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Enhancement of cisplatin-induced cytotoxicity against cervical cancer spheroid cells by targeting long non-coding RNAs

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ABSTRACT

Cervical cancer (CVC) is one of the most common types of gynecologic malignant tumor in the world. Unfortunately, current treatments including chemo-/radiotherapy still show the limitation on CVC progress. It is well known that cancer stem cells (CSCs) plays a critical role in drug resistance and metastasis. Furthermore, dysregulation of long non-coding RNA (LncRNA) shows close association to tumorigenesis and development of multiple cancers. In this study, we investigated the cytotoxic effect of cisplatin, a clinical chemotherapeutic for cervical cancer treatment, on parental and spheroid CVC cells and surveyed the effect of LncRNA on drug-resistance. We found that spheroid CVC cells showed much more resistant to cisplatin-induced cytotoxicity compared with parental CVC cells. Furthermore, cisplatin significantly induced apoptotic cell death, while it induced cell cycle arrest in G0/G1 phase at the same dose (10 μg/ml). We also found the significant expression of EGFR in spheroid instead of parental CVC cells. Interestingly, we revealed that protruding target lncRNAs were up-regulated in cisplatin-treated spheroid CVC cells, and inhibition of these lncRNAs enhanced the cytotoxicity of cisplatin against spheroid CVC cells. These data suggest that LncRNA might act as a critical modulator on drug-resistant capability of cervical CSCs and would be a novel target for cervical cancer treatment.

1. Introduction

The worldwide incidence of cervical cancer (CVC) ranks 4th among female malignancies [1]. Cervical cancer can be found early by having regular Papanicolaou test and sometimes even prevented completely. It has been reported that cervical cancer is the second most common cause of cancer death among women worldwide [2]. In China, CVC is one of the major malignancies in women, ranking first in incidence among urinary system tumors and showing a trend of increasing yearly [3].

Human papillomavirus (HPV) infection is detected in over 90% of cervical cancer patients and is concerned to be the major risk factor for cervical cancer [4,5]. Epidemiological studies have shown that the estimated lifetime risk of HPV infection is more than 75%, while the lifetime risk of developing cervical cancer is only around 0.7% [6]. It is already suggested that HPV integration and expression is insufficient for the development of cervical epithelium [7]. The exact molecular mechanisms underlying cervical cancer initiation and progression are still unclear. Epidermal growth factor (EGF) family of receptor kinases play significant roles in the survival, development and homeostasis in multicellular organisms through activating the intracellular signaling transduction or through the translocating the receptor to the nucleus [8]. It has been reported that the E5 protein of HPV type 16 activates EGFR through binding to a subunit of the protein pump ATPase that resulting in a reduced degradation of EGFR receptors, an increase in EGFR recycling, and an overexpression of EGFR [9]. Previously, it has also been revealed that the HPV-negative head and neck cell line has an activating mutation in EGFR that resulting in phosphorylation of Akt [10]. Furthermore, as compared to HPV-transformed head and neck cells, HPV-negative cells were more resistant to radiotherapy. However, there is relative less evidence about the drug-resistance of HPV-negative cervical cancer cells.

Accumulating evidence has suggested that cancer stem cells (CSCs) has a tumorigenic capability and play critical roles in metastasis,

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progression and chemo/radio-resistance [11]. Besides leukemia, CSCs can be observed in several solid cancers including brain, oral, lung, breast, liver, colon, and melanoma, etc. [12]. Recently, it is suggested that EGFR/Notch antagonists can enhance the response to inhibitors of the PI3K/Akt by targeting tumor-initiating cells [13].

