MiR-181a upregulation is associated with epithelial-to-mesenchymal transition (EMT) and multidrug resistance (MDR) of ovarian cancer cells

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Abstract. – OBJECTIVE: Elevated miR-181a is associated with the transition of ovarian tissues from normal into a cancerous state. However, its regulative effect on multidrug resistance (MDR) of ovarian cancer is not quite clear. Therefore, this study aimed to investigate its regulative effects on epithelial-to-mesenchymal transition (EMT) and MDR in ovarian cancer.

MATERIALS AND METHODS: The expression profile of miR-181a in normal and ovarian cancer tissues was firstly quantified using qRT-PCR analysis. Then, human ovarian cancer cell line SKOV3 were transfected for miR-181a overexpression and the paclitaxel-resistant variant SKOV3/PTX cells were transfected for miR-181a knockdown. The effect of miR-181a overexpression/knockdown on EMT and PTX sensitivity were studied.

RESULTS: MiR-181a level in chemoresistant (CR) cancer tissues were significantly higher than in chemosensitive (CS) cancer tissues and in normal tissue. SKOV3/PTX cells had significantly higher expression of miR-181a and N-cadherin than SKOV3 cells. SKOV3 cells had decreased E-cadherin expression and increased N-cadherin expression after enforced miR-181a expression, while SKOV3/PTX cells had increased E-cadherin expression and decreased N-cadherin expression after miR-181a knockdown. SKOV3 cells had increased P-gp expression after enforced miR-181a expression. Following MTT assay and flow cytometry analysis both confirmed that miR-181a overexpression decreased the PTX sensitivity of SKOV3 cells and while miR-181a inhibition increased the sensitivity of SKOV3/PTX cells.

CONCLUSIONS: MiR-181a is an important oncomiR significantly increased in chemoresistant ovarian cancer. Its upregulation is associated with increased level of EMT and decreased cell apoptosis induced by PTX treatment.

Key Words:
 miR-181a, Ovarian cancer, MDR, EMT.

Introduction

Epithelial ovarian cancer (EOC) is one of the most common gynecologic malignancy across the world[1]. Currently, a platinum-containing compound in combination with paclitaxel (PTX) is recommended as the first-line chemotherapy for ovarian cancer patients[2]. Although the initial responsive rate for the chemotherapy can reach up to 80%, a large proportion of the patients eventually relapses and develops multidrug resistance (MDR)[3-5]. This is one of the major obstacles in ovarian cancer therapy[6]; however, the mechanisms involved in MDR of ovarian cancer is still not fully revealed.

The epithelial-to-mesenchymal transition (EMT) is an important physiological process involved in progression and metastasis of EOC[7]. The major characteristic of EMT is the decreased expression of epithelial markers (such as E-cadherin) and enhanced expression of mesenchymal markers (such as N-cadherin)[8,9]. Recent studies[8,9] suggest that EMT also contributes MDR of EOC. miRNAs, as a family of small non-coding RNA that exerts a silencing effect through binding to the 3'UTR of target genes can regulate a wide range of physiological processes[10]. Multiple miRNAs can regulate EMT in ovarian cancer through targeting different genes. For example, miR-150 can suppress transcriptional repressor ZEB1, thereby inhibiting EMT[11]. MiR-506 can simultaneously target the E-cad transcriptional repressor SNAI2, vimentin and N-cadherin, leading to suppressed EMT[12]. Elevated miR-181a is associated with the endometrial transition from normal into cancerous state[13]. It is also an important miRNA contributing to enhanced EMT in ovarian cancer[14]. However, its regula-

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tive effect on MDR of ovarian cancer is not quite clear. In this study, we firstly explored the expression profile of miR-181a in ovarian cancer tissues. Then, its regulative effects on EMT and MDR in ovarian cancer were further investigated by using in-vitro cell model based on SKOV3 and SKOV3/PTX cells.

