.05$ vs. NC.

3. Discussion

Cisplatin-based chemotherapy has been found to improve the prognosis of patients with TSCC, the most common oral cancer with a poor prognosis (Xing et al. 2013; Peng et al. 2015). Chemoresistance to cisplatin appears to an important clinical problem for cisplatin-based TSCC chemotherapy (Gu et al. 2011). Accumulating evidence has indicated that lncRNAs play important roles in regulating tumor cells' sensitivity to chemotherapeutic agents (Liu et al. 2015; Wu et al. 2016; Li et al. 2015). A recent study has shown that the expression of lncRNA UCA1 is significantly enhanced in TSCCs, suggesting that UCA1 may play a role in TSCC progression (Fang et al. 2014). In the present study, we have found that UCA1 knockdown markedly increases cisplatin chemosensitivity in TSCC cells.

In agreement with a previous study showing that the expression levels of lncRNA UCA1 were markedly elevated in TSCC tissues compared to adjacent normal tissues (Fang et al. 2014), we found that UCA1 was highly overexpressed in human TSCC cell lines, approximately 18-25 fold of that in normal human tongue tissues. Therefore, we only employed knockdown experiments in this study, for overexpression of UCA1 in TSCC cells that already have a very high basal expression level of UCA1 could not exert significant effects on cell viability, as revealed in a previous study (Fang et al. 2014). Transient transfection of commercially available Silencer Select UCA1 siRNA (Thermo Fisher Scientific) with high-efficiency transfection regent (Lipofectamine 3000; Thermo Fisher Scientific) effectively knocked down 68%-75% of the

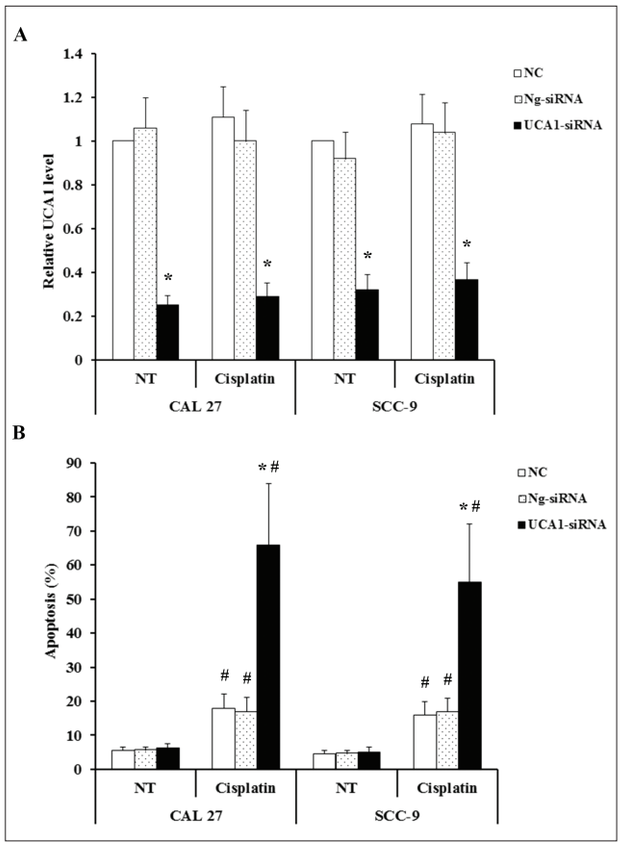

Fig. 3: Effect of UCA1 knockdown on apoptosis in tongue squamous cell carcinoma (TSCC) cells in the presence and absence of cisplatin.
CAL27 and SCC-9 cells were transfected with human UCA1 siRNA to knock down UCA1. Nontransfected cells (NC) and cells transfected with negative control (Ng) siRNA were used as controls. Twenty-four hours after transfection, the cells were treated at the half maximal inhibitory concentration (IC_{50}) of cisplatin (8.9 mM for CAL cells and 20.7 mM for SCC-9) for 24 h. (*A*) The expression level of UCA1 was determined with real-time quantitative RT-PCR in nontreated (NT) cells and cisplatin-treated cells and expressed as fold changes to that of NC in NT (designated as 1). $^{*}p<0.05$ vs. NC. (*B*) Cell apoptosis was determined with a microplate reader-based apoptosis detection kit (R&D Systems) in nontreated (NT) cells and cisplatin-treated cells and shown as percentages of total cells. $^{\#}p<0.05$ vs. NT; $^{*}p<0.05$ vs. NC.

