# Uca1 in cisplatin induced ovarian cancer cell resistance

The Expression of Long Non-coding RNA UCA1 in Human Ovarian Cancer Cells and Its Role in Cisplatin Cytotoxicityin Vitro

Running title: UCA1 in cisplatin induced ovarian cancer cell resistance

Highlights

- Increasing expression of UCA1 RNA was found in ovarian cancer tissues.
- UCA1 can increase the cell migration, invasion and cisplatin resistance.
- The effect of UCA was extended through targeting SRPK1 and apoptosis related pathway.

Abstract

Objective: The therapeutic potential of cisplatin in ovarian cancer treatment is limited by the occurrence of cellular resistance. To explore the role of long non-coding RNA UCA1 in cisplatin induced ovarian cancer cell resistance.

Methods: Twenty-four ovarian cancer tissues and sixteen normal tissues were used to assess the expression of UCA1 RNA. After expression UCA1 in SKOV3 cells, the cell migration, invasion and cisplatin resistance was assessed. Furthermore, the related mechanism was also explored. In addition, SRPK1 knockdown cell line was established and the effect of SRPK1 on cell migration, invasion and cisplatin resistance was also evaluated.

Results: The increased expression of UCA1 RNA was identified in 24 ovarian cancer tissue compared with normal tissue. Expression of UCA1 RNA in SKOV3 cells increased the cell migration, invasion and cisplatin resistance. Alternated expression of SRPK1 and apoptosis related proteins were found in

SKOV3/pcDNA-UCA1 cells. The effect of UCA1 expression on cell migration, invasion and cisplatin resistance was reversed by knocking-down SRPK1 in SKOV3 cells.

Conclusions: Increasing expression of UCA1 RNA was found in ovarian cancer tissues. UCA1 can increase the cell migration, invasion and cisplatin resistance. The effect of UCA was extended through targeting SRPK1 and apoptosis related pathway.

Key words: Long non-coding RNA, UCA1, SRPK1, cisplatin resistance, cell migration, invasion

Introduction

Ovarian cancer is the second most commonly diagnosed gynecological cancer in the world, and causes more deaths per year than any other the female reproductive system related cancer(1). More than 200, 000 cases are newly diagnosed and 120, 000 women die of ovarian cancer annually all over the world(2). Platinum based chemotherapy is active in ovarian cancer treatment. However, intrinsic or acquired cellular resistance to cisplatin is encountered regularly and severely limits the therapeutic potential of the drug(3). Multiple biological processes, such as dose accumulation, metabolism, apoptosis, DNA damage, are involved in the mechanisms of cellular resistance(4). Conquering cisplatin resistance remains therefore a critical goal for anticancer therapy and considerable efforts have been undertaken to solve this problem throughout the past three decades.

Previous studies have shown that serine/arginine-rich protein-specific kinase 1(SRPK1) and apoptosis related protein are closely related with cisplatin resistance. SRPK1 is a kinase which belongs to SR kinase family (5). Through regulating the phosphorylation of SR splicing factors, SRPK1 can afftect the pre-mRNA splicing and consequently gene expression (6). Increasing attentions have been paid on the role of SRPK1 in cisplatin resistance(7-8). The apoptosis resistance induced by anticancer drug treatment has been suggested as another important mechanism in cellular resistance(4).

More and more studies have shown that abnormal expression of long non-coding RNA (lncRNA) is involved in tumor development and progression(9). In a previous study, we obtained lncRNA UCA1 using RACE and found that higher expression of lncRNA UCA1 in bladder tumor tissues than normal tissues(10). Here, we tried to assess the expression of UCA1 and SRPK1 in ovarian cancer tissue and normal tissue using RT-PCR and explore the role of UCA1 in cisplatin induced ovarian cancer cell resistance. Our results might provide theoretical basis for chemotherapy selection in clinic and a novel cisplatin resistance related target was also suggested.

Methods and materials

Cell culture, Patients and Ovarian tumor specimens

The human ovarian cancer cell line SKOV3 was maintained at 37°C and 5% $CO_2$ incubator in RPMI-1640 media with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Flash frozen tissue specimens (n= 40) were obtained from patients undergoing debulking surgery for ovarian cancer at People's hospital of Shaanxi Providence, Shaanxi, China from January 2010 to January 2013. Among the specimens, epithelial ovarian cancer (n= 24) were obtained from primary lesion of the patients without radiochemotherapy while normal ovarian samples (n= 16) were obtained from patients undergoing hysterectomies for benign conditions. The pathological examination on all tissues was confirmed by two experienced physician. Written consent was provided by each patient and the whole protocol was proved by the Review Board of the hospital.

