MiR-182-5p functions as a tumor suppressor to sensitize human ovarian cancer cells to cisplatin through direct targeting the cyclin dependent kinase 6 (CDK6)

Li Duan¹, Ying Yan², Guan Wang¹, Yan-ling Xing¹, Jie Sun¹, Li-li Wang¹

¹Department of Obstetrics & Gynaecology, Heilongjiang Provincial Hospital, Harbin, Heilongjiang 150001, China. ²Department of Oncology, the First Hospital of Harbin, Harbin, Heilongjiang 150001, China.

Summary

Purpose: Ovarian cancer has a difficult diagnosis and high mortality rate. Cisplatin, a platinum compound agent which has been widely used in the clinical treatment of ovarian cancer. However, development of chemoresistance is a major obstacle that limits the therapeutic efficacy. The precise roles and molecular mechanisms of cisplatin resistance in ovarian cancer remain unclear.

Methods: The expressions of microRNA (miR)-182-5p and CDK6 mRNA from ovarian tumors and cell lines were detected by qRT-PCR. MiR and siRNA were transfected into ovarian cancer cells using Lipofectamine 2000 transfection reagent. Cisplatin resistant ovarian cancer cell line was established by exposing parental cells to gradually increased cisplatin doses. The binding of miR-182-5p on CDK6 3'UTR was predicted from Targetscan.org and validated by Western blot and dual luciferase reporter assay. The cell viability was determined by MTT assay.

Results: miR-182-5p is downregulated in ovarian cancer tissues and cells. Overexpression of miR-182-5p significantly sensitized ovarian cancer cells to cisplatin. By creating cisplatin resistant cell line SKOV3, we observed miR-182-5p was apparently downregulated in cisplatin resistant cells. In addition, we identified cyclin-dependent kinase-6 (CDK6) as a direct target of miR-182-5p in both ovarian cancer cell line and patient tissues. Moreover, CDK6 was found to be upregulated in ovarian cancer and displayed an inverted expression pattern with miR-182-5p in ovarian cancer tissues. Silencing CDK6 by siRNA significantly increased the cisplatin sensitivity. Importantly, restoration of CDK6 in miR-182-5p overexpressed ovarian cancer cells successfully recovered the cisplatin resistance.

Conclusions: miR-182-5p plays a tumor suppressive role in cisplatin resistance via direct targeting the CDK6, showing miR-182-5p-CDK6 axis as a promising therapeutic target against chemoresistant ovarian cancer.

Key words: chemosensitivity, cisplatin resistance, cyclin dependent kinase 6, miR-182-5p, ovarian cancer

Introduction

Ovarian cancer has a poor diagnosis and highest mortality rate [1]. This disease includes heterogeneous subtypes that exhibit diverse etiology, molecular biology, and characteristics [2]. Ninety percent of ovarian cancers are epithelial [3]. Most cancer patients are diagnosed in stage III (51%) or IV (29%), resulting in a low 5-year cause-specific survival rate [4]. Currently, the primary treatment strategy for ovarian cancer is surgical resection with pre or postoperative chemotherapy with platinum- and taxane-based anticancer agents, especially for advanced and/or metastatic disease [5]. Investigation of novel molecular markers for early diagnosis and specific targeting malignant pathways remains an urgent task.
Cisplatin (CDDP) is a widely used anticancer drug for the treatment of diverse human cancers including breast, bladder, head and neck, lung, ovarian, and colon cancers [6]. It acts through crosslinking with the purine bases on the DNA to interfere DNA repair mechanisms, leading to DNA damage, and subsequently inducing cancer cell death [7]. Despite the impressive anticancer effects that are observed in cisplatin treatments, a fraction of patients develops drug resistance and numerous undesirable side effects, leading to a major obstacle for the clinical application of cisplatin [8]. Currently, improved approaches by combination of cisplatin with other therapeutic agents have been widely studied for overcoming cisplatin resistance and reducing tissue toxicity.

MicroRNAs (miRs) are a class of short (~20 nt), non-coding RNAs, which bind with 3'-UTR of their target mRNAs to regulate gene expressions at the post-transcriptional level [9]. Accumulating evidence revealed that miRs were involved in various cancer processes as indispensable regulators including carcinogenesis, cancer cell proliferation, apoptosis, metastasis and chemoresistance [10]. A recent report showed that miR-182-5p was down-regulated in lung cancer [11], bladder cancer [12] and breast cancer [13], suggesting a tumor suppressive role of miR-182-5p. Moreover, miR-182-5p was known to enhance the anticancer effects of cisplatin in lung cancer [14] and bladder cancer [15]. Therefore, these data suggest that miR-182-5p may be involved in the modulating the cisplatin sensitivity of ovarian cancer cells. However, the precise roles and the underlying molecular mechanisms of miR-182-5p in ovarian cancer chemosensitivity have not been fully elucidated.

