miR-106a confers cisplatin resistance by regulating PTEN/Akt pathway in gastric cancer cells

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Recent studies have shown that microRNA-106a (miR-106a) is overexpressed in gastric cancer and contributes to tumor growth. In this study, we investigated whether miR-106a mediated resistance of the gastric cancer cell line SGC7901 to the chemotherapeutic agent cisplatin (DDP). MiR-106a expression was up-regulated in the DDP resistant cell line SGC7901/DDP compared with its parental line SGC7901. Transfection of miR-106a induced DDP resistance in SGC7901, while suppression of miR-106a in SGC7901/DDP led to enhanced DDP cytotoxicity. Further study indicated that the mechanism of miR-106a-induced DDP resistance involved the expression of phosphatase and tensin homolog deleted from chromosome 10 (PTEN) protein and its downstream phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) pathway. This study provides a novel mechanism of DDP resistance in gastric cancer.

Keywords gastric cancer; miR-106a; PTEN

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Introduction

Gastric cancer is the fourth most commonly diagnosed cancer worldwide and ranks second in global cancer mortality [1]. The outcome among patients with advanced gastric cancer is poor. Surgery combined with chemotherapy is the current treatment of choice. The combination of epirubicin, cisplatin (DDP), and 5-fluouracil has been considered as one of the current standard chemotherapies for advanced gastric cancer [2–4]. However, the acquisition of resistance to DDP is a major clinical obstacle to the successful treatment of gastric cancer. Therefore, it is necessary to understand the molecular mechanism involved in DDP resistance, which will be helpful for designing new and targeted therapeutic strategies that can overcome drug resistance.

The process of DDP resistance appears to be multifactorial, which includes changes in drug transport leading to decreasing drug accumulation and increasing drug detoxification, and changes in DNA repair and damage and/or alterations in the apoptotic cell death pathways. However, the mechanisms underlying DDP resistance have not been fully characterized. MicroRNAs (miRNAs) are a class of small non-coding RNAs, which act as post-transcriptional regulators by inhibiting gene expression through either cleavage of the target mRNA or translational repression [5]. Generally, one miRNA can regulate multiple target genes and one gene can be repressed by multiple miRNAs, which results in the formation of complex regulatory pathways [6]. Several studies have suggested that miRNAs are novel players in the development of chemoresistance. miRNAs are differentially expressed in chemosensitive and chemoresistant cells. For example, miR-98, miR-21, and miR-125b have been shown to potentiate chemoresistance [7–9].

MiR-106a belongs to the miR-17 family, which is made up of six family members: miR-17-5p, miR-20a, miR-20b, miR-93, miR-106a, and miR-106b. Wang et al. [10] have found that miR-106a is among the most highly expressed miRNAs in human gastric cancer cell lines and in primary gastric tumors. It also has been reported that the up-regulation of miR-106a promotes survival of esophageal adenocarcinoma cells and confers resistance to DDP [11]. Furthermore, we have found that the level of miR-106a is higher in DDP-resistant gastric cancer cells than in parent gastric cancer cells. However, whether miR-106a can lead to drug resistance in gastric cancer remains unknown. Therefore, we hypothesize that the acquisition of DDP resistance by cancer cells may be modulated via the changes in miR-106a levels. Our present study for the first time showed that miR-106a regulates the expression of the phosphatase and tensin homolog deleted from chromosome 10 (PTEN), a crucial factor in drug resistance, and this interaction may...
have an important functional consequence in the formation of gastric cancer cell resistance to DDP.

Materials and Methods

Cell lines and cell culture
Human gastric cancer cell line SGC7901 was maintained in our laboratory and its DDP-resistant variant SGC7901/DDP was obtained from Keygen Biotech (Nanjing, China). The cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, USA) supplemented with 10% fetal calf serum (Gibco), 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. To maintain the DDP-resistant phenotype, DDP was added to the culture media with a final concentration of 1 µg/ml for SGC7901/DDP cells. SGC7901/DDP cells were cultured for 1 week in DDP-free medium prior to their use in the experiment.