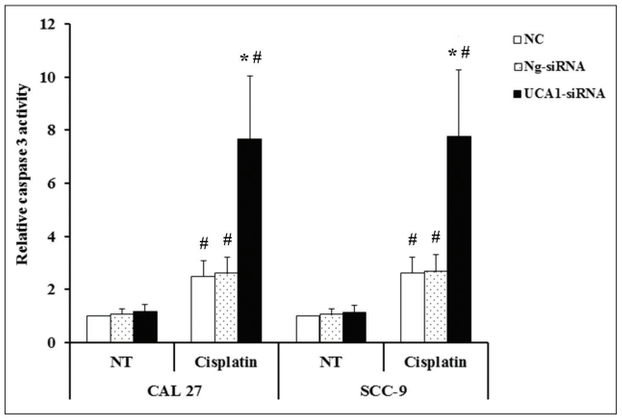

Fig. 4: Effect of UCA1 knockdown on caspase 3 activity in tongue squamous cell carcinoma (TSCC) cells in the presence and absence of cisplatin.
CAL27 and SCC-9 cells were transfected with human UCA1 siRNA to knock down UCA1. Nontransfected cells (NC) and cells transfected with negative control (Ng) siRNA were used as controls. Twenty-four hours after transfection, the cells were treated at the half maximal inhibitory concentration (IC_{50}) of cisplatin (8.9 mM for CAL cells and 20.7 mM for SCC-9) for 24 h. The activities of caspase 3 were determined with a colorimetric caspase 3 assay kit (Abcam) in nontreated (NT) cells and cisplatin-treated cells and expressed as fold changes to that of NC in NT (designated as 1). $^{\#}p<0.05$ vs. NT; $^{*}p<0.05$ vs. NC.

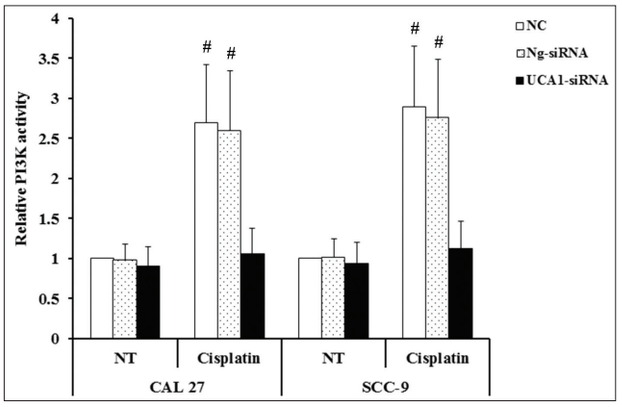

Fig. 5: Effect of UCA1 knockdown on phosphatidylinositol 3-kinase (PI3K) activity in tongue squamous cell carcinoma (TSCC) cells in the presence and absence of cisplatin.
CAL27 and SCC-9 cells were transfected with human UCA1 siRNA to knock down UCA1. Nontransfected cells (NC) and cells transfected with negative control (Ng) siRNA were used as controls. Twenty-four hours after transfection, the cells were treated at the half maximal inhibitory concentration (IC_{50}) of cisplatin (8.9 mM for CAL cells and 20.7 mM for SCC-9) for 24 h. PI3K activities were determined with a PI3K activity ELISA kit (Echelon Biosciences) in nontreated (NT) cells and cisplatin-treated cells and expressed as fold changes to that of NC in NT (designated as 1). $^{\#}p<0.05$ vs. NT; $^{*}p<0.05$ vs. NC.

UCA1 expression level in TSCC cells, proving to be a simple and effective method to knock down UCA1 .

