miR-3622b-5p regulates cisplatin resistance of human gastric cancer cell line by targeting BIRC5

Ping Zhu¹,△, Xia Shan²,³,△, Jinhui Liu⁴,△, Xin Zhou², Huo Zhang², Tongshan Wang², Jianqing Wu¹,✉, Wei Zhu²,⁵,△, Ping Liu²,✉¹  

¹Jiangsu Provincial Key Laboratory of Geriatrics, Department of Geriatrics, the First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, China; ²Department of Oncology, the First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210000, China; ³Department of Respiration, the Affiliated Jiangning Hospital of Nanjing Medical University, Nanjing, Jiangsu 210000, China; ⁴Department of Obstetrics and Gynecology, the First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, China; ⁵Department of Oncology, the Affiliated Jiangsu Shengze Hospital of Nanjing Medical University, Suzhou, Jiangsu 215000, China.

Abstract

Many evidences showed that drug resistance of gastric cancer cells could be regulated by the abnormal expression of microRNAs (miRNAs), a post-transcriptional regulator of gene expression. Thus, we investigated the role of miR-3622b-5p in the development of cisplatin (DDP) resistance in human gastric cancer cell lines. A set of biochemical assays were used to elucidate the mechanism by which miR-3622b-5p regulates drug resistance in cancer cells. The expression of miR-3622b-5p was measured by quantitative real-time PCR and showed that miR-3622b-5p was significantly downregulated in the plasma of patients with acquired drug resistance to platinum-based chemotherapy for gastric cancer. miR-3622b-5p was also found significantly downregulated in DDP-resistant gastric cancer cell line SGC7901/DDP, compared with the parental SGC7901 cells. An in vitro drug sensitivity assay showed that overexpression of miR-3622b-5p sensitized SGC7901/DDP cells to DDP. The luciferase activity of reporters constructed by BIRC5 3′-untranslated regions in SGC7901/DDP cells suggested that BIRC5 was target gene of miR-3622b-5p. Ectopic miR-3622b-5p expression in SGC7901/DDP cells significantly repressed the expression of the BIRC5 and sensitized the cells to DDP-induced apoptosis. By contrast, treatment with miR-3622b-5p inhibitor increased the protein expression of BIRC5 and led to a lower proportion of apoptotic cells in the SGC7901 cells. In conclusion, our findings suggest that miR-3622b-5p regulates DDP resistance of human gastric cancer cells at least in part by repressing the expression of BIRC5. Altering miR-3622b-5p expression may be a potential therapeutic strategy for the treatment of chemoresistance in gastric cancer in the future.

Keywords: miR-3622b-5p, gastric cancer, BIRC5, cisplatin resistance
Introduction

Gastric cancer is the fifth most common malignancy in the world with a high mortality rate. However, due to the low rate of early diagnosis, many patients are diagnosed at advanced stages. Nowadays, the standard treatment method for gastric cancer includes surgery, chemotherapy and radiotherapy. For patients at advanced stages, chemotherapy is still one of the main treatments. However, tumor cells may become resistant to chemotherapeutic drugs, which often eventually leads to failure of chemotherapy. Thus, it is important to identify the molecular mechanisms underlying the drug resistance of gastric cancer cells.

MicroRNAs (miRNAs), an important type of non-coding RNA, are single-stranded and consist of 19–23 bp nucleotides in length. The mechanism of miRNA function is that miRNA can cause partial or complete bind to the 3′-UTR of target mRNAs, resulting in translational repression or target mRNA degradation. miRNAs are involved in the post-transcriptional regulation of more than 30% of the encoded genes in the human body, involving a wide range of biological processes including signal transduction, and cell proliferation, differentiation and apoptosis. In addition, abnormal miRNA expression is associated with tumor proliferation, invasion and metastasis. Therefore, miRNAs can act as a marker for tumor diagnosis and prognosis and as a new target for tumor therapy. Aberrant miRNAs have been found to be associated with chemoresistance in different types of tumors, including ovarian and breast cancer. However, there is limited available knowledge on the potential role of miRNAs in the chemotherapeutic resistance of gastric cancer.

