



Long non-coding RNA ENST00000457645 reverses cisplatin resistance in CP70 ovarian cancer cells

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ABSTRACT. The objective of this study was to investigate the effect of downregulating long non-coding RNAs (lncRNAs) on the reversal of cisplatin resistance in CP70 ovarian cancer cells, and to identify the underlying mechanism(s) of action. An lncRNA microarray was performed to screen for downregulated lncRNAs in cisplatin-resistant CP70 cells. Expression levels of these lncRNAs were then verified in SKOV3 and SKOV3/DDP cells. Quantitative polymerase chain reaction was conducted to identify the lncRNA most downregulated, which was then synthesized and transfected into CP70 cells. To assess the viability and migration ability of these transfected CP70 cells, methyl thiazolyl tetrazolium and Transwell assays were carried out. In addition, expression levels of apoptosis-related proteins were examined by western blotting. The lncRNA microarray analysis and qPCR identified seven lncRNAs that were significantly downregulated. Transfection of lncRNA ENST00000457645 into CP70 cells markedly inhibited

viability and migration ability, and significantly increased expression of apoptotic proteins such as Bax and cleaved caspase-3. lncRNA ENST00000457645 negatively affects the viability and migration of cisplatin-resistant CP70 ovarian cancer cells. The mechanism responsible involves modification of apoptotic protein expression.

Key words: CP70 ovarian cancer cell; Cisplatin resistance; lncRNA; Apoptotic protein

INTRODUCTION

Ovarian cancer is one of the most common gynecologic malignant tumors. Previous epidemiological studies have indicated that approximately 200,000 new cases are diagnosed every year worldwide, and the associated mortality rate is over 60% (Harter et al., 2014). Debulking surgery combined with chemotherapy is the currently preferred treatment for this disease (Tentes et al., 2012); however, over 70% of patients exhibit resistance to chemotherapy following surgery, critically restricting curative care (Mantia-Smaldone et al., 2011; Huang et al., 2016). Recently, long non-coding RNAs (lncRNAs) have been proposed as a novel approach to tumor treatment (Chen et al., 2016), and a number of studies have suggested a close relationship between aberrant lncRNA expression and the development and progression of cancer (Meseure et al., 2015; Li et al., 2016). lncRNAs are a class of RNAs similar in structure to mRNAs, and are over 200 bp in length. These molecules do not encode proteins, but are able to regulate the expression of a variety of genes (Zhao and Lin, 2015).

lncRNAs are known to be involved in tumor formation and progression (Qiu et al., 2015; Cao et al., 2016). Numerous lncRNAs demonstrate modified expression during the development of drug resistance, suggesting that they play an important role in this process in tumor cells (Wang et al., 2015). At present, the role of lncRNAs in platinum resistance in ovarian cancer is not fully understood. Therefore, in this study, we aimed to screen for lncRNAs significantly downregulated in cisplatin-resistant ovarian cancer cell lines, investigate the effect of these lncRNAs on the reversal of drug resistance, and identify the underlying molecular mechanism(s) of action.

MATERIAL AND METHODS

Cells

All cell lines (A2780, CP70, SKOV3, and SKOV3/DDP) were cryopreserved in the Department of Gynecology and Obstetrics at Linyi Central Hospital.

Reagents

Fetal bovine serum (FBS) was obtained from Gibco (Waltham, MA, USA); RPMI-1640 medium was purchased from HyClone (Logan, UT, USA); polymerase chain reaction (PCR) primers were designed and synthesized by Sangon Biotech (Shanghai, China); RNA extraction and RNeasy Mini kits were purchased from QIAGEN (Dusseldorf, Germany); a RevertAid first strand complementary DNA (cDNA) synthesis kit was acquired from Fermentas

(Burlington, Canada); SYBR *Premix Ex Taq* II was provided by TaKaRa (Mountain View, CA, USA); TransFast transfection reagent was manufactured by Promega (Madison, WI, USA); a cell proliferation and cytotoxicity assay kit was purchased from CellChip Biotech (Beijing, China); Transwell plates were obtained from Millipore (Billerica, MA, USA); and all antibodies were produced by Cell Signaling Technology (Danvers, MA, USA).

