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Exosomes mediated transfer of lncRNA UCA1 results in increased tamoxifen resistance in breast cancer cells

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Abstract. – OBJECTIVE: In this study, we firstly compared the loading of urothelial carcinoma-associated 1 (UCA1) in exosomes between tamoxifen sensitive and tamoxifen resistant breast cancer cells and further investigated the role of exosomal transfer of UCA1 in the development of tamoxifen resistance in estrogen receptor (ER) positive breast cancer cells.

MATERIALS AND METHODS: Exosomes were isolated from the culture medium of tamoxifen sensitive MCF-7 cells and tamoxifen resistant LCC2 cells. QRT-PCR was performed to analyze UCA1 expression in cells and exosomes. CCK-8 assay, immunofluorescence staining of cleaved caspase-3 and flow cytometric analysis of annexin V/PI staining were used to assess tamoxifen sensitivity.

RESULTS: UCA1 is significantly increased not only in LCC2 cells, but also in exosomes released from LCC2 cells. The increase in exosomes is more evident than in cells. MCF-7 cells pretreated with exos/LCC2 had a significantly increased cell viability, a decreased expression of cleaved caspase-3 and a lower ratio of apoptosis after tamoxifen treatment. The exos/LCC2 with impaired UCA1 loading had significantly suppressed capability to promote tamoxifen resistance in MCF-7 cells.

CONCLUSIONS: UCA1 is significantly loaded in exosomes from tamoxifen resistant LCC2 cells. Exosomes mediated transfer of UCA1 can significantly increase tamoxifen resistance in ER-positive MCF-7 cells.

Key Words:
UCA1, Tamoxifen, Breast cancer, Exosomes.

Introduction

Tamoxifen, an antagonist of the estrogen receptor (ER), is a therapeutic agent currently used for the breast cancer patients with ER-positive tumors[1,2]. The use of this endocrine therapeutic drug has significantly improved disease free survival and overall survival of the patients[1,3]. But acquired tamoxifen resistance is still the main reason for endocrine therapy failure and subsequent cancer recurrence and cancer-related death[1,4]. Actually, the mechanism of tamoxifen resistance is quite complex and is far from been fully understood.

Extracellular vesicles, such as exosomes and microvesicles can transport coding and non-coding RNAs, proteins and lipids, thereby acting a potential mode of intercellular communication[5]. Some recent papers reported that exosomes are involved in the regulation of chemosensitivity of the recipient cells. In human hepatocellular cancer, exosomes mediated transfer of long non-coding RNA (lncRNA) ROR can increase chemoresistance[6,7]. In breast cancer, exosomal transfer of miR-221/222 can enhance tamoxifen resistance in recipient ER-positive breast cancer cells[8].

LncRNAs are evolutionarily conserved non-protein-coding RNAs greater than 200 nucleotides[9]. Dysregulated lncRNAs RNAs is also a mechanism of tamoxifen resistance development in breast cancer. One recent study found HOTAIR overexpression can activate the ER transcriptional program even under hormone-deprived conditions and promote

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tamoxifen-resistance[10]. Urothelial carcinoma-associated 1 (UCA1) is an lncRNA with three exons that encode a 1.4 kb isoform and a 2.2 kb isoform[11]. The oncogenic role of UCA1 in breast cancer is identified via multiple mechanisms, such as suppression of p27[11] and acting as miR-143 sponge[12]. In addition, the association between aberrant UCA1 expression and acquired drug resistance has also been reported in bladder cancer cells[13], in gastric cancer[14], and in colorectal cancer[15].

In this study, we firstly compared the loading of UCA1 in exosomes released from tamoxifen sensitive and tamoxifen resistant breast cancer cells and further investigated the role of exosomal transfer of UCA1 in the development of tamoxifen resistance in ER-positive breast cancer cells.

Materials and Methods

Cell Culture

The ER positive and tamoxifen sensitive human breast cancer cell line MCF-7 cells were obtained from ATCC (Manassas, VA, USA). The MCF-7 derived tamoxifen-resistant LCC2 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All of the cancer cells were grown in Roswell Park Memorial Institute-1640 (RPM-640) medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units of penicillin/ml and 100 μg of streptomycin/ml and were cultured in an incubator with humidified atmosphere and 5% CO_2 at 37 °C.