In the previous study, we found that compared with that in normal gastric tissues, miR-3622b-5p was significantly downregulated in gastric cancer tissues. This downregulation in ERBB2-positive gastric cancer tissues was more obvious than that in ERBB2-negative gastric cancer tissues. miR-3622b-5p directly targeted HER2 and increased the sensitivity of HER2-negative gastric cancer tissues. miR-3622b-5p was found significantly downregulated in gastric cancer tissues compared to that in normal gastric tissues, miR-3622b-5p was found significantly downregulated in gastric cancer tissues. This downregulation in ERBB2-positive gastric cancer tissues was more obvious than that in ERBB2-negative gastric cancer tissues. miR-3622b-5p directly targeted HER2 and increased the sensitivity of HER2-negative gastric cancer tissues. miR-3622b-5p was found significantly downregulated in gastric cancer tissues compared to that in normal gastric tissues.

Materials and methods

Clinical samples

Fourteen patients with advanced gastric cancer were recruited. They underwent at least 4 cycles of platinum-based chemotherapy until acquired drug resistance was developed. Plasma samples of these patients were gathered for miRNA detection before chemotherapy and at the development of acquired drug resistance. All the procedures were approved by Institutional Review Boards of the First Affiliated Hospital of Nanjing Medical University, and the written informed consents were obtained from each participant.

Peripheral venous blood (2 mL) samples from gastric cancer patients were collected with ethylenediaminetetraacetic acid (EDTA) containing tubes (Becton, Dickinson and Company, USA). The blood plasma was separated according to the two-step protocol [350 g for 10 minutes, 20 000 g for 10 minutes (Beckman Coulter, USA)]. Plasma samples were then stored at −80 ℃ for further analyses.

Cell culture

Gastric adenocarcinoma cell lines SGC7901 were purchased from the National Institute of Cells (Shanghai, China). DDP-resistant variant SGC7901/DDP was obtained from KeyGEN Biotechnology Company (Nanjing, China). All the cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, USA) in a humidified atmosphere containing 5% CO₂ at 37 °C. To maintain the DDP-resistant phenotype, DDP (with final concentration of 1 μg/mL) was added to the culture media for SGC7901/DDP cells.

RNA extraction

Trizol (Invitrogen, Carlsbad, USA) was used to lyse cells according to the manufacturer’s instructions. The mirVana PARIS Kit (Ambion, Austin, USA) was used to extract total RNA from cell lysis products and 200 μL plasma according to the manufacturer's protocol. Synthetic C. elegans miR-39 (5 μL; 5 nmol/L, RiboBio, Guangzhou, China) was added to each sample for normalization. The acquired RNA was finally dissolved in 100 μL RNase-free water and stored at −80 ℃ for future analysis. The concentration and purification of RNA were measured using ultraviolet spectrophotometer (NanoDrop Technologies, Wilmington, USA).

Quantitative real-time PCR analysis for miRNA

The amplification of miRNA was conducted via
specific primers of reverse transcription (RT) and the following polymerase chain reaction (PCR) using Bulge-Loop™ miRNA qRT-PCR Primer Set (RiboBio). According to the previous protocol[11], RT reaction was conducted at 42 °C for 60 minutes followed by 70 °C for 10 minutes. Then, quantitative real-time PCR (qRT-PCR) was performed in triplicate in 384-well plates on the LightCycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany) at 95 °C for 20 seconds, followed by 40 cycles at 95 °C for 10 seconds, 60 °C for 20 seconds and then 70 °C for 10 seconds.

As previously described, expression levels of plasma miRNAs were evaluated by a standard curve constructed by using synthetic miRNAs (micrON™ miRNA mimic, RiboBio)[12]. For the evaluation of miRNA expression in cells the 2ΔΔCt method normalized with RNU6B (U6) was applied (ΔCt = average Ct_{assay}−average Ct_{normalizer assay}; Ct: the cycle number at the threshold level of fluorescence)[13].