Long non-coding RNAs (lncRNAs) are non-coding RNA molecules of over 200 nucleotides long and playing key roles in regulating gene expression through post-transcriptional gene regulation [14]. LncRNAs are reported to be involved in diverse biological processes, tissue formation as well as development, and also play a critical role in various human diseases including cancer [15]. Moreover, lncRNAs are highly conserved among species, suggesting that they may be of great significance to gene expression [16]. Previous studies have reported that LncRNA NEAT1 inhibits the radiotherapy of cervical cancer cell via upregulating the expression of cyclin D1 [17]. Recently, it is disclosed that suppression of lncRNA NCK1-AS1 increases chemosensitivity to cisplatin in HPV-infected cervical cancer cell [18]. However, the underlying regulatory role of lncRNAs in chemoresistance of cervical CSCs has not yet been illustrated. Our findings revealed that as compared to parental cells, cervical CSCs were more resistant to cisplatin and EGFR was markedly high expressed in cervical CSCs. Additionally, silence of cisplatin-induced lncRNAs significantly enhanced the cytotoxicity of cisplatin against cervical CSCs spheres. These data suggested that lncRNAs might play critical role in chemoresistance of CSC and provide combination therapeutic strategies for cervical cancer treatment.

2. Materials and methods

2.1. Tissue samples

A total of twenty-eight pairs of fresh cervical cancer (CVC) and adjacent normal tissues were collected from patients without chemotherapy or radiotherapy at the Shaoxing Women and Children's Hospital (Shaoxing, China). The tissues were collected within 30 min after resection and frozen at −80 °C until use.

Cell culture. Human cervix carcinoma cell lines C-33-A and DoTC2 4510 were obtained from the Cell Research Institute of the Chinese Academy of Sciences (Shanghai, China). C-33-A cells were cultured in Eagle's Minimum Essential Medium (Thermo Fisher Scientific, Waltham, MA) and DoTC2 4510 cells were in Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific). The media were supplemented with fetal bovine serum (Thermo Fisher Scientific) and 100 units/ml penicillin/streptomycin antibiotics (Thermo Fisher Scientific) followed by the cell culture manual. Cells were cultured in a humidified atmosphere of 5% CO_2 incubator at 37 °C.

2.2. CSCs sphere culture

The study was executed following previously mentioned [19]. Cells were maintained in stem cell media consisting of related basal media, N2 and B27 supplements (Thermo Fisher Scientific), 20 ng/ml human recombinant epidermal growth factor and 20 ng/ml basic fibroblastic growth factor (PeproTech Inc., Rocky Hill, NJ). For the CSCs sphere formation assay, cells were plated at a density of 200 cells/well on 24-well ultra-low attachment plates and maintained in stem cell medium. CSCs spheres that arose within one week were recorded. The experiments were performed in triplicate for each cell line, and the spheres were counted by two individuals in a blinded fashion.

2.3. Cell viability assay

Cell proliferation was measured by the Cell Counting Kit-8 assay kit (Dojindo Molecular Technologies, Kumamoto, Japan). Cells were seeded in 96-well plates at a density of 5×10 [3] cells per well. After overnight incubation, the medium was changed with 2% FBS medium containing cisplatin with various concentrations. After 72 h of incubation, a batch of cells was stained with 10 µl CCK-8 dye at 37 °C for 1 h. Optical density value (450 nm) was then assessed by spectrophotometry.

2.4. RNA extraction and reverse transcription

Total RNA was extracted from the frozen cervical cancer tissues and corresponding adjacent normal tissues by RNAiso Plus (TAKARA, Shaoxing, China). Each specimen was quantified with the NanoDrop Lite spectrophotometer (Thermo Scientific, MA) and RNA integrity was assessed by standard denaturing agarose gel electrophoresis. Total-RNA (1000 ng) was converted to cDNA by reverse-transcription with ReverTra Ace qPCR RT Kit (TAKARA, Shaoxing, China). The reverse transcription procedure was performed at 65℃ for 5 min, 42℃ for 18 min, and 98℃ for 5 min.

2.5. Long non-coding RNA array

The study was performed based on the protocol of the Human lncRNA Profiler™ qPCR Array Kit (SBI, Mountain View, CA) as previously described [20]. In brief, RNA was mixed with PolyA buffer, $MnCl_2$, ATP, and PolyA polymerase and incubated for 30 min at 37 °C. This mixture was then added to Oligo dT adapter and heated for 5 min at 60 °C. Subsequently, reverse transcription buffer, dNTP, DTT, random primer mix, and reverse transcriptase were added into the mixture and incubated for 1 h at 42 °C. Finally, the lncRNA-cDNA was heated for 10 min at 95 °C and stored at −80 °C for further study. Mastermix (contained SYBR Green qPCR Mastermix buffer, lncRNA cDNA and RNase-free water) and lncRNA primer were mixed and the qPCR standard protocol was followed: one cycle of 50 °C for 2 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. Data were read at the step of 60 °C for 1 min and the relative lncRNA level was calculated according to the comparative $2^{-\Delta\Delta Ct}$ method as compared to related endogenous housekeeping RNAs.

