Original Article
Overexpression of miR-199a-5p confers cisplatin resistance in ovarian cancer cells by regulating GSK-3β

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Abstract: Ovarian cancer is the most common cause of death from gynecological malignancies in the world. Aberrantly expression of MiRNAs in ovarian cancer has been confirmed that is associated with histological subtypes, tumor stage and prolong. Chemotherapy resistance is the major obstacle to the effective therapy of cancer. While the mechanism of chemotherapy resistance is still not fully revealed. Accumulating evidences showed that microRNAs (miRNAs) may have a crucial function in response to chemotherapy through modulating cellular pathways. In this study, we found that the expression level of miR-199a-5p was lower in ovarian cancer cell line A2780 cells compared with its cisplatin resistant variant A2780/DDP cells. Overexpression of miR-199a-5p in A2780 cells significantly attenuated cisplatin-induced apoptosis and antiproliferative effects, while knockdown of miR-199a-5p in A2780/DDP cells increased the sensitivity to cisplatin. Moreover, expression levels of GSK-3β and its downstream protein Bcl-2 were both modulated by miR-199a-5p. The luciferase report assay indicated that GSK-3β is the target gene of miR-199a-5p. In addition, interference of GSK-3β expression enhanced miR-199a mimics-induced cell drug resistance in A2780 cells. These results conclude that miR-199a-5p is able to confer cisplatin resistance in ovarian cancer cells through inhibiting GSK-3β expression.

Keywords: Chemotherapy resistance, cisplatin, ovarian cancer, microRNAs, apoptosis, GSK-3β

Introduction
Ovarian cancer is the most common cause of death from gynecological malignancies in the world [1]. Cytotoxic chemotherapy has contributed to dramatic improvements of survival rate of ovarian cancer patients. While, chemotherapy resistance is the main limitation to the effective therapy of ovarian cancer. Cisplatin is a widely used chemotherapeutic agent in ovarian cancer and other solid tumors [2]. Clinically relevant levels of resistance can develop quickly after cisplatin treatment. The process of cisplatin resistance is multifactorial, including changes in drug accumulation or drug-target interaction, increased DNA-repair, or alteration of apoptosis signal pathway [3]. However, the mechanism for cisplatin resistance is not fully characterized.

MiRNAs are a large group of conserved small non-coding RNA that bind to the 3’ untranslated region (3’UTR) of target RNA acting as regulators of gene expression at the posttranscriptional level [4]. Thus, miRNAs could participate in the regulation of critical processes, such as cell proliferation, apoptosis and differentiation. Numerous studies have shown the involvement of microRNAs in tumorigenesis, tumor progression and response to therapy [5, 6]. There is a significant association between miRNA expression profiling and clinical pathological characteristics [7]. For instance, miR-199a has been shown to be participated in multiple malignancy relative processes, including cycle process, apoptosis and invasion [8, 9]. MiR-199a expression exerts different levels in various human cancers and might be associated with tumor development. MiR-199a is low expressed in hepatocellular carcinoma [10], but highly expressed in gastric cancer [11, 12]. In the different genotype breast cancer cell lines, miR-199a-5p shows dual differential roles in autophagy which is important in cancer therapeutic resistance, e.g. miR-199a-5p inhibits radiation-induce autophagy in MCF-7 cells, but
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upregulates autophagy in MDA-MB-231 cells [13]. Moreover, miR-199a-5p, upregulated in cetuximab-resistant colorectal cancer cells, is able to affect cell resistance by targeting PHLPP1 [14]. Hence, the role of miRNAs in cancer shows cell specificity.

In this study, we explored the expression and function of miR-199a-5p in cisplatin-treated ovarian cancer cell lines and examined the mechanisms underlying its effects. We found that ovarian cancer cell line resistant to cisplatin A2780/DDP exhibits a significant up-regulation of miR-199a-5p expression compared with its parental line A2780. Re-expression of MiR-199a-5p enhanced cisplatin resistance in ovarian cancer cells. Moreover, miR-199a-5p could regulate the expression of GSK-3β, a crucial factor in drug resistance and tumor progress, by binding GSK-3β 3’UTR. Interference of GSK-3β expression enhanced miR-199a mimics-induced drug resistance in A2780 cells. Our results suggest that miR-199a-5p acts as an enhancer of cisplatin resistance in ovarian cancer cells via targeting downregulation of GSK-3β, suggesting miR-199a-5p/GSK-3β axis as potential therapeutic targets for the treatment of cisplatin-resistant ovarian cancer.