RNA extraction and quantitative real-time polymerase chain reaction analysis of mRNA
Small RNAs were isolated from cultured cells using the RISOTM RNA ISolation Reagent (Biomics, Nantong, China) according to the manufacturer’s protocol. Quantitative real-time polymerase chain reaction (qRT-PCR) for mature miR-106a was conducted with the TaqMan stem-loop kit and the TaqMan universal PCR master mix from Applied Biosystems (Foster City, USA) using the Applied Biosystems AB 7500 real-time PCR system as described previously [12]. The PCR conditions were: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. We used U6 snRNA for normalization of the relative amount of each miRNA. The primers for miR-106a and U6 have been described previously [13]. All of the qRT-PCR assays were conducted in triplicate. The expression levels of each analyte compared with the untreated controls were assessed by using the 2−ΔΔCT method.

qRT-PCR analysis of mRNA expression
Total RNA was extracted with Trizol® reagent (Invitrogen, Carlsbad, USA) in accordance with the manufacturer’s protocol. cDNA was synthesized with the RevertAid First-Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada) according to the manufacturer’s instructions. After the reverse transcription reaction, qRT-PCR was conducted in ABI 7500HT fast real-time PCR System (Applied Biosystems), with U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as endogenous control for data normalization. Primer sequences used for qRT-PCR were as follows: PTEN forward and reverse primers: 5’-CCGGCAG CATCAAATG TTTCAG-3’ and 5’-AAGGGCTGACTGTTAGGAGC-3’, respectively; GAPDH forward and reverse primers: 5’-AAGAGGTTGGAACGAGCAGG-3’ and 5’-TCCACCACCTGTGTGCTGTA-3’, respectively. The PCR conditions were: 95°C for 2 min followed by 35 cycles (95°C for 45 s, 60°C for 45 s, and 72°C for 60 s) and final extension (72°C for 10 min). All of the reactions were run in triplicate and the gene expression was calculated using the 2−ΔΔCT method.

Transfection of miR-106a mimic and inhibitor
The effect of miR-106a on chemosensitivity and apoptosis was evaluated by transfecting the gastric cells with miR-106a mimic or inhibitor purchased from GenePharma (Shanghai, China). The sequences were as follows: miR-106a mimic sequences: 5’-GAUGGACGUGACAUUCGUGAAA-3’, miR-106a inhibitor sequences: 5’-CUACGU GCACUGUAAGCACUUU-3’. The cells were plated into a six-well plate at a density of 1 × 10⁵ cells per well in RPMI-1640 media containing 10% fetal bovine serum (FBS), without antibiotics. After a 12 h incubation, the medium was replaced with Opti-MEM® (Invitrogen), without serum and antibiotics. When the cells reached 50% confluence, the cells were transfected with 50 nM of miR-106a mimic or inhibitor using Lipofectamine® 2000 regent (Invitrogen) according to the manufacturer’s protocol. The medium was replaced with fresh RPMI-1640 containing 10% FBS and antibiotics after a 6 h post-transfection. For RT-PCR and western blot, the cells were collected after an additional 48 h. Three independent experiments were performed.

Cell viability assay
The Cell Counting Kit 8 (CCK-8) was conducted as follows: after a 48 h transfection, the cells were plated at 0.5 × 10⁴ per well in a 96-well plate in RPMI-1640 medium containing 10% FBS, without antibiotics. After 8 h, the cells were treated with various doses of DDP (Qilu Pharmaceutical Factory, Jinan, China) (0.125, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 µg/ml) in combination with or without the Akt inhibitor LY294002 (10 µM) (Beyotime, Shanghai, China). Approximately 48 h after DDP treatment, 10 µl CCK-8 solution was added to the medium, and the cells were incubated for 3 h at 37°C. The optical density at 570 nm was measured with a microplate spectrophotometer. Each experiment was conducted in triplicate and repeated three times.