2.6. Transfection of lncRNA oligonucleotides

The lncRNAs antisenses and their negative controls (NC) were obtained from GenePharma (Shanghai, China). C-33-A and DoTC2 4510 cells were transfected using Lipofectamine™ 3000 (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol.

Western blot assay. C-33-A and DoTC2 4510 total protein lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The membranes were incubated with anti-EGFR antibodies (sc-373746, 1:1000, Santa Cruz) or anti-β-actin antibodies (sc-58673, 1:1000, Santa Cruz) at 4 °C overnight. After incubation with secondary antibody for 1 h, the membranes were developed with an ECL detection system (Thermo Fisher Scientific, Waltham, MA).

2.7. Cell cycle analysis by flow cytometry

After treatment, the adherent and floating cells were all collected and stained with Propidium Iodide Flow Cytometry Kit (ab139418, Abcam, Cambridge, UK) following the manufacturer's protocal, followed by flow cytometric analysis with a BD Accuri C6 plus (BD Biosciences, San Jose, CA).

Statistical analysis. All data were analyzed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA) and SPSS software ver. 19.0 (SPSS, Inc., Chicago, IL). Data are represented as the mean ± standard of deviation of the mean (SEM) for triple independent experiments. Probability (p) value of < 0.05, calculated using one-way ANOVA followed by the Student's t-test, was considered statistically significant.

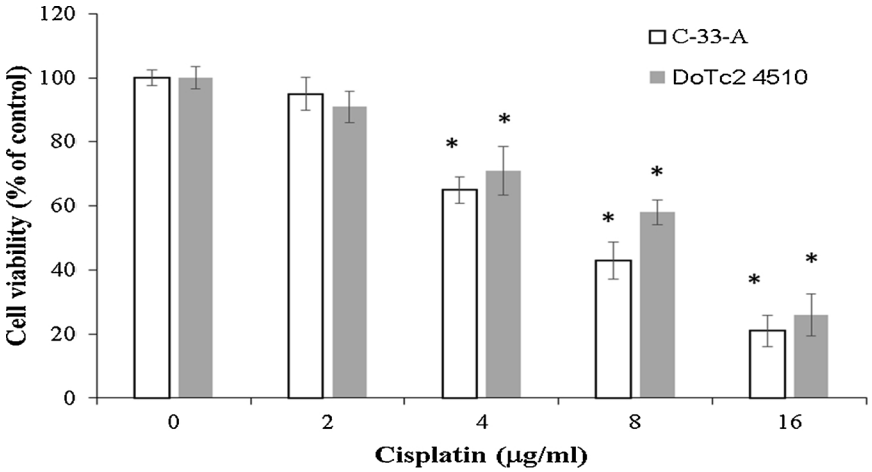

Fig. 1. Effects of cisplatin on the cell viability of cervical cancer cells. (A) Cervical cancer cell lines including C-33-A and DoTC2 4510 were treated with indicated concentration of cisplatin for 72 h and the cell viability was evaluated by CCK-8 assay kit. *, $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Cisplatin inhibits cervical cancer cell growth

First, the effect of cisplatin on ovarian cancer cells was investigated. The human cervix carcinoma cell lines C-33-A and DoTC2 4510 were examined. It was revealed that cisplatin exhibited cytotoxicity against two cervical cancer cell lines, and C-33-A cell was more sensitive (Fig. 1, $IC_{50} = 7 \pm 1.5$ μM for 72 h of incubation).

