miR-20a induces cisplatin resistance of a human gastric cancer cell line via targeting CYLD

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Abstract. The dysregulation of microRNAs (miRNAs) has been demonstrated to contribute to drug resistance of cancer cells, and sustained nuclear factor (NF)κB activation is also pivotal in tumor resistance to chemotherapy. In the present study, an essential role for miRNA (miR)-20a was identified in the regulation of gastric cancer (GC) chemoresistance. The expression level of miR-20a was assayed by reverse transcription-quantitative polymerase chain reaction. Additionally, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was used to detect the drug-resistance phenotype changes of cancer cells associated with upregulation or downregulation of miR-20a. Protein expression levels were measured by western blotting and immunohistochemistry. Flow cytometry was used to detect cisplatin-induced apoptosis. It was found that miR-20a was markedly upregulated in GC plasma and tissue samples. Additionally, miR-20a was upregulated in GC plasma and tissues from patients with cisplatin (DDP) resistance, and in the DPP-resistant gastric cancer cell line (SGC7901/DDP). The expression of miR-20a was inversely correlated with the expression of cylindromatosis (CYLD). Subsequently, the assessment of luciferase activity verified that CYLD was a direct target gene of miR-20a. Treatment with miR-20a inhibitor increased the protein expression of CYLD, downregulated the expression levels of p65, livin and survivin, and led to a higher proportion of apoptotic cells in the SGC7901/DDP cells. By contrast, ectopic expression of miR-20a significantly repressed the expression of CYLD, upregulated the expression levels of p65, livin and survivin, and resulted in a decrease in the apoptosis induced by DDP in the SGC7901 cells. Taken together, the results of the present study suggested that miR-20a directly repressed the expression of CYLD, leading to activation of the NFκB pathway and the downstream targets, livin and survivin, which potentially induced GC chemoresistance. Altering miR-20a expression may be a potential therapeutic strategy for the treatment of chemoresistance in GC in the future.

Introduction

Gastric cancer (GC) is one of the most common malignancies worldwide (1). Currently, rates of early detection and diagnosis for GC are low, therefore, chemotherapy remains one of the major treatment methods for GC. However, the effectiveness of chemotherapy is often limited by the development of drug resistance (2). Cytological mechanisms, which have contributed to the chemoresistance include enhanced DNA repair activity, alterations in cell cycle and proliferation, and defective apoptosis (3). In addition, molecular mechanisms, including increased rates of drug efflux, alterations in drug metabolism and mutation of drug targets may be important in drug resistance (4,5). Previous studies have found that the activation of certain signaling pathways, including the, phosphatase and tensin homolog/phosphoinositide 3-kinase/AKT, nuclear factor (NF)κB/inhibitor of NFκB, Ras/Raf/mitogen-activated protein kinase kinase and P53/murine double minute-2 (6-9), are also associated with drug resistance.

MicroRNAs (miRNAs), a class of short, non-coding RNAs of 19-22 nucleotides in length, function as post-transcriptional regulators by directly cleaving target messenger RNA or inducing translational repression (10). Previously, aberrant miRNAs have been found to be involved in drug resistance in different types of tumor, acting as oncogenes or tumor suppressors (11-14). Accumulating evidence has revealed that miRNA regulates NFκB signaling (15), and activation of the
NFkB pathway enhances cell resistance to chemotherapeutic drugs (16). Therefore, the present study hypothesized that miRNA may induce chemoresistance through activation of the NFkB pathway.

In the present study, the potential function of miR-20a in GC chemoresistance was investigated. It was found that miR-20a was significantly upregulated in samples from patients with GC, particularly in those with chemoresistance. miR-20a was also found to be upregulated in a cisplatin (DDP)-resistant GC cell line (SGC7901/DDP) miR-20a contributed to DDP resistance when overexpressed in the SGC7901 GC cell line. Furthermore, the present study demonstrated that miR-20a may activate the NFkB pathway and upregulate the downstream targets, survivin and lvin, by repressing the expression of cyclin-dependent kinase (CYLD), leading to the resistance to DDP. The results of the study may aid the development of personalized treatment for patients with GC that exhibit abnormal levels of miR-20a.

Materials and methods

Clinical sample collection. A total of 121 patients (age range, 46-75; males, 66; females, 45) with histopathologically confirmed GC and 56 healthy donors (age range, 35-71; males, 33; females, 23) were recruited between 2012 and 2014 at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). A total of 28 GC plasma samples and an additional 28 formalin-fixed paraffin embedded (FFPE) sections of GC were obtained from preoperative patients who had not received chemotherapy. In addition, 30 GC plasma samples and an additional 35 FFPE sections of GC were collected from patients with advanced disease, who had undergone two cycles of platinum-based chemotherapy. Response to chemotherapy was graded by the standards of Evaluation Criteria in Solid Tumors recommended by the World Health Organization (WHO), defined as complete remission (CR), partial remission (PR), stable disease (SD), and progressive disease (PD). All procedures were approved by the Institutional Review Boards of the First Affiliated Hospital of Nanjing Medical University. Written informed consent was obtained from each participant.

