miR-100 resensitizes resistant epithelial ovarian cancer to cisplatin

PENG GUO1,2, XIANGPENG XIONG2, SAINAN ZHANG2 and DONGXIAN PENG2

1Department of Obstetrics and Gynecology, Zhongshan People's Hospital, Zhongshan, Guangdong 528403; 2Department of Obstetrics and Gynecology, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong 510515, P.R. China

Received May 4, 2016; Accepted September 5, 2016

DOI: 10.3892/or.2016.5140

Abstract. Epithelial ovarian cancer (EOC) is one of the malignant tumors that seriously affects women's health and chemotherapy resistance is an important reason for the poor prognosis. The present study was conducted to investigate whether microRNA-100 (miR-100) can be used to modulate the tolerance to cisplatin in EOC. Expression of miR-100 was compared between ovarian cancer cells tolerant and not tolerant to cisplatin. Mimic and antisense were used to study the roles and related mechanisms of miR-100 in cisplatin sensitivity in EOC. The alternation in the cisplatin sensitivity was investigated using grafted tumors derived from SKOV3/DDP cells with upregulated or downregulated miR-100 expression. miR-100 was lower in cisplatin resistant cell line SKOV3/DDP than in cisplatin sensitive cell line SKOV3. miR-100 might increase cisplatin sensitivity by inhibiting cell proliferation and conversion from G1 to S phase and increasing apoptosis. We showed that mTOR and PLK1 are targets of miR-100 and the cells were resensitized probably due to targeted downregulation of mTOR and PLK1 by miR-100. In vivo study with nude mice showed that tumors derived from miR-100 mimic-transfected cells were more sensitive to cisplatin and had reduced expression of mTOR and PLK1. miR-100 resensitizes resistant epithelial ovarian cancer to cisplatin probably by inhibiting cell proliferation, inducing apoptosis and arresting cell cycle and by targeted downregulation of mTOR and PLK1 expression.

Introduction

Epithelial ovarian cancer (EOC) is one of the malignant tumors that seriously affects women's health. Because of insidious onset and rapid development, its mortality rate ranks the first among gynecological cancers (1). At present, cytoreductive surgery combined with platinum-based chemotherapy is effective in early stage of 70% of advanced stage patients, but the subsequent relapse and chemotherapy resistance often result in unimproved overall survival in ovarian cancer patients (2). The occurrence of chemotherapy resistance is an important reason for the poor prognosis (3,4). Therefore, a better understanding of the mechanism underlying the drug resistance in ECO is essential to improve the poor prognosis.

The mechanisms of drug resistance to chemotherapy is very complex, and the possible mechanisms of cisplatin resistance include the pharmacokinetic factors (drug exposure and vascular tumor), micro-environmental factors (which are related to the impact on the cell cycle and apoptosis signaling) and cellular factors (such as the change in expression of proteins related to drug resistance, point mutations at drug target, enhanced cellular repair system and reduced apoptosis) (5-7). Genetic and epigenetic variations in the key genes in these pathways can result in drug resistance in cancer cells as well. A large number of studies have also demonstrated that micro-RNAs are playing regulatory role in the expression of these key genes (8,9).

Micro-RNA (miRNA) is a group of endogenous and non-coding nucleotides of approximately 22 nucleotides long and functions through complementary pairing with 3'-untranslated region (3'-UTR) of target gene to regulate the expression of the target gene (10). Increasing evidence has demonstrated that the abnormal expression of miRNA is associated with the occurrence, development, metastasis and chemotherapy resistance of cancer (11). miR-100 is a miRNA identified earlier to be associated with tumor. It represses the post-transcription of mammalian target of rapamycin (mTOR) and Polo-like kinase 1 (PLK1) to control the growth, metastasis and chemotherapy sensitivity of tumor cells. Nagaraja et al (12) reported that in ovarian clear carcinoma cell line miR-100 is targeted to the (3'-UTR) of mTOR to suppress its expression, which results in increased sensitivity of the cell line to everolimus. Feng et al (13) found that in lung adenocarcinoma, the downregulation of miR-100 can activate target protein PLK1, thereby promoting taxol resistance (13). However, it is not clear whether miR-100 is associated with cisplatin resistance in EOC.

