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MiR-106a Targets Mcl-1 to Suppress Cisplatin Resistance of Ovarian Cancer A2780 Cells*

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Summary: Resistance to chemotherapy is a major obstacle for the effective treatment of advanced ovarian cancer. The mechanism of chemoresistance is still poorly understood. Recently, more and more evidence showed microRNAs (miRNAs) modulated many key molecules and pathways involved in chemotherapy. microRNA-106a (miR-106a) has been implicated in many cancers, but its role in ovarian cancer and drug resistance still remains unexplored. This study was to investigate whether miR-106a mediated resistance of the ovarian cancer cell line A2780 to the chemotherapeutic agent cisplatin (DDP). The different levels of miR-106a in A2780 cells and their resistant variant A2780/DDP cells were identified by using real-time PCR. MTT assay and flow cytometry were used to analyze the effect of miR-106a on cisplatin resistance of these paired cells. Real-time PCR, Western blotting and luciferase reporter assay were applied to explore whether Mcl-1 was a target of miR-106a. As compared to A2780 cells, the expression of miR-106a was down-regulated in the cisplatin resistant cell line A2780/DDP. Moreover, knockdown of miR-106a dramatically decreased antiproliferative effects and apoptosis induced by cisplatin in A2780 cells, while overexpression of miR-106a significantly increased antiproliferative effects and apoptosis induced by cisplatin in A2780/DDP cells. Furthermore, miR-106a inhibited cell survival and cisplatin resistance through downregulating the expression of Mcl-1. Mcl-1 was a direct target of miR-106a. These results suggest that miR-106a may provide a novel mechanism for understanding cisplatin resistance in ovarian cancer by modulating Mcl-1.
Key words: miR-106a; ovarian cancer; cisplatin resistance; Mcl-1

Epithelial ovarian cancer is a common gynecologic malignancy and causes more deaths than any other cancer of the female reproductive system[1]. The treatment of ovarian cancer has been improved over the last 20 years because of more effective surgery and optimized combinational chemotherapy. However, the overall curative rate is only 30%. The major patients will eventually develop resistance to combined chemotherapy, such as platinum-based drugs, cisplatin or carboplatin coupled with paclitaxel[2, 3]. Therefore, understanding of the molecular mechanisms of drug resistance involved in ovarian cancer cells has the potential to improve the therapy of this disease.

The recently discovered microRNAs (miRNAs) constitute a novel regulatory layer of gene expression and may play a role in the etiology of various kinds of human cancers, including ovarian cancer[4, 5]. Although recent reports suggest that miRNAs may influence ovarian cancer cell response to chemotherapy[6, 7], so far, the role of them and their target mRNAs in determination of ovarian cancer chemosensitivity remains to be completely elucidated. It was reported that miR-106b is invovled in cisplatin resistance in human testicular cancer[8], and its seed family member miR-106a[9] is highly expressed in ovary[10]. So, we wanted to know whether the miR-106a took part in cisplatin resistance chemotherapy in ovarian carcinoma.

In this report, we found the miR-106a was down-expressed in cisplatin-resistant A2780 ovarian cancer cell line, and it was involved in the resistance of A2780 cells to cisplatin by negatively regulating target gene. We postulate that the miR-106a may develop new strategies for targeted cisplatin resistance chemotherapy in ovarian carcinoma patients.

1 MATERIALS AND METHODS

1.1 Chemicals and Antibodies

Cisplatin was purchased from Sigma (USA). RPMI-1640, fetal bovine serum (FBS), and Lipofectamine2000 reagent were purchased from Life Technologies Inc. (USA). MTT was obtained from Sigma (USA). MiR-106a mimics or inhibitors and negative miRNA control mimics or inhibitors were from Dharmacon, Inc. (USA). Psi-CHECK-2TM plasmid and dual luciferase reporter assay kit were from Promega (USA). Mcl-1 rabbit polyclonal and β-actin antibodies were obtained from Santa Cruz Biotechnology (USA). Real time-PCR prim-

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ers were from Invitrogen Corporation (USA).