We used IC_{50} as an indicator of cisplatin chemosensitivity/chemoresistance. A lower IC_{50} value was considered to correspond to higher chemosensitivity or lower chemoresistance to cisplatin. Notably, knockdown of UCA1 led to an over one-magnitude decrease in cisplatin IC_{50}. This, together with the technical efficiency of knocking down UCA1 in TSCC cells by transient transfection of siRNA, suggests that UCA1 knockdown could be a promising new strategy to increase cisplatin chemosensitivity and thereby improve the therapeutic outcomes of cisplatin-based chemotherapy for TSCC. Future in vivo studies are needed to investigate its clinical application potential.

UCA1 knockdown markedly enhanced cisplatin-induced caspase 3 activity and apoptosis in TSCC cells, providing a mechanistic explanation how UCA1 knockdown led to increased cisplatin chemosensitivity in TSCC cells. Cisplatin treatment reportedly activates the PI3K/Akt survival pathway in cancer cells to suppress apoptosis (Belyanskaya et al. 2005). In this study, we found that UCA1 knockdown significantly inhibited cisplatin-activated PI3K/Akt signaling in TSCC cells. Thus, cisplatin-activated PI3K/Akt signaling could be a survival response of TSCC cells to apoptotic stress, with UCA1 being a major mediator required in the process. As UCA1 knockdown largely abolished this cell survival response to cisplatin-induced apoptotic stress, it resulted in enhanced cell apoptosis, which was manifested as increased cisplatin chemosensitivity. In addition, as UCA1 showed no significant effect on nontreated control cells, certain cisplatin-induced/activated factors should be needed for UCA1 to take effect in cisplatin-treated TSCC cells. How UCA1 mediates cisplatin-induced activation of PI3K activity in TSCC cells will be explored in our future studies.

Cell survival against apoptotic stress is critical for cancer progression and metastasis (Wu et al. 2013). The proposed notion that UCA1 is a major mediator for tumor cells' survival response to apoptotic stress is in line with previous reports suggesting an important role of UCA1 in the invasion and progression of a variety of cancers, including TSCC (Wang et al. 2008; Yang et al. 2012; Fang et al. 2014; Li et al. 2014). It will be intriguing to explore in future studies whether manipulation of UCA1 may affect tumor cell survival under apoptotic stress induced by radiotherapy, hypoxia, and chemotherapeutic agents besides cisplatin in TSCC and other cancers.

In conclusion, we have shown in this study that UCA1 knockdown markedly increased cisplatin-induced apoptosis and chemosensi-

