Upregulation of microRNA-375 increases the cisplatin-sensitivity of human gastric cancer cells by regulating ERBB2

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Received January 11, 2015; Accepted September 25, 2015

DOI: 10.3892/etm.2015.2920

Abstract. Resistance to chemotherapy is a major challenge in the effective treatment of patients with gastric cancer; however, the mechanisms underlying chemoresistance in gastric cancer cells are yet to be elucidated. MicroRNAs (miRNAs) have previously been associated with cancer progression and sensitivity to chemotherapy; therefore, the present study aimed to identify miRNAs that may influence the sensitivity of human gastric cancer cells to cisplatin (DDP) treatment. Initially, miRNAs that were differentially expressed between the DDP-sensitive SGC7901 human gastric cancer cell line and DDP-resistant SGC7901/DPD cell line were identified using high-throughput sequencing technology. miRNA-375 (miR-375), which was shown to be downregulated in the SGC7901/DPD cells, has previously been associated with numerous types of cancer; however, to the best of our knowledge, a role for miR-375 in the DDP-sensitivity of gastric cancer cells has yet to be explored. In the present study, the expression levels of miR-375 were significantly downregulated in the SGC7901/DPD cells, as compared with the SGC7901 cells, as demonstrated by reverse transcription-quantitative polymerase chain reaction. In addition, upregulation of miR-375 significantly enhanced the anti-proliferative and apoptosis-inducing effects of DDP, whereas downregulation of miR-375 decreased these effects. Subsequently, western blot analysis demonstrated that upregulation of miR-375 in the SGC7901/DPD cells increased their sensitivity to DDP treatment via regulating the protein expression levels of erb-b2 receptor tyrosine kinase 2 and phosphorylated-Akt. The results of the present study indicate that the expression levels of miR-375 may influence the sensitivity of human gastric cancer cells to the effects of DDP; thus suggesting that a combination of miR-375 regulation and DDP may be considered a novel strategy in the treatment of patients with chemoresistant gastric cancer.

Introduction

Gastric cancer is the fourth most common cancer worldwide, and the second leading cause of cancer-associated mortality (1). Surgery is considered the primary treatment for patients with early-stage gastric carcinoma; however, recurrence is a common phenomenon (2,3). A combination of surgery and chemotherapy has emerged as an effective strategy in the treatment of patients with gastric cancer, and has been shown to improve the disease-free survival rates and reduce the risks of recurrence and metastasis, as compared with surgery only, in various clinical trials (4,5). Cisplatin (DDP) is the most widely used first-line chemotherapeutic agent for the treatment of patients with gastric cancer. Anti-cancer drugs typically kill tumor cells by inducing apoptosis; however, it has been suggested that the majority of solid tumors are resistant to chemotherapy-induced apoptosis (6-8). Furthermore, long-term and repetitive administration of DDP has previously been associated with severe side effects (9). Therefore, the development of a strategy for improving the sensitivity of gastric cancer cells to DDP is required, in order to enhance the effectiveness of chemotherapy for the treatment of patients with chemoresistant gastric cancer.

MicroRNAs (miRNAs) are a series of small (19-24 nt) non-coding RNAs, which have roles in post-transcriptional gene regulation and target RNA degradation (10,11). In addition, miRNAs have been associated with various plant and animal processes, including cell proliferation, differentiation and metabolism (12,13). miRNAs bind to target mRNAs at the 3'-untranslated region (UTR) and/or 5'-UTR, in order to block translation or promote target mRNA degradation (14). Previous studies have demonstrated that dysregulation of miRNAs may contribute to a DDP chemoresistance phenotype in human tumor cells (15,16). Furthermore, downregulated expression levels of miRNA-375 (miR-375) have previously been detected in numerous types of human cancer, including head and neck squamous cell carcinoma, esophageal cancer, and hepatocellular carcinoma (17-20). In addition, miR-375 has previously been associated with the progression of gastric cancer (21,22); thus suggesting that a combination of miRNA regulation and chemotherapy may be considered a potential therapeutic strategy in the treatment of patients with chemoresistant tumors in the future.