Materials and Methods

Human Tissue Collection

This study was approved by the Ethics Committee of the Third Affiliated Hospital of Zhengzhou University. The ovarian cancer samples (n=20) were collected from 20 patients who received hysterectomy and the following standard platinum-taxane therapy. Normal ovarian tissues were obtained from 15 women who underwent surgery for benign or malignant gynecological diseases other than ovarian carcinoma. Informed consent was obtained from all of the patients before the study. Patients with progressive disease (PD) or recurrence within 6 months after completion of the chemotherapy were defined as chemoresistant. The patients with complete remission (CR), partial remission (PR) and stable disease (SD) within 6 months after completion of the chemotherapy were defined as chemosensitive. Pathological examinations were done by two experienced pathologists according to the criteria laid down by the World Health Organization (WHO).

Cell Culture and Treatment

Human ovarian cancer cell line SKOV3 and the paclitaxel-resistant variant SKOV3/PTX were obtained from Shanghai Institute of Cell Biology, China Academy of Sciences (Shanghai, China). The cells were grown in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 To maintain the PTX resistance of SKOV3/PTX cells, the culture medium was additionally supplemented with 2 nM PTX.

MiR-181a mimics, miR-181a inhibitors (anti-miR-181a) and the scramble negative controls were purchased from RiboBio (Guangzhou, China). SKOV3 cells were transfected with 100 nM miR-181a mimics and SKOV3/PTX cells were transfected with 100 nM anti-miR-181a using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The levels of mature miR-181a in the cells were measured using qRT-PCR analysis 24 hours after transfection.

QRT-PCR Analysis

QRT-PCR analysis was performed according to the method described in one previous study. Briefly, after total RNA extraction, miRNA-specific cDNA was firstly synthesized via reverse transcription (RT) using the stem-loop primers and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Then, the expression of mature miR-181a in tissue or cell samples were detected using the TaqMan MicroRNA Assays Kit (Applied Biosystems). The relative expression of miR-181a was calculated using the $2^{-\Delta\Delta CT}$ method.

Western Blot Analysis

Cell samples were firstly lysed using the RIPA Lysis Buffer (Beyotime, Shanghai, China). Then, the protein concentration was quantified using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Denatured proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 12 % acrylamide gels and were then transferred to polyvinylidene difluoride membranes (Bio-Rad). After blocking and washing, the membranes were probed using primary antibodies against E-cadherin (1:500, ab76055, Abcam, Cambridge, UK), N-cadherin (1:1000, ab18203, Abcam), P-glycoprotein (P-gp) (1:2000, ab129450, Abcam) and β-actin (1:5000, A5316, Sigma-Aldrich, St. Louis, MO, USA). After three times of washing using TBST, the membranes were incubated with the secondary horseradish peroxidase-conjugated antibodies (Abcam). The blotted protein bands were visualized using the enhanced chemiluminescence (ECL) Western blotting system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Fluorescence Microscopy

SKOV3 cells after transfection of miR-181a mimics and SKOV3/PTX cells after transfection of miR-181a inhibitors (anti-miR-181a) were grown on coverslips. Then, the cells were fixed in methanol, permeabilized in 0.1% Triton X-100 and blocked with 1% BSA. To detect the expression of E-cadherin and N-cadherin, the coverslips were probed with primary antibodies against E-cadherin (1:500, ab40772, Abcam) and N-cadherin (1: 100, ab76011, Abcam) respectively at 4°C overnight. After the incubation, the coverslips were washed and further incubated with secondary Alexa Fluor®555-conjugated donkey anti-rabbit IgG H&L (1: 500, ab150074, Abcam) and Alexa Fluor®488-conjugated Donkey anti-rabbit polyclonal antibody (1:

500, ab150073, Abcam) respectively for 1 hour at room temperature. Nuclei were stained with Gold Antifade Reagent with DAPI (Invitrogen). Digital immunofluorescent images were obtained using an Eclipse Ti-S inverted phase/fluorescent microscope (Nikon, Tochigi, Japan).