Isolation of Exosomes

In brief, 1×10^6 MCF-7 or LCC2 cells were cultured in vesicle-depleted medium for 3 days. Cell culture was then collected for exosomes isolation by sequential centrifugations according to the method introduced in one previous study[16]. The exosomes collected from the culture medium of MCF-7 cells were termed as exos/MCF-7, while that from the culture medium of LCC2 cells were named as exos/LCC2. The exosomes from LCC2 cells with knockdown of UCA1 were also isolated. The exosomes were used immediately, or were resuspended in 50-100 μL of PBS and stored at -80°C.

Nanoparticle tracking analysis (NTA) was performed using a Nanosight LM10-HS (NanoSight, Amesbury, UK) to determine size and quantity of EVs isolated as described in one previous study[17]. Five recordings of 30 sec each were captured, analyzed and the data from at least 5,000 individual particle tracks were analyzed per sample.

Cell Treatment

To investigate the effect of exosomes on tamoxifen sensitivity in MCF-7 cells, MCF-7 cells were cultured in exosomes depleted medium and incubated with exos/LCC2 (0, 1 and 10 μg/ml) for 24 h before tamoxifen treatment.

Two UCA1 siRNAs were chemically synthesized by Ribobio (Guangzhou, China) with the following sequence: si-UCA1-1: 5'-GTTAATCCAGGAGACAAAGA-3', si-UCA1-2: 5'-TCTTTGTCTCCTGGATTAAC-3'. LCC2 cells were transfected with 100 nM UCA1 siRNAs using Hiperfect transfection reagent (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instruction.

QRT-PCR

Total RNA in cell samples was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. cDNA was reversely transcribed using the PrimeScript® RT reagent kit (TaKaRa, Dalian, Liaoning, China). The UCA1 expression level was quantified using the following primers: forward: 5'-TTTGCCAGCCTCAGCTTAAT-3'; reverse: 5'-TTGTCCCCATTTTCCATCAT-3' and SYBR® Premix DimerEraser kit (TaKaRa, Dalian, Liaoning, China) in an ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA). GAPDH was used as the endogenous control gene. The results of QRT-PCR analysis were calculated using the $2^{-\Delta\Delta CT}$ method.

CCK-8 Assay of Cell Viability

MCF-7 cells were seeded in a 96-well plate (3000 cells per well) and were cultured in exosomes depleted medium supplemented with 0, 1 and 10 μg/ml exos/LCC2 with or without UCA1 knockdown for 24 h and then were further cultured with varying concentrations of tamoxifen (0.1, 0.5, 1, 5, 10, 20 50 μmol/L) for 3 days. Then, cell viability was measured using WST-8 assay using Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan) according to manufacturer's instruction.

Immunofluorescent Staining

LCC2 cells were grown on coverslips (174950, Thermanox, Thermo Fisher, Waltham, MA, USA) and were cultured in exosomes depleted medium supplemented with 0, 1 and 10 μg/ml exo/LCC2 for 24 h and then were treated with 5 μM Tamoxifen for 72 h. Then, the cells were fixed, permeabilized in 0.1% Triton X-100 and blocked with 1% bovine serum albumin (BSA). Then, the co-