1.2 Cell Line and Cell Culture

A cisplatin-sensitive ovarian cancer cell line (A2780) and its resistant variant (A2780/DDP) were gifts from Cancer Biology Research Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology[11]. Cells were maintained in RPMI-1640 complete medium supplemented with 2 mmol/L L-glutamine and 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2. The cells were passaged once every 1 to 2 day(s) and used for following experiments in their logarithmic phase.

1.3 MiRNA Mimics or Inhibitors Transfection

Lipofectamine 2000 (Invitrogen, USA) was used for the transfection of miRNA mimics or miRNA inhibitors in the A2780 or A2780/DDP cell lines according to the manufacturer's protocol. Forty-eight h later, the cell lysates were prepared and the expression of miR-106a was detected by using real-time PCR, and the target gene of miR-106a Mcl-1 expression was tested by Western blotting.

1.4 Real-Time PCR

Cultured cells were harvested in Trizol reagent (Invitrogen Corp, USA) to extract total RNA. For analysis of miRNA expression by real-time PCR, reverse transcription and PCR were carried out using Bulge-LoopTM miRNA qPCR Primer Set for hsa-miR-106a and U6 snRNA (RiboBio, China) according to the manufacturer's instructions. Real-time PCR was carried out using SYBR green PCR master mix (TaKaRa, Japan). Amplification and detection were performed using ABI Prism 7700 system (Applied Biosystems, USA). The relative amounts of miRNA expression were calculated by using the comparative CT method. Each measurement was performed in triplicate.

1.5 Western Blotting

Cells were harvested and lysed in lysis buffer. The lysates were incubated at 4°C for 30 min and centrifuged at 12 000 r/min at 4°C for 20 min to collect the supernatants. The protein concentrations were determined by using the Bicinchoninic acid (BCA) methods. Proteins were loaded to each well of a 10% SDS-PAGE gel and electrotransferred onto PVDF membranes. After blocking with 5% non-fat milk for 1 h at room temperature, the membranes were incubated with primary antibodies specific for the following proteins: Mcl-1 (1:1000), and β-actin (1:1000). After three washes with TBST for 15 min each at room temperature, the membranes were incubated with horseradish peroxidase-labeled secondary antibody for 1 h at 37°C. Then they were washed again in TBST three times for 15 min each at room temperature. Protein was visualized with NBT/BCIP/buffer (1:1:50). Protein loading was assessed by blotting the same membrane with an antibody against β-actin.

1.6 Measurement of Cell Viability by MMT

The thiazolyl blue tetrazolium blue (MTT)-based cell proliferation assay was carried out. Forty-eight h after transfection, A2780 and A2780/DDP cells were seeded in triplicate in a 96-well plate at the density of 5×10^3 per well. After being cultured for 24 h, A2780 cells were transfected with miR-106a inhibitor, or miR-inhibitor negative control (inhibitor NC), at a dose of 50 nmol/L using Lipofectamine 2000. A2780/DDP cells were transfected with miR-106a mimics at a dose of 50 nmol/L, or miR-mimics negative control (mimics NC), similarly. After incubation for 24 h, cells were treated with increasing concentrations (0, 10, 20, 30, 40, 50 μmol/L) of cisplatin for 48 h. The inhibition of cell viability was detected by using MTT. A total of 15 μL of thiazolyl blue (5 mg/mL) was added to each well and incubated at 37°C for 4 h until formazan was formed. After the supernatants were discarded, 100 μL DMSO was added into each well. The plates were then gently agitated for 20 min to dissolve the crystals. Finally, absorbance (*A*) was detected at 570 nm using a microplate reader. All experiments and measurements were repeated three times.

1.7 Cell Apoptosis Rate Assayed by Flow Cytometry

Twenty-four h after transfection with miR-106a mimics or inhibitors, the cells were allowed to grow for additional 48 h in cisplatin-free medium or treated with DDP at a final concentration of 20 μmol/L in A2780 cells and 50 μmol/L DDP in A2780/DDP cells. For apoptosis analysis by annexin V/propidium iodide, 1×10^5 treated cells were incubated with annexin V/propidium iodide for 15 min at room temperature. Cells were sorted using a FACScalibor (BD Biosciences, USA) and analyzed with CellQuest version 3.3 software. The apoptotic cells were calculated after FACS analysis. The experiments were repeated three times.