Reverse transcription PCR analysis

Total RNA extraction of cancer tissue or cells were performed with Trizol (Life Tech, US) and the reverse transcription reaction were performed with ImProm II reverse transcriptase(Promega, US) according to the manufacturer's instruction. UCA1, SRPK1, 18S rRNA specific sequences were amplified during 30 cycles of 30 s denaturing at 95°C, 60 s annealing at 57°C, and 60 s extension at 72°C, with the primers listed in Table 1.

Table 1Primer sequences used in the study

| Name | Forward primer | Reverse primer |
|------|----------------|----------------|
| UCA1 | CTCTCCATTGGGGTTCACCATTC | GCGGCAGGTCTTAAGAGATGAG |
| SRPK1 | TAACGGACCACTGGACAACAAA | TTCCTGCGACCACTCATACTTC |
| 18S | CAGCCACCCGAGATTGAGCA | TAGTAGCGACGGGCGGTGTG |

rRNA

UCA1

(full

length            CGGGATCCTGACATTCTTCTGGACAATGAG   CCGGAATTCGCATATTAGCTTTAATG

)

Expression of UCA1 in SKOV3 cells

The full length of UCA1 was expanded by PCR (The primer was showed in Table 1) at an annealing temperature of 53 °C. After digested with BamHI and EcoRI, the PCR fragment was subcloned into pcDNA3. 1 to construct the pcDNA-UCA plasmid. Transient transfection of cells with plasmid was performed with Lipofectamine® 2000 (Life Tech, US). Twenty-four hours later, G418 selection(500 µg/mL) was processed for 3 weeks. The characterization of the positive clone was confimed by RT-PCR. The pcDNA3. 1 without UCA1 fragment was used as negative control.

RNAi

The shRNA sequences of SRPK1 were obtained according to previous description(11). SH1 and SH3, encoding shRNA targeting nucleotides 1423 to 1443 (GGTCAGTCATTCAGTGAACAA) and 288 to 308 (CAAGAAGATCCTAATGATTA), respectively, of the SRPK1 mRNA, were processed with annealing, subcloning into PRNAT-U6. 1/Neo plasmid (GenScrpt Corp., Piscataway, NJ, US), plasmid expansion and media amount extraction. Transient transfection of cells with plasmid was performed with Lipofectamine® 2000 (Life Tech, US) and 3 different batch of cells were used

for knockdown efficacy examination. Stable cell lines were obtained by G418 selection for 3 weeks. The expression of SRPK1 was confirmed by western-blot analysis.

Western-blot analysis

The frozen myocardial tissues were lysed in RIPA buffer (Beyotime, China), followed by high speed centrifugation and BCA quantification. Cellular protein was separated by electrophoresis on SDS-PAGE gel and then transferred onto PVDF membrane. After blocking, the blots were incubated with the antibodies to SRPK1 (BD), Bcl-2 (Cell Signaling Technology), BAX (Cell Signaling Technology), caspase-3(Cell Signaling Technology), aspase-3(Cell Signaling Technology). And β-Actin(Cell Signaling Technology) was used as loading control. The appropriate HPR conjugated secondary antibodies were applied. The protein bands detected with SuperSignal Ultra Chemiluminescent Substrate (Pierce) on X-ray films (Koda).

MTT

After preparing the single cell suspension, $4\times10^{3}$ cells in 100 μL culture media were seeded in 96-well plate in quadruplicate overnight. MTT was added for 4 hr, and formazan dye was dissolved with DMSO and read at 490 nm in a microplate reader (Molecular Device, US). All the experiments were performed for three times.

Clonogenic Survival Assay

Cells ($5 \times 10^2$) were seeded in 6-well plates overnight and incubated with RPMI1640 + 10%FBS + 500 µg/ml G418 for 14 day. After removing the media, cells were washed with PBS, fixed with 95% ethanol for 30 min and stained with Giemsa for 15 min. Colonies with > 50 cells were counted under microscope. Percentage cell survival is expressed relative to untreated control.

Scratch assay

3. $0 \times 10^5$ cells were seeded in 6-well plates and the cells were allowed to grow until 90% confluence was reached. Then the cells were grown in 0. 2% FBS RPMI1640 media overnight for resting and a scratch was made by using the 200 µL pipette tip. The photos were taken at 0 h and 24 h under a microscopy and the relative migrating distances of the wound areas were measured on the images.