Drug Sensitivity Assay

SKOV3 cells after transfection of miR-181a mimics and SKOV3/PTX cells after transfection of anti-miR-181a were plated in 96-well plates and incubated at 37 °C in a humidified 5% CO_2 atmosphere for 24 hours. Then, PTX in various concentrations (0, 0.1, 0.5, 1, 2, 5, 10, 20 and 50 nM) were added. 48 hours later, cell viability was assessed using a methyl thiazolyl tetrazolium (MTT) assay. Three independent experiments were performed in triplicate.

Flow cytometry analysis of cell apoptosis

SKOV3 cells after transfection of miR-181a mimics and SKOV3/PTX cells after transfection of anti-miR-181a were further treated with 10 nM PTX for 48 hours. Then, the ratio of apoptotic cells was determined using the Annexin V-FITC Apoptosis Detection Kit (V13241, Invitrogen) according to manufacturer's instruction in a FACS-Caliber (BD Biosciences, Franklin Lakes, NJ, USA). Data acquisition was done using CellQuest Pro Software (BD Biosciences). Each test was performed with at least three repeats.

Statistically Analysis

Statistical analysis was performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was performed to compare means of multiple group experiments. Between group difference was compared by using unpaired t-test. $p < 0.05$ was considered as statistically significant.

Results

MiR-181a Upregulation and EMT are Associated with PTX Resistance in Ovarian Cancer

6 months after the chemotherapy, there were 11 chemosensitive (CS) cases and 9 chemoresistant (CR) cases among the 20 ovarian cancer patients recruited. Based on normal healthy ovarian tissues and ovarian cancer tissues, we observed that miR-181a level in CR tissues was significantly higher than that in CS cancer tissues and normal tissues (n=15) (Figure 1A). Therefore, we decided to explore further the role of miR-181a in the regulation of PTX sensitivity in ovarian cancer. By performing qRT-PCR analysis, we found that the expression of miR-181a in PTX resistant SKOV3/PTX cells was over 4 times higher than that in PTX sensitive SKOV3 cells (Figure 1B). Previous studies[15,16] suggested that the increased chemoresistance in cancer cells might be related to enhance EMT. By performing Western blot analysis, we also found that SKOV3/PTX cells had significantly higher expression of N-cadherin, but with a substantially lower expression of E-cadherin than SKOV3 cells (Figure 1C).

MiR-181a Modulates EMT in SKOV3 and SKOV3/PTX Cells

Then, we further investigated whether miR-181a has a regulative role in EMT of SKOV3
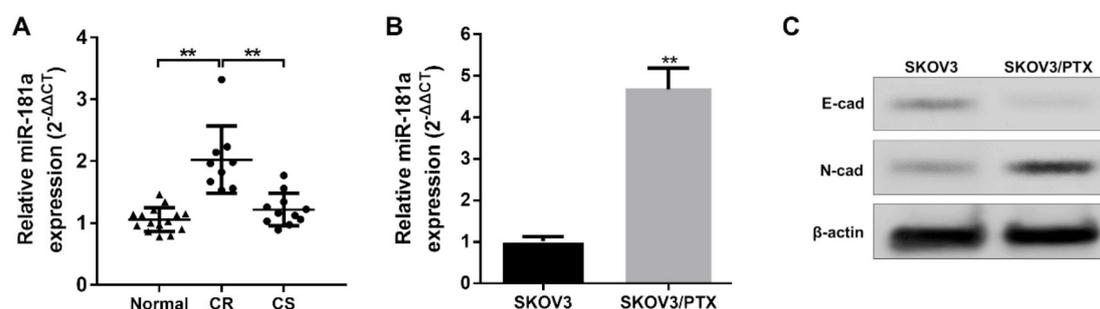