This study aimed to investigate the functions of miR-182-5p in the progression of cisplatin sensitivity of ovarian cancer.

**Methods**

**Ovarian cancer patient samples**

A total of 30 pairs of resected ovarian cancer tissues and their adjacent normal tissues were collected. No ovarian cancer patients had preoperative chemoradiotherapy. Upon dissection, tissues were frozen immediately in liquid nitrogen (-80°C) and stored until use. Written informed consent was obtained from all patients. The present study was approved by the Medical Ethics Committee of Heilongjiang Provincial Hospital.

**Cell culture and reagents**

Four human ovarian cancer cell lines (HO-8910, A2780, SKOV3 and Caov-3) were obtained from the American Type Culture Collection (ATCC). The human normal ovarian epithelial cell line OEC was purchased from the Shanghai Library of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) complemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA). Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. The cisplatin resistance ovarian cancer cell line was established from SKOV3 according to previous descriptions [16]. In brief, cells were exposed to gradually increased concentrations of cisplatin for 3 months to select surviving cells. Cell clones were pooled and frozen in liquid nitrogen for the experiments of this study. Cisplatin, and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Shanghai, China). Rabbit monoclonal anti-CDK6 (#13351) and anti-β-actin (#4970) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

**Transfections of miR and plasmid DNA**

Transfection of negative control miR (25 nM) or MiR-182-5p (25 nM); control siRNA (50 nM) or siCDK6 (50 nM); control plasmid or CDK6 overexpression vector (2 μg) into ovarian cells was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. After 48 h, cells were collected and subjected to downstream experiments.

**RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)**

Ovarian cancer cells were seeded into a 6-well plates at a density of 5×10⁴ cells/well. After 24 h, the total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and quality of RNA samples were detected using an ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA). Complementary DNA (cDNA) was synthesized from 500 ng total RNA using a SuperScript First-Standard Synthesis System (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. qRT-PCR experiments were performed using the SYBR Green qPCR Master Mix (ThermoFisher Scientific, Shanghai, China). Primer sequences used in this study were as follows: miR-182-5p, forward: 5’-TTAGGAAAACTTCTCTCTC-3’; reverse: 5’-CGGTGATGTGAAGAAGGA-3’; β-actin, forward: 5’-CTGAGAGGGAAATCGTGCGT-3’; reverse: 5’-CACTCCAGGCTCTGGAACTT-3’; U6, forward: 5’-CTCGCTTCGGCAGCACA-3’; reverse: 5’-AACGCTTCACGTCAACTAAAT-3’; H3, forward: 5’-TTAGGAACCCTCCTCTCTC-3’; reverse: 5’-AACGCTTCAGTTTCTAG-3’. The thermal cycle was set as follows: 95°C for 1 min and 40 cycles at 95°C for 15 s, 58°C for 20 s, and 72°C for 20 s. The relative expressions were analyzed using the 2^ΔΔCt method. Experiments were performed in triplicate.
Luciferase reporter assays

The predicted binding sequence of miR-182-5p on CDK6 3'UTR through searching TargetScan and miR.org was mutated using a point mutation kit (Agilent Technologies, Santa Clara, CA, USA). Then, the wild type and mutant 3'UTR of CDK6 were cloned onto the pGL3-Basic Luciferase reporter vector (Promega, Madison, WI, USA). Cells were seeded in a 24-well plate at 5×10^4 cells/well and co-transfected with negative control or miR-182-5p and luciferase vector containing wild type or mutant 3'UTR of CDK6, respectively. The luciferase activity was measured using Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). The experiments were performed in triplicate.

Cell viability

Cell viability was measured using the MTT assay (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s instructions. Cells were seeded into 96-well plates for 24 h for attachment. Cells were incubated with MTT solution at 0.5 mg/ml for 4 h at 37°C. After washing with PBS, cells were incubated with 100 μl DMSO for 2 h at 37°C. The optical density (OD) was measured at 540 nm using a microplate spectrophotometer (Bio-Tek, Winooski, VT). Relative viability was calculated to the ratio of control group and normalized by cell numbers. The experiments were performed in triplicate.