To explore the possible relationship between miR-100 expression and cisplatin resistance in EOC, we examined the expression of miR-100 in human ovarian cancer cell line SKOV3 and its cisplatin resistant cell line SKOV3/DDP. We upregulated and downregulated the expression of miR-100 in SKOV3/DDP to gain insights into the role of miR-100 in...
regulating apoptosis, proliferation, cell cycle and cisplatin sensitivity and in regulating the expression of mTOR and PLK1 in EOC. Mouse models were used to validate the sensitization of implanted cisplatin resistant tumor to cisplatin by miR-100. The findings will provide new strategies to overcome drug resistance.

Materials and methods

Cell lines and mice. Human ovary cancer cell line SKOV3 was a gift from the Cancer Research Institute of Southern Medical University; cisplatin resistant human ovary cancer cell line (SKOV/DDP) was a gift from the Central Laboratory, Qingdao University Medical College Affiliated Hospital. Female BALB/c- nu/nu nude mice (aged 4-5 weeks) were purchased from the Experimental Animal Center, Zhongshan University, under permit number SCKK (Guangdong) 2011-0029 and housed in the Animal Center on the North Campus of Zhongshan University. The animal experiments were approved by the Animal Care and Use Committee of Southern Medical University.

Cell culture. SKOV3 and SKOV3/DDP cells were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS) at 37°C and 5% CO2 in incubator with saturated humidity.

Cell transfection. SKOV3/DDP cells were transfected with lentivirus containing miR-100 mimics, (LV-miR-100), antisense (LV-anti-100) and empty vector (LV-NC) (Shanghai GenePharma, Co., Ltd., Shanghai, China). After 12 h, virus solution was removed and the cells were cultured in RPMI-1640 containing 10% FBS for another 48 h and examined for infection efficiency using a fluorescence microscope, and further confirmed using real-time quantitative PCR (qRT-PCR). The cells were cultured in complete culture medium containing puromycin (2 µg/ml) for two weeks to obtain SKOV3/DDP cells with miR-100 stably downregulated or upregulated.

qRT-PCR. RNA (3 µg) was reverse transcribed using random primers (Applied Biosystems, Carlsbad, CA, USA). miRNA expression was determined using TaqMan® miRNA assays (Applied Biosystems) according to the manufacturer’s protocol. In brief, 50 ng of total RNA was reverse transcribed using specific miRNA primers. U6 expression was used for normalization using the comparative CT method (14). The primers (Shanghai GenePharma) were forward, 5'-ATCATTTAAC CGTAGATCCGAA and reverse, 5'-GGAAACCGTCACGATTTG for miR-100; and forward, 5'-ATTGGAAACGATA CGAGAGATT and reverse, 5'-GGAAACCGTCACGATTT G for U6.

Cell counting assay. Transfected SKOV3/DDP cells were seeded in wells of 96-well plates at a density of 3x10³ cells/well, cultured overnight and added with different concentrations of cisplatin from 0 (control) to 64 µg/ml. For each concentration, 5-wells were used. The cells were cultured for 48 h, added with 10 µl CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) and cultured for another 4 h to measure the absorbance (A) at 450 nm. The values were used to calculate the IC50 of cisplatin. In addition, the transfected SKOV3/DDP cells were seeded in wells of 96-well plates at a density of 2x10³ and measured for absorbance at 450 nm wavelength at different time-points (12-72 h) using a plate reader to plot growth curves.