Apoptosis assay
After a 24 h transfection, the cells were incubated for an additional 48 h without DDP or treated with a final concentration of 5 µg/ml DDP in SGC7901/DDP and 0.5 µg/ml in SGC7901. The apoptosis ratio was analyzed using the Annexin V Apoptosis Detection Kit (Beyotime) according to the manufacturer’s instruction. Early apoptotic cells were defined as Annexin-V-positive, propidium iodide-negative cells. Analyses were performed on BD FACSCalibur (BD
Biosciences, San Diego, USA). The experiments were repeated three times.

**Luciferase assay**
To establish the PTEN 3’-untranslation region (UTR) luciferase reporter, full-length human PTEN 3’-UTR was inserted into the XhoI and NotI sites in the PGL3 vector (Promega, Madison, USA) downstream of the Renilla luciferase coding sequence. The 3’-UTR sequence of PTEN was generated from human genomic DNA by PCR with the following primers: forward, 5’-TCGCTCGAGATTTTTTTATCAA GAGGG-3’ and reverse, 5’-TCGGCGGCCGCGACAA GAATGAGACTTTAATC-3’. Mutations within the putative miR-106a binding sites were performed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, USA). SGC7901 cells were transfected with PGL3 containing either the wild-type or mutant PTEN 3’-UTR, in combination with 10 nM miR-106a mimics, and 20 ng Renilla luciferase control reporter vector (Promega) as transfection control using Lipofectamine 2000. Assays were performed after 48 h of transfection, using the Dual-Luciferase® Reporter Assay System kit (Promega) according to the manufacturer’s instructions. Firefly luciferase activity was normalized to Renilla luciferase activity. Experiments were performed three times.

**Statistical analysis**
The data were presented as mean ± standard error. The difference between means was analyzed with Student’s t-test. All of the statistical analyses were performed by using SPSS12.0 software (SPSS, Chicago, USA). The differences were considered significant when \( P < 0.05 \).

**Results**

**miR-106a is up-regulated in the DDP-resistant SGC7901/DDP cells**
We detected the survival curves of the SGC7901/DDP cell lines and the parental SGC7901 cell lines [Fig. 1(A)]. The results showed that the SGC7901/DDP cell lines were 8.99-fold resistant to DDP compared with the SGC7901 cell lines based on IC_{50} values (4.263 ± 0.357 vs. 0.474 ± 0.021 \( \mu g/ml \), \( P < 0.05 \)). To further investigate the involvement of miR-106a in DDP-resistant gastric cancer cell lines SGC7901/DDP, we performed qRT-PCR analysis. The

**Western blot analysis**
The cells were scraped and lysed in radioimmunoprecipitation assay buffer (Sigma, St Louis, USA). Protein samples (50 \( \mu g \)) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Millipore Corp., Billerica, USA). The membranes were incubated at 4°C overnight with mouse monoclonal antibodies against PTEN (1 : 400, Santa Cruz, Santa Cruz, USA) and rabbit monoclonal antibodies of phospho-Akt (p-Akt) (1 : 500, Cell Signaling, Beverly, USA) and anti-Akt (1 : 500, Cell Signaling). GAPDH (1 : 500, Santa Cruz) was used as an internal control for protein loading. The resulting immunoblots were visualized with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit immunoglobulin using an enhanced chemiluminescence substrate system.

**siRNA transfection**
The transfection was performed using the SignalSilence PTEN siRNA kit (Cell Signaling) according to the manufacturer’s protocol. Forty-eight hours after transfection, the cells were prepared for further analysis. SiRNA transfection efficiency was measured with flow cytometry by calculating the percentage of fluorescein-labeled cells. The experiments were repeated three times. The transfection efficiency was \( \sim 75\% \).