Immunohistochemistry

The staining of CDK6 in the paraffin-embedded human ovarian cancer tissues was performed according to a previous report [17]. Briefly, samples were de-paraffinized with xylene and dehydrated with graded series of alcohol. Heat-induced epitope retrieval was performed using a pressure cooker in 0.1 M citrate buffer. Slides were blocked with 5% bovine serum albumin (BSA), followed by CDK6 antibody incubation overnight. The experiments were repeated three times.

Western blot

Total proteins were extracted from cells using the RIPA buffer (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) containing 1x protease inhibitor cocktail (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) on ice. Proteins were separated by centrifugation at 10,000 g for 10 minutes at 4°C. Protein concentration was measured by bicinchoninic acid (BCA) assay (Bio-Rad, Hercules, CA, USA). 40 μg protein of each experimental group were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS/PAGE) and then transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA) followed by blocking with 5% non-fat milk buffer for 1 h at room temperature. Membranes were then exposed to primary antibody (1:1000) at 4°C overnight. After the membranes were completely washed by tris-buffered saline with Tween-20 (TBST) 3 times, they were incubated with the corresponding secondary antibody at room temperature for 1 h. After washing by TBST, proteins were detected using an enhanced chemiluminescence (ECL) kit (Bio-Rad, Hercules, CA, USA). β-actin was used as internal control. The experiments were repeated 3 times.

Statistics

Statistical analysis was performed with the Student’s t-test (between two groups) or two-way ANOVA (among multiple groups) using the Prism GraphPad 6.0 software (La Jolla, CA, USA). Data were presented as mean ± SD and p<0.05 was considered as statistically significant.

Results

miR-182-5p is downregulated in ovarian cancer patients

Results from qRT-PCR clearly demonstrated that miR-182-5p was significantly downregulated in ovarian cancer tissues compared with normal tissues (Figure 1A). In addition, we compared the expressions of miR-182-5p in human normal ovarian epithelial cells (OEC) and the other four ovarian cancer cell lines (SKOV3, HO-8910, Saov-3 and A2780). Consistently, miR-182-5p was significantly downregulated in ovarian cancer cells (Figure 1B). These clinical and in vitro results indicate miR-182-5p is negatively associated with ovarian cancer progression and contributes to enhancing the cytotoxicity of chemotherapy.

Figure 1. miR-182-5p is downregulated in ovarian cancer tissues and ovarian cancer cell. A: Downregulation of miR-182-5p levels between 30 ovarian cancer tissues and corresponding adjacent normal ovarian tissues. B: Expressions of miR-182-5p in normal ovarian epithelial cell line (OEC) and five ovarian cancer cell lines were detected by qRT-PCR. Data shown are mean ± SD, *p<0.05; **p<0.01; ***p<0.001.
miR-182-5p sensitizes cisplatin resistant ovarian cancer cells to cisplatin

Given the evidence that miR-182-5p plays suppressive roles in ovarian cancer, we investigated whether miR-182-5p could modulate cisplatin sensitivity. Control miR or miR-182-5p precursor were transfected into two ovarian cancer cell lines, HO-8910 and SKOV3. The results from Figure 2A showed remarkable overexpression of miR-182-5p. Cells were then exposed to gradually increasing concentrations of cisplatin. As expected, HO-8910 and SKOV3 cells with higher miR-182-5p expressions were more sensitive to cisplatin treatment (Figure 2B, 2C). The cisplatin IC$_{50}$ of HO-8910 was dropped from 1.57 μg/ml (control cells) to 0.582 μg/ml. Similarly, cisplatin IC$_{50}$ of SKOV3 cells was