Figure 1. MiR-181a upregulation and EMT are associated with PTX resistance in ovarian cancer. A. QRT-PCR analysis of miR-181a expression in chemoresistant (CR) cancer tissues (n=9), chemosensitive (CS) cancer tissues (n=11) and in normal tissues (n=20). B. QRT-PCR analysis of miR-181a expression in human ovarian cancer cell line SKOV3 and the paclitaxel-resistant variant SKOV3/PTX cells. C. Western blot analysis of E-cadherin (E-cad) and N-cadherin (N-cad) expression in SKOV3 and SKOV3/PTX cells. **$p<0.01$.
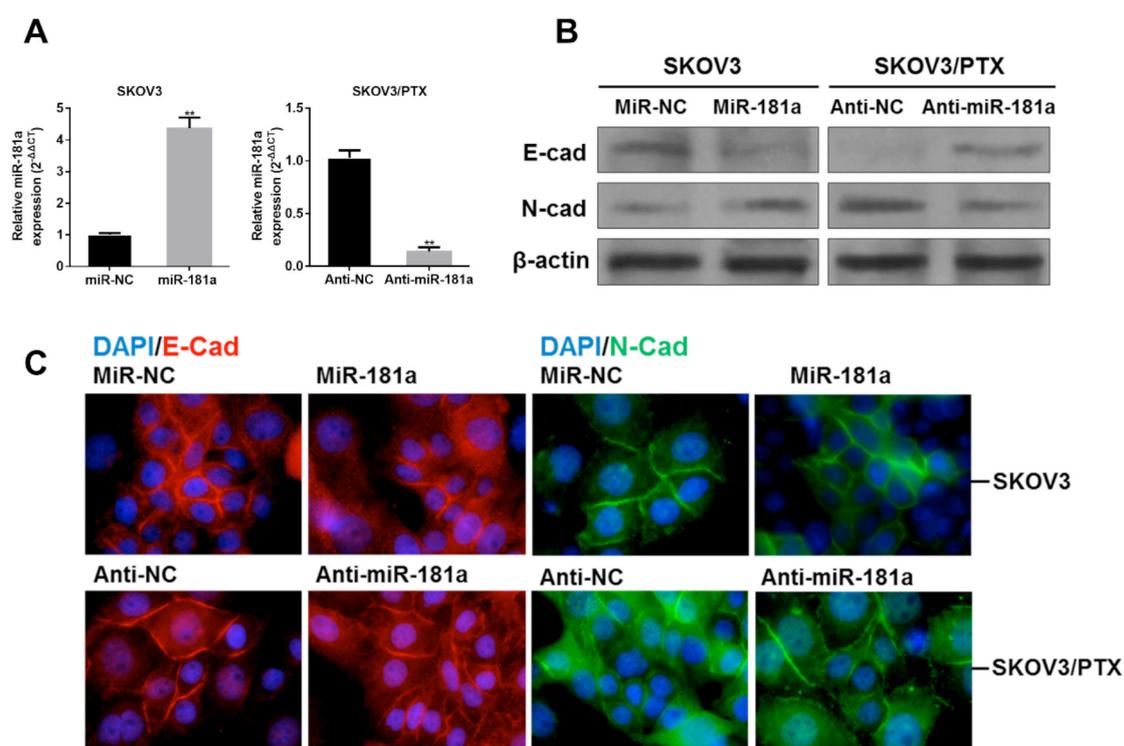
Figure 2. MiR-181a modulates EMT in SKOV3 and SKOV3/PTX cells. **A,** SKOV3 cells were transfected with 100 nM miR-181a mimics and SKOV3/PTX cells were transfected with 100 nM miR-181a inhibitors. 24 hours after transfection, QRT-PCR analysis was performed to measure miR-181a expression in the cells. **B,** Western blot analysis of E-cadherin and N-cadherin expression in SKOV3 cells with miR-181a overexpression and in SKOV3/PTX cells with miR-181a knockdown. **C,** Representative immunofluorescent images of the epithelial marker E-cadherin (E-cad) and mesenchymal marker N-cadherin (N-cad) in SKOV3 cells with miR-181a overexpression and in SKOV3/PTX cells with miR-181a knockdown. Red: E-cad; Green: N-cad; blue: DAPI. **p<0.01.

and SKOV3/PTX cells. SKOV3 cells were firstly transfected with miR-181a mimics and SKOV3/PTX cells were transfected with miR-181a inhibitors (Figure 2A). Western blot analysis showed that SKOV3 cells had decreased E-cadherin expression and increased N-cadherin expression after enforced miR-181a expression (Figure 2B). In contrast, after transfection with miR-181a inhibitors, SKOV3/PTX cells had increased E-cadherin expression and decreased N-cadherin expression (Figure 2B). Then, we performed immunofluorescence study, the results of which further verified the alteration of E-cadherin and N-cadherin expression in the cells (Figure 2C). These results suggest that miR-181a modulates EMT in SKOV3 and SKOV3/PTX cells.