In the present study, high-throughput miRNA sequencing was used to compare the expression levels of specific miRNAs between the DDP-sensitive SGC7901 human gastric cancer
cell line and the DDP-resistant SGC7901/DDP cell line. The results of the present study suggested that miR-375 was downregulated in the SGC7901/DDP cells, as compared with the SGC7901 cells. Furthermore, upregulation of miR-375 expression levels in the DDP-resistant SGC7901/DDP cell line was demonstrated to enhance the DDP sensitivity of the cells via regulation of the erb-b2 receptor tyrosine kinase 2 (ERBB2)/phosphoinositide-3-kinase (PI3K)/Akt pathway. Therefore, altering the expression levels of miR-375 may represent a novel strategy for resolving the DDP resistance associated with human gastric cancer.

Materials and methods

Cell culture. The SGC7901 human gastric cancer cell line was purchased from American Type Culture Collection (Manassas, VA, USA). The DDP-resistant SGC7901/DDP cell line was purchased from Nanjing KeyGen Biotech. Co., Ltd. (Nanjing, China). The SGC7901 and SGC7901/DDP cells were cultured in Gibco RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 IU/ml streptomycin (all Gibco; Thermo Fisher Scientific, Inc.) at 37°C, in a humidified incubator containing 5% CO2. In order to maintain the DDP-resistant phenotype of the SGC7901/DDP cells, DDP (final concentration, 1 µg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to the culture media of the SGC7901/DDP cells. Prior to the experiments, the SGC7901 cells were cultured and harvested.

Cell viability assay. Cell viability was assessed using the Cell Counting kit (CCK)-8 assay (Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's protocol. Briefly, cells were seeded into 96-well plates (5x10^3 cells/well). After 12 h, the cells were treated with serial dilutions of DDP (0, 0.02, 0.06, 0.2, 0.63, 2, 6.32 and 20 µg/ml), and CCK-8 reagent was added to each well (dilution, 1:10) ~48 h after treatment with DDP, after which the cells were incubated for 2 h at 37°C. Absorbance was measured at a 450 nm using the MD VersaMax microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA) and was expressed as the viability percentages of the cells, as compared with the controls. All tests were performed in triplicate, and the data were presented as the mean ± standard deviation (SD). Cell survival curves were generated and the half maximal inhibitory concentration (IC50) was calculated using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) by plotting the concentration of the DDP versus cell survival as previous described (23). The IC50 values were calculated as the concentration when the cell survival was 0.5, relative to a cell survival value of 1 when the concentration of DDP was 0 µg/ml.

High-throughput sequencing. SGC7901 and SGC7901/DDP cells were cultured and harvested. RNA was isolated from the harvested cells using the TruSeq Small RNA-Seq Preparation kit (Illumina, Hayward, CA, USA). Subsequently, RNA sequencing was performed using the Illumina HiSeq 2000 sequencing platform, according to the manufacturer's instructions (GEO accession no., GSE59565). The differentially expressed miRNAs were determined as previous described (24).

Transfection of cells with a miR-375 mimic or inhibitor. The effects of miR-375 on the DDP sensitivity of human gastric cancer cells were evaluated by transfecting SGC7901/DDP cells with an miR-375 mimic (assay ID, MCI0327; Thermo Fisher Scientific, Inc.) and SGC7901 cells with an miR-375 inhibitor (assay ID, MH10327; Thermo Fisher Scientific, Inc.), at a density of 2x10^5 cells/well. The U54 sequence was used as a control (Thermo Fisher Scientific, Inc.; GenBank no. AB061842). Briefly, the cells were cultured in RPMI-1640 medium, supplemented with 10% FBS, in the absence of antibiotics. After 24 h, the cells were treated with Invitrogen Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The miR-375 mimic and inhibitor were transfected at a final concentration of 10 nM, in antibiotic-free Gibco Opti-MEM medium (Thermo Fisher Scientific, Inc.). After 6 h, the medium was replaced with RPMI-1640 medium, supplemented with 10% FBS, in the absence of antibiotics.