**In vitro drug sensitivity assay**

SGC7901/DDP and SGC7901 cells were plated in 6-well plates (6 × 10^4 cells/well), 100 nmol/L miR-3622b-5p mimic or 100 nmol/L miRNA mimic control were transfected in SGC7901/DDP cells, while 100 nmol/L miR-3622b-5p inhibitor or 100 nmol/L miRNA inhibitor control were transfected in SGC7901 cells, using lipofectamine 2000 (Invitrogen, Long Island, USA) according to the manufacturer's protocol. The miR-3622b-5p mimic, miRNA mimic control, 2′-O-methyl (2′-O-Me) modified miR-3622b-5p inhibitor and miRNA inhibitor control were chemically synthesized with Shanghai GenePharma Company (Shanghai, China). Twenty-four hours after transfection cells were seeded into 96-well plates (5 × 10^3 cells/well). After cellular adhesion, freshly prepared DDP was added at the final concentration 0.01, 0.1, 1 and 10 times of the human peak plasma concentration of DDP as previously described[14]. The peak serum concentration of DDP was 2.0 μg/mL[14]. Forty-eight hours after the addition of drugs, cell viability was assessed by MTT assay. The absorbance at 490 nm of each well was read on a spectrophotometer. The concentration at which DDP produced 50% inhibition of growth (IC_{50}) was estimated by the relative survival curve. Three independent experiments were performed in triplicate.

**Dual-luciferase activity assay**

The 3′-UTR of human BIRC5 cDNA containing the putative target site for the miR-3622b-5p (sequence shown in Supplementary Data, available online) was chemically synthesized and inserted at the Xba I site, immediately downstreaming the luciferase gene in the pGL3-control vector (Promega, Madison, USA) by Integrated Biotech Solutions Co, Ltd (Shanghai, China). Twenty-four hours before transfection, cells were plated at 1.5 × 10^4 cells/well in 24-well plates. 200 ng of pGL3-BIRC5-3′-UTR plus 80 ng pRL-TK (Promega) were transfected in combination with 60 pmol/L of the miR-3622b-5p mimic or miRNA mimic control using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol[14]. Luciferase activity was measured 24 hours after transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to renilla luciferase activity for each transfected well. Three independent experiments were performed in triplicate.

**Immunohistochemistry**

Tissue samples were obtained from surgical specimens of 15 cases diagnosed with gastric cancer between May 2016 and December 2017 at the First Affiliated Hospital of Nanjing Medical University. Tissue samples were formalin-fixed and paraffin embedded, cut into 4-μm thick slides, and stained using the avidin-biotin complex method. Tissue slides were subjected to antigen retrieval using microwave irradiation in 10 mmol/L citrate buffer (pH 6.0), followed by incubation with anti-BIRC5 primary antibody (1: 1 000 dilution of rabbit mAb, clone 71G4B7, Cell Signaling Technology, Danvers, USA) overnight at 4 °C. Following three washes with PBS, the sections were incubated with goat anti-rabbit IgG (1: 1 000, cat No. A0277, Beyotime Institute of Biotechnology, Shanghai, China) for 2 hours at 27 °C. The slides were scored by two independent observers blinded to the clinical data. They evaluated the immunostaining of the slides under an optical microscope at a magnification of 200×. The staining intensity of the expression of the above proteins was scored on a scale of 1–3 as follows: 0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. The percentage of positive cancer cells was scored as follows: 0 for 0%, 0.1 for 1%–9%, 0.5 for 10%–49%, and 1.0 for 50% or more. We multiplied the staining intensity by the proportion score of the percentage of positive cancer cells.

**Immunofluorescence staining**

SGC7901 and SGC7901/DDP cells were grown on glass coverslips and fixed with 4% paraformaldehyde at 4 °C for 15 minutes and were further permeabilized
and blocked with 0.5% Triton X-100 and 5% bovine serum albumin in phosphate-buffered saline for 30 minutes. The coverslips were then exposed to primary antibodies as mentioned above at 4 °C overnight, followed by incubation with the appropriate secondary antibodies. The cells were visualized using an Olympus IX70 fluorescence microscope.