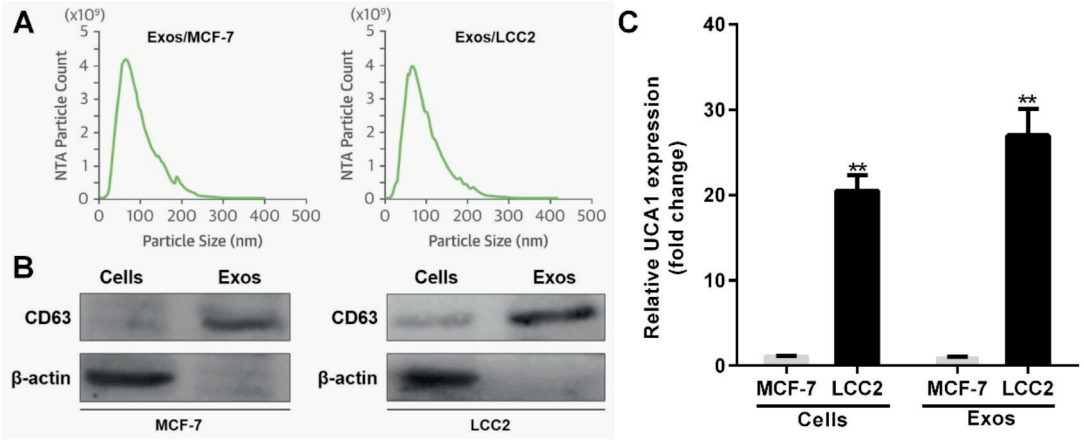

Figure 1. LncRNA UCA1 is significantly upregulated in the tamoxifen-resistant breast cancer cells. ***A***, NTA (analysis of the number and diameters of the Exosomes isolated from the culture medium of MCF-7 cells (left) and LCC2 cells (right). ***B,*** Western blot analysis of CD63 and β-actin in cell and exosome samples. ***C,*** QRT-PCR analysis of UCA1 expression in tamoxifen sensitive MCF-7 cells and tamoxifen resistant LCC2 cells and in exosomes released from the cells. $^{**}p<0.01$.

verslips were incubated with antibodies against cleaved caspase-3 at Asp175 (1: 500, #P42574, Cell Signaling, Danvers, MA, USA) at 4°C overnight. After washing, the coverslips were incubated in a secondary Alexa Fluor-555-labeled goat anti-rabbit IgG (#4413, Cell Signaling, Danvers, MA, USA) for 30 min at room temperature in darkness. Coverslips were mounted with mounting media (ab104139, Abcam, Cambridge, MA, USA), which contains DAPI to stain the nuclei. The number of positively stained cells was scored by counting of three sets of at least 100 cells under the microscope.

Flow Cytometric Analysis

Cell apoptosis was assessed using the Annexin V-FITC Apoptosis Detection Kit (JingMei Biotech, Beijing, China), according to the manufacturer's instructions. In brief, 100 μL cell suspension containing 1×10^5 cells were prepared. Then, 5 μL Annexin V-FITC and 10 μL propidium iodide (PI) (20 μg/mL) were added and incubated in the dark for 15 min at room temperature. Then, the ratio of apoptotic cells was analyzed using a FACSCaliber flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). Each test was performed with at least three repeats.

Western Blot Analysis

Cell samples were lysed using a lysis buffer (P0013, Beyotime, Shanghai, China). Then, the protein concentration was quantified using a BCA protein assay kit (Beyotime, Shanghai, China). Then, a conventional western blot was performed. Primary antibodies used included anti-CD63 (sc-5275, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin (1: 2000, ab8227, Abcam, Cambridge, UK). After that, the membranes were incubated with the corresponding HRP conjugated secondary antibodies. The blot signals were visualized using the ECL Western blotting substrate (Promega, Madison, WI, USA).

Statistical Analysis

Data were presented in the form of means ± standard deviation (SD) based on at least three repeats of three independent experiments. Comparison between groups was performed using the unpaired *t*-test. A two-sided *p*-value of <0.05 was considered statistically significant.

Results

UCA1 is Significantly Increased in Exosomes from Tamoxifen Resistant Cells than that from Tamoxifen Sensitive Cells

Exosome has been recently demonstrated as an important modulator of cell-cell signaling. In this study, we firstly isolated exosomes from tamoxifen sensitive MCF-7 cells and tamoxifen resistant LCC2 cells. Nanoparticle tracking analysis showed that the size distribution of the exosomes is mainly from 50-100 nM in diameter (Figure 1A). Following Western blot analysis detected very strong signals of CD63 and no β-actin in exosome samples (Figure 1B). However, β-actin was detectable in cell samples (Figure 1B). These results suggest that exosomes were