Methods

Cell culture

Cells of lines A2780, CP70, SKOV3, and SKOV3/DDP were rapidly thawed and maintained in RPMI-1640 medium supplemented with 10% FBS at 37°C in a humidified 5% CO₂ atmosphere. Cells were passaged 2-3 days after seeding, when 70-80% confluent. Cells in the log growth phase were used for the experiments described below.

Extraction and purification of total RNA

When in the log phase, A2780, CP70, SKOV3, and SKOV3/DDP cells were harvested and centrifuged at 300 g for 5 min. Cells were lysed in TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) for 5 min, then lysates were mixed with 0.2 mL chloroform for 15 s and left to settle at 20°C for 2-3 min. Subsequently, this mixture was centrifuged at 2000 g for 15 min. The upper aqueous layer was collected, mixed with 0.5 mL isopropanol, and left to stand for 10 min. Following centrifugation at 12,000 g for 10 min, the supernatant was discarded and the pellet resuspended in 1 mL 75% ethanol. After thorough vortexing, the solution was centrifuged at 7500 g for 5 min. The resulting RNA pellet was air-dried and resuspended in RNase-free water, before being left to dissolve for 10 min. RNA concentration was determined and RNA samples were stored at -20°C. The purity of total RNA extracts was measured using a QIAGEN RNeasy Mini Kit according to the manufacturer instructions.

Analysis of lncRNA microarray

Total RNA extracted from A2780 and CP70 cells was labeled with a fluorochrome using the Quick Amp Labeling Kit (Agilent, Santa Clara, CA, USA) according to the manufacturer instructions. A Gene Expression Hybridization Kit (Agilent) was then used to hybridize the RNA to probes on the microarray. The microarray was washed with Gene Expression Wash Buffer (Agilent) using a magnetic stirrer (Corning, Corning, NY, USA), and signals were detected with a Microarray Scanner (Agilent). The microarray experiment was conducted by KangChen Biotech (Shanghai, China; test No. H1312088).

Quantitative PCR (qPCR)

Significantly downregulated lncRNAs were verified by qPCR in SKOV and SKOV3/DDP cells. Reverse transcription of total RNA was conducted using a cDNA synthesis kit and the following conditions: 25°C for 5 min, 42°C for 60 min, and 70°C for 5 min. DNA amplification was subsequently carried out using a SYBR *Premix Ex Taq* II kit in an ABI 7500 fluorescent qPCR system (Applied Biosystems, Foster City, CA, USA). β -actin was used as an internal reference.

Transfection of lncRNA

CP70 cells were harvested, counted, and seeded on cell culture dishes at a density of 0.8×10^6 cells per dish. lncRNA was mixed with liposomes at an appropriate ratio (1:1-2, liposome volume:lncRNA mass) in tubes containing serum-free medium, and the mixture was allowed to settle for 30 min. Following addition of an appropriate volume of transfection reagent, the mixture was oscillated and left to settle for 10-15 min at room temperature. After removal of medium from the culture dishes, the transfection mixture and complete medium were added and incubated with cells for 24 h at 37°C. The medium was then replaced with fresh culture medium.

Methyl thiazolyl tetrazolium (MTT) assay of cell viability

A2780, CP70, SKOV3, and SKOV3/DDP cells in the log growth phase were harvested, adjusted for density, and seeded on 96-well plates (2×10^4 cells/well). The cells were then exposed to different concentrations (20, 40, 60, and 80 μM) of cisplatin, and an untreated control group was processed simultaneously. Cells transfected with the lncRNA of interest were exposed to 60 μM cisplatin, and negative and CP70 control groups were treated alongside. All cells were incubated at 37°C for 48 h, before being exposed to 5 g/L MTT for 4 h. After discarding supernatants, 150 μL dimethyl sulfoxide was added to each well. For thorough dissolution of formazan, the plates were incubated with shaking for 10 min. Optical density at 570 nm was then determined using an automatic plate reader.