Figure 2. miR-182-5p contributes to cisplatin sensitization in ovarian cancer. A: HO-8910 and SKOV3 cells were transfected with negative control or miR-182-5p precursor for 48 hours, followed by detection of miR-183-5p expressions by qRT-PCR. B, C: The above transfected cells were treated with cisplatin at the indicated concentrations for 48 hours. Cell viability was determined by MTT assay. D: The cisplatin resistant cell line from SKOV3 was established. The cisplatin resistance was verified by treating parental and resistant cells with cisplatin at 0, 1, 2, 4, 8 and 16 μg/ml for 48 hours, followed by cell viability measurement. E: Expressions of miR-182-5p were detected by qRT-PCR from SKOV3 parental and cisplatin resistant cells. F: The SKOV3 parental and cisplatin resistant cells were transfected with negative control miRNAs or miR-182-5p precursor for 48 hours, cells were treated with cisplatin at 0, 1, 2, 4, 8, 16 μg/ml for 48 hours. Cell viability was then determined by MTT assay. Data shown are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Figure 3. CDK6 is directly targeted by miR-183-5p. A: Prediction of putative miR-182-5p binding sites on the 3’-UTR of CDK6 by sequence alignment from TargetScan. B: HO-8910 and SKOV3 cells were transfected with negative control miR or miR-182-5p precursor for 48 h and the protein expressions of CDK6 were measured by western blotting. β-actin was an internal control. C: Luciferase activities were shown from cells with co-transfection of control miRNAs or miR-182-5p with luciferase vector carrying wild type or mutant 3’-UTR of CDK6. D: A negative correlation between miR-182-5p and CDK6 mRNAs was found in ovarian cancer tissues. Data shown are mean ± SD. **p<0.01.
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significantly decreased from 1.73 μg/ml (control cells) to 0.491 μg/ml. To further elucidate the association between miR-182-5p and cisplatin sensitivity, we established cisplatin resistant ovarian cancer cell line originating from SKOV3 cells [16]. To validate the resistance, parental SKOV3 cells and the cisplatin-resistant subline SKOV3/CDDP cells were treated with cisplatin. The results from MTT assay demonstrated the cisplatin resistant cells could tolerate higher dosage of cisplatin treatment (Figure 2D). The cisplatin IC\textsubscript{50} of SKOV3/CDDP cells was 15.08 μg/ml, which was undoubtedly higher than that of parental cells (Figure 2E). In addition, we detected miR-182-5p expression was significantly downregulated in cisplatin resistant cells compared with parental cells (Figure 2E), suggesting miR-182-5p could be selected as a new molecule against chemoresistance. To test this, we performed in vitro chemosensitivity assay by treating the miR-182-5p overexpressed or not SKOV3 cisplatin resistant cells with cisplatin at 0, 1, 2, 4, 8 or 16 μg/ml. Cells expressing exogenous miR-182-5p showed apparent sensitization to cisplatin (Figure 2F). Taken all together, these results demonstrated a negative association between miR-182-5p and cisplatin resistance in ovarian cancer cells.

CDK6 is a direct target of miR-182-5p in ovarian cancer

Accumulating evidence elucidated that miRNAs function through direct binding to 3'UTR region of their target mRNAs can regulate gene expressions [9,10]. To investigate the molecular mechanisms for the miR-182-5p-promoted cisplatin sensitivity, we performed bioinformatics analysis through two publicly available algorithms: TargetScan and microRNA.org. Interestingly, 3'UTR of CDK6 was found to have a predicted complementary sequence with the miR-182-5p (Figure 3A). Literature research suggested an oncogenic role of CDK6 in multiple malignant tumors [18]. Thus, control miRNAs or miR-182-5p were transfected into HO-8910 and SKOV3 cells. Results from Western blot demonstrated that overexpression of miR-182-5p effectively inhibited CDK6 protein expressions (Figure 3B). To determine whether miR-182-5p could directly bind to the predicted 3'UTR region of CDK6, we performed luciferase reporter assay by establishing the luciferase vector containing the wild type or binding site mutant 3'UTR of CDK6. Co-transfection of miR-182-5p significantly decreased the luciferase activity of luciferase vector containing wild type 3'-UTR of CDK6 in two cells. However, transfection

Figure 4. CDK6 is positively associated with ovarian cancer and renders ovarian cancer cells resistant to cisplatin. A: IHC staining of CDK6 in normal ovarian tissue and ovarian cancer tissue. B: Expressions of CDK6 mRNA from 30 ovarian cancer tissues and corresponding adjacent normal ovarian tissues. C: HO-8910 (upper) and SKOV3 (lower) were transfected with control siRNA or siCDK6 for 48 h and the expressions of CDK6 were detected by Western blot. The above cells were treated with cisplatin at the indicated concentrations for 48 h and cell viability was determined by MTT assay. D: Protein expressions of CDK6 from SKOV parental and cisplatin resistant cell were measured by Western blot. β-actin was an internal control. E: SKOV3 cisplatin resistant cell were transfected with control siRNA or siCDK6 for 48 h, followed by treatment of cisplatin at 0, 1, 2, 4, 8 and 16 μg/ml for 48 h. Cell viability was measured by MTT assay. Data shown are mean ± SD, *p<0.05; **p<0.01; ***p<0.01.
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of miR-182-5p could not affect the luciferase activity of luciferase vector containing binding site mutant 3’-UTR of CDK6. To evaluate the clinical relevance of miR-182-5p and CDK6, we analyzed the expression pattern between them from the ovarian cancer tissues. Expectedly, a negative correlation was found that higher mRNA levels of CDK6 were associated with lower miR-182-5p expressions in tumor tissues (Figure 3F). In summary, the above results demonstrated miR-182-5p could directly target 3’UTR of CDK6 in ovarian cancer.