**Figure 1 miR-106a expression in SGC7901 and SGC7901/DDP cells** (A) Survival curves of SGC7901/DDP and SGC7901 cells. The cells were treated with various doses of DDP (0.125, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 \( \mu g/ml \)). After 48 h of incubation, the viability of the cells was measured with the CCK-8 assay. \( P < 0.05 \). (B) qRT-PCR analysis of miR-106a mRNA expression in SGC7901 and SGC7901/DDP cells. \*\( P < 0.05 \).
results showed that the expression level of miR-106a was higher in the SGC7901/DDP cells than in the SGC7901 cells [Fig. 1(B)], which suggested that miR-106a may be associated with DDP resistance in gastric cancer cells.

**Overexpression of miR-106a in the SGC7901 cells confers resistance to DDP**

To investigate the relationship between miR-106a and DDP resistance in SGC7901 cells, we tested the effect of overexpression of miR-106a on the DDP sensitive cells. qRT-PCR revealed that miR-106a mimic significantly increased miR-106a level, suggesting that miR-106a is efficiently transfected into the cells [Fig. 2(A)]. The miR-106a mimic transfected the SGC7901 cells showing a significantly higher survival than the negative control (NC) group (IC50, 3.597 ± 0.259 vs. 0.549 ± 0.019 μg/ml, P < 0.01) [Fig. 2(B)]. The SGC7901 cells transfected with miR-106a mimic had a significantly lower apoptosis rate than the NC group (P < 0.01) [Fig. 2(C,D)]. These results demonstrated that miR-106a confers DDP resistance in the SGC7901 cells.

**Knockdown of miR-106a in SGC7901/DDP cells partially restores DDP sensitivity**

To further detect the effects of miR-106a down-regulation on DDP-induced cell death in SGC7901/DDP cells, we transfected 80 nM of miR-106a inhibitor or NC into the SGC7901/DDP cells. qRT-PCR revealed that the miR-106a inhibitor could reduce miR-106a expression in SGC7901/DDP cells compared with the NC group (P < 0.05) [Fig. 3(A)]. The miR-106a inhibitor transfecting the SGC7901/DDP cells had a significantly lower survival than the NC group (IC50, 0.694 ± 0.265 and 5.563 ± 0.273 μg/ml respectively, P < 0.05) [Fig. 3(B)]. The SGC7901/DDP cells transfected with miR-106a inhibitor group showed a significantly higher apoptosis than the NC group (P < 0.05) [Fig. 3(C,D)]. These results suggested that the knockdown of miR-106a could partially sensitize the SGC7901/DDP to DDP.

**PTEN is a target gene of miR-106a**

TargetScanHuman 6.2 (http://www.targetscan.org) predicted that PTEN was a conserved target gene of miR-106a.

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Figure 2 Overexpression of miR-106a confers DDP resistance in SGC7901 cells (A) qRT-PCR showed that the expression of miR-106a significantly increased in the cells transfected with the miR-106a mimic. *P < 0.05. (B) After 48 h of transfection with the miR-106a mimic or NC, the SGC7901 cells were incubated with various doses of DDP (0.125, 0.25, 0.5, 1.0, and 2.0 μg/ml) for 48 h. The CCK-8 assay was performed to determine the cell viability. *P < 0.05. (C,D) Overexpression of miR-106a in the SGC7901 cells rendered resistance to DDP-induced apoptosis after 48 h of 0.5 μg/ml DDP treatment.
To further detect whether PTEN is the target gene of miR-106a, we constructed a luciferase reporter vector with the target site or the mutant target site downstream of the luciferase gene (PTEN-3'-UTR-wild and PTEN-3'-UTR-mut). Then, we transfected the luciferase reporter vector together with the miR-106a mimic or the NC into the SGC7901 cells, respectively. In the SGC7901 cells, a significant decrease was noted in PTEN-3'-UTR-wild and miR-106a mimic co-transfected group compared with the PTEN-3'-UTR-mut and miR-106a mimic co-transfected group [Fig. 4(B)]. These results indicated that PTEN is the target gene of miR-106a. Then, we further explored whether miR-106a-regulated PTEN expression in SGC7901 cells [Fig. 4(D,F, and H)]. PTEN mRNA and protein levels were increased in the SGC7901/DDP cells transfected with the miR-106a inhibitor [P < 0.05, Fig. 4(C,E, and G)]. These results showed that there was a consistent and strong inverse correlation between the miR-106a levels and PTEN.