Western blot analysis of apoptosis-related proteins

lncRNA-transfected cells were administered 60 μM cisplatin for 48 h at 37°C. Negative and CP70 control groups were treated simultaneously. Cells were subsequently harvested and lysed to extract total protein, the concentration of which was measured using a bicinchoninic acid protein quantification kit. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked with blocking buffer for 1 h, and incubated overnight at 4°C with a primary antibody against Bax, Bcl-2, caspase-3, cleaved caspase-3, or β -actin (diluted 1:1000). After being washed, membranes were incubated with a secondary antibody (1:500) for 1 h, washed again, and visualized. Densitometric analysis of target bands was performed using Quantity One software (Bio-Rad, Hercules, CA, USA).

Transwell assay of cell migration

After being harvested, 1×10^5 transfected CP70 cells were placed in the upper Transwell chamber, along with 60 μM cisplatin. RPMI-1640 medium (600 μL) containing 10% FBS was added to the lower chamber, and the cells were incubated for 48 h at 37°C. Cells on the inner surface of the upper chamber membrane were subsequently gently wiped off, and those on the lower surface were fixed in methanol for 30 min, air-dried, and stained with hematoxylin. Cells were counted under a microscope in five random fields of view (at 200X magnification).

Data analysis

Data were analyzed using the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Pairwise comparisons were conducted using the Student *t*-test, and comparisons between multiple groups were carried out by one-way analysis of variance. P values < 0.05 were considered statistically significant. Microarray data were analyzed using the GeneSpring GX v12.0 software package (Agilent).

RESULTS

Differential expression of lncRNAs in A2780 and CP70 cells

In this study, we examined the cisplatin sensitivity of A2780 and CP70 ovarian cancer cells. As shown in Figure 1A, the apoptosis rate of CP70 cells was significantly lower than that of A2780 cells ($P < 0.05$), indicating greater cisplatin resistance in the former.

Microarray analysis of A2780 and CP70 cells revealed that a large number of lncRNAs were differentially expressed to a statistically significant degree ($P < 0.05$). Among the 1902 differentially expressed lncRNAs, 1033 were upregulated and 869 downregulated in A2780 compared to CP70 cells. Of the lncRNAs identified, 298 and 307 exhibited increases and decreases in expression of more than two-fold, respectively, while levels of 14 and 11 sequences were raised and lowered more than five-fold, respectively (as partially listed in Table 1). Principal component analysis was used to simplify the microarray data into fewer dimensions, classifying it into clusters. This analysis confirmed the reliability of our experimental design and microarray data (Figure 1B).

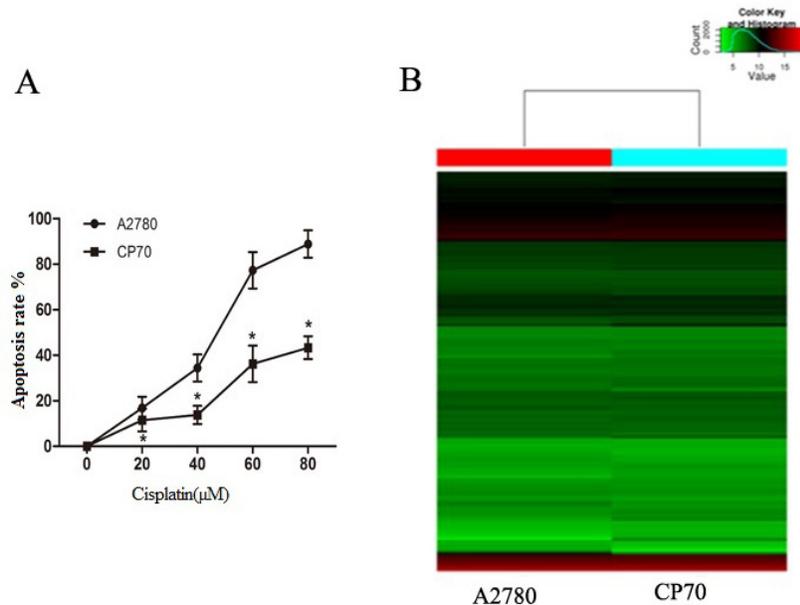


Figure 1. A. Cisplatin sensitivity in A2780 and CP70 cells. B. Differential expression of long non-coding RNAs in A2780 versus CP70 cells. * $P < 0.05$ compared with A2780 cells.