1.8 Construction of Reporter Plasmids and Luciferase Reporter Assay

Target prediction algorithms such as MiRanda, PicTar and TargetScan predicted that miR-106a is a putative regulator of Mcl-1. The whole sequence of Mcl-1 3′-UTR was PCR-amplified using human genome DNA as template. The PCR product was linked into the PGM-T vector (Tiangen, China). Plasmid DNA was subsequently isolated from recombinant colonies and sequenced to certify reliability. The Mcl-1 3′-UTR (3'-Mcl-1) inserts were removed from the PGM-T plasmid and inserted into the downstream of the Renilla luciferase reporter gene (psiCHECK-2™, Promega, USA). The authenticity of the inserts gene was confirmed by sequencing. The new recombinant plasmid was named psiCHECK/Mcl-1. The control vector (psiCHECK/con) was generated by cloning the same 3'-UTR in reverse orientation (3'-Mcl-1-rev) to the psiCHECK-2 reporter vector. For the luciferase assay, A2780/DDP cells were co-transfected with psiCHECK/Mcl-1 or psiCHECK/con and miR-106a mimics or mimics NC using Lipofectamine 2000 reagent at 70%—80% cell confluence in 12-well plates. Twenty-four h after transfection, firefly and renilla luciferase activities were measured consecutively using dual-luciferase assays (Promega, USA) according to the manufacturer's protocol.

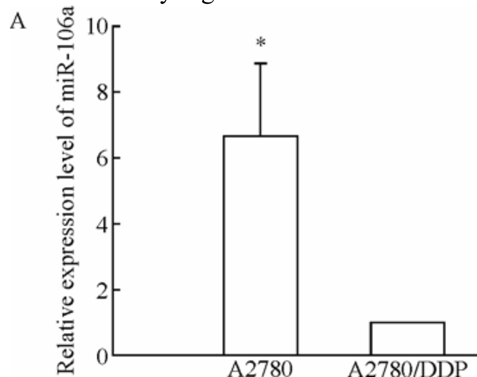
1.9 Statistical Analysis

Statistical analysis was conducted with SPSS 12.0 software. Student's *t* test and ANOVA analysis were used to compare mean values. Data were expressed as $\bar{x}\pm s$ of at least triplicate samples. A $P<0.05$ was considered to be statistically significant.

2 RESULTS

2.1 Expression Levels of MiR-106a and Mcl-1 in A2780 and A2780/DDP Cells

There was statistically significant difference in the expression levels of miR-106a and Mcl-1 between A2780 and A2780/DDP cells (fig. 1), indicating that miR-106a and Mcl-1 had a possible correlation with cisplatin resistance in ovarian cancer cells.



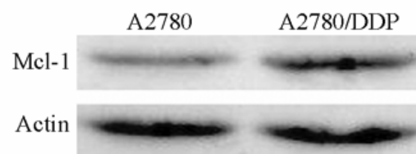


Fig. 1 The expression levels of miR-106a and Mcl-1 in A2780 and A2780/DDP cells

A: The expression level of miR-106a was significantly higher in A2780 cells than in A2780/DDP cells ($^{*}P<0.05$); B: The expression of Mcl-1 was remarkably higher in A2780/DDP cells than in A2780 cells.

2.2 Correlation between Change of MiR-106a Expression and Cisplatin-induced Apoptosis in A2780 and A2780/DDP Cells

We further studied the role of miR-106a and its mechanism in cisplatin-induced apoptosis in ovarian cancer cells. The miR-106a inhibitor and inhibitor NC were transfected into A2780 cells respectively. The transfection efficiency was tested by real-time PCR (fig. 2A). Then, cells were treated with increasing concentrations of cisplatin for 48 h. The cell viability was assessed by using MTT assay. We found that knockdown of miR-106a by miR-106a inhibitors decreased cisplatin-induced cytotoxicity in A2780 cells as compared with negative control (fig. 2C). Conversely, the miR-106a mimics and mimics NC were transfected into A2780/DDP cells respectively. Real-time PCR was used to test the transfection efficiency (fig. 2B). Similarly, transfected cells were treated with the increasing concentrations of cisplatin. It was found that overexpression of miR-106a enhanced cisplatin-induced cytotoxicity in A2780/DDP cells as compared with negative control (fig. 2D).