MiR-181a Modulates PTX Sensitivity of SKOV3 and SKOV3/PTX Cells

Considering the role of miR-181a in EMT of the cancer cells, we then investigated wheth-er it is involved in MDR regulation. P-gp is an ATP-binding cassette (ABC) transporter, which plays a critical role in MDR regulation[17]. In this study, we observed that SKOV3 cells had increased P-gp expression after enforced miR-181a expression, while SKOV3/PTX cells had decreased P-gp expression after transfection with miR-181a inhibitors (Figure 3A). By performing MTT assay to predict the IC50 value of the cancer cells, we found that miR-181a overexpression desensitized SKOV3 cells to PTX, while miR-181a suppression sensitized SKOV3/PTX cells to PTX (Figure 3B). Then, we performed flow cytometry analysis to study how miR-181a affects cell apoptosis. The results showed that miR-181a overexpression decreased the ratio of apoptotic SKOV3 cells after PTX treatment (Figure 3C-D), while miR-181a inhibition increased the ratio of apoptotic SKOV3/PTX cells after PTX treatment (Figure 3C-D).
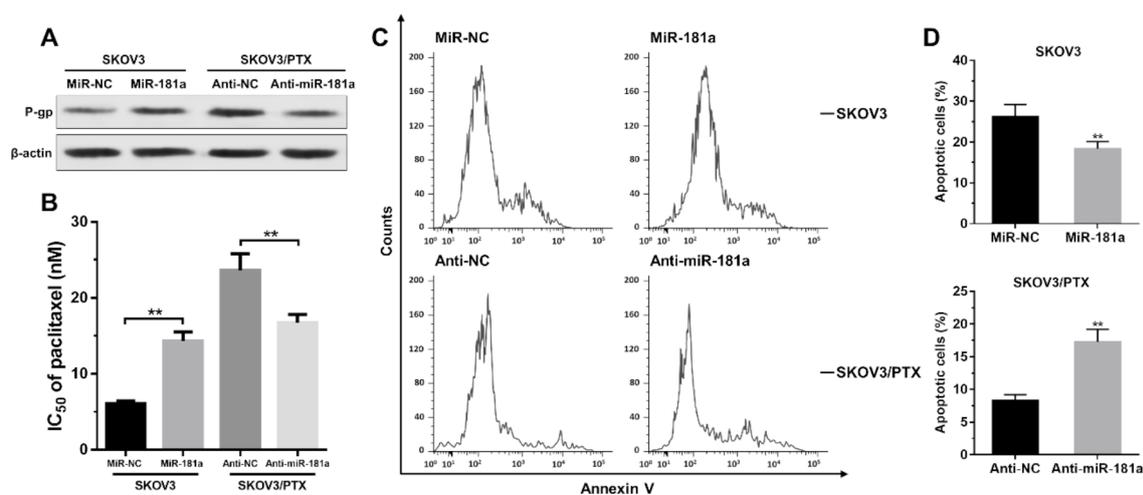
Figure 3. MiR-181a modulates PTX sensitivity of SKOV3 and SKOV3/PTX cells. **A,** Western blot analysis of P-gp expression in SKOV3 cells with miR-181a overexpression and in SKOV3/PTX cells with miR-181a knockdown. **B,** PTX IC50 value of SKOV3 cells with miR-181a overexpression and SKOV3/PTX cells with miR-181a knockdown. **C,** Representative images of flow cytometric analysis of cells with positive Annexin V staining 48 hours after 10 nM PTX treatment. **D,** Quantification of apoptotic SKOV3 and SKOV3/PTX cells showed in figure C. $^{**}p<0.01$.