Table 1. Differential expression of long non-coding RNAs in A2780 versus CP70 cells.

Probe name	FC (abs)	Regulation
TCONS_00003617	17.45006	Up
ENST00000424251	9.3812	Up
ENST00000446495	682495	Up
ENST00000547898	6.696175	Up
ENST00000555539	7.950607	Up
UC002rlw.3	5.9099	Up
TCONS_00006578	3.69445	Up
ENST00000424410	3.7696	Up
NR_028308	8.285297	Up
TCONS_00003974	5.634886	Up
ENST00000449694	9.42863	Down
NR_038435	7.26851	Down
TCONS_00021730	5.30137	Down
ENST00000457645	19.38386	Down
ENST00000511064	9.37823	Down
ENST00000558952	10.376053	Down
ENST00000400178	14.298271	Down
NR_046438	9.692917	Down
ENST00000458468	8.127298	Down
ENST00000422352	7.609907	Down

FC (abs) = absolute fold change.

Verification of downregulated lncRNAs in SKOV3 and SKOV3/DDP cells

Figure 2A shows the cisplatin sensitivity of SKOV3 and SKOV3/DDP ovarian cancer cells. Our results indicated that the former were sensitive to cisplatin, whereas the latter were highly resistant. Therefore, both cell lines were used for further screening.

The downregulated lncRNAs identified by microarray analysis were screened in SKOV3 and SKOV3/DDP cells. As shown in Figure 2 (which does not feature all of the lncRNAs tested), seven lncRNAs exhibited significant differences in expression consistent with the microarray data: ENST00000449694, ENST00000413580, NR_038435, ENST00000457645, NR_038464, ENST00000458468, and TCONS_00021730. Of these, the most pronounced downregulation was observed in relation to ENST00000457645.

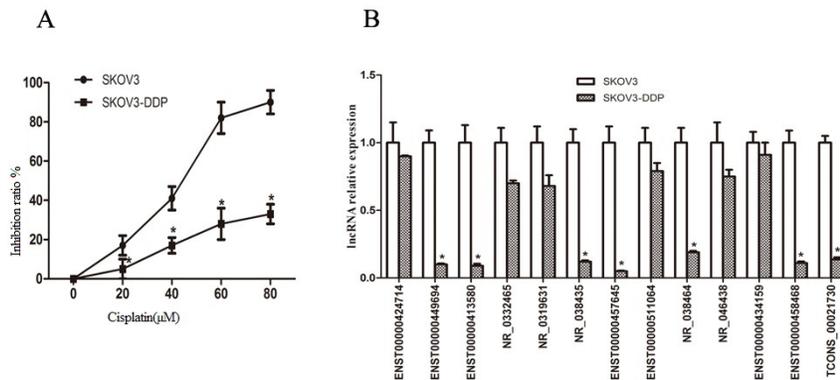


Figure 2. A. Inhibitory effect of cisplatin on SKOV3 and SKOV3/DDP cells. **B.** Verification of downregulated long non-coding RNAs by quantitative polymerase chain reaction. *P < 0.05 compared with SKOV3 cells.

Effect of ENST00000457645 on apoptosis of CP70 cells

ENST00000457645 was synthesized and added to a transfection cocktail, which was administered to CP70 cells to assess the effect of this lncRNA on cisplatin resistance. The MTT assay indicated that the viability of ENST00000457645-transfected cells was significantly lower than that of negative control (NC) cells transfected with the empty vector ($P < 0.05$; Figure 3A), implying that ENST00000457645 promoted CP70 cell apoptosis.