Western blot analysis. Total protein was extracted 72 h after transfection, separated on SDS-PAGE, transferred to membrane and incubated with primary antibodies (rabbit anti-human PLK1, anti-human mTOR and anti-human GAPDH monoclonal antibodies; Cell Signaling Technology, Beverly, MA, USA) for 1 h, and secondary antibody (goat anti-rabbit IgG; Abcam, Cambridge, UK) for 1 h. The membrane was developed using enhanced chemiluminescence (ECL) kit (Pierce, Rockford, IL, USA) and the gray value of the bands was quantified using Quantity One software to calculate the relative expression of mTOR and PLK1. The experiments were repeated 3 times.

FACS analysis. SKOV3/DDP cells with stably upregulated and downregulated miR-100 expression and control cells were cultured for 48 h, harvested and stained with FITC Annexin V and propidium iodide (PI) (FITC Annexin V apoptosis detection kit; BD Biosciences, San Jose, CA, USA) for cell cycle analysis, they were fixed in 70% ethanol on ice, washed with phosphate-buffered saline (PBS), then stained with PI containing RNase A (20 µg/ml in PBS). The cells were analyzed on FACSVerse flow cytometry (BD Biosciences).

Crystal violet staining. Cells were inoculated in wells of 6-well plates at a density of 1x10³ cells/well and cultured for 14 days in RPMI-1640 medium with 10% FBS, fixed in methanol and downregulated miR-100 expression and control cells stained with 0.1% crystal violet solution. Samples were photographed and counted for the number of visible colonies.

Allograft and immunohistochemistry. Cells in logarithmic growth were adjusted to a cell density of 5x10⁶/ml and injected subcutaneously into the nude mice at 200 µl/mouse. When the tumors grew to 5 mm in diameter cisplatin was injected at 4 mg/kg every 3 days for a total of 7 times. The size of tumor was measured every 3 days to draw the growth curve. Three days after the end of treatment, the mice were sacrificed, tumor tissue removed, fixed in 10% formalin and used in immunohistochemistry assays for PLK1 and mTOR.

Statistical analyses. The SPSS 20 software was used for statistical analysis, measurement data were expressed as mean ± standard derivation (SD). Paired groups were analyzed using Student's t-test, and one-way analysis of variance (ANOVA) was applied to compare the difference among multiple groups. Values with P<0.05 was considered statistically significant.

Results

Resistance of SKOV3/DDP cells to cisplatin. We first determined the IC50 to measure the resistance to cisplatin. For SKOV3/DDP and SKOV3 cells, IC50 was 8.29 and 3.72 µg/ml, respectively. Therefore, SKOV3/DDP was 2.23 times more resistant to cisplatin than SKOV3 (Fig. 1A).

Expression of miR-100. We then profiled the expression of miR-100 in the two cell lines. It was found that the expression of
miR-100 was 25 times less in SKOV3/DDP cells as compared with SKOV3 cells (Fig. 1B). However, after transfection with the miR-100 mimics, the level of miR-100 in transfected SKOV3/DDP cells was significantly higher than that in LV-NC transfected cells (P<0.05; Fig. 2A), while transfection with the miR-100 antisense resulted in significantly reduced miR-100 level in SKOV3/DDP cells (P<0.01; Fig. 2A).

**Change in IC₅₀ after transfection.** The sensitivity of resistant SKOV3/DDP cells to cisplatin was determined after the cells were upregulated by miR-100 mimics. Compared with the cells transfected with LV-NC, cells transfected with LV-miR-100 had significantly low IC₅₀ to cisplatin (P<0.001). On the other hand, when transfected with LV-anti-100, the IC₅₀ was significantly increased (P<0.001; Fig. 2B).

**Expression of mTOR and PLK1.** Next, we examined the expression of mTOR and PLK1 in the cell lines before and after transfection using western blot analysis. Compared with SKOV3 cells, mTOR and PLK1 protein levels were significantly higher in the resistant SKOV3/DDP cells (P<0.05; Fig. 3A). After transfection with LV-miR-100, the levels of the two proteins were significantly lower than these in the LV-NC-transfected cells (P<0.05; Fig. 3B), while transfection with LV-anti-100 resulted in significantly higher expression of the two proteins (P<0.05; Fig. 3B).