3.2. Cisplatin inhibits CSCs sphere formation

To investigate the cytotoxic effect of cisplatin on cervical CSCs, we further cultured cervical parental cells in serum free CSCs culture medium. Two to three weeks later, it was observed that spheroid CSCs formed (Fig. 2A). We harvested an amorphous mass of spheroid CSCs and disaggregated gently with 0.25% trypsin/EDTA and re-seeded the cells to study the effect of cisplatin on CSC sphere formation. After incubation of the cells with various concentrations of cisplatin for one week (added cisplatin-containing fresh medium directly and gently twice a week), the number of CSCs spheres were counted. We found that cisplatin inhibited the formation of CSCs sphere in a dose-dependent manner, while cisplatin at a higher concentration (> 10 μM) showed significant ($p < 0.05$) reducing efficacy (Fig. 2B).

3.3. Cisplatin induced apoptotic cell death and cell cycle arrest of parental and CSCs cervical cancer cells respectively

To investigate the cytotoxic effect of cisplatin on parental and CSCs cervical cancer cells, we treated both cells with cisplatin for, then harvested the cells and stained with PI for flow cytometric analysis. Differentially, cisplatin (10 μM) treatment for 72 h significantly ($p < 0.05$) induced apoptotic cell death in the parental cells (P), while cisplatin at the same dose only induced cell cycle arrested in G0/G1 phase in the CSCs (S) (Fig. 3A and B). This indicates that cervical cancer CSCs cells are resistant to cisplatin-induced cell apoptosis.

3.4. EGFR is markedly high expressed in cervical CSCs cells and patients with high-EGFR is accompanied with low survival rate

EGFR is reported to be abundant in cervical cancer tissues. We further investigated the expression of EGFR in cervical parental and CSCs cells. As shown in Fig. 4A, as compared to cervical parental cancer cells, EGFR was obviously high expressed in CSC. Furthermore, as

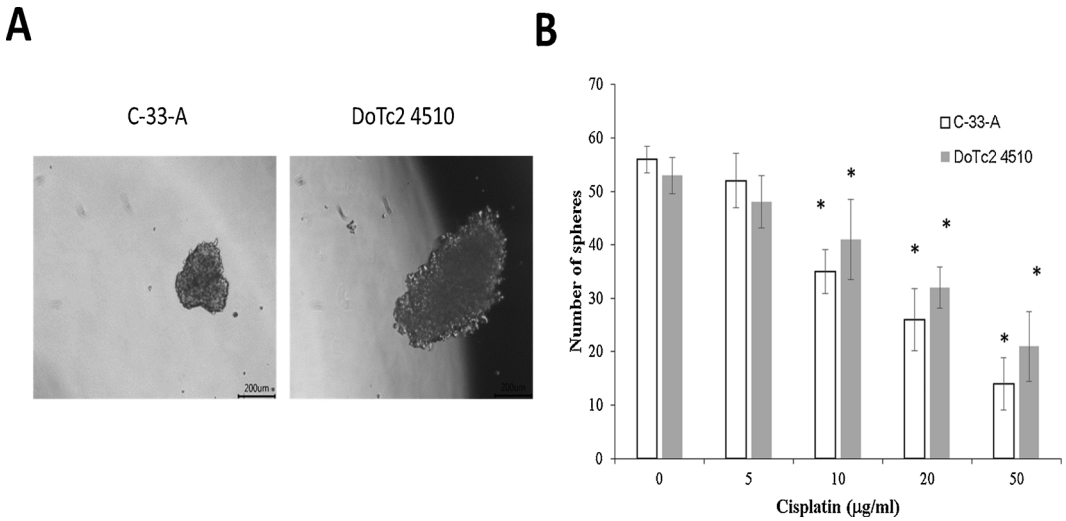

Fig. 2. Effects of cisplatin on cervical cancer stem spheroid cells. (A) Morphology of spheroid cervical cancer stem cells for two weeks incubation. (B) Cytotoxicity of cisplatin on cervical CSCs. Cervical CSCs were treated with various concentrations of cisplatin for one week and the CSCs sphere formation was evaluated. *, $p < 0.05$ was considered to indicate a statistically significant difference.