Expression levels of apoptosis-related proteins were examined by western blotting, which showed that Bax was significantly upregulated in ENST00000457645-transfected cells, resulting in an increased Bax/Bcl-2 ratio (Figure 3B and C). In addition, levels of cleaved caspase-3 were markedly elevated in ENST00000457645-transfected cells, and the ratio of cleaved caspase-3 to caspase-3 was also significantly increased ($P < 0.05$; Figure 3B and D). These results suggest that overexpression of ENST00000457645 may promote apoptosis of cisplatin-resistant CP70 cells, and that the mechanism responsible may involve the regulation of apoptotic proteins.

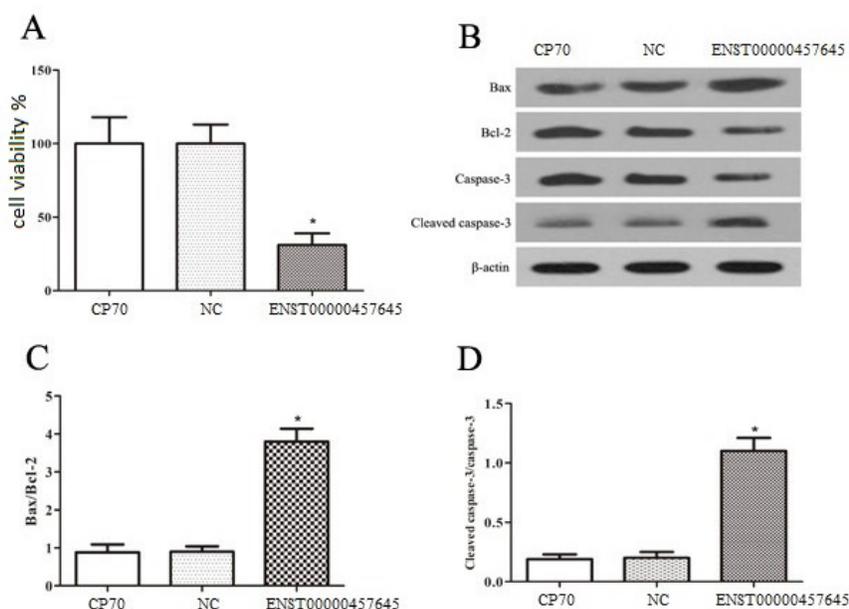


Figure 3. Effect of ENST00000457645 transfection on apoptosis of CP70 cells. **A.** Viability of CP70 cells. **B.** Western blot analysis of apoptosis-related protein expression. **C.** Bax/Bcl-2 ratio. **D.** Cleaved caspase-3/caspase-3 ratio. CP70: normal CP70 cell group; N C: empty vector-transfected negative control group; ENST00000457645: ENST00000457645-transfected group. * $P < 0.05$ compared with the N C group.

Effect of ENST00000457645 on CP70 cell migration

A Transwell assay was performed to explore the effect of ENST00000457645 on CP70 cell migration and invasion. Compared with the NC group, the number of migrating CP70 cells was significantly decreased following transfection of ENST00000457645, indicating that this lncRNA may inhibit cisplatin resistance-associated migration of CP70 cells (Figure 4).