**Effect of miR-100 on the proliferation of SKOV3/DDP cells.** The numbers of colony were reduced or increased after SKOV3/DDP cells were transfected with LV-miR-100 upregulation or downregulation constructs. Compared with SKOV3 cells, the growth of SKOV3/DDP cells was reduced as revealed by the proliferation index (Fig. 4C); on the other hand, transfection with LV-anti-100 increased the proliferation index, as compared with LV-NC (Fig. 4C).

**Effect of miR-100 on early apoptosis of SKOV3/DDP cells.** After 48 h of cisplatin treatment at 3 µg/ml, we examined the early apoptosis rate in SKOV3/DDP cells. The results showed that the apoptosis rate was significantly higher after the cells were transfected with LV-miR-100 as compared with LV-NC-transfected cells (P<0.01), while the apoptosis rate in SKOV3/DDP cells transfected with LV-anti-100 was significantly lower (P<0.05; Fig. 5A and C).

**Effect of miR-100 on SKOV3/DDP cell cycle.** The proportions of G1 and S phase cells in LV-miR-100 transfected cells was significantly higher or lower than those in LV-NC-transfected cells (P<0.01; Fig. 5B and D), respectively; in contrast, the proportions of G1 and S phase cells in LV-anti-100 transfected cells were significantly lower or higher than those in LV-NC-transfected cells (P<0.05; Fig. 5B and D), respectively.

**Effect of miR-100 on efficacy of cisplatin in grafted resistant tumors in mice.** We then used mouse models with grafted cells to assess the efficacy of cisplatin. When the mice were grafted...
Figure 4. (A) Colonies, (B) their numbers and (C) growth curves of SKOV3/DDP transfected with miR-100 mimic and antisense constructs. *P<0.05 vs. LV-NC.

Figure 5. Early apoptosis rate and proportion of SKOV3/DDP cells at different cell cycle after transfection with miR-100 mimic and antisense constructs. (A) Flow cytometry of apoptotic cells; (B) flow cytometry of cells at different cell cycle; (C) apoptosis rate; (D) cell percent at different cell cycles. *P<0.05 and **P<0.01 vs. LV-NC.
with SKOV3/DDP cells transfected with LV-miR-100, the tumors grew slower than those grafted with LV-NC-transfected SKOV3/DDP cells. On other hand, tumors grew faster in mice grafted with LV-anti-100 than with LV-NC-transfected SKOV3/DDP cells (Fig. 6A). Twenty-one days after cisplatin treatment, the tumor volumes were smaller from SKOV3/DDP cells stably transfected with LV-miR-100 than with LV-NC (Fig. 6A and B; P<0.01). In contrast, the tumors derived from SKOV3/DDP cells stably transfected with LV-anti-100 were significantly larger than with LV-NC (P<0.01; Fig. 6A and B). Immunohistochemical analysis indicated that compared with tumors derived from LV-NC transfected cells, mTOR and PLK1 were downregulated in the tumors derived from LV-miR-100 transfected-cells and upregulated in the tumors derived from LV-anti-100 transfected cells (P<0.05; Fig. 6C), respectively.

Discussion

Recent studies have shown that the abnormal expression of miRNAs is closely related to the occurrence, development, metastasis and prognosis of tumor, suggesting that miRNAs play a pivotal and regulatory role in the occurrence and development of tumors (15). Various miRNAs are demonstrated to impact the sensitivity of ovarian cancer to chemotherapy (16). For example, Chan et al (17) found that the suppression of miR-21 expression increases the chemosensitivity of ovarian cancer to cisplatin and paclitaxel and induces apoptosis of ovarian cancer cells. Wang et al (18) showed that the expression of miR-182 is associated with the tolerance of ovarian cancer cells to platinum-based drugs and paclitaxel and that suppression of miR-182 expression sensitizes ovarian cancer cells to these drugs. Abnormally expressed miRNAs in ovarian cancer may regulate the expression of genes that are responsible for drug tolerance, thereby influencing the outcome and prognosis of chemotherapy. Zhang et al (19) found that in ovarian cancer cell line A2780/DDP, upregulation of miR-182 expression results in targeted suppression of XIAP and restores the sensitivity of the cell line to platinum drugs; Cochrane et al (20) found that upregulation of miR-200c in ovarian cancer cell line Hey leads to targeted repression of TUBB3 and sensitizes the cell line to paclitaxel. These studies demonstrate that miRNAs may be used as a biomarker for the treatment of ovarian cancer and have potential in clinical applications and may serve as important targets for reversing drug resistance in ovarian cancer.