A2780 cells transfected with miR-106a inhibitor or inhibitor NC were treated with 20 μmol/L cisplatin for 48 h. Apoptosis assays showed as many as 48.25%±1.94% and 50.83%±2.04% of the cells underwent apoptosis in A2780 and inhibitor NC groups, respectively. In contrast, only 24.57%±2.43% of the cells underwent apoptosis in A2780 cells transfected with miR-106a inhibitor ($P<0.05$) (fig. 2E). However, when transfected with miR-106a mimics and negative control in A2780/DDP cells and treated with 50 μmol/L cisplatin for 48 h, nearly 57.23%±2.56% of the cells underwent apoptosis in A2780/DDP mimics group, but 38.47%±2.08% and 40.39%±2.32% of the cells underwent apoptosis in mimics NC and blank groups respectively ($P<0.05$) (fig. 2F). These results apparently demonstrated that miR-106a may play an important role in cisplatin resistance in ovarian cancer cells.

2.3 Inverse Correlation between Mcl-1 and MiR-106a in Ovarian Cancer Cells

Candidate miR-106a targets were predicted with TargetScan, Pictar, and Mirnada sequence analysis software, and Mcl-1 was chosen for further examination. We found differential expression levels of Mcl-1 in A2780 and A2780/DDP cells (fig. 1B). Then, we found that in A2780 cells in which the miR-106a inhibitor was introduced, the endogenous miR-106a activity was suppressed (fig. 2A), and the expression of Mcl-1 was notably increased (fig. 3A). However, the up-regulation of endogenous miR-106a by the miR-106a mimics led to the significant down-regulation of Mcl-1 protein in A2780/DDP cells (fig. 3B). This inverse correlation between miR-106a and Mcl-1 protein levels provided evidence to support our conclusions.

2.4 Mcl-1: a Direct Target of MiR-106a in Ovarian Cancer Cells A2780

The three public miRNA databases (TargetScan, Pictar, and Mirnada) all predicted that Mcl-1 might be a target gene for miR-106a, and the 3′-UTR of Mcl-1 contains a binding site for miR-106a (fig. 4A). In order to further identify whether Mcl-1 in A2780 cells responded to miR-106a through direct interactions with its 3'-UTR, we cloned the Mcl-1 3'-UTR sequences into a luciferase reporter plasmid, and cloned its reverse orientation sequences into the luciferase reporter plasmid as control. miR-106a mimics or mimics NC and psiCHECK/Mcl-1 or psiCHECK/con were co-transfected into A2780/DDP cells transiently. A luciferase reporter assay system was used to detect luciferase expression 48 h after transfection. The results showed that the miR-106a mimic attenuated the fluorescence driven by the Mcl-1 by more than 2-fold compared with the negative control, whereas the psiCHECK/con, 3'-Mcl-1-rev was not affected by miR-106a (fig. 4B).

