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Downregulation of microRNA-630 inhibits cell proliferation and invasion and enhances chemosensitivity in human ovarian carcinoma

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ABSTRACT. MicroRNAs (miRNAs) are a family of small non-coding RNAs (approximately 21-23 nt long) that can target genes for either degradation of mRNA or inhibition of translation. miRNAs have not been comprehensively studied in human epithelial ovarian carcinoma (EOC). MicroRNA-630 (miR-630) has been frequently observed to be aberrantly expressed in various types of tumors. The present study explored the functions of miR-630 in the proliferation, apoptosis, chemosensitivity, and invasion of EOC. Using real-time polymerase chain reaction, we detected the miR-630 expression in cancerous, benign, and normal human ovarian tissues. Then, we evaluated the role of miR-630 in cell proliferation, chemosensitivity, apoptosis, and invasion by using the Cell Counting Kit-8, Annexin-V/FITC, and transwell assay on A2780 and SKOV3 cells. Western blotting was performed for analyzing the phosphatase and tensin homolog gene (PTEN) protein expression.

The miR-630 expression level was higher in ovarian cancerous tissues than in benign and normal ovarian tissues. Decreased expression of miR-630 suppressed EOC cells' proliferation, migration, and invasion as well as significantly enhanced cell apoptosis and chemosensitivity to cisplatin. Furthermore, PTEN expression was increased in A2780 cells transfected by miR-630 inhibitor in comparison with inhibitor-negative control-transfected cells. In conclusion, downregulation of miR-630 dramatically increased apoptotic cell death chemosensitivity to cisplatin and decreased the proliferation, invasion, and migration of EOC cells. MiR-630 may thus play an important role in the biological behaviors of EOC cells through negative control of the PTEN expression.

Key words: MicroRNA-630; Proliferation; Invasion; Chemosensitivity; Phosphatase and tensin homolog; Epithelial ovarian cancer

INTRODUCTION

Epithelial ovarian carcinoma (EOC) is one of the leading causes of morbidity and mortality due to gynecological malignancies in the world. In most cases, this cancer is not diagnosed until the disease reaches an advanced stage. Despite great advances in chemotherapy and surgical treatment of ovarian cancer, 70-90% patients with ovarian cancer develop relapse or metastasis, and the 5-year survival rate of patients with advanced ovarian cancer with peritoneal metastasis remains at approximately 30% (Vaughan et al., 2011). Ovarian cancer patient suffer from poor prognosis, largely due to the aggressive metastasis in the peritoneal cavity and the emergence of drug resistance (Williams et al., 2007). Improved understanding of the mechanism involved in ovarian cancer metastasis and drug resistance is of great importance in overcoming this malignance.

MicroRNAs (miRNAs) are a class of small, endogenous, non-coding RNAs (21-23 nt) that act as negative regulators of gene expression at the post-transcription levels by either preventing translation or causing degradation (Bartel, 2004; Suzuki et al., 2012). These small RNAs negatively regulate approximately one-third of the human coding genes by binding to the complementary sites of the 3'-untranslated region (3'-UTR) of their target genes (Meister et al., 2004). Accumulating evidence has shown that miRNAs play vital roles in the progression of various cancers, including human ovarian cancer (Lu et al., 2005; Garzon et al., 2006; Jansson and Lund, 2012; Nana-Sinkam and Croce, 2013). In human ovarian cancer, several miRNAs with aberrant expression have been identified, including miR-21, miR-200, miR-22, miR-183, and miR-141, which play oncogenic or suppressive roles (Leskela et al., 2011; Mateescu et al., 2011; Li et al., 2012; Chan et al., 2014).

Recent reports have indicated that deregulation of miR-630 occurs in various cancers. Patnaik (2010) investigated the expression profiles of miRNA in stage I non-small cell lung cancer to identify patterns that may predict recurrence after surgical resection and found miR-630 to be one of the most common miRNA among the 1000 identified classifiers. Another study demonstrated that the miR-630 expression was an independent prognostic factor for patients with gastric cancer, which may be a potential valuable biomarker for gastric cancer (Chu et al., 2014). Huang et al. (2011) reported that miR-630 is induced by cisplatin and 3-Cl-

AHPC and that it causes apoptosis in the head and neck squamous cell carcinoma cells by targeting different molecules such as BCL2 and BCL2L2. It has been reported that miR-630 acts as a regulator downstream of phospho-ΔNp63α in autophagy (Huang et al., 2012). However, only few studies have reported about the role of miR-630 in human ovarian cancer until date.

The aim of the present study was to investigate the potential role of miRNA-630 in ovarian cancer that could be related to tumor progression and used as a prognostic marker or even as a potential target for developing new therapies.

MATERIAL AND METHODS

Cell lines and cell cultures

Human epithelial ovarian cancer cell lines A2780 and SKOV3 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (SiJiQing, Beijing, China), 100 IU/mL penicillin, and 100 μg/mL streptomycin under a humidified atmosphere incubator of 5% CO_2 at 37°C.

Tissue samples

A total of 53 cancerous, 25 benign, and 20 normal human ovarian tissues were sampled from patients who had undergone surgery at the second affiliated hospital of the Harbin Medical University between 2012 and 2014. The normal ovarian tissues were obtained from patients who had undergone ovarian wedge biopsy or adnexectomy due to myoma or adenomyosis. After surgery, the tissues were snap-frozen in liquid nitrogen and stored at -80°C until further use. None of the patients had ever undergone chemotherapy or biotherapy, and the diagnoses were confirmed pathologically in all cases.

The study was approved by the ethics committee of the hospital, and informed consent was obtained from each patient by the research team prior to performing surgery.

Cell transfection of the microRNA inhibitor

A2780 and SKOV3 cells were transfected with miR-630 inhibitor (miR-630i) and microRNA inhibitor negative control (NCi) (GenePharma Co., Ltd., Shanghai, China) with Lipofectamine 2000 transfection reagent (Invitrogen, Grand Island, NY) according to the manufacture protocols. The sequences were as follows: miR-630i sequence was 5'-ACCUUCCCUGGUACAGAAUACU-3' and NCi sequence was 5'-CAGUACUUUUGUGUAGUACAA-3'. The transfection efficiency was evaluated by real-time polymerase chain reaction (PCR) after 48 h of transfection.

RNA extraction and real-time PCR analysis

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer specification. The miR-335 expression level was analyzed by using the miRcute miRNA First-Strand cDNA Synthesis Kit (Tiangen, Beijing, China). Real-time PCR (qRT-PCR) was performed by using the SYBR Green miRcute miRNA qPCR Detection Kit

(Tiangen) with miR-630 specific primer and U6 specific primer (Tiangen). Human U6 served as the reference gene for normalization. QRT-PCR was performed by the C100™ Thermal Cycler (Bio-Rad, Richmond, CA, USA). The expression of miR-630 was defined from the threshold cycle, and the relative expression level was measured by using the $2^{-\Delta\Delta CT}$ method. All real-time PCR were run in triplicates.

Cell proliferation analysis

Cell proliferation was determined by using the cck-8 Kit (Dojindo Laboratories, Kumamoto, Japan) according to manufacturer protocols. Briefly, cells were seeded (10^5 cells/well) on 6-well plates and cultured for 24 h before transfection. After 24 h of transfection of the miR-630i or NCi, the cells were digested and plated on 96-well plates at the density of 3,000 cells/well. After 48 h, 10 µL of the CCK-8 solution was added to each well for measuring the cell viability. After 2 h incubation, the absorbance of the plate was measured at 450 nm on a microplate reader (Model 680, Bio-Rad). Each assay was performed in 5 replicates in 3 independent experiments.

Chemosensitivity assay

The rates of sensitivity to cisplatin were determined by the cell proliferation reagent CCK-8. After 48 h of transfection, the cells were digested and plated on 96-well plates at a density of 3000 cells/well. After overnight incubation, the cells were treated with cisplatin (10 nM) (Mayne Pharma Pty Ltd, Australia). The cell viability was measured after 72 h by CCK-8 method, as described above. The number of viable cells was evaluated by measuring the absorbance at 450 nm. Each assay was performed in 5 replicates in 3 independent experiments.

Migration and invasion assays

The migration of A2780 and SKOV3 cells were measured on matrigel-uncoated transwell chambers (BD Biosciences, Bedfold, MA, USA) with 8-µm pores on 24-well cell culture plates. Approximately 1 x 10^4 cells transfected with miR-630i or NCi were seeded on the upper chambers with 100 µL serum-free RPMI1640. The lower chamber filled with 600 µL RPMI1640 supplemented with 5% FBS served as the chemoattractant solution. After 24 h, cells that invaded the lower surface of the transwell membrane were fixed with 100% methanol for 15 min, followed by staining with 0.1% crystal violet solution for 20 min. Pictures were captured under a wild-field microscope. The cell numbers in 5 random fields were counted for each chamber, and the average value was calculated.

For invasion assays, the infected cells (4 x 10^5) were plated in the top chamber with matrigel-coated membrane, whereas the bottom chambers were filled with 10% FBS. After 48 h-incubation, the number of migrated cells (at the lower side of the membrane) was counted, as described above.

Cell apoptosis assays

Cells were plated on 6-well plates and transfected with miR-630i or NCi. The apopto-

sis ratio was analyzed at 48 h and 72h after transfection by using the AnnexinV FITC Apoptosis Detection Kit (BD Biosciences) according to manufacturer instructions. Briefly, 1 x 10^5 treated cells were incubated with annexin V/propidium iodide for 15 min at room temperature. Cells were sorted by using a FACScalibor (BD Biosciences) and analyzed with the CellQuest (version 3.3) software. The apoptotic cells were calculated after FACS analysis. The tests were repeated in triplicates.

Western blotting

The A2780 cells were transfected for 72 h as previously described. The cells were lysed with RIPA lysis buffer, and the proteins were harvested. The protein concentrations of lysates were measured by using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Samples (40 mg) were loaded in 10% SDS-PAGE and then transferred on nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline, pH 7.4, containing 0.05% Tween-20, and then incubated with phosphatase and tensin homolog gene (PTEN) antibody (Santa Cruz Biotechnology, Santa Cruz, USA) and GAPDH polyclonal antibody (Santa Cruz Biotechnology) at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (ZSGB-Bio, Beijing, China). The protein was visualized with ECL western blot detection reagents and analyzed by scanning densitometry using the Smart View Analysis software (Shanghai FURI Science & Technology Co., China).

Statistical analysis

Statistical analysis data shown in the graphs are reported as means ± SD of 3 independent experiments. The results of the assay were analyzed by Student *t*-test and one-way ANOVA. The values were considered to be statistically significant at $P < 0.05$. All statistical analyses were conducted by using the SPSS software version 13.0 (SPSS, Chicago, IL, USA).

RESULTS

Evaluation of miR-630 expression in human ovarian cancer tissues

In our study, the miR-630 expression was detected in all 98 cases with real-time RT-PCR, and the relative amount of miR-630 was described by using $2^{-\Delta\Delta CT}$. The results shown in Figure 1 indicate that, among the 53 ovarian cancer samples analyzed, the relative expression of miR-630 was significantly upregulated as compared with that of the benign ovarian cysts and normal ovarian tissues ($P < 0.05$), respectively. No differences were observed in the miR-630 relative expression between the benign ovarian cysts and normal tissue controls.

miR-630 expression in relation to clinicopathological features

As shown in Table 1, the relative expressions of miR-630 were significantly higher in advanced-stage tumor samples than in the early stage tumor samples ($P < 0.05$). The same differences were observed between the lymph node positive group and negative group ($P < 0.01$). However, we did not observe any significant differences in the 3 grades and the 4 histotypes analyzed.

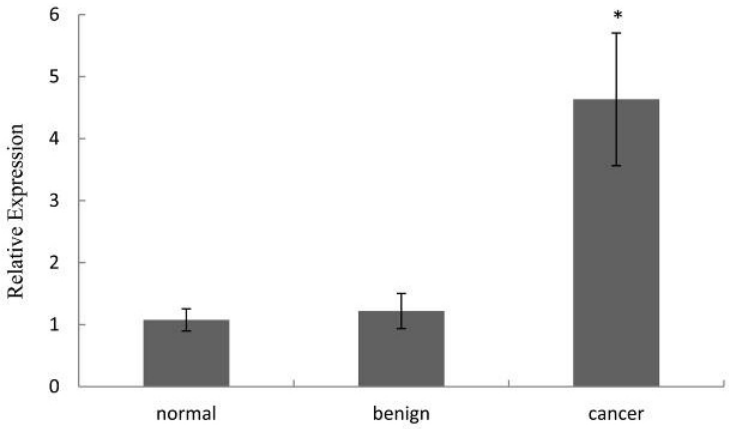

Figure 1. Relative expression of miR-630 in epithelial ovarian cancer, benign ovarian cysts, and normal ovarian tissue. Relative expression of miR-630 was detected by real-time RT-PCR, and the relative amount of miR-630 was determined by using $2^{-\Delta\Delta CT}$. MiR-630 was significantly overexpressed in EOC (*P < 0.05).

Table 1. miR-630 expression in relation to the clinicopathological features in epithelial ovarian cancer.

Parameter	Cases (N)	miR-630 (means ± SD)	P value
FIGO stage			
I, II	15	3.933 ± 1.183	<0.05
III, IV	38	4.911 ± 0.896	
Grade			
G1, G2	26	4.630 ± 1.29	>0.05
G3	27	4.638 ± 0.818	
Lymph node			
Negative	37	4.395 ± 1.060	<0.05
Positive	16	5.246 ± 0.839	
Histology			
Serous	31	4.578 ± 1.180	>0.05
Mucinous	16	4.627 ± 0.899	
Endometrioid	3	4.717 ± 0.964	
Clear cell	3	5.180 ± 1.181	

Validation for miR-630i transfection in A2780 and SKOV3 cell lines

The qRT-PCR results revealed that miR-630i decreased the expression in A2780 and SKOV3 cells (Figure 2; P < 0.005) than in the control cells. However, no statistical significance of miR-630 expression was noted between the miR-630i or NCi and the blank control groups. These results indicated that miR-630i could regulate miR-630 expression effectively in both A2780 and SKOV3 cells. These strategies were used as the basis of the remaining experiments.

Downregulation of miR-630 suppresses cell proliferation and improves sensitivity of the A2780 and SKOV3 cells to cisplatin

The effects of ectopic expression of miR-630 on cell proliferation in A2780 and SKOV3 cells were examined by CCK-8 assay. The CCK-8 results demonstrated that cell pro-

liferation was significantly suppressed by downregulation of miR-630 48 h after transfection as compared with the NCi groups in A2780 and SKOV3 cells (Figure 3; $P < 0.05$). These results demonstrated that miR-630 could modulate cell proliferation.

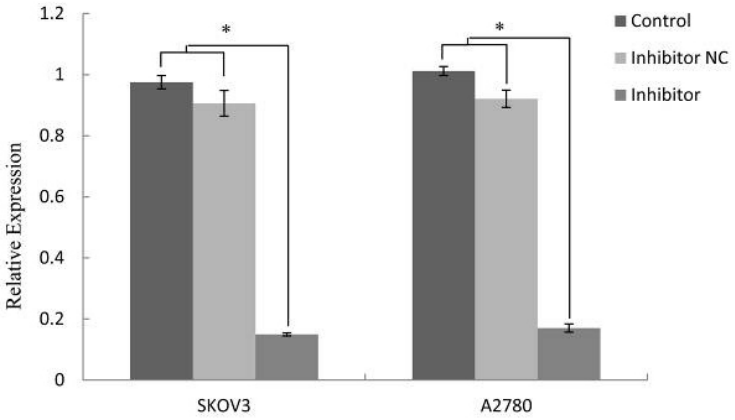

Figure 2. miR-630 expression was significantly downregulated in A2780 and SKOV3 cells transfected with miR-630 inhibitor. Relative expression of miR-630 was significantly downregulated in A2780 and SKOV3 cells transfected with miR-630 inhibitor as compared with the inhibitor NC group and control group (*$P < 0.05$). Relative amount of miR-630 was determined by using $2^{-\Delta\Delta Ct}$.

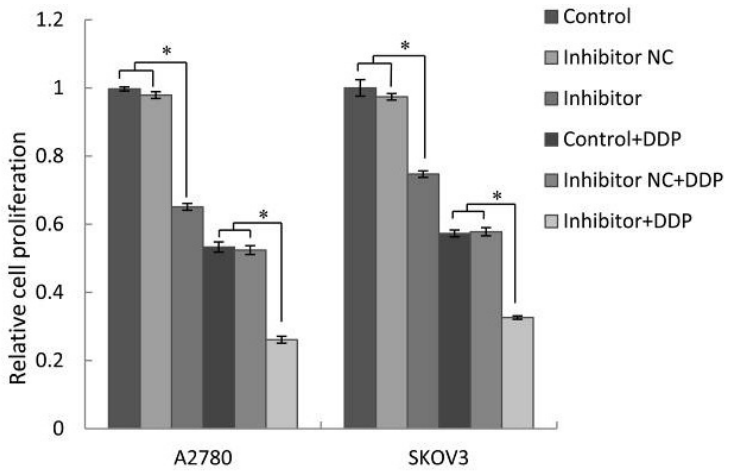

Figure 3. Downregulation of miR-630 inhibits proliferation and enhances sensitivity to cisplatin. Transfection with miR-630 inhibitor resulted in a significant decrease in cell proliferation of the A2780 and SKOV3 cell lines (*$P < 0.05$). miR-630-inhibitor-treated A2780 and SKOV3 cells showed greater chemosensitivity to 10 nM cisplatin (*$P < 0.05$).

In addition, it was found that miR-630i-treated-A2780 and -SKOV3 cells demonstrated higher chemosensitivity to 10 nM cisplatin than the NCi-treated cells (Figure 3; $P < 0.05$), suggesting that downregulation of miR-630 improved A2780 and SKOV3 cell chemosensitivity to cisplatin.

Downregulation of miR-630 suppresses the migration and invasion of A2780 and SKOV3 cells

We further investigated the effects of miR-630 on the migration and invasion of A2780 and SKOV3 cells - the 2 essential steps for malignant progression and metastasis. A2780 and SKOV3 cells transfected with miR-630i and NCi were applied to Matrigel-uncoated transwell assays. The results showed that downregulation of miR-630 significantly decreased the migration of A2780 and SKOV3 cells (Figure 4A; $P < 0.05$) as compared with the migration of the control cells.

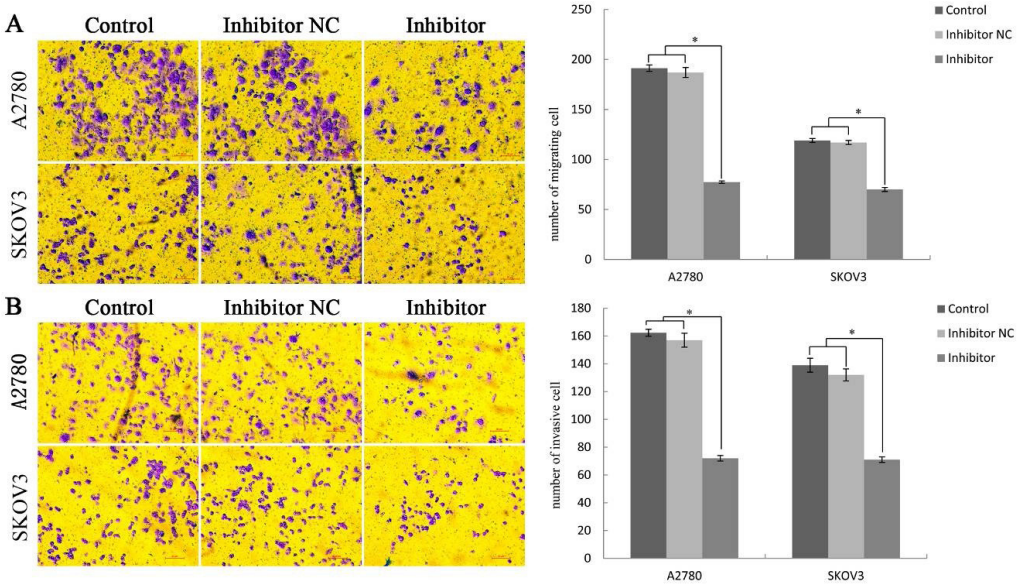

Figure 4. Downregulation of miR-630 suppresses A2780 and SKOV3 cells migration (**A**) and invasion (**B**) Images (left panel) are representative of blinded random fields. Histograms (right panel) represent the average of three independent experiments (*$P < 0.05$).

Matrigel invasion assays results showed that A2780 and SKOV3 cells transfected with miR-630i displayed a remarkable decrease as compared with those transfected with NCi cells (Figure 4B; $P < 0.05$). These results suggest that miR-630 can promote A2780 and SKOV3 cell motility.

MiR-630 regulates apoptosis in A2780 and SKOV3 cells

Apoptosis was detected by Annexin-V/propidium iodide-double staining-based flow cytometry analysis after 48-h of transfection. Our results showed that A2780 and SKOV3 cells transfected with miR-630i significantly promoted apoptosis as compared with the control cells (Figure 5; $P < 0.005$).

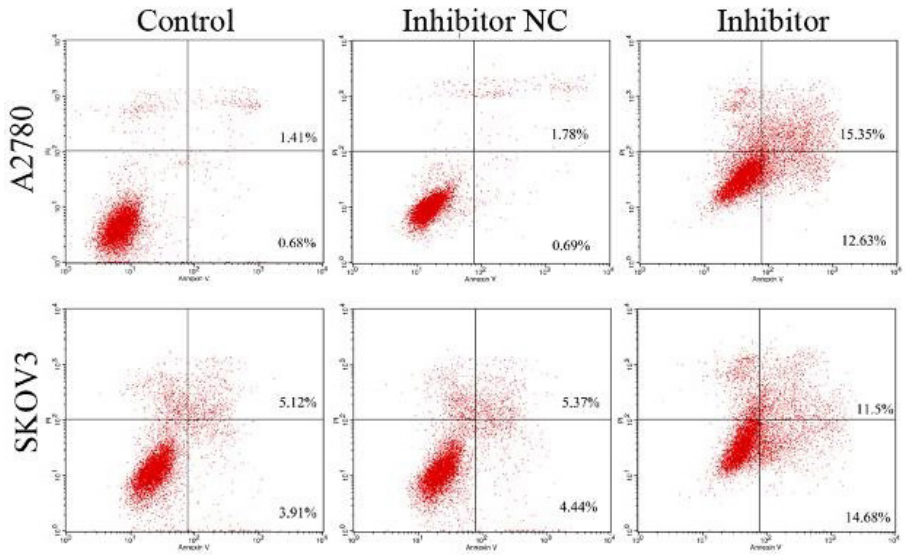

Figure 5. Detection of apoptosis in A2780 and SKOV3 cells transfected with miR-630 inhibitor. Apoptotic cell death rate in the miR-630 inhibitor group 48 h after transfection was significantly higher than that in the control groups ($P < 0.05$).

MiR-630 modulates PTEN expression in A2780 cells

To evaluate whether PTEN was directly modulated by miR-630, western blotting was performed to detect PTEN expression in A2780 cells after transfection. The result showed that PTEN expression was increased in A2780 cells transfected by miR-630i as compared with the control cells (Figure 6).

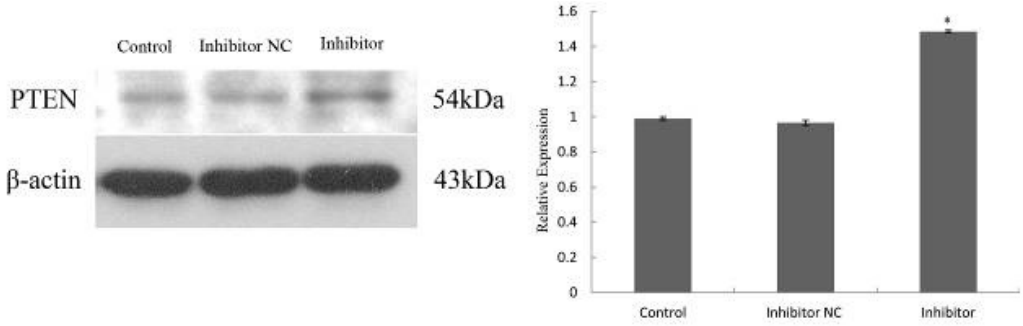

Figure 6. Effects of miR-630 inhibitor on PTEN expression in A2780 cells. Western blotting (left panel) revealed that the PTEN protein level was increased in the miR-630 inhibitor-transfected A2780 cells. Histograms (right panel) represent the average of three independent experiments (*$P < 0.05$).

DISCUSSION

It has become increasingly evident that the dysregulation of miRNAs and their functional target genes are widely involved in causing malignancy. However, the potential role and regulation mechanism of miRNAs in ovarian cancer are not well demonstrated. Elucidation of abnormally expressed miRNAs in cancers may facilitate deeper understanding of the molecular mechanisms of cancer initiation and progression.

miRNAs can function as either tumor oncogenes or suppressors during tumorigenesis by regulating diverse biological processes such as cell proliferation, cell cycle, apoptosis, invasion, metastasis, and chemosensitivity (Esquela-Kerscher and Slack, 2006; Zhang et al., 2007).

Previous studies have confirmed that, in non-small cell lung cancer cells, miR-630 can regulate cisplatin-induced cancer cell death and that, in pancreatic cancer cells, miR-630 can induce apoptosis by targeting IGF-1R (Galluzzi et al., 2010; Farhana et al., 2013). A recent study showed that the motility and invasion of the ANGPTL1-overexpressing lung cancer cell line (CL1-5/ANGPTL1) is restored following miR-630 inhibition (Kuo et al., 2013). Another study recently reported that CIS-induced miR-630 can be attributed to the promotion of pri-miR-630 processing by E2F1-regulated DROSHA (Cao et al., 2014a). These authors also showed that miR-630 was upregulated in a majority of ccRCC patients and that miR-630 expression was an independent prognostic factor for patients with renal cancer (Zhao et al., 2014). Moreover, Cao et al. (2014b) demonstrated that miR-630 targets CDC7, thereby inhibiting CDC7-mediated initiation of DNA synthesis and inducing G1 arrest. However, miR-630 has a bimodal role in the regulation of apoptosis because of its multiple target effects, which maintains apoptotic balance under genotoxic stress.

In this study, to further explore the roles of miR-630 in the biological pathways of EOC, the cell proliferation, apoptosis, migration, invasion, and drug sensitivity of A2780 and SKOV3 cells were studied. Quantitative real-time RT-PCR was performed to detect the precise expression of miR-630 in the ovarian cancer tissues. The results showed that the level of miR-630 is remarkably overexpressed in ovarian cancer tissues, suggesting that miR-630 may be involved in human ovarian cancer progression. Moreover, miR-630 overexpression was closely correlated with advanced clinical stage and lymph node metastases, which are the main prognostic factors for EOC, implying that miR-630 may be involved in the development and metastasis of cancer and that it has a prognostic implication for EOC. We further decreased the miR-630 level in ovarian cancer cells A2780 and SKOV3 to estimate its influence on mechanisms such as cell proliferation, drug sensitivity, apoptosis, migration, and invasion. As expected, decreasing the level of miR-630 in ovarian cancer cells attenuated the A2780 and SKOV3 cell proliferation and promoted cell apoptosis and drug sensitivity. Transwell assay revealed that downregulation of miR-630 significantly diminished the migration and invasion ability of A2780 and SKOV3 cells. Taken together, the expression level of miR-630 was inversely correlated with the metastatic potential of ovarian cancer, which may influence the pathways governing ovarian cancer cell metastasis, invasion, and drug sensitivity.

PTEN, or mutated in multiple advanced cancers (MMAC-1), was identified in 1997 as a tumor-suppressor gene and located at the human chromosome band 10q23. Several mechanisms such as genetic mutation, promoter methylation, and post-transcriptional modification may contribute to PTEN inactivation. PTEN can encode a series of specificity proteins, which

regulate cellular processes such as cell growth, survival, proliferation, and migration (Schneider et al., 2011; Sarkar et al., 2013; Shukla et al., 2013). Moreover, PTEN is one of the most frequently inactivated tumor suppressors in a wide range of human cancers, including gastric, osteosarcoma, hepatocellular carcinomas, as well as ovarian cancer (Wu et al., 2008a; Yang et al., 2013; Sui et al., 2014). Previous studies have shown that abnormal expression of the PTEN/PI3K/pAkt pathway has been associated with poor prognosis in ovarian carcinomas (Woenckhaus et al., 2007; de Graeff et al., 2008; Guo et al., 2008). PTEN, which is well-known for its role in tumor growth, invasion, and metastasis, has been reported as a tumor suppressor gene in ovarian cancer (Kurose et al., 2001; Lee and Park, 2009) with implications in ovarian cancer cell resistance to cisplatin (Wu et al., 2008b; Singh et al., 2013). MiR-630 gets involved in tumorigenesis by targeting different molecules such as BCL2, BCL2L2, CDC7, and IGF-1R (Galluzzi et al., 2010; Huang et al., 2011; Cao et al., 2014b). As an miRNA may possess multiple targets, we speculated that miR-630-induced cell proliferation and migration and that apoptosis may be linked to PTEN. According to our results, PTEN overexpression in miR-630-knockdown A2780 cell suggests a potential mechanism of PTEN regulation by miR-630, which led to PTEN underexpression in EOC.

Our study shows that downregulation of miR-630 significantly inhibited the migration and invasion abilities of A2780 and SKOV3 cells, in addition to the inhibition of cell proliferation and increase in apoptotic cell death. All these results support our conclusion that miR-630-induced downregulating of PTEN leads to an increase in cell proliferation, invasion, and metastasis of A2780 and SKOV3 cells. However, additional studies are required to confirm whether PTEN is a direct target gene of miR-630 in ovarian cancer cells.

In summary, we showed for the first time that miR-630 is markedly overexpressed in human ovarian cancer tissues and that it is not only involved in the regulation of cell proliferation but is also an important regulator in cell migration and invasion processes, probably through negative regulation of PTEN. It will be important to determine the target genes of miR-630 involved in the regulation of cell invasion and chemosensitivity in the future.

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