Materials and methods

Cell lines and cell culture

Human ovarian cancer cell line A2780 (ATCC, USA) and its cisplatin resistant variant A2780/DDP (YRbio, China) were cultured in DMEM medium (GIBCO, USA) supplemented with 10% fetal calf serum (GIBCO, USA), 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified incubator with 5% CO₂ at 37°C.

Transfection of miRNA and siRNA

The miR-199a-5p mimics and inhibitors and specific siRNA of GSK-3β were all purchased from GenePharm, China. For miRNA transfection, cells were plated at 3×10⁵ per well in 6 well plates and cultured for 24 h. Then the cells were transfected with the mimics or inhibitors of miR-199a-5p or negative control (NC) RNA, at a final concentration of 50 nM, using Li-pofectamine 2000 (Invitrogen, USA) and serum-free Opti-MEM medium (GIBCO, USA). For siRNA transfection, the cells were transfected with the siRNA GSK-3β or siRNA NC at a final concentration of 20 nM, using si-Mate transfection reagent (GenePharm, China) and serum-free Opti-MEM medium. After 6 h, the medium was replaced with DMEM with 10% FBS. Total RNAs and proteins were extracted after 48 h of transfection.

Construction of vector and luciferase reporter assay

The 3’-UTR of GSK-3β gene (position 61-67) were predicted to be complementary to the sequence of miR-199a-5p according to an analysis of the miRNA target gene prediction database, TargetScan. The fragment sequence of wild-type and mutant GSK-3β 3’-UTR was amplified by PCR and cloned into the pYr-MirTarget luciferase vector (Yrbio, China), named pYr-GSK-3U and pYr-GSK-3Um. The primers for the wild-type 3’UTR were: forward primer: ccgctc gag cctaac cag tctagcttg; reverse primer: aaagccggccgcatgacagatga, containing XhoI and NotI restriction sites. The primers for the mutant 3’UTR were: ctagctctggtgatgact ctc ttaa cta ttttttcctc; reverse primer: aagggaaaaagatgagatgaggtg acctgtcatacc acc ggctg a. HEK293 cells (ATCC, USA) were plated into a 96 well plate and co-transfected with 100 ng pYr-GSK-3U or pYr-GSK-3Um and miRNA mimics (50 nM), using Lipofectamine 2000. Luciferase activity was determined by the Dual-Luciferase reporter assay kit (Promega, USA) following the manufacturer’s instructions. Each experiment was repeated at least three times in triplicate.

Cell viability assay

The cytotoxic effect of cisplatin was measured by MTT assay as previously described [15]. Briefly, cells were plated in 96-well plate at 2×10⁴ per well and cultured for 24 h, the A2780 and A2780/DDP cells were transfected with the mimics or inhibitor of miR-199a-5p or co-transfected with siRNA GSK-3β for 24 h, then the cells were treated with different concentration of cisplatin (0, 5, 10, 20, 40, 60, 80, 100 μg/ml) for 48 h. The MTT (Sigma, USA) was added to a final concentration of 0.5 mg/ml, and the cells were incubated for 4 h at 37°C. The absorbance at 570 nm were measured by microplate reader (Bio-rad, USA). Each experiment was repeated at least three times in triplicate.
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Apoptosis assay

Apoptotic cells were performed with Annexin V-FITC apoptosis detection kit (Keygen, China). After 24 h post-transfection, the A2780 and A2780/DDP cells were treated with cisplatin (20 μg) for 48 h. Then the cell samples were harvested with 0.25% trypsin without EDTA washed twice with ice-cold PBS and re-suspended in 500 μl binding buffer. Then cells were incubated with 5 μl Annexin V-FITC specific antibodies and 5 μl propidium iodide (PI) then incubated for 15 minutes in dark and detected by BD C6 cytometer (BD, USA) with the excitation wavelength of Ex = 488 nm and emission wavelength of Em = 530 nm. Each experiment was repeated three times in triplicate.

qRT-PCR analysis

Total microRNAs were extracted by the miRNeasy Kit (Qiagen, German) according to the manufacturer’s instructions. RNA was converted to cDNA using the miScriptRT kit (Qiagen, German). QRT-PCR was performed using a miScript SYBR Green PCR kit (Qiagen, German) on the ABI 7900HT Real-time PCR System (Applied Biosystem, USA). The expression of miRNAs was normalized using the RNU6 as endogenous control. The primers of miR-199a and U6 were purchased from Yrbio Biotech (China).