RNA extraction and quantification of miR-375 expression levels. Total RNA was extracted from the SGC7901 and SGC7901/DDP cells (5x10^4), using Invitrogen TRIZol® reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Stem-loop reverse transcription-quantitative polymerase chain reaction (RT-qPCR), for the specific quantification of miRNA-375 expression levels, was performed using the Applied Biosystems TaqMan® miRNA assay (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Briefly, total RNA (10 ng) was reverse transcribed into cDNA using a looped RT primer specific to miRNA, within the TaqMan® miRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR of the generated cDNA (2 µl) was performed using the Applied Biosystems TaqMan® Universal PCR Master mix (Thermo Fisher Scientific, Inc.; GenBank no. AB061842) and miRNA-specific TaqMan® minor groove binder probes. The qPCR primers used were commercially available (purchased from Thermo Fisher Scientific, Inc.) and qPCR was performed at 95°C for 20 sec, followed by 40 cycles at 95°C for 10 sec and at 60°C for 20 sec. RNA U6 (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used as an internal control. The relative expression levels were calculated using the comparative cycle quantification (Ct) method (25). All RT-qPCR were performed in triplicate and the data were presented as the mean ± SD.

Flow cytometric analysis of apoptosis. The transfected SGC7901 and SGC7901/DDP cells were treated with DDP (0.5 µg/ml or 5 µg/ml) for 48 h, after which they were harvested for double staining with fluorescein isothiocyanate-Annexin V and propidium iodide (Beyotime Institute of Biotechnology), and analyzed by flow cytometry (BD FACSCalibur; BD Biosciences, San Jose, CA, USA), equipped with BD CellQuest™ Pro software (BD Biosciences). The relative ratio of apoptotic cells was compared to the control from each experiment. All of the samples were tested in triplicate and the data were presented as the mean ± SD.

Western blot. Cells were harvested and homogenized with RIPA lysis buffer (Beyotime Institute of Biotechnology). Whole extracts were prepared, and protein concentration was detected using a BCA protein assay kit (Beyotime Institute of Biotechnology). Total protein from the SGC7901 and
SGC7901/DDP cells (40 µg) was separated by 15% SDS-PAGE (Invitrogen; Thermo Fisher Scientific, Inc.) and electrophoretically transferred onto a polyvinylidene fluoride membrane (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Subsequently, the membranes were blocked with 5% non-fat dried milk for 2 h, after which they were incubated with specific rabbit primary antibodies against ERBB2 (cat. no. 2242), phosphorylated (p)-Akt (cat. no. 13461), Akt (cat. no. 14702) and β-actin (cat. no. 4967; all from Cell Signaling Technology, Inc., Danvers, MA, USA, and used at a dilution of 1:1,000), for 2 h. The membranes were washed with Tris-buffered saline, supplemented with 0.1% Tween 20 (Beyotime Institute of Biotechnology), after which they were incubated with horse-radish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (dilution, 1:1,000; catalog no. A0208; Beyotime Institute of Biotechnology) for 1 h at room temperature. The membranes were washed and the proteins were visualized using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology) and exposed to X-ray film (Kodak, Rochester, NY, USA). All of the samples were performed in triplicate.

Statistical analysis. The data are expressed as the mean ± SD of triplicate experiments. SPSS 13.0 software (SPSS, Inc., Chicago, USA) was used to analyze data. Statistical differences were detected using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

**DDP-resistant SGC7901/DDP cell line.** In order to confirm the DDP resistance of the SGC7901/DDP cell line, parental SGC7901 cells and DDP-resistant SGC7901/DDP cells were cultured and treated with DDP for 48 h, after which the cell viability was assessed using the CCK-8 assay. The IC_{50} values of SGC7901/DDP and SGC7901 cells were found to be 6.46 and 0.49 µg/ml, respectively (calculated from Fig. 1). The SGC7901/DDP cells exhibited ~13.1-fold acquired resistance to DDP, as compared with the parental SGC7901 cells, based on calculation of the IC_{50} values (P<0.01; Fig. 1).

**miR-375 is downregulated in the DDP-resistant SGC7901/DDP cells.** In order to investigate the differential expression levels of miRNAs between the SGC7901 and SGC7901/DDP cells, the cells were cultured and harvested for RNA isolation, after which high-throughput miRNA sequencing was performed. A total of 27 miRNAs were upregulated and 19 miRNAs were downregulated in the SGC7901/DDP cells, as compared with the SGC7901 parental cells (data not shown). miR-375 was downregulated in the DDP-resistant SGC7901/DDP cells, and was selected for further study as its effects on DDP resistance in gastric cancer cells have not, to the best of our knowledge, previously been reported. miRNA RT-qPCR analysis corroborated that the expression levels of miR-375 were downregulated in the SGC7901/DDP cells, as compared with the SGC7901 cells (P<0.001; Fig. 2), thus suggesting that the expression levels of miR-375 may influence the sensitivity of human gastric cancer cells to treatment with DDP.