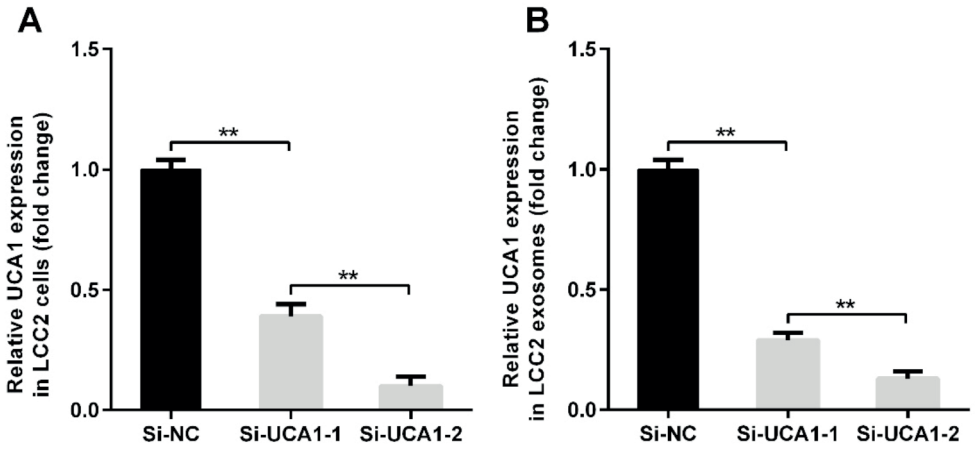

Figure 2. UCA1 knockdown significantly inhibits UCA1 loading in exosomes. ***A-B.*** QRT-PCR analysis of UCA1 expression in LCC2 cells ***(A)*** and LCC2 exosomes ***(B)*** after transfection of 100 nM si-UCA1-1 or si-UCA1-2. $^{**}p<0.01$.

isolated successfully. By performing qRT-PCR, we found that UCA1 expression increased more than 20 folds in LCC2 cells than in MCF-7 cells, while the UCA1 level in exosomes from LCC2 cell culture was about 27 folds higher than that from MCF-7 cells (Figure 1C).

UCA1 Knockdown Significantly Inhibits UCA1 Loading in Exosomes

To inhibit UCA1 expression, we designed two siRNAs. LCC2 cells were firstly transfected with 100 nM si-UCA1-1 or si-UCA1-2. QRT-PCR analysis showed both si-UCA1-1 and si-UCA1-2 significantly reduced UCA1 level in LCC2 cells, while si-UCA1-2 had a better inhibiting effect than si-UCA1-1 (Figure 2A). QRT-PCR based on exosomes samples also confirmed that si-UCA1-1 and si-UCA1-2 significantly inhibited UCA1 loading in exosomes, while si-UCA1-2 had a stronger inhibitive effect than si-UCA1-1 (Figure 2B).

MCF-7 Cells Pretreated with Exosomes from LCC2 Cells have Increased Tamoxifen Resistance

Then, we investigated how exosomes isolated from LCC2 cells modulate tamoxifen sensitivity in MCF-7 cells. MCF-7 cells were pretreated with different concentration of exos/LCC2. By performing a CCK-8 assay of cell viability, we found that exos/LCC2 significantly increased the viability of MCF-7 cells after tamoxifen treatment in a dose-dependent manner (Figure 3A). Then, we performed immunofluorescent staining to examine the expression of cleaved caspase-3 in MCF-7 cells with or without pretreatment of exos/LCC2. The results also showed that exos/LCC2 significantly decreased tamoxifen-induced activation of caspase-3 in MCF-7 cells in a dose-dependent manner (Figure 3B). The following flow cytometric analysis showed that exos/LCC2 suppressed tamoxifen-induced cell apoptosis in MCF-7 cells (Figure 3C-D).

UCA1 Knockdown Impaired the Exosomes Mediated Transfer of Tamoxifen resistance

To further investigate the regulative effect of UCA1 on tamoxifen resistance, MCF-7 cells were pretreated with exosomes isolated from LCC2 cells with or without knockdown of UCA1 before tamoxifen treatment. The CCK-8 assay showed that the exos/LCC2 with impaired UCA1 loading had significantly suppressed capability to promote cell viability of MCF-7 cells (Figure 4A). The following flow cytometric analysis also confirmed that UCA1 knockdown substantially weakened the exos/LCC2's capability to reduce tamoxifen-induced cell apoptosis in MCF-7 cells (Figure 4B).