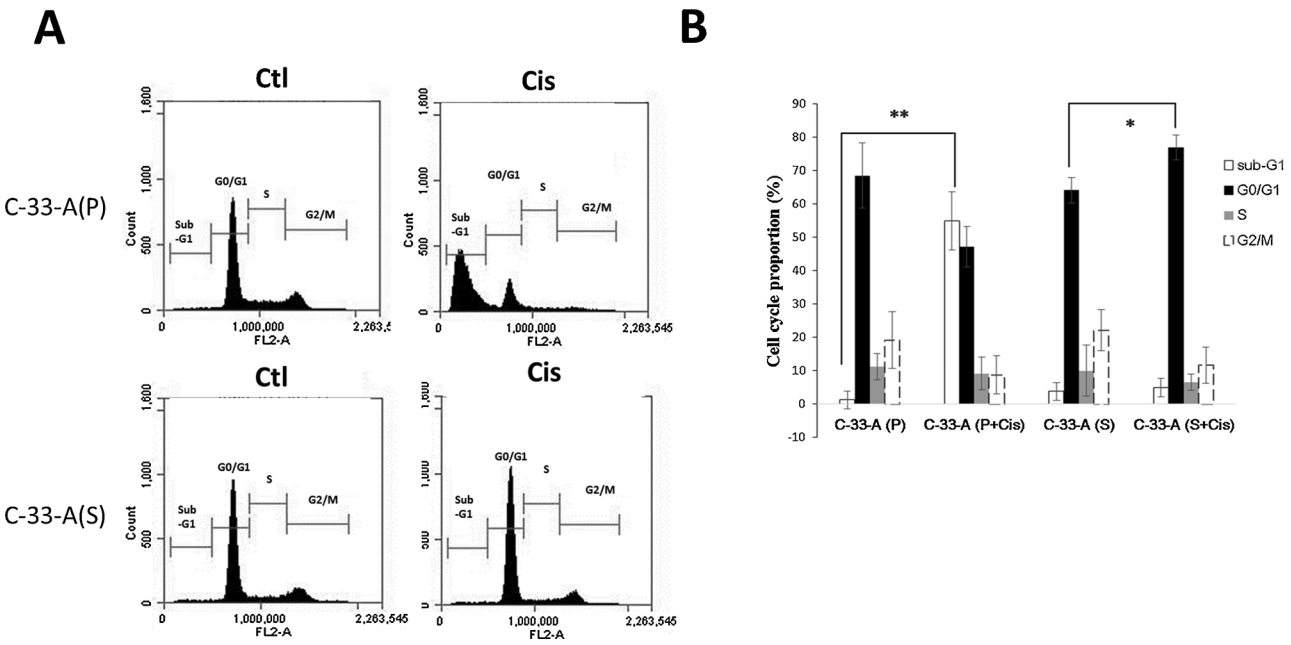

Fig. 3. Effects of cisplatin on apoptotic cell death of cervical parental and CSCs cancer cells. (A) The C-33-A parental (P) and CSCs spheroid (S) cells were treated with cisplatin (10 μM) for 72 h and the cells were harvested for PI staining and then analyzed by flow cytometer. (B) Data were presented as mean fluorescence intensity (MFI) ± SEM from three independent studies. * and ** represent $p < 0.05$ and $p < 0.01$ as a statistically significant difference, respectively.

shown in Fig. 4B, EGFR expression is associated with a poorer prognosis in cervical patients by analyzing public database from 263 patients (OncoLnc database, which links TCGA survival data to mRNA, miRNA, or lncRNA expression levels, http://www.oncolnc.org). The relapse-free survival rate of patients with higher EGFR expression was significantly worse than those with lower expression ($p = 0.0209$, Fig. 4B).