**Western blotting analysis**

SGC7901/DDP cells and SGC7901 were plated in 6-well plates (6 × 10⁵ cells/well), 72 hours after being transfected with miR-3622b-5p mimic or miR-3622b-5p inhibitor, respectively, cells were harvested and homogenized with lysis buffer. Total protein was separated by denaturing 10% SDS–polyacrylamide gel electrophoresis. Total protein of SGC7901 and SGC7901/DDP was also extracted and separated as described above. Western blotting analysis was performed as described [14]. The primary antibodies for BIRC5 (1 : 1 000 dilution of rabbit mAb, Catalog: 71G4B7) and GAPDH (1 : 1 000 dilution of rabbit mAb, Catalog: BS6945) were purchased from Cell Signaling Technology and Bioworld Technology, respectively. Protein levels were normalized to GAPDH. Fold changes were determined.

**Apoptosis assay**

The cells were transfected with miR-3622b-5p mimic or inhibitor. After 24 hours, the cells were treated with DDP at a final concentration of 10 μg/mL. After 48 hours, apoptosis was assessed via the counting of annexin V–fluorescein isothiocyanate (Annexin V-FITC)-positive and propidium iodide-negative cells using flow cytometry, as described previously[14–15].

**Statistical analysis**

Each experiment was repeated at least 3 times. Numerical data were presented as mean±SD. The difference between means was analyzed with Student's t-test. All statistical analyses were performed using SPSS22.0 software. Differences were considered significant when P<0.05.

**Results**

**miR-3622b-5p was downregulated in gastric cancer patient plasma with chemotherapeutic resistance and DDP resistant cell line**

To investigate the potential role of miR-3622b-5p in gastric cancer chemoresistance, we first detected the plasma level of miR-3622b-5p in 14 gastric cancer patients with acquired DDP resistance and matched gastric cancer patients before chemotherapy. The plasma expression of miR-3622b-5p was significantly decreased in DDP resistance group compared with that in matched gastric cancer group (Fig. 1A). We further found that the expression of miR-3622b-5p was significantly lower in SGC7901/DDP cells than that in the parental cell line SGC7901 (Fig. 1B).

**miR-3622b-5p regulated DDP resistance of the SGC7901/DDP cell line**

In SGC7901/DDP cells, MTT assay revealed that those transfected with miR-3622b-5p mimics exhibited greatly decreased resistance to DDP compared with the miRNA mimic control transfected cells (Fig. 2A), while in SGC7901 cells, those transfected with miR-3622b-5p inhibitor exhibited greatly enhanced resistance to DDP compared with the miRNA inhibitor control transfected cells.

![Fig. 1](image-url)  
**Expression of miR-3622b-5p was associated with chemoresistance in gastric cancer.** A: Plasma level of miR-3622b-5p significantly lower in DDP resistance group compared with that in matched gastric cancer group (*P=0.016). B: Expression levels of miR-3622b-5p were also significantly downregulated in SGC7901/DDP cells, compared with that in the parental SGC7901 cell line (*P=0.002).
These results suggested that miR-3622b-5p might modulate DDP resistance of SGC7901/DDP cells.

**BIRC5 was the target gene of miR-3622b-5p**

TargetScanHuman (http://www.targetscan.org) predicted that BIRC5 was a potential direct target gene of the miR-3622b-5p (Supplementary Fig. 1, available online). To explore whether the BIRC5 was the target gene of the miR-3622b-5p, we constructed the luciferase reporter vectors with the putative BIRC5 3′-UTR target sites for the miR-3622b-5p downstream of the luciferase gene (pGL3-BIRC5-3′-UTR). Luciferase reporter vectors together with the miR-3622b-5p mimic or the miRNA mimic control were transfected into SGC7901/DDP cells. In SGC7901/DDP cells, significant decrease in relative luciferase activity was observed when pGL3-BIRC5-3′-UTR was cotransfected with the miR-3622b-5p mimic but not with the miRNA mimic control (Fig. 3).

**BIRC5 was differently expressed in gastric cancer tissues and upregulated in DDP-resistant gastric cancer cell line SGC7901/DDP**

We observed BIRC5 protein expression in 15 gastric cancer tissue samples by immunohistochemistry. BIRC5 was significantly highly expressed in 4 samples, including 2 diffuse gastric cancer tissues and 2 intestinal gastric cancer tissues, with the positive rate of 26.7% (4/15) (Supplementary Fig. 2, available online). Meanwhile, we also detected the expression levels of BIRC5 in DDP-resistant gastric cancer cell line SGC7901/DDP and gastric cancer cell line SGC7901 using immunofluorescence staining and Western blotting, respectively. We found that the BIRC5 was obviously overexpressed in DDP-resistant gastric cancer cell line, compared with that in the gastric cancer cell line (Fig. 4A and B).