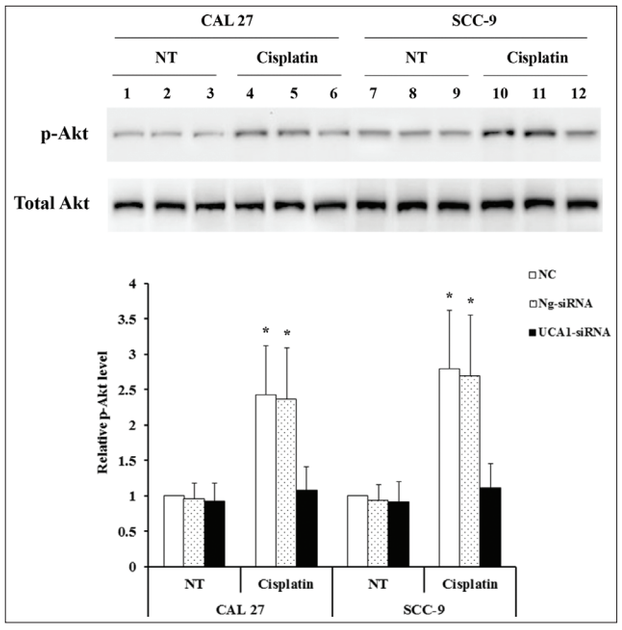

Fig. 6: Effect of UCA1 knockdown on phosphorylated Akt (p-Akt) levels in tongue squamous cell carcinoma (TSCC) cells in the presence and absence of cisplatin. CAL27 and SCC-9 cells were transfected with human UCA1 siRNA to knock down UCA1. Nontransfected cells (NC) and cells transfected with negative control (Ng) siRNA were used as controls. Twenty-four hours after transfection, the cells were treated at the half maximal inhibitory concentration (IC_{50}) of cisplatin (8.9 mM for CAL cells and 20.7 mM for SCC-9) for 24 h. Levels of total Akt and p-Akt at serine 473 (ser473) were determined with Western blot analyses in nontreated (NT) cells and cisplatin-treated cells. The arrangement of test samples were as follows: *lanes 1-6*, CAL 27 cells; *lanes 7-12*, SCC-9 cells; *lanes 1-3 and 7-9, NT; lanes 4-6 and 10-12, cisplatin-treated; lanes 1, 4, 7* and 10, NC; *lanes 2, 5, 8* and 11, Ng-siRNA*; lanes 3, 6, 9* and 12, UCA1-siRNA. The total Akt level was not significantly changed in all experimental groups in CAL 27 and SCC-9 cells, respectively. Density of the p-Akt (ser473) blot was normalized against that of total Akt to obtain a relative p-Akt level, which was expressed as fold changes to that of NC in NT (designated as 1). $^{\#}p<0.05$ vs. NT; $^{*}p<0.05$ vs. NC.

tivity in TSCC cells, likely through inhibiting cisplatin-activated PI3K/Akt signaling. This study provides new insights into the functional role of UCA1 in cancer cells and suggests that UCA1 knockdown could be a new strategy to increase cisplatin chemosensitivity and thereby improve the therapeutic outcomes of cisplatin-based chemotherapy for TSCC.

4. Experimental

4.1. Cell culture

Human TSCC cell lines CAL 27 (Cat. No. CRL-2095) and SCC-9 (Cat. No. CRL-1629) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in DMEM medium (Thermo Fisher Scientific, Beijing, China) containing 10% heat-inactivated FBS (Thermo Fisher Scientific) and 100 U/mL penicillin-streptomycin (Sigma-Aldrich, Beijing, China) in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Normal human tongue tissues were obtained from the Human Tissue Bank of Liaocheng People's Hospital, Liaocheng, China.

4.2. siRNA transfection

Silencer Select human UCA1 siRNA (Cat. No. 4390771) and negative control siRNA (Cat. No. 4390843) were purchased from Thermo Fisher Scientific and transfected into CAL 27 and SCC-9 cells using Lipofectamine 3000 (Cat. No. L3000008; Thermo Fisher Scientific) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were subject to subsequent experiments.

4.3. Real-time quantitative RT-PCR

RNA was prepared from cells using TRIzol reagent (Thermo Fisher Scientific) followed by purification with TURBO DNA-free System (Ambion, Austin, TX, USA). cDNA was synthesized using SuperScript II reverse transcriptase (Thermo Fisher Scientific) and random hexamer primers (Thermo Fisher Scientific). RT-qPCR was performed using an ABI-PRISM 7700 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific) and the fluorescent dye SYBR Green Master Mix (Thermo Fisher Scientific) as described by the manufacturer. The primers used were as follows: Forward, 5'-CATGCTTGACACTTGGTGCC-3' and reverse, 5'-GGTC-GCAGGTGGATCTCTTC-3' for UCA1; forward, 5'-GACTCATGACCACAGTC-CATGC-3' and reverse, 5'-AGAGGCAGGGATGATGTTCTG-3' for GAPDH. The PCR amplification condition was: 20 s at 95 °C followed by 40 cycles of 3 s at 95 °C and 30 s at 60 °C. Relative quantification of the UCA1 level was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and normalized against that of GAPDH in the same sample. Each experiment was repeated three times in duplicate.