Discussion

Exosome mediated transfer of non-coding RNAs, including miRNAs and lncRNAs have been gradually demonstrated as an important mechanism of acquired drug resistance in some cancer cells. For example, in ovarian cancer, exo-

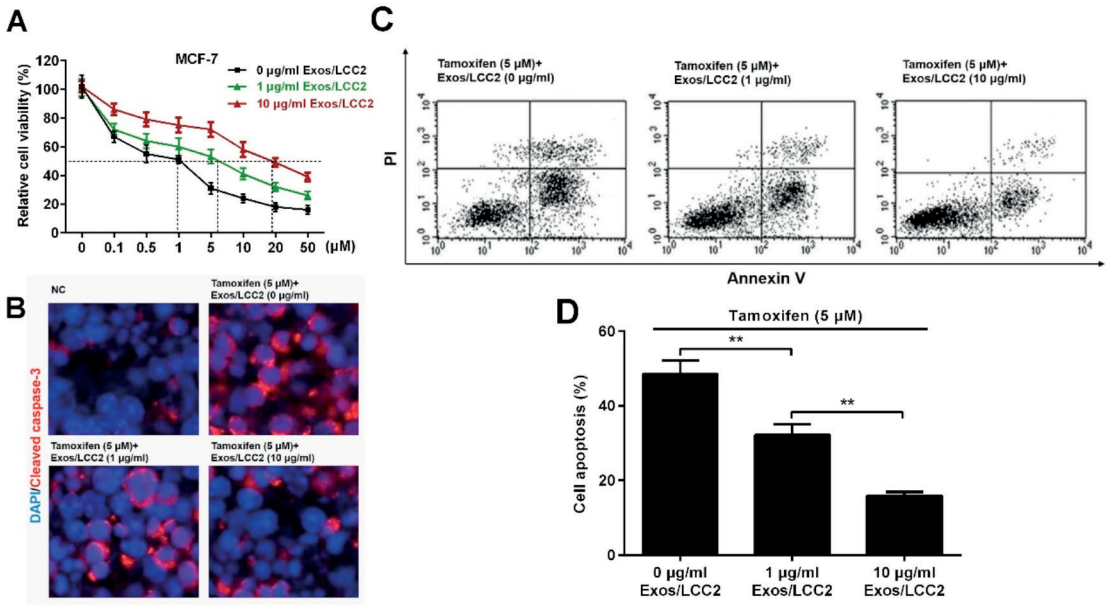

Figure 3. MCF-7 cells pretreated with exosomes from LCC2 cells have increased tamoxifen resistance. ***A,*** CCK-8 assay of cell viability of MCF-7 cells pretreated with 0, 1 or 10 μg/ml exos/LCC2 for 24 h and then treated with varying concentrations of tamoxifen (0.1, 0.5, 1, 5, 10, 20 and 50 μmol/L) for 3 days. B. Typical images of the cleaved Caspase-3 labeled by Alexa Fluor-555-labeled antibody (red color) and the nuclei stained with DAPI (blue color). ***C-D.*** Representative images ***(C)*** and quantitation ***(D)*** of flow cytometric analysis of apoptotic MCF-7 cells pretreated with 0, 1 or 10 μg/ml exos/LCC2 for 24 h and then treated with 5 μM of tamoxifen for 3 days. $^{*}p<0.05$.

somal transfer of miR-433 between resident cells can promote resistance to paclitaxel through the induction of cellular senescence[18]. Exosome mediated transferring of P-glycoprotein from paclitaxel-resistant A2780 cells to paclitaxel-sensitive A2780 cells led to a redistribution of and resistance to paclitaxel and adriamycin in recipient cells[19]. One recent work founds that exosomes released from tamoxifen-resistant MCF-7 cells could enter into wild-type MCF-7 cells and release miR-221/222. The elevated miR-221/222 in the recipient cells can effectively reduce the target genes expression of P27 and ERalpha, leading to enhanced tamoxifen resistance[8].

Besides the oncogenic effect, UCA1 also exerts a regulative effect on drug resistance in multiple types of cancer. For example, UCA1 can induce acquired resistance to EGFR-TKIs in EGFR-mutant non-small cell lung cancer by activating the AKT/mTOR pathway[20]. It can also increase chemoresistance of bladder cancer cells via activating the Wnt signaling pathway in a Wnt6-dependent manner[21]. Knockdown of UCA1 in Adriamycin-resistant SGC7901/ADR cells can significantly decrease the resistance[14]. In this study, we firstly compared the expression of UCA1 between exosomes from MCF-7 cells and LCC2 cells. The results confirmed that UCA1 is significantly increased not only in LCC2 cells, but also in exosomes released from LCC2 cells. Actually, the increase in exosomes is more evident than in cells. Therefore, we decided to investigate the whether the exosomes can modulate tamoxifen sensitivity of the cancer cells. The results showed that MCF-7 cells pretreated with exos/LCC2 had significantly increased cell viability, decreased expression of cleaved caspase-3 and lower ratio of apoptosis after tamoxifen treatment. Then, we further investigated whether this phenomenon is a direct result of UCA1 loading in the exosomes. By performing CCK-8 assay and flow cytometric analysis, we confirmed that the exos/LCC2 with impaired UCA1 loading had significantly suppressed capability to promote tamoxifen resistance in MCF-7 cells. Therefore, we infer that exosomes mediated transfer of UCA1 might be an important mechanism of acquitted tamoxifen resistance in breast cancer cells.