3.5. *LncRNAs activation by cisplatin involves in drug resistance and CSCs sphere formation*

To investigate the role of lncRNA against cisplatin-induced cytotoxicity, we further treated CSCs with cisplatin for 48 h and then perform a lncRNA array to evaluate the expression of lnRNAs. As shown in Fig. 5A, top five considerably up-regulated lncRNAs (HOXA-AS2, UCA1, HOTAIR, HIF1A-AS1, and IPW) were observed. To confirm the role of these target lncRNAs in cisplatin-induced cytotoxicity, we further transfected the anti-sense inhibitors against these lncRNAs to examine the tumor sphere formation cervical CSCs. The inhibition of these lncRNAs by their anti-sense inhibitors showed a decrease in the formation of cervical CSCs spheres for one-week incubation (Fig. 5B).

4. Discussion

It has been suggested that long non-coding RNAs play as key regulators of epithelial-mesenchymal transition, drug resistance and cancer stemness [21]. However, there are only few studies exploring the relationship of lncRNAs and EGFR pathway. According to the current studies, we demonstrated that lncRNAs, which significantly induced by cisplatin might play critical roles in drug resistance and spheroid formation of cervical CSCs. It was reported that a rare population of $CD44^+/CD24^+$ cervical cancer resist cell apoptosis induced by irradiation and possessed the characteristics of stem cells [22]. Furthermore, these stem cells were more resistant to radiotherapy than the parental cells. As compared to cervical parental cancer cells, we found that cervical CSCs spheres are more resistant to cisplatin, and EGFR were higher expressed in CSCs spheres. It was suggested that EGFR/SRC/AKT signaling pathway can modulate SOX-2 expression and self-renewal of stem-like side-population cells in non-small cell lung cancer [23]. Thus, the combination of EGFR antagonists and AKT inhibitors would be benefit for targeting cancer stem cells [13].

Recently, it has been reported that ubiquitin-specific protease 22 promotes resistance to EGFR-TKIs by preventing ubiquitination-mediated EGFR degradation through AKT/mTOR, and MEK/ERK pathways

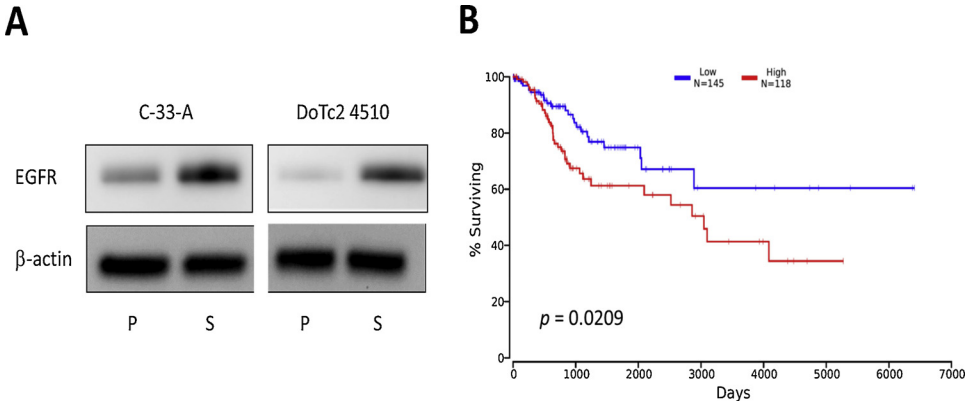

Fig. 4. EGFR expression in CVC parental and spheroid cells and the correlation between EGFR and survival rate in patients with CA. (A) Comparison of EGFR in CVC parental and CSCs spheroid cells by Western blot assay. (B) Higher EGFR expression is associated with poor prognosis in cervical cancer patients. A web-based software package, OncoLnc database (http://www.oncolnc.org), consisting of 263 cervical cancer patients' EGFR expression and survival information was used for this analysis.