3-D migration and Invasion assay

Cells ($5 \times 10^5$) were seeded in triplicate in upper chamber of the Millicell (8 µm pore diameter) which was pre-coated with Matrigel (Becton Dickinson Labware, Bedford, MA). After the lower chamber of the Millicell was added with 900 µL RPMI 1640 +20% FBS, the Millicell was incubated at 37°C and 5% $CO_2$ for 24 hrs. Then the Matrigel was removed by cotton tip, fixed with 95% ethanol for 30 min, stained with Giemsa staining. The membrane was checked with microscopy. The migration assay was similar with invasion assay but with 12 hr incubation time.

Cisplatin resistance assay

Cells ($3 \times 10^4$) were seeded in quadruplicate in 24-well plate and allowed to adhere overnight. Then the cells were treated with serious concentration of cisplatin(0, 2. 5, 5, 10, 20, 40, 80 μM) for 48 hr. Cell viability was determined by MTT assay at 490 nm wavelength.

Statisticalanalysis

All statistical analyses were performed using the SPSS13. 0 software. The results were presented as means ± SD. Two-tailed Student's t-test was used to examine the differences between groups. $P < 0.05$ was considered as statistically significant.

Results

The expression of UCA 1 RNA and SRPK1 mRNA in ovarian tissues

Twenty-four ovarian epithelial cancer tissue and sixteen normal ovarian tissue was used to assess the UCA1 and SRPK1 expression. And we found higher expression of UCA 1 RNA and SRPK1 mRNA in ovarian cancer tissue while no significant expression of UCA1 and SRPK1 was found in normal ovarian tissue(Figure 1A).

Theeffect of UCA1 RNA expression on SKVO3 migration and invasion

Cell lines establishingAfter constructing of pcDNA-UCA1, the stable cell lines with or without UCA1 RNA expression were established. The positive control was confirmed by RT-PCR and the result showed that a length of 1442 bp UCA1 RNA was expanded from SKOV3/pcDNA-UCA1 while no UCA1 was found in negative control SKOV3/pcDNA 3. 1(Figure 1B).

2-D and 3-D Migration and invasion assayThe scratch assay suggested that cell migration ability of SKOV3/pcDNA-UCA1 was significantly increased that of SKOV3/pcDNA 3. 1(Figure 1C). The 3-D migration and invasion assay with millicell chamber showed that the migration and invasion abilities were significantly increased in SKOV3/pcDNA-UCA1 cell than SKOV3/pcDNA 3. 1 cells(Figure 2A).

Cisplatin resistance assayThe cisplatin resistance assay was performed with SKOV3/pcDNA-UCA1 and SKOV3/pcDNA 3. 1 cells by MTT. Increased cisplatin resistance was found in SKOV3/pcDNA-UCA1 cell. The IC $_{50}$ of SKOV3/pcDNA-UCA1 cells increased 2. 41 times than that of SKOV3/pcDNA 3. 1 cells(Figure 2B).

Western blot analysis of SRPK1 and apoptosis pathwayTo explore the mechanism we analyzed the expression of SRPK1, Bcl-2, Bax, Caspase3 and Caspase9 in SKOV3/pcDNA-UCA1 and SKOV3/pcDNA 3. 1 cells and found that increased expression of SRPK1 and Bcl-2 and decreased expression of Bax, Caspase3 and Caspase9 in SKOV3/pcDNA-UCA1 cells (Figure 2C).

The effect of SRPK1 knockdown on SKOV3 cells

Knockdown cell line establishingThe knockdown efficacy of pRNAT-SH1 and pRNAT-SH3 were firstly examined by transient transfestion and western-blot. And the results showed that SKOV3/ pRNAT-SH3 was extend a better effect of knocking down SRPK1 (Figure 3A $_1$ ). Stable cell lines of SKOV3/pRNAT-SH3 and SKOV3/pRNAT-U6. 1 were also established and the effect of pRNAT-SH3 on SRPK1 knockdown was showed in Figure 3A $_2$ .

The proliferation, colongenic, migration, invasion abilities of SRPK1 knockdownThe result of MTT assay was showed that decreased proliferation was found in SKOV3/pRNAT-SH3(Figure 3B). The colongenic ability of SKOV3/pRNAT-SH3 was significantly decreased than that of SKOV3/pRNAT-U6. 1(Figure 3C). The 3-D migration and cell invasion assay showed that the cell migration and invasion were decreased in SKOV3/pRNAT-SH3 cells than SKOV3/pRNAT-U6. 1 cells(Figure 4A).