**miR-3622b-5p regulated DDP resistance by repressing BIRC5 protein expression**

Worth of note, in this study the downregulation of miR-3622b-5p in SGC7901/DDP cells was accompanied by the upregulation of BIRC5 protein level compared to that in the SGC7901 cells. Since it was illustrated that BIRC5 may be the target of miR-3622b-5p and that BIRC5 was related to antiapoptosis, it was hypothesized that miR-3622b-5p may play a role in the development of drug resistance, at least in part through modulation of apoptosis by targeting BIRC5. Western blotting analysis was employed to analyze BIRC5 levels in the SGC7901/DDP cells transfected with miR-3622b-5p.
mimic or miRNA mimic control. In the SGC7901/DDP cells, 72 hours after transfection, Western blotting analysis showed a significantly decreased BIRC5 protein expression level in the miR-3622b-5p mimic-transfected cells compared with that in the miRNA mimic control transfected cells. By contrast, the expression of BIRC5 in the SGC7901 cells transfected with the miR-3622b-5p inhibitor was upregulated (Fig. 4C). These results demonstrate that miR-3622b-5p modulates the resistance of gastric cancer cell to DDP, at least in part by suppressing BIRC5 protein expression.

Fig. 4 miR-3622b-5p regulated BIRC5 protein level in SGC7901/DDP cell line. A: Immunofluorescence staining showed that anti-apoptotic BIRC5 protein was overexpressed in SGC7901/DDP cells compared with that in the parental SGC7901 cells (original magnification × 200). B: Western blotting showed that BIRC5 protein was overexpressed in SGC7901/DDP cells compared with that in the parental SGC7901 cells (*P=0.012). C: In the SGC7901/DDP cells, 72 hours after transfection, Western blotting analysis demonstrated a significantly decreased BIRC5 protein level in the miR-3622b-5p mimic-transfected cells compared with that in the miRNA mimic control-transfected cells (*P=0.007). Expression of BIRC5 in the SGC7901 cells transfected with miR-3622b-5p inhibitor was upregulated (*P=0.018). Representative image from Western blotting analysis is shown on panel.
**miR-3622b-5p sensitized SGC7901/DDP cells to DDP-induced apoptosis**

Moreover, the development of drug resistance in various cancer cells had been linked to a reduced susceptibility to drug-induced apoptosis, which was shown to be a consequence, at least in some cases, of overexpression of anti-apoptotic proteins, such as BCL2, IAPs, and survivin. Since the miR-3622b-5p might regulate DDP resistance of gastric cancer cells at least in part by repressing the anti-apoptotic BIRC5 protein expression, we hypothesized that miR-3622b-5p might also play a role in the development of DDP resistance at least in part by regulation of apoptosis of gastric cancer cells. To confirm this hypothesis, we evaluated DDP-induced apoptosis after transfecting SGC7901/DDP cells with the miR-3622b-5p mimic and or miRNA mimic control. In SGC7901/DDP cells, a marked increase in apoptosis, as assessed by flow cytometry, was observed in the miR-3622b-5p mimic transfected cells after DDP treatment, compared with that in the miRNA mimic control transfected cells (*Fig. 5A*). On the contrary, reduced expression of miR-3622b-5p led to a decrease in the apoptosis induced by DDP in SGC7901 cells (*Fig. 5B*).