4.4. Cisplatin chemosensitivity assay

Cells were plated in 96-well plates at a density of 2000 cells. After 6 h of incubation, the medium was replaced by fresh medium with or without various concentrations of cisplatin (0.1, 0.25, 0.60, 1.60, 3.30, 6.20, 15.00, 32.00, and 60.00 mM) (Sigma-Aldrich). Then cell viability was assayed 24 h later using a modified MTT assay as previously described (Ding et al. 2011). The half maximal inhibitory concentration (IC_{50}) was defined as the concentration resulting in a 50% reduction in growth compared to control cell growth. Each experiment was repeated for three times in duplicates. The IC_{50} dose-response curves were plotted with GraphPad Prism Version 5.0 (GraphPad Software, La Jolla, CA, USA).

4.5. Cell apoptosis assay

Cells were cultured at 7×10^4 cells per well in 96-well tissue culture plates and treated with cisplatin at the IC_{50} concentration (8.9 mM for CAL cells and 20.7 M for SCC-9) at 37 °C for 24 h. Cell apoptosis was measured after 24 h with a microplate reader-based TiterTACS in situ apoptosis detection kit (Cat. No. 4822-96-K; R&D systems, Minneapolis, MN, USA) as described by the manufacturer (Byun et al. 2012). Each experiment was repeated for three times in duplicates.

4.6. Caspase 3 activity assay

The activity of caspase 3 was determined using a colorimetric caspase 3 Assay Kit (Cat. No. ab39401) from Abcam (Cambridge, MA, USA). Briefly, the assays were performed in 96-well plates by incubating 20 ml cell lysate protein per sample in 70 ml reaction buffer (1% NP-40, 20 mM Tris-HCl (pH 7.5), 137 mM Nad, and 10% glycerol) containing 10 ml caspase-3 substrate (2 mM). The lysates were then incubated at 37 °C for 6 h, after which the samples were assayed using a spectrophotometer (Cat. No. 1702525; Bio-Rad Laboratories, Beijing, China) at 405 nm.

4.7. Phosphatidylinositol 3-kinase (PI3K) activity assay

PI3K activity was determined with a PI3K Activity ELISA kit (Cat. No. K-1000s; Echelon Biosciences, Salt Lake City, UT, USA) according to the manufacturer's protocol. For direct functional assessment of PI3K activity, PI3K was isolated by immunoprecipitation using a rabbit polyclonal anti-human PI3K antibody (Cat. No. 06-195; Millipore, Billerica, MA, USA) to the p85 adapter subunit, and the ability of the co-precipitated catalytic p110 catalytic subunit to convert a standard PIP2 to PIP3 in a kinase reaction assessed by measuring the generated PIP3 by the ELISA kit. Each experiment was repeated for three times in duplicates.

4.8. Western blot analysis

Cells was lysed with a hypotonic buffer containing 2% Nonidet-P and a protease inhibitor cocktail (Sigma-Aldrich) by sonication three times for 3 s on ice and the supernatant was obtained after centrifugation at 2000 *g* for 15 min at 4 °C. Equal amounts of proteins for each sample were separated by 10% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride microporous membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk powder in TBS-T for 2 h and incubated for 1 h with a 1:1000 dilution of rabbit polyclonal anti-human phosphorylated Akt (Ser 473) antibody (Cat. No. sc-101629; Santa Cruz Biotechnology, Dallas, TX, USA) or mouse monoclonal anti-human Akt antibody (Cat. No. sc-81434; Santa Cruz Biotechnology), and then washed and revealed using bovine anti-rabbit (sc-2370; Santa Cruz Biotechnology) or anti-mouse (sc-2371; Santa Cruz Biotechnology) secondary antibody (1:5000, 1 hour). Peroxidase was revealed with an ECL kit (GE Healthcare, Shanghai, China). Three independent experiments were performed.

4.9. Statistical analysis

Statistical analyses were performed with SPSS for Windows 10.0 (SPSS Inc., Chicago, IL, USA). All data values were expressed as means±SD. Comparisons of means among multiple groups were performed with one-way ANOVA followed by *post hoc* pairwise comparisons using Tukey's tests. A two-tailed $p<0.05$ was considered statistically significant in this study.

Conflicts of interest: None declared.

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