**Manipulation of miR-375 expression levels in the SGC7901 and SGC7901/DDP human gastric cancer cells.** In order to selectively regulate the miR-375 expression levels in human gastric cancer cells, a miR-375 mimic or inhibitor transfection assay was performed. miRNA RT-qPCR demonstrated that the expression levels of miR-375 in the SGC7901/DDP cells significantly increased following transfection with the miR-375 mimic, as compared with the miR-control and SGC7901/DDP parental cells (P<0.001; Fig. 3A). In addition, the miR-375 expression levels in the SGC7901 cells were significantly downregulated following transfection of the cells with a miR-375 inhibitor, as compared with the miR-control and SGC7901 parental cells (P<0.001; Fig. 3B). Therefore, the miR-375 mimic or inhibitor transfection assay may be considered an effective strategy for manipulating the expression levels of miR-375 in human gastric cancer cells, in order to investigate its biological effects.

**Downregulation of miR-375 renders the SGC7901 cells resistant to DDP.** In order to investigate the association between down-regulated expression levels of miR-375 and DDP resistance in

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**Figure 1.** Survival curves for the DDP-resistant SGC-7901 and parental SGC7901 human gastric cancer cells, following treatment with DDP. The SGC7901/DDP cells exhibited ~13.1-fold acquired resistance to DDP, as compared with the parental SGC7901 cells. The cells were incubated in various concentrations of DDP for 48 h, after which the viability of the cells was measured using the Cell Counting kit-8 assay. Data are presented as the mean ± standard deviation of triplicate experiments. ***P<0.001 vs. SGC7901 parental cells. DDP, cisplatin.

**Figure 2.** Expression levels of miR-375 in the DDP-sensitive SGC7901 and DDP-resistant SGC7901/DDP human gastric cancer cells. Reverse transcription-quantitative polymerase chain reaction demonstrated that miR-375 was significantly downregulated in the SGC7901/DDP cells, as compared with the SGC7901 parental cells. Data are presented as the mean ± standard deviation of triplicate experiments. **P<0.001 vs. the SGC7901 cells. DDP, cisplatin; miR, microRNA.
human gastric cancer cells, the SGC7901 cells transfected with the miR-375 inhibitor or miR-control were cultured and treated with DDP for 48 h, after which cell viability was measured using the CCK-8 assay. The SGC7901 cells that were transfected with the miR-375 inhibitor had a significantly increased survival rate, as compared with the miR-control and SGC7901 parental cells (P<0.01; Fig. 4A). In addition, FACS analysis indicated that the SGC7901 cells transfected with the miR-375 inhibitor had a significantly decreased rate of apoptosis when treated with 0.5 µg/ml DDP, as compared with the miR-control and SGC7901 parental cells (P<0.001, Fig. 4B). These results indicate that the upregulation of miR-375 may increase the sensitivity of DDP-resistant SGC7901/DDP human gastric cancer cells to treatment with DDP.

**Upregulation of miR-375 sensitizes SGC7901/DDP human gastric cancer cells to DDP.** In order to investigate the effects of upregulating the expression levels of miR-375 on the sensitivity of SGC7901/DDP cells to DDP-induced cytotoxicity, the cells transfected with a miR-375 mimic or miR-control were cultured and treated with DDP for 48 h, after which cell viability was measured using the CCK-8 assay. The SGC7901/DDP cells that were transfected with the miR-375 mimic exhibited significantly reduced survival rates, as compared with the miR-control and SGC7901/DDP parental cells (P<0.01, Fig. 5A). FACS analysis demonstrated that the SGC7901/DDP cells transfected with the miR-375 mimic had a significantly increased rate of apoptosis when treated with 5 µg/ml DDP, as compared with the miR-control and SGC7901/DDP parental cells (P<0.001, Fig. 5B). These results indicate that the upregulation of miR-375 may increase the sensitivity of DDP-resistant SGC7901/DDP human gastric cancer cells to treatment with DDP.