Total RNA was isolated with Trizol Reagent (Invitrogen, USA), for GSK-3β and Bcl-2 mRNA analyses according to the manufacturer’s instructions. RNA was converted to cDNA using the PrimeScript® 1st Strand cDNA Synthesis Kit (Takara, Japan). QRT-PCR was performed using SYBR Premix Ex Taq™ (TaKaRa, Japan) on ABI7500 Real-time PCR System (Applied Biosystem, USA). GSK-3β primers were: forward 5’GGA GAA CTG GTC GCC ATC AAG3’; and reverse 5’ACA TTG GGT TCT CCT CGG ACC3’. Bcl-2 primers were: forward 5’GTG GAG GAG CTG TTC ATG GA-3’; and reverse 5’TGG TG CTC GCA ATT AGT GG3’. β-actin primers were: 5’GGG AAA TCG TGC GTG AC3’; and reverse 5’TGG TGT TGG GCA ATT AGT GG3’. All of the qRT-PCR assays were performed in triplicate and the change was calculated using the 2⁻ΔΔCt method.

Figure 1. Expression levels of miR-199a-5p and GSK-3β in A2780 and A2780/DDP cells. A: Survival curves of A2780 and A2780/DDP cells. The cells were exposed to different concentration of cisplatin (DDP) (0, 5, 10, 20, 40, 60, 80, 100 μg) for 48 h. Cell viability was determined by MTT assay. B: Expression level of miR-199a-5p was 4.1 fold higher in A2780/DDP cells compared with A2780 cells. C: Expression of GSK-3β in A2780 and A2780/DDP cells. Experiments were performed in triplicate and expressed as means ± SD and statistically analyzed by t-test, **P<0.01 vs. A2780 cells.
Western blot analysis

After transfection and cisplatin treatment, cells were harvested and homogenized with lysis buffer (Beyotime, China). The protein concentrations were measured using the BCA protein assay kit (Beyotime, China). Proteins (30 to 50 μg) were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane (Millipore, USA). After blocking with 5% non-fat milk, the membranes were incubated with primary antibodies specific to GSK-3β, Bcl-2 and β-actin (1:1000, Santa Cruz, USA). The membranes were further incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (1:5000, Santa Cruz, USA). The immunoblots were detected by western luminescent detection kit (Thermo, German) and quantified by ImageJ software.

Statistical analysis

Data was expressed as means ± SD of at least three independent experiments and statistically analyzed by one way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test or independent sample t-test using the SPSS Statistics 17.0 software. The level of significance was based on the probability of P<0.05 and P<0.01.

Results

Expression level of miR-199a-5p in A2780 and A2780/DDP cells

The cell viability of A2780 and A2780/DDP cells under cisplatin treatment for 48 h was shown in Figure 1A. The estimating IC_{50} of A2780/DDP cells showed 4 fold higher than
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that of A2780 cells (20 μg/ml vs. 87 μg/ml, P<0.01). To investigate the involvement of miR-199a-5p in cisplatin resistant ovarian cancer cell lines A2780/DDP, the levels of miR-199a-5p were detected by qRT-PCR. Comparing with A2780 cells, the expression levels of miR-199a-5p were 4.1 fold higher in A2780/DDP cells (Figure 1B, P<0.01). Moreover, western blot results showed that the expression of GSK-3β was higher in A2780 cells compared with A2780/DDP cells Therefore, the effect of miR-199a-5p and GSK-3β on cisplatin resistance in A2780 cells was further investigated.

Re-expression of miR-199a-5p confers the A2780 cells resistant to cisplatin treatment

To investigate the effect of miR-199a-5p on regulating A2780 cells resistant to cisplatin, the A2780 cells were transfected with miR-199a-5p mimics or NC and then measured the cell survival to cisplatin. The transfection of miR-199a-5p mimics significantly up-regulated miR-199a-5p expression (Figure 2A). Up-regulated miR-199a-5p attenuated cisplatin-induced anti-proliferation in A2780 cells (Figure 2B). Moreover, after treated with 20 μg cisplatin for 48 h, the A2780 cells transfected with miR-199a-5p mimics had a lower apoptotic rate than NC (Figure 2C).