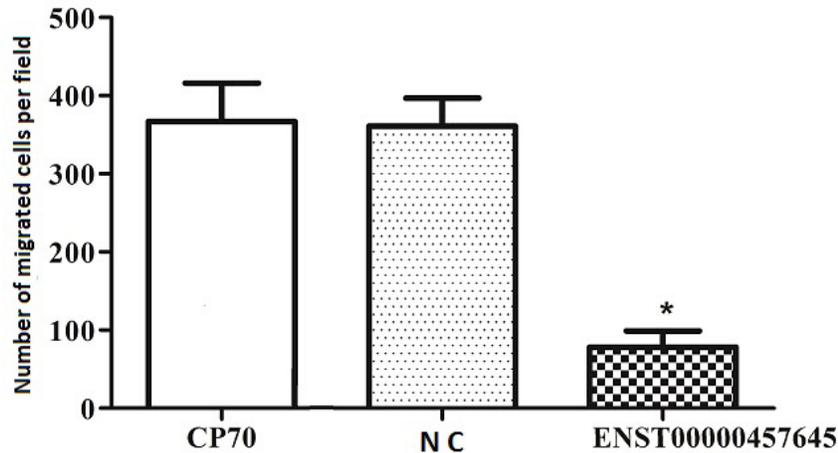


Figure 4. Effect of ENST00000457645 transfection on the migration of CP70 cells. CP70: normal CP70 cell group; NC: empty vector-transfected negative control group; ENST00000457645: ENST00000457645-transfected group. * $P < 0.05$ compared with the NC group.

DISCUSSION

As the development of biochips and sequencing technologies has advanced, it has become apparent that lncRNAs play critical roles in the pathogenesis of multiple diseases (Archer et al., 2015; Kawasaki et al., 2016). Although lncRNAs do not encode proteins, they do regulate a variety of genes that participate in cell signaling, controlling the expression of further genes, and cellular activities such as chromatin modification, epigenetic marking, and protein folding and activation (Froberg et al., 2013; Bassett et al., 2014). Moreover, lncRNA expression is regulated at both transcriptional and post-transcriptional levels. The selection pressure to which lncRNAs are exposed is relatively low, resulting in a high degree of sequence variation and diversity (Peng and Jiang, 2016). Aberrant lncRNA expression may cause numerous diseases (Bhartiya et al., 2012). In a number of investigations, abnormal lncRNA levels have been observed in tumor cells (Gao et al., 2015), which may influence tumor formation, progression, and metastasis (Bhartiya et al., 2012; Xu et al., 2014). Among the tumor-associated lncRNAs, HOTAIR, H19, HOST2, LSINCT5, and OVAL have been reasonably well studied (Gao et al., 2015; Zhang et al., 2015). Differential expression of tumor-associated genes is closely related to tumorigenesis and tumor progression. For example, certain lncRNAs can act as oncogenes when upregulated via unique mechanisms, thereby promoting the survival of tumor cells (Yang et al., 2012). Furthermore, downregulated lncRNA expression can inhibit the transcription of tumor-suppressor genes by restricting protein activity, thereby contributing to drug resistance in tumor cells (Huang et al., 2013). Under normal circumstances, gene expression tends towards a state of metabolic equilibrium in response to an anti-tumor drug. As the duration of chemotherapy extends, tumor cells may alter mechanisms of intracellular transport, DNA repair, or inhibition of apoptosis, resulting in drug resistance (Augoff et al., 2012). Such resistance during anti-tumor therapy has become a major clinical challenge, and better understanding of tumor-specific lncRNAs may lead to the identification of novel therapeutic targets.

In the present study, analysis of microarray data revealed a number of significantly

up- and downregulated lncRNAs in CP70 cells. These lncRNAs may be involved in the development of cisplatin resistance in ovarian cancer. Secondary screening using cisplatin-resistant SKOV3/DDP cells revealed seven lncRNAs significantly downregulated in a manner consistent with the microarray data. Transfection of the lncRNA ENST00000457645 into CP70 cells remarkably reversed their cisplatin resistance and reduced their viability and migration ability. These changes were closely associated with altered levels of apoptotic proteins.

In this study, we investigated the lncRNA ENST00000457645, which is aberrantly expressed in cisplatin-resistant cells. We demonstrated that ENST00000457645 can augment the reduced viability and migration of CP70 ovarian cancer cells induced by cisplatin. We suggest that the underlying mechanism may be related to apoptotic protein expression levels. Our study provides the foundation for further investigation of lncRNAs and their effects on drug resistance in tumors. However, such resistance also involves lncRNA-related proteins and cell phenotypes other than those examined here. Moreover, this study focused on *in vitro* assays, whereas the extremely complicated milieu of the human body necessitates consideration of multiple interactions among myriad factors. Therefore, further investigation is clearly warranted to validate the effect and mechanism of action of lncRNA ENST00000457645 on drug resistance in ovarian cancer.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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