**miR-375 regulates the expression levels of ERBB2 in human gastric cancer cells.** Previous studies have demonstrated that ERBB2 is a target gene of miR-375 (20), that the PI3K/Akt signal pathway is associated with ERBB2 regulation, and that the activation of the ERBB2/PI3K/Akt signal pathway may promote drug resistance (26); however, to the best of our knowledge, whether miR-375 alters the sensitivity of gastric cancer cells to DDP via regulation of the ERBB2/PI3K/Akt pathway is unknown. Western blotting demonstrated that upregulation of miR-375 expression levels in the SGC7901/DDP cells markedly
decreased the protein expression levels of ERBB2 and p-AKT, as compared with the miR-control and SGC7901/DDP parental cells (Fig. 6A). Conversely, downregulation of miR-375 expression levels in the SGC7901 cells markedly increased the protein expression levels of ERBB2 and p-AKT, as compared with the miR-control and SGC7901 parental cells (Fig. 6B). These results suggest that overexpression of miR-375 may sensitize SGC7901/DDP cells to the effects of DDP via inactivation of the ERBB2/PI3K/Akt pathway.

Discussion

miRNAs are small noncoding RNAs that regulate the expression of numerous intracellular target genes, which have been shown to be associated with various biological processes, including developmental timing, differentiation, cell proliferation, apoptosis and cancer prognosis (27,28). Previous studies have demonstrated that miRNAs have important roles in the progression of cancer (29), and may also be associated with the development of resistance in cancer cells to chemotherapeutics (30-34). Yang et al. (35) previously demonstrated that the downregulation of miR-21 altered the survival rates of gastric cancer cells and sensitized the cells to DDP. Cao et al. (36) also reported that miR-34a was able to regulate the DDP-induced gastric cancer cell death process via the PI3K/AKT/survivin pathway. miR-375 was initially identified in murine pancreatic β-cells, and its expression was shown to be upregulated in human pancreatic islet cells (20). In addition, previous studies have demonstrated an association between downregulated miR-375 expression levels and gastric carcinogenesis (21,22,37).

To the best of our knowledge, the present study is the first to indicate an association between miR-375 expression levels and the DDP-sensitivity of gastric cancer cells. Significantly reduced miR-375 expression levels were detected in the DDP-resistant SGC7901/DDP cells, as compared with the SGC7901 cells. Furthermore, overexpression of miR-375 in the SGC7901/DDP cells increased their sensitivity to DDP-induced apoptosis. These results suggested that down-regulation of miR-375 may contribute to the development of a DDP-resistant phenotype in human gastric cancer cells.

ERBB2 is a member of the epidermal growth factor receptor family, which has previously been associated with the enhanced proliferation rates of tumor cells (38). In addition, previous studies have demonstrated that ERBB2 is a target gene of miR-375 (20), that the PI3K/Akt signal pathway is associated with ERBB2 regulation, and that the activation of the ERBB2/PI3K/Akt pathway may promote resistance of cancer cells to drugs (26). In the present study, upregulation of miR-375 expression levels in the DDP-resistant SGC7901/DDP human gastric cancer cells decreased the protein expression levels of ERBB2 and p-Akt. Therefore, overexpression of miR-375 may have sensitized the SGC7901/DDP cells to DDP by inactivating the ERBB2/PI3K/Akt pathway; thus suggesting that a
combination of miR-375 regulation and DDP may have potential in the treatment of patients with DDP-resistant gastric cancer.

Various concerns must be addressed prior to the application of this therapeutic strategy: Firstly, there is the possibility that upregulating the expression levels of miR-375 in patients with gastric cancer may initiate abnormal gene expression patterns in normal cells, which may result in the abnormal cell proliferation, cell cycle arrest or apoptosis of these cells. Furthermore, overexpressed miR-375 may bind non-specifically to off-target mRNAs, which may lead to undesirable side-effects. Future studies should endeavor to elucidate the biological effects of altering the expression levels of miR-375 in both cancer and normal cells, and this may be achieved using the miR-375 mimic or inhibitor transfection assay demonstrated in the present study.

In conclusion, miR-375 expression levels were down-regulated in the DDP-resistant SGC7901/DDP human gastric cancer cell line, as compared with the DDP-sensitive SGC7901 cell line. Furthermore, overexpression of miR-375 was able to enhance the sensitivity of the SGC7901/DDP cells to DDP; thus suggesting that a combination of DDP administration, alongside miR-375 overexpression, may be considered a potential strategy in the treatment of patients with DDP-resistant gastric cancer in the future.

References