Knockdown of miR-199a-5p enhances the A2780/DDP cells sensitivity to cisplatin

We further investigated the effects of down-regulated miR-199a-5p on cisplatin-induced cytotoxicity in A2780/DDP cells. A2780/DDP cells transfected with miR-199a inhibitor effectively reduced the miR-199a expression and enhanced the sensitivity of A2780/DDP cells to
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Figure 4. GSK-3β is a target gene of miR-199a-5p. (A) GSK-3β’s UTR mRNA includes a highly-conserved binding site for miR-199a-5p. (B) HEK293 cells were co-transfected with pYr-GSK-3β-3U and miR-199a-5p mimics or NC. Dual-Luciferase activity was detected. (C) HEK293 cells were co-transfected with pYr-GSK-3U or pYr-GSK-3β-3UMt and miR-199a-5p mimics. Dual-Luciferase assays showed that miR-199a-5p mimics repressed pYr-GSK-3β-3U luciferase, but not pYr-GSK-3β-3UMt luciferase. (D) Expression of GSK-3β and Bcl-2 in A2780 cells transfected with miR-199a-5p mimics and (E) in A2780/DDP cells transfected with miR-199a inhibitor. The densitometric analysis results were shown in the right panels. Experiments were performed in triplicate and expressed as means ± SD and statistically analyzed by t-test and ANOVA. *P<0.05 and **P<0.01 vs. miR-199a-5p NC.

cisplatin (Figure 3A and 3B). Moreover, miR-199a inhibitor transfected cells had a significantly higher apoptotic rate than NC (Figure 3C). These results indicated that miR-199a-5p is involved in the cisplatin resistance mechanism of ovarian cancer cells.

GSK-3β is a direct target gene of 199a-5p

GSK-3β is a widely expressed serine/threonine kinase which regulates numerous cellular functions, including cell cycle progression, differentiation, migration and metabolism [16]. It has
been reported that the activation of GSK-3β exerts antitumor effects through negatively regulating several downstream targets, such as Bcl-2, Mcl-1, β-catenin and c-Myc [17]. Overexpression of antiapoptotic Bcl-2 occurs frequently in cancers, resulting in enhanced cell survival and drug resistance [18]. Above results had confirmed that the expression of GSK-3β was significantly higher in A2780 cells compared with A2780/DDP cells (Figure 1C). To identify whether GSK-3β is the target gene of miR-199a-5p, the miRNA target prediction public databases (Targetscan, USA) were used for analysis. It was observed that the 3’-UTR mRNA of GSK-3β contained highly-conserved binding sites for miR-199a-5p (Figure 4A). To investigate the association between GSK-3β and miR-199a-5p, the HEK293 cells were co-transfected with pYr-GSK3-3U and pYr-GSK3-3Umt and mir-21 mimics or NC. The results showed that mir-199a mimics dramatically reduced the luciferase activity of pYr-GSK-3β-3U compared with miR-199a NC (Figure 4B). Moreover, there was no difference in the luciferase activity of pYr-GSK-3β-3Umt (Figure 4C). These data suggested that GSK-3β is a direct target gene of miR-199a-5p.

To determine whether miR-199a-5p is involved in the modulation of GSK-3β expression, the miR-199a mimics and inhibitor were transfected into the A2780 and A2780/DDP cells, respectively. The expression of GSK-3β was reduced following miR-199a mimics transfection in A2780 cells, while, its downstream gene Bcl-2 expression was increased dramatically (Figure 4D). In contrast, the increased GSK-3β expression and decreased Bcl-2 expression were observed in A2780/DDP cells transfected with miR-199a inhibitor compared with the NC group (Figure 4E).

GSK-3β is involved in A2780 cells resistance to cisplatin

To further confirm the relationship between miR-199a/GSK-3β axis and A2780 cells resistance to cisplatin, we suppressed GSK-3β expression by specific siRNA in A2780 cells and cell viability was tested. As showed in Figure 5A, siRNA GSK-3β effectively reduced GSK-3β expression in A2780 cells and cell viability was tested. As showed in Figure 5A, siRNA GSK-3β effectively reduced GSK-3β expression in A2780 cells. Knockdown of GSK-3β further enhanced cell resistance to cisplatin induced by miR-199a mimics (Figure 5B).