PTEN is a key signal molecule in miR-106a-regulated DDP resistance in SGC7901/DDP cells

To determine whether PTEN plays a key role in miR-106a-regulated DDP resistance, we transfected the PTEN siRNA or NC into the miR-106a inhibitor transfected SGC7901/DDP cells, and detected the cell survival rate under various concentrations of DDP. Western blot results showed that PTEN siRNA effectively reduced the protein level of PTEN [Fig. 5(A,B)], and PTEN knockdown significantly increased the survival rate of the SGC7901/DDP cells transfected with the miR-106a inhibitor than the NC group [Fig. 5(C)], which suggested that miR-106a may modulate DDP resistance in the SGC7901/DDP cells by down-regulating PTEN.

Overexpression of miR-106a activates the PI3K/Akt pathway

Western blot results showed that the miR-106a mimic overexpression reduced the expression of PTEN and increased phosphorylation of its downstream kinase Akt [Fig. 4(E,F)],
Figure 4: PTEN is the target gene of miR-106a. (A) The predicted binding site of miR-106a in the 3’-UTR of PTEN. (B) The effect of miR-106a on PTEN was assessed with the luciferase reporter system. The miR-106a mimic together with the luciferase reporter vector or control vector, was co-transfected into the SGC7901 cells, respectively. *P < 0.05. (C) qRT-PCR measured the levels of PTEN mRNA in the SGC7901/DDP cells and SGC7901/DDP transfected with the miR-106a inhibitor or NC. *P < 0.05. (D) qRT-PCR measured the levels of PTEN mRNA in the SGC7901 cells and SGC7901 transfected with the miR-106a mimic or NC. *P < 0.05. (E,G) The PTEN, p-AKT, and AKT protein levels in the SGC7901/DDP cells and SGC7901/DDP cells transfected with the miR-106a inhibitor or NC. *P < 0.05. (F,H) The PTEN, p-AKT, and AKT protein levels in the SGC7901 cells and SGC7901 cells transfected with the miR-106a mimic or NC. *P < 0.05.
As we know, PTEN is a tumor suppressor that is mutated in multiple tumor types with high frequency, and a negative regulator of the PI3 K/Akt pathway. Therefore, we hypothesized that the overexpression of miR-106a may activate the phosphatidylinositol 3 kinase (PI3K)/Akt pathway by reducing the protein level of PTEN, resulting in the SGC7901 cells resistance to DDP. To further investigate the relevance of the PI3K/Akt pathway in miR-106a-induced cell survival and DDP resistance, we treated the miR-106a mimic transfected SGC7901 cells with various doses of DDP and/or PI3K inhibitor LY294002. The results showed that LY294002 partially abrogated the phosphorylation of AKT induced by miR-106a mimic transfection [Fig. 6(A,B)]. Meanwhile, miR-106a-induced cell survival and DDP resistance were significantly inhibited [Fig. 6(C)]. These results suggested that overexpression of miR-106a could activate the PI3K/Akt pathway by down-regulating PTEN in the SGC7901 cells.

**Discussion**

One major mechanism of drug resistance in cancer cells is the dysregulation of the apoptosis pathway [14]. Recently, increasing evidence has indicated that miRNAs could modulate drug resistance of cancer cells, at least in part, through this mechanism [15–17]. Several studies have shown that PTEN is involved in drug resistance in different types of cancer [18,19]. However, to our knowledge, it is still unclear about the role of PTEN in DDP resistance in SGC7901/DDP cells. In this study, the mechanistic connection of miR-106a dysregulation with the establishment of DDP resistance in SGC7901/DDP cells was evidenced by the correlation between the exogenous overexpression of miR-106a and the corresponding changes in the protein levels of its target PTEN, which play an important role in regulating the formation of cancer cells drug resistance.