Cisplatin resistance assayThe cisplatin resistance assay was performed with SKOV3/pRNAT-SH3 and SKOV3/pRNAT-U6. 1 cells by MTT. Increased cisplatin resistance was found in SKOV3/pRNAT-SH3 cell. The IC $_{50}$ of SKOV3/pRNAT-SH3 cells was increased 2. 64 times than that of SKOV3/pRNAT-U6. 1 cells(Figure 4B).

Western blot analysis of SRPK1 and apoptosis pathwayTo explore the mechanism we analyzed the expression of SRPK1, Bcl-2, Bax, Caspase3 and Caspase9 in SKOV3/pRNAT-SH3 and SKOV3/pRNAT-U6. 1 cells and found that increased expression of SRPK1 and Bcl-2 and decreased expression of Bax, Caspase3 and Caspase9 in SKOV3/pRNAT-SH3 cells (Figure 4C).

Discussion

The lnc RNA UCA1 was cloned in our lab using SMAT-RACE from the bladder cancer cell line BLZ-211. And UCA1 RNA showed an expression pattern of increasing expression in early stage of human embryonic development, differential expression at 28 week of embryonic development, no expression in normal adult tissues. However, the expression of UCA1 RNA was increased

in bladder cancer tissues(10). In addition, the increasing expression of UCA1 RNA than the normal or para-carcinoma tissueswas also found in breast cancer, liver cancer, thyroid cancer, cervical cancer, lung cancer, esophagus cancer, gastric cancer and so on(12). We didn't observe an obvious expression of UCA1 RNA in normal tissues and did observe the expression of UCA1 RNA in ovarian cancer tissues. This suggested UCA1 RNA may extent a critical role in the development and progression of ovarian cancer.

The previous study showed that the abilities of cell proliferation, cisplatin resistance, invasion and migration were increased in bladder cancer cell line(13). Yang et al showed that UCA1 can regulate the cell cycle through CREB and PI3K pathway(14). Wang et al found that overexpression of UCA1a(also named as CUDR) in bladder cancer cells would increase the abilities of cell proliferation, invasion and cisplatin resistance and decrease cell apoptosis(15). Wing et al showed that increased expression of UCA1a could increase the cellular resistance and decrease the apoptosis in A431 squamous cancer cells. However, the mechanism is still unknown(16).

The cisplatin resistance of ovarian cancer is the main cause of tumor recurrence and the failure of chemotherapy. The mechanisms of cisplatin resistance included dose accumulation of the drug, metabolism, apoptosis and DNA damage and it is a complicate process of multi-factor, multi-level and multi-gene. SKOV3 was used to assess the cisplatin resistance effect in ovarian cancer. We established SKOV3 cell lines expressing UCA1 RNA and found that cell abilities of migration, invasion and cisplatin resistance were increased, which was consistent with the results obtained from the bladder cancer cell lines. Since SRPK1 was proved to involve in the cisplatin

resistance(17-18), we also tried to analyze the association between UCA1 RNA and SRPK1. And the western blot results showed that increased expression of SRPK1 and Bcl-2 while decreased expression of Bax, Caspase 3 and Caspase 9. SRPK1 is specific kinase belonged to SR family. It can specifically phosphorylate the SR splice factor and regulate the gene expression by alternative splicing of pre-mRNA of target gene(6). Hayes et al found decreased expression of SRPK1 in pancreas, colon and breast cancer could lead to increasing and decreasing expression of Bcl-2 and Bax. The decreasing on cell proliferation and increasing on cell apoptosis were found (19). Furthermore, increased sensitivities of Gemcitabine and Cisplatin were also found (11, 19). In order to confirm whether SRPK1 is involved in the mechanism of UCA1 regulating ovarian cancer proliferation and migration, we employed RNAi to decrease the expression of SRPK1 and found that increased expression of Bcl-2 and decreased expression of Bax, Caspase 3 and Caspase 9 after downregulating the expression of SRPK1. In addition, we found the increasing abilities on cell proliferation, migration and invasion after SRPK1 knockdown.

In conclusion, we found UCA1 RNA may increasing of cell proliferation, decreasing apoptosis and lead to the cisplatin resistance by increasing the expression of SRPK1 and affecting the expression of apoptosis related proteins(such as Bcl-2, Bax, Caspase 3 and Caspase 9). Our results will add novel insight on cisplatin resistance and provide novel molecular target to the treatment.