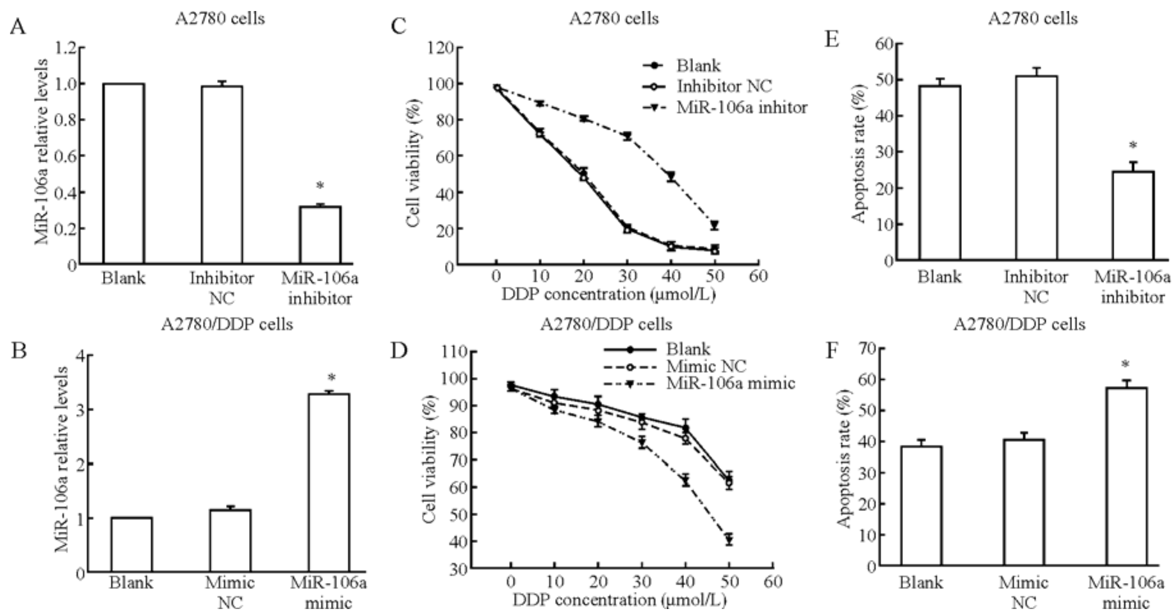


Fig. 2 The miR-106a expression and cisplatin-induced apoptosis in A2780 and A2780/DDP cells

A: The relative expression of miR-106a in A2780 and control cells after transfected with miR-106a inhibitor ($^*P$<0.05); B: The relative level of miR-106a in A2780/DDP and control cells after transfected with miR-106a mimics ($^*P$<0.05); C: Down-regulation of miR-106a by miR-106a inhibitor decreased cisplatin-induced cytotoxicity in A2780 cells as compared with inhibitor NC and blank groups (P<0.05); D: Over-expression of the miR-106a by miR-106a mimics increased cisplatin-induced cytotoxicity in A2780/DDP cells as compared with mimics NC and blank groups (P<0.05); E: MiR-106a inhibitor or inhibitor NC was transfected into A2780 cells for 24 h and treated with 20 μmol/L cisplatin for 48 h. The percentage of apoptosis in cells transfected with miR-106a inhibitor was significantly lower than in inhibitor NC and blank groups ($^*P$<0.05); F: MiR-106a mimics or mimics NC was transfected into A2780/DDP cells for 24 h and treated with 50 μmol/L cisplatin for 48 h. The percentage of apoptosis in cells transfected with miR-106a mimics was significantly higher than in mimics NC and blank groups ($^*P$<0.05).

Fig. 3 Inverse correlation between Mcl-1 and miR-106a in ovarian cancer cells

A: After A2780 cells were transfected with miR-106a inhibitor and inhibitor NC for 48 h, the Mcl-1 protein expression was tested by Western blotting; B: The Mcl-1 protein expression levels in A2780/DDP cells transfected with an miR-106a mimic and mimic NC 48 h later were verified by Western blotting.