Increasing number of studies have found that miRNAs may exert functions as oncogenes or tumor suppressors in human cancers by regulating their targets [20,21]. miRNAs play an important role in the genesis and progression of cancer and are also responsible for the resistance of cancer cells to chemotherapeutics. Kovalchuk et al. [22] have demonstrated that miR-451 confers resistance to doxorubicin in breast cancer cells. miR-214 could promote cell survival and induce resistance to DDP in ovarian cancer cells [23]. In this study, we focused on the function of miR-106a in human gastric cancer cells. As an initial step, we found that the expression of miR-106a was significantly increased in DDP-resistant SGC7901/DDP cell lines when compared with SGC7901 cells, which indicated that increased miR-106a levels may be associated with DDP resistance in gastric cancer. To further investigate the function of miR-106a, we transfected the chemically synthesized oligonucleotides mimic or inhibitor into the SGC7901 cells and SGC7901/DDP cells. The experimental results showed that these oligonucleotides could be efficiently transfected into cells and could significantly increase or decrease the levels...
of miR-106a expression. According to the present study, we confirmed for the first time that the overexpression of miR-106a regulates apoptosis and DDP resistance in gastric cancer cell.

Computational algorithms predicted up to hundreds of putative targets for miR-106a, but only a few of them have been experimentally validated. PTEN, which locates on 10q23 and encodes a phosphatase, is involved in tumor suppression [24]. PTEN is associated with gastric cancer pathology and its down-regulation can lead to chemotherapeutic drug including DDP resistance in gastric cancer patients [25]. The PI3K/Akt pathway is well known as a major cell survival pathway, and its activation enhances resistance to apoptosis [26–29]. Cenni et al. [30] have shown that inhibition of the Akt pathway could up-regulate the sensitization of multidrug-resistant human osteosarcoma cells to Apo2 Ligand/TRAIL-induced apoptosis. Inoue et al. [31] have demonstrated that alendronate could inhibit the PI3K/Akt pathway, which caused the apoptosis of osteosarcoma cells. These studies suggested that the PI3K/Akt pathway is also associated with drug resistance in cancer cells. The tumor suppressor PTEN specifically regulates the PI3K/AKT signaling pathway, playing a key role in tumor formation and drug resistance [32]. PTEN could antagonize PI3K activity by dephosphorylating PIP3 and thereby negatively regulate the activity of the Akt pathway [33,34], while loss of PTEN function in human cancer cell lines results in accumulation of PIP3 mimicking the effect of PI3K activation and triggering the activation of its downstream Akt pathway. However, little is known about the impact of miR-106a on the activity of the PI3K/Akt pathway in gastric cancer cells. In the present study, we predicted binding sites for miR-106a in the PTEN 3′-UTR by bioinformatics analysis. Western blot assay indicated that increased expression of miR-106a might have an impact on PTEN expression. Furthermore, we demonstrated that PTEN was a target gene of miR-106a by the luciferase reporter assay. Consistent with these findings, we also found that the overexpression of miR-106a in the SGC7901 cells when used in combination with DDP. Similarly, when the expression of miR-106a and PTEN were inhibited at the same time, the SGC7901/DDP cells showed a high survival than the miR-106a inhibitor alone intervention group. LY294002 abrogated miR-106a activated Akt and significantly inhibited miR-106a-induced cell survival and DDP resistance. To the best of our knowledge, this is the first study which described an association between miR-106a, PTEN expression, and DDP resistance in gastric cancer cells.

In summary, our study demonstrated that miR-106a-induced cell survival and DDP resistance at least through the
PI3K/Akt pathway in human gastric cancer by directly targeting PTEN. We also provided direct evidence that the miR-106a inhibitor may be used as a therapeutic approach for gastric cancer. The exact roles and mechanisms underlying the effects of miR-106a and other miRNAs need to be further studied in more gastric cancer cell lines and clinical gastric cancer biopsies in the future.

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References

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