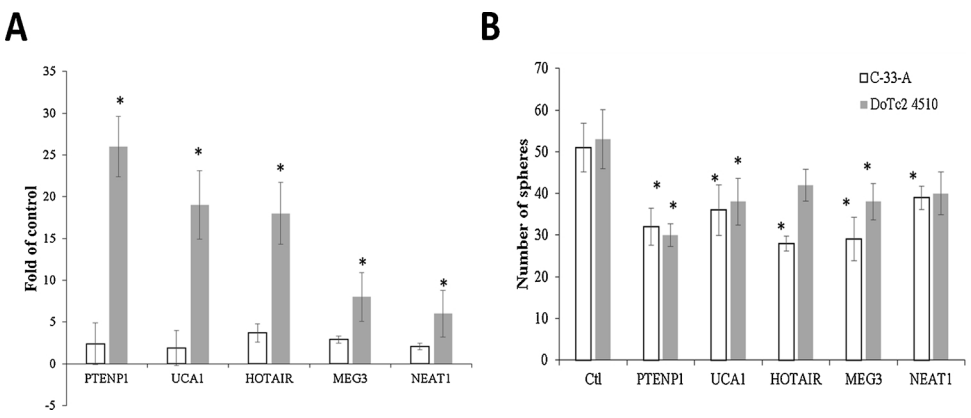

Fig. 5. Effects of cisplatin on long non-coding RNA expression in cervical cells. (A) The C-33-A cells were treated with or without cisplatin (10 μM) for 48 h and the cells were harvested for LncRNA array assay. The data were normalized to untreated cervical parental cells. Gray and blank bars represent cervical CSCs sphere cells treated with or without cisplatin. (B) Before the treatment of cisplatin (10 μM), the cervical C-33-A and DoTC2 4510 CSCs cells were treated with targeted lncRNA anti-sense inhibitors and then treated with cisplatin for one week and the CSCs sphere formation was evaluated. * and ** represent $p < 0.05$ and $p < 0.01$ as a statistically significant difference, respectively.

[24]. It is also revealed that lncRNA HOXD cluster antisense RNA1 (HOXD-AS1) was downregulated in most colorectal cancer (CRC) specimens and cell lines, while MAPK/AKT signalling pathways were activated and resulted in the CRC tumourigenesis and metastasis [25]. Furthermore, the data from the LncRNA array assay revealed that the lncRNAs activated by cisplatin are all involved in the central regulator, AKT, and the results were similar to previous studies related to other solid tumors [26–29]. In non-small cell lung cancer, it is suggested that a positive correlation between the expression of PI3K/AKT pathway and EGFR [30]. Furthermore, it is suggested that tumour suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN) negatively regulates EGRF endocytic trafficking and degradation by converting phosphatidylinositol -3,4,5-trisphosphate (PIP3) to phosphatidylinositol-4,5-bisphosphate (PIP2) at the cellular membrane and thereby negatively regulates oncogenic PI3K-AKT signaling [31].

Non-coding RNA dysregulation affects the metastatic process, including the epithelial-mesenchymal transition, tumor stemness, metastasis, and oncogene and tumor suppressor expression. Non-coding RNAs also affect angiogenesis in the microenvironment of cancer. Furthermore, some microRNAs act as potential biomarkers for use in clinical screening, early diagnosis and prognosis of cancer [32]. Unfortunately, relatively less ncRNAs have been exactly identified as targets for the treatment of CVC [33]. Cisplatin is one of the most potential and conventionally used drugs for the treatment of various solid cancers including cervical cancer. However, side effects and drug resistance are two of the inevitable challenges of cisplatin which limit its application and effectiveness [34]. To decrease cisplatin side effects and resistance by lowering the dose of cisplatin and applying the combination therapies are used and have proven more effective to defect cancers. It has been suggested that radiotherapy plus platinum-based doublet therapy can improve survival rate in patients with locally advanced cervical cancer [35]. Furthermore, as compared to HPV-positive CVC, HPV-negative tumors are diagnosed at advanced stages, show higher prevalence of lymph nodes metastases and have an impaired prognosis [36].

Interestingly, it has been revealed that lncRNA-mediated cytotoxicity against cervical squamous cell carcinoma was in an HPV-dependent manner [37]. Thus, targeting ncRNAs might have potential in CVC treatment and diagnosis. Furthermore, targeting CSCs would improve the outcome of radio-/chemotherapy to CVC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The study was approved by the ethics committee of Shaoxing Maternity and Child Health Care Hospital and All patients provided written informed consent.

Patient consent for publication

Written informed consent was obtained from all participants for the publication of any data and associated images.

Declaration of Competing Interest

The authors declare that they have no competing interests

Acknowledgements

Not applicable.

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