**Discussion**

Chemoresistance, whether primary or acquired, is the leading cause of failure in the treatment of advanced gastric cancer. Recent studies found that the mechanism of tumor chemoresistance mainly involves genetic and epigenetic abnormalities. Genetic abnormality means drug-mediated gene mutations, deletions and amplifications. Epigenetic changes are drug-induced, non-mutational control of genes such as abnormal methylation of promoter regions, abnormal histone modifications, and abnormalities in non-coding RNA regulation. In recent years, increasing evidence have showed that epigenetic abnormalities play an important role in the development of chemoresistance in cancers. Therefore, it is of great significance to deeply study epigenetic, non-coding RNA regulation and reveal the mechanism of tumor resistance. DDP inhibits the DNA replication process and has been used for many years in the chemotherapy of tumors, including gastric cancer. However, the effectiveness of DDP is greatly reduced by primary or acquired drug resistance. Many studies have reported that the mechanism of cancer chemoresistance involves decreased intracellular drug concentration, increased cis-dexteric detoxification, increased DNA damage repair, increased glutathione and anti-apoptotic protein levels, decreased pro-apoptotic protein expression, and etc.

miRNAs are non-coding small RNAs that regulate gene expression at the post-transcriptional level and are drug-induced, non-mutational control of genes such as abnormal methylation of promoter regions, abnormal histone modifications, and abnormalities in non-coding RNA regulation. In recent years, increasing evidence have showed that epigenetic abnormalities play an important role in the development of chemoresistance in cancers. Therefore, it is of great significance to deeply study epigenetic, non-coding RNA regulation and reveal the mechanism of tumor resistance. DDP inhibits the DNA replication process and has been used for many years in the chemotherapy of tumors, including gastric cancer. However, the effectiveness of DDP is greatly reduced by primary or acquired drug resistance. Many studies have reported that the mechanism of cancer chemoresistance involves decreased intracellular drug concentration, increased cis-dexteric detoxification, increased DNA damage repair, increased glutathione and anti-apoptotic protein levels, decreased pro-apoptotic protein expression, and etc.

**Fig. 5** miR-3622b-5p mimic enhanced DPP-induced apoptosis in SGC7901/DDP cells. A: In SGC7901/DDP cells, a marked increase in apoptosis in cells transfected with miR-3622b-5p mimic was detected, compared with that in cells transfected with mimic control (*P*<0.005). B: SGC7901 cells were transfected with the miR-3622b-5p inhibitor or control. There were fewer cells undergoing apoptosis in the cells under-expressing miR-3622b-5p (*P*<0.008). Representative flow cytometry results are shown adjacent to the graphs. Data are presented as the mean±SD.
are suggested to be associated with drug resistance in a variety of tumors. For example, the downregulation of miR-181b can promote chemoresistance in DDP-resistant H446 small cell lung cancer cells by targeting Bcl-2[24]. miR-142-3p directly targets sirtuin 1 to enhance DDP sensitivity of ovarian cancer[25]. microRNA-223-3p regulates cell chemo-sensitivity by targeting FOXO3 in prostate cancer[26]. It has been reported that miR-3622b-5p acts as a tumor suppressor by repressing ERBB2 expression in gastric and breast cancer. In addition, miR-3622b-5p made ERBB2-positive cancer cells more vulnerable to the apoptosis induced by DDP and 5-FU. Therefore, miR-3622b-5p can be taken as a novel target of chemotherapeutic agents[10]. However, there has not been any thorough research investigating the association between miR-3622b-5p and resistant gastric cancer.

BIRC5, also known as survivin, is a member of the IAP family that plays an important role as an anti-apoptotic protein in the inhibition of apoptosis[27]. The overexpression of survivin is significantly associated with a poor clinical outcome and cancer chemoresistance[28]. As a consequence, survivin is a very promising biomarker for drug resistance[29]. Dysregulation of survivin in human cancers can be the result of epigenetic mechanisms due to promoter methylation[30]. In addition, several survivin-targeting miRNAs have been described in different cancers[31], including miR-218 in nasopharyngeal cancer and miR-485-5p in breast cancer[9]. Our research found that miR-3622b-5p was downregulated and negatively associated with anti-apoptotic protein BIRC5 in DDP-resistant gastric cancer cells.

Our results showed that miR-3622b-5p could modulate the expression of BIRC5. Overexpression of miR-3622b-5p could sensitize SGC7901/DDP cells to DDP-induced apoptosis, suggesting that miR-3622b-5p might also be associated with development of DDP resistance by regulating the apoptosis of gastric cancer cells.

**Acknowledgments**

The work was supported by the National Natural Science Foundation of China (Grant No. 81672400 and 81672788) and Jiangsu Provincial Key Discipline of Medicine (ZDXKA2016003).

**References**