Cell culture and transfection. The SGC7901 human gastric cancer cell line was purchased from the National Institute of Cells (Shanghai, China). The DPP-resistant variant (SGC7901/DDP) was obtained from KeyGen Biotechnology Company (Nanjing, China). All cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified atmosphere with 5% CO2 at 37°C, as previously described (17,18). To maintain the DPP-resistant phenotype, DDP, with final concentration of 1 μg/ml, was added to the culture media of the SGC7901/DDP cells.

miRNA mimics and their appropriate negative control were obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). All transfection experiments were performed using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. UCSC Genome Browser (genome.ucsc.edu/) was used to search the promoter regions of miR-20a.

Bioinformatics analysis. TargetScan (www.targetscan.org), a bioinformatics tool for miRNA target screening, was used to predict the target genes of miR-20a.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Following tissue homogenization, total RNA from the cells was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. For the plasma samples, total RNA was extracted using the miVana Paris kit (Ambion, Austin, TX, USA), according to the manufacturer's protocol. Total RNA from the FFPE tissues was isolated using the High Pure FFPE RNA Micro kit (Ambion), according to the manufacturer's protocol. The amplification of miRNA was performed using the specific primers for the RT-qPCR analysis in the Bulge-Loop™ miRNA qRT-PCR primer set (RiboBio Co., Ltd.), as previously described (19,20). RT-qPCR was performed, according to the manufacturer's protocol. The expression levels of the PCR products were determined by the level of fluorescence emitted by SYBR Green (SYBR® Premix Ex Taq™ II, Takara Bio, Inc., Otsu, Japan). The relative expression levels of the target miRNAs from the cells and tissue samples were calculated using the comparative 2ΔΔCt method, relative to U6 snRNA, as previously described (17,18). The levels of miRNA in the plasma samples were determined based on a standard curve constructed with using synthetic miRNAs (microON miRNA mimic; RiboBio Co., Ltd.), as previously described (19,20).

In vitro drug sensitivity assay. The SGC7901 cells and SGC7901/DDP cells were seeded into 6-well plates (6x104 cells/well). Either the miR-20a mimic (100 nM) or mimic control (100 nM) were transfected into the SGC7901 cells, whereas the SGC7901/DDP cells were transfected with either miR-20a inhibitor (100 nM) or inhibitor control (100 nM). The miR-20a mimic, inhibitor and relative controls were purchased from Gene Pharma (Shanghai, China).

At 24 h post-transfection, the cells were seeded into 96-well plates (5x103 cells/well). Following cellular adherence, freshly prepared DDP was added at final concentrations 10, 1, 0.1 and 0.01 times that of the human peak plasma concentration for DDP (2.0 μg/ml). After 48 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyl-tetrazolium bromide (MTT) was added to each well to measure the viability of the cells, and the plate was incubated for 2 h in a humidified incubator. The absorbance at 490 nm of each well was measured on a spectrophotometer. The relative survival curve was used to calculate the concentration of cisplatin, which caused 50% inhibition of growth.

Dual-luciferase activity assay. The 3′-untranslated region (3′UTR) sequences of human CYLD cDNA containing the predicted target site of miR-20a, were chemically synthesized by RiboBio Co., Ltd. HEK293T human embryonic kidney cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were plated in 24-well plates (1.5x105 cells/well) for 24 h, following which 200 ng of the pGL3-CYLD-3′-UTR, 60 pmol of the miR-20a mimic or miRNA mimic control, and 80 ng of pRL-TK (Promega Corporation, Madison, WI, USA) were co-transfected into
miR-20a is significantly upregulated in GC plasma samples and GC tissue samples. (A) Expression levels of miR-20a were detected in 28 normal plasma samples and 28 preoperative GC plasma samples using reverse transcription-quantitative polymerase chain reaction analysis. Expression levels of miR-20a in the tumor group were higher, compared with those in the normal group. Y-axis data are presented as log10 (concentration; fmol/l). (B) Expression levels of miR-20a were also upregulated in GC formalin-fixed paraffin embedded samples. Y-axis data are presented as relative expression (normalized to U6; calculated using the 2^−ΔΔCq method). Data are expressed as the mean ± standard deviation. *P<0.01. N, normal; T, tumor; GC, gastric cancer; miR, microRNA.

Immunohistochemistry. For immunohistochemical analysis, tissue sections (4-μm-thick) were formalin-fixed and paraffin-embedded, and were stained using the avidin-biotin complex method (21). Antigen retrieval was performed using microwave irradiation in 10 mM citrate buffer (pH 6.0), followed by incubation with primary antibodies at 4°C overnight. The antibody for CYLD (cat. no. 8462; dilution 1:200) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The biotinylated goat anti-rabbit secondary antibody (cat. no. A0227) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Specimens were incubated in the secondary antibody (1:50) for 30 min at room temperature. Staining was repeated if the result was uncertain. The slides were scored independently by two observers blinded to the clinicopathological characteristics. The immunostaining of the slides was evaluated under an optical microscope (magnification x400). Discordant scores were reevaluated to reach a consensus. The staining intensity of the expression of CYLD was scored (scores 0-3 for negative, weak, moderate and strong expression, respectively), as was the percentage of positive cancer cells (0 for 0%; 0.1 for 1-9%; 0.5 for 10-49%; and 1.0 for ≥50%). The staining intensity was multiplied by the proportion score of the percentage of positive cancer cells.

Western blot analysis. Total protein was prepared from the cells using radioimmunoprecipitation assay buffer in the presence of proteinase inhibitor. The nuclear proteins were extracted from the cells using a nuclear cytoplasmic extraction kit (Beyotime Institute