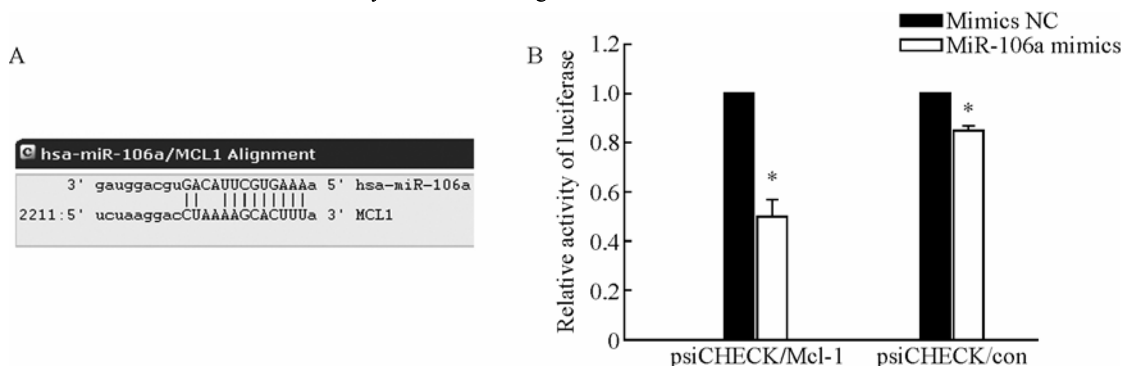


Fig. 4 Mcl-1: a direct target of miR-106a in A2780 ovarian cancer cells

A: TargetScan, PicTa and MiRanda analyses all indicated that miR-106a has one binding site on Mcl-1 3′-UTR. Mcl-1 was the possible target of miR-106a. This picture was downloaded from MiRanda base data. B: A2780/DDP cells co-transfected with luciferase reporter plasmids and with 3'-UTR or with its reverse orientation sequences of Mcl-1. Luciferase activity was measured by using a dual luciferase reporter assay.

3 DISCUSSION

Drug resistance is a multifactorial process which is responsible for the absence of chemoresponse in primary and secondary tumors. The first line of chemotherapy is the combination of carboplatin/cisplatin with paclitaxel in advanced ovarian cancer. But around 20% of patients do not respond to this regimen at the first cycle and pro-

gress upon treatment in the first year and with poor outcome[12, 13]. The recently discovered miRNAs constitute a novel regulatory layer of gene expression. Increasing studies have indicated that aberrant miRNAs are associated with chemotherapy response in ovarian cancer[14–18]. They can regulate different cellular processes: proliferation, differentiation, apoptosis, and drug resistance by targeting hundreds of genes[19]. However, the role of miRNAs in drug-resistant ovarian cancer remains poorly understood.

MiR-106a is located in Xq26.2. Previous reports showed that miR-106a is up-regulated in gastric and colon cancers, and glioblastoma, ect.[20–23], and it is involved in the migration and invasion of these cancers. There are seldom reports about miR-106a expression and function in ovarian cancer and its correlation with cancer chemotherapy. In this report, we first demonstrated that miR-106a was down-regulated in A2780/DDP cells as compared with their parental A2780 cells. Furthermore, we have shown that the up-regulation of miR-106a in A2780/DDP cells increased sensitivity and apoptosis induced by cisplatin, whereas the down-regulation of miR-106a expression in A2780 cells led to a significant reduction of anti-proliferative effects and apoptotic index induced by cisplatin.

To explore the possible mechanism by which miR-106a mediates chemoresistance of cisplatin involved in ovarian cancer, we predicted and tested the activity of miR-106a on a downstream target. It was found that miR-106a expression was negatively correlated with Mcl-1 protein expression in A2780 cells and A2780/DDP cells. In fact, the up-regulation of miR-106a was associated with the decreased expression of Mcl-1, a common feature of aggressive disease and chemoresistance in ovarian cancer. Mcl-1 is a member of the Bcl-2 family which contributes to the ability of the cells to survive and evade the toxic effects of a drug. Reports showed that the Mcl-1 gene is involved in cisplatin and paclitaxel resistance in ovarian cancer[24–28]. Here, we proved that Mcl-1 is a direct target of miR-106a in A2780 cells. So, we conclude that miR-106a might take part in the cisplatin drug-resistance through the regulation of Mcl-1 expression in A2780 cells. To the best of our knowledge, this study firstly described an association between miR-106a/Mcl-1 expression and drug resistance in DDP-resistant ovarian cancer cells.

This novel finding may provide drug targets for the sensitivity of ovarian tumor cells and could be used for treating chemotherapy resistance in ovarian cancer patients. However, future researches are needed to further support the function of miR-106a in cisplatin resistance of ovarian cancer cells. These reports may help patients who have abnormal levels of miR-106a or Mcl-1 to develop individualized treatment protocols.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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