miR-100 was found early to be related to tumor. Studies have shown that it is lowly expressed in malignant tumors such
as nasopharyngeal carcinoma, non-small cell lung, bladder cancer, hepatocellular carcinoma and ovarian cancer (21-26), where it functions as tumor repressor. Studies also show that it is possible to regulate the chemosensitivity and prognosis of cancer by targeted regulation of expression of genes involved in drug sensitivity (27-29). In our earlier study, we found that there is an association between the expression of miR-100 in EOC patients and clinical or pathological outcomes. We found that miR-100 is negatively correlated with the clinical stage, metastasis, relapse and prognosis of ovarian cancer after chemotherapy (30). In this study, we show that the expression level of miR-100 was lower in drug tolerant SKOV3/DDP cells than in their parent cells, suggesting that miR-100 may be associated with cisplatin resistance in EOC. Upregulation of miR-100 in SKOV3/DDP cells with miR-100 mimics reduced the IC_{50} of the tolerant cells to cisplatin, while downregulation of miR-100 with antisense miR-100 increased the IC_{50}. These results indicate that the expression level of miR-100 is a factor affecting the sensitivity of EOC cells to cisplatin. We then investigated the mechanism underlying the regulation of cisplatin sensitivity by miR-100. Our data showed that miR-100 increases the sensitivity probably by inhibiting cell proliferation, promoting cell apoptosis and cell cycle arrest.

Previous studies have indicated that mTOR and PLK1 are the target molecules of miR-100 (31,32). mTOR and PLK1 are members of highly conserved serine/threonine kinase family, which are widely present in eukaryotic cells, and are involved in cell proliferation, metabolism, metastasis and other biological processes (33). PLK1 and PLK1 are highly expressed in many human cancers, including ovarian cancer processes (34), and play an important role in regulating the growth and metastasis of tumor cells and in determining the sensitivity to chemotherapy (35,36). Yang et al (37) showed that the mTOR pathway is an intracellular signaling pathway important in regulating the sensitivity of ovarian cancer cells to cisplatin. Gao et al (38) suggested that PLK1 is an independent prognostic factor for ovarian cancer and silencing PLK1 increases the sensitivity of ovarian cancer cells to cisplatin chemotherapy. In this study, the expression of mTOR and PLK1 was significantly higher in SKOV3/DDP cells than in SKOV3 cells, suggesting that the overexpression of the mTOR and PLK1 genes may be associated with cisplatin resistance in EOC. Once miR-100 expression was upregulated, mTOR and PLK1 expression was decreased, vice versa. These findings demonstrate that miR-100 may sensitize EOC to cisplatin by targeted downregulation of mTOR and PLK1 expression.

In summary, the present study demonstrates that upregulation of miR-100 can inhibit cell proliferation, promote cell apoptosis and cell cycle arrest, and downregulate mTOR and PLK1 expression in EOC; as a result, it sensitizes the resistant EOC cells to cisplatin, resulting in reversed drug resistance. These findings provide new insights into the mechanism of drug resistance in EOC, solution to overcome the drug resistance and help to identify new targets for the gene therapy of drug resistant ovarian cancer.

Acknowledgements

The present study was supported by the Science and Technology Funds of Guangdong Province (2013B0218000303).

References