Discussion

MiRNAs play a crucial role in many biological processes, such as proliferation, apoptosis and differentiation, through modulating gene expression at the posttranscriptional level [4]. Aberrant miRNAs could affect the expression of target genes, which may modulate cell death signal pathway, drug targets protein and cell cycle related proteins. This may lead to resistance of cancer cells to chemotherapy [19]. Upregulation of miR-214 and miR-93 induces resistance of ovarian cancer cell to cisplatin through modulating PTEN signal pathway [20, 21]. While, miR-152 and miR-185 co-contribute to ovarian cancer cells sensitivity to cisplatin [22]. In the present study, we first demonstrat-
ed that miR-199a-5p higher expressed in cisplatin resistant ovarian cancer cell line A2780/DDP compared with the parental A2780 cells. Furthermore, knockdown of miR-199a-5p in A2780/DDP cells increased GSK-3β expression and decreased Bcl-2 expression, promoting the sensitivity of A2780/DDP cells to cisplatin. Overexpression of miR-199a-5p in A2780 cells led to reducing GSK-3β and increasing Bcl-2 expression, while the sensitivity to cisplatin was decreased. Suppression of GSK-3β expression by specific siRNA in A2780 cells enhanced the cell resistant to cisplatin. These findings indicate that upregulation of miR-199a-5p may contribute to cisplatin chemoresistance in ovarian cancer through negative regulation of GSK-3β expression.

Accumulating evidences have confirmed that miR-199a is involved in cancer development by targeting cancer oncogenes or tumor suppressors [9, 23]. The expression of miR-199a was diversely deregulated in several types of cancer. For instance, miR-199a was downregulated in prostate cancer and bladder cancer, but it was upregulated in gastric cancer and cervical carcinoma [24, 25]. Moreover, it has been demonstrated that miR-199a are the differential expressed between chemosensitive and chemoresistant cells which may participate in the cell chemoresistance. Downregulation of miR-199a-3p and miR-199a-5p is involved in hepatoma cells resistant to cisplatin [26, 27]. In contrast, miR-199a-5p is higher expressed in cetuximab resistant colon cancer cells than its parental line, and enforced expression of it promotes cetuximab resistance [14]. These researches indicated that miR-199a also has diverse roles in chemotherapy resistance. Here we found that miR-199a-5p expression was significantly increased in A2780/DDP cells compared with its parental cells (Figure 1B). Inhibitor of miR-199a-5p significantly promoted cisplatin-induced apoptosis and antiproliferative effect (Figure 2).

To further investigate the mechanism of miR-199a-5p in the cisplatin resistance, we predicted possible target mRNA for miR-199a-5p using computational algorithms (TargetScan). The intersection of algorithms and luciferase reporter assay indicated that GSK-3β was a target gene of miR-199a-5p (Figure 4). The modulation of GSK-3β has been involved in tumorigenesis and cancer progression, while the role of GSK-3β is controversial and cell types dependent [28]. In ovarian cancer cells, GSK-3β regulates ovarian cancer cell proliferation by control of cell cycle [29]. Activation of GSK-3β by adipo-nectin could induce apoptosis and cell cycle arrest in breast cancer cells [30]. Evidences indicate that GSK-3β could regulate cellular sensitivity to chemotherapy. It has been reported that GSK-3β activation sensitizes human breast cancer cells to chemotherapy drugs-induced apoptosis (cisplatin, taxol and 5-fluoro-uracil, etc.) [31, 32]. However, in some cases, downregulation of GSK-3β promotes chemotherapy-induced apoptosis in human colorectal cancer cells [33]. Recent studies indicate that the suppression of tumors by GSK-3β is mediated by Bcl-2 family members, such as Mcl-1, Bax and Bcl-2 [17]. Bcl-2 is an antiapoptotic protein which inhibits most types of apoptotic cell death [34]. Aberrant expression of Bcl-2 has been shown to be associated with drug resistance in cancer chemotherapy [35]. In the current study, we demonstrated that overexpression of miR-199a-5p in A2780 cells led to increasing Bcl-2 expression and reduction of GSK-3β expression and apoptosis. Knockdown of miR-199a-5p by its inhibitor caused the opposite trends (Figures 2 and 3). The modulation of GSK-3β by miR-199a-5p mediated Bcl-2 expression which might contribute to the change of cell sensitivity to cisplatin (Figure 4). Similar results were also found in other studies that inhibition of GSK-3β blocks estoposide-induced apoptosis via an increased Bcl-2 expression and blockade of caspase-3 activity [36].

In summary, our finding showed that miR-199a-5p could confer the cisplatin resistance by blocking the expression of GSK-3β in cisplatin-resistant ovarian cancer cells. Moreover, we demonstrated that GSK-3β is a target gene of miR-199a-5p and play an important role in sensitivity of ovarian cancer cells to cisplatin. These results suggest miR-199a-5p/ GSK-3β axis might be potential therapeutic targets for the treatment of cisplatin-resistant ovarian cancer.

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Disclosure of conflict of interest

None.

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