Discussion

Aberrant expression of miR-181a and its oncogenic role in ovarian cancer has been reported in previous studies[13,14]. One recent study[14] found that the level of miR-181a and phosphorylated Smad2 are significantly higher in recurrent than in matched-primary ovarian tumors. Also, their expression is also associated with worse recurrence-free survival and poorer overall survival of the patients. The underlying mechanisms have also been gradually revealed. MiR-181a, together with miR-98 can decrease the expression of PGRMC1, PGR, CYP19A1, TIMP3, and DDX3X, leading to a wide range of dysregulated endometrial cellular activities during normal menstrual cycle and transition into endometrial cancer[13]. In addition, it can also promote TGF-beta-mediated EMT via repression of its functional target, Smad7[14]. However, its role in chemosensitivity of ovarian cancer is not clear.

Based on normal ovarian tissues and cervical cancer tissues, we observed that the chemoresistant cervical cancer tissues had significantly higher expression of miR-181a. Then, we decided to further investigate its regulative effect on PTX sensitivity of the cancer cells. A previous study[18] observed that the resistance to platinum-based chemotherapy might be associated with EMT in epithelial ovarian cancer. Marchini et al[18] compared the molecular profiles of 23 tumor biopsies

of stage III-IV patients and found that the platinum resistance is associated with activation of EMT by transforming growth factor (TGF)-beta pathway. Several miRNAs are involved in this process of regulation[19,20]. One recent study[21] observed that the miR-200 family members in cellular sensitivity to paclitaxel and carboplatin in ovarian cancer cell lines, OVCAR-3 and MES-OV. This study found that the paclitaxel-resistant variants OVCAR-3/TP and MES-OV/TP cells had significantly decreased expression of miR-200c and miR-141. Enforced expression of miR-200 family members in OVCAR-3/TP cells initiated mesenchymal to epithelial transition (MET). Transfection of miR-200c and miR-141 mimics conferred resistance to carboplatin in MES-OV/TP cells but sensitized MES-OV to paclitaxel[21]. Overexpression of miR-15a inhibited EMT and decreased the expression of the cisplatin transporter ATP7B, while overexpression of miR-16 led to decreased degradation of the extra-cellular matrix and enhanced sensitization of ovarian cancer cells to cisplatin[22]. Overexpression of miR-30a decreased EMT marker levels and re-sensitized platinum-resistant EOC cells to cisplatin-induced apoptosis[15]. MiR-186 can decrease Twist1 expression, thereby decreasing EMT and promoting chemosensitivity of ovarian cancer cells[16]. These findings suggest that the miRNAs may play cell-context dependent roles in regulating EMT and MDR.

In this study, we observed that the PTX resistant SKOV3/PTX cells had significantly higher expression of miR-181a and also presented more mesenchymal cell phenotypes than SKOV3 cells. Therefore, we further investigated the regulative effect of miR-181a in these physiological processes. The results showed that SKOV3 cells had decreased E-cadherin expression and increased N-cadherin expression after enforced miR-181a expression, while SKOV3/PTX cells had increased E-cadherin expression and decreased N-cadherin expression after miR-181a knockdown. Then, we further investigated the regulative effect of miR-181a on PTX sensitivity. We have found that SKOV3 cells had increased P-gp expression after enforced miR-181a expression, while SKOV3/PTX cells had decreased P-gp expression after transfection with miR-181a inhibitors. Following MTT assay and flow cytometry analysis both confirmed that miR-181a overexpression decreased the PTX sensitivity of SKOV3 cells and while miR-181a inhibition increased the sensitivity of SKOV3/PTX cells.

Conclusions

MiR-181a is an important oncomiR significantly increased in chemoresistant ovarian cancer. Its upregulation is associated with increased level of EMT and decreased cell apoptosis induced by PTX treatment.

Conflicts of interest

The authors declare no conflicts of interest.

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