Previous researches showed that dysregulated lncRNAs could modulate drug sensitivity in breast cancer via multiple mechanisms. For example, the lncRNA ATB can competitively bind with miR-200c and lead to upregulation of miR-200c target gene ZEB1 and ZNF-217, re-

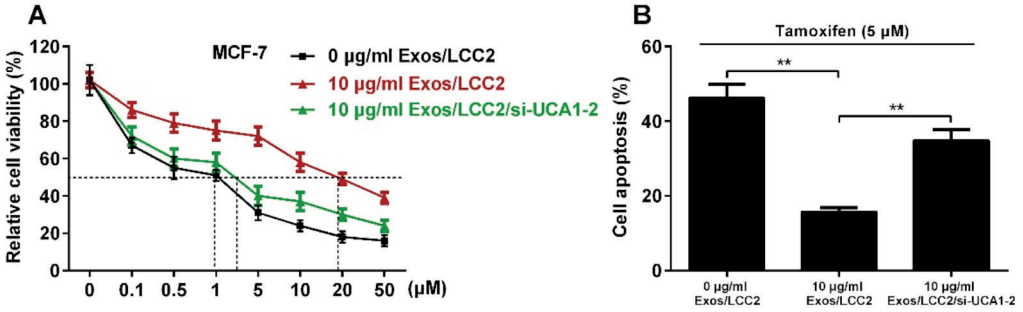

Figure 4. UCA1 knockdown impaired the exosomes mediated transfer of tamoxifen resistance. ***A,*** CCK-8 assay of cell viability of MCF-7 cells pretreated with 0 or10 μg/ml exosomes from LCC2 cells with or without UCA1 knockdown for 24 h and then treated with varying concentrations of tamoxifen (0.1, 0.5, 1, 5, 10, 20 and 50 μmol/L) for 3 days. ***B,*** Quantitation of flow cytometric analysis of apoptotic MCF-7 cells pretreated with 0 or10 μg/ml exosomes from LCC2 cells with or without UCA1 knockdown for 24 h and then treated with 5 μmol/L tamoxifen for 3 days. $^{*}p<0.05$.

sulting in enhanced epithelial-to-mesenchymal transition and subsequent trastuzumab resistance[22]. GAS5 can decrease trastuzumab-resistance via acting as a molecular sponge for miR-21 and decreasing the expression of phosphatase and tensin homologs (PTEN)[23]. The major limitation of this study is the lack of investigation of the downstream regulation of UCA1 in ER-positive breast cancer cells. Some recent studies suggest that UCA1 upregulation can lead activation of the AKT/mTOR pathway in multiple cancers[20,24]. mTOR inhibition can effectively restore the susceptibility of ER-positive breast cancer cells to tamoxifen[25]. Inhibition of the AKT/mTOR signaling can effectively reduce tamoxifen resistance in breast cancer cells[26,27]. Therefore, we hypothesized that mTOR signaling might be an important downstream signaling pathway of UCA1 in tamoxifen resistance. Actually, some non-coding RNAs, such as miRNAs with regulative effect on mTOR signaling pathway can modulate tamoxifen sensitivity of breast cancer cells. For example, miR-21 inhibition can sensitize breast cancer cells to tamoxifen by enhancing autophagic cell death through inhibition of the PI3K-AKT-mTOR pathway[28]. Enforced miR-451a expression can also increase the sensitivity of breast cancer cells to tamoxifen by reducing the activation of p-AKT and p-mTOR[29]. However, further studies are required to validate our hypothesis.

Conclusions

UCA1 is significantly loaded in exosomes from tamoxifen resistant LCC2 cells. Exosomes mediated transfer of UCA1 can significantly increase tamoxifen resistance in ER-positive MCF-7 cells.

Conflicts of interest

The authors declare no conflicts of interest.

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