CDK6 is upregulated in ovarian cancer tissues and positively correlated with cisplatin resistance

To explore the functions of CDK6 in the miR-182-5p-mediated chemosensitivity, we examined the expressions of CDK6 in normal and ovarian cancer tissues. IHC and qRT-PCR experiments consistently demonstrated that both protein and mRNA levels of CDK6 were significantly upregulated in cancer tissues compared with normal tissues (Figure 4A, 4B). To test whether the upregulation of CDK6 was response to cisplatin resistance, we transfected HO-8910 and SKOV3 cells control siRNA or CDK6 siRNA. Western blot results demonstrated CDK6 siRNA transfection successfully blocked the CDK6 protein expressions (Figure 4C). Furthermore, we observed a significantly decreased cell viability in CDK6 silenced ovarian cancer cells under cisplatin treatments (Figure 4C). To further explore the direct evidence for the CDK6-mediated cisplatin resistance, we examined the expressions of CDK6 in SKVO3 parental and cisplatin resistant cells. CDK6 was found to be upregulated in cisplatin resistant cells (Figure 4D). Moreover, similar results were found that silencing CDK6 in SKOV3 cisplatin resistant cell clearly overcame the cisplatin resistance (Figure 4E).

Recovery of CDK6 rescues the miR-182-5p-promoted cisplatin sensitivity of ovarian cancer cells

Given that miR-182-5p and its direct target CDK6 exhibited reverse roles in cisplatin sensitiv-
study clearly demonstrated that miR-182-5p was negatively associated with cisplatin resistance in ovarian cancer. Overexpression of miR-182-5p significantly sensitized ovarian cancer cells to cisplatin. Importantly, the cisplatin resistant cells displayed apparent downregulated miR-182-5p levels. These results indicated that delivering miR-182-5p to ovarian tumor tissues will facilitate to enhance the cytotoxicity of antitumor drugs.

MiRs function through binding to 3’UTR of their target mRNAs [9]. Through bioinformatics analysis, we focused on CDK6 as a candidate of miR-182-5p target since recent studies indicated CDK6 was involved in chemoresistance [17]. What we discovered was that CDK6 was positively correlated with ovarian cancer progression with the evidence that CDK6 was significantly upregulated in ovarian cancer tissues. CDK6 was upregulated in cisplatin ovarian resistant cells and silencing CDK6 effectively sensitized ovarian cancer cells to cisplatin. Furthermore, an invert correlation between CDK6 and miR-182-5p was observed in ovarian cancer tissues. Importantly, rescue experiments demonstrated that restoration of CDK6 in miR-182-5p overexpressed ovarian cancer cells successfully recovered cisplatin resistance, suggesting the miR-182-5p-mediated sensitivity in ovarian cancer cells was through targeting CDK6. However, these in vitro molecular mechanisms which contributed to drug resistance need to be verified from in vivo animal models. Our current project is creating an in vivo xenograft mice model to test the synergistical anti-chemoresistant effects by delivering a lentivirus-based miR-182-5p into tumors under cisplatin treatments. In summary, this study reported a tumor suppressive role of miR-182-5p in ovarian cancer. By establishing the CDDP-resistant SKOV-3 cell line, we showed decreased miR-182-5p and increased CDK6 in cisplatin resistant cells. Moreover, we found that CDK6 is a direct target of miR-182-5p in ovarian cancer. Rescue experiments demonstrated the miR-182-5p-promoted cisplatin sensitization was through targeting CDK6. Taken all together, our study indicates that targeting the miR-182-5p-CDK6 axis contributes to overcoming cisplatin resistance in ovarian cancer.

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Conflict of interests

The authors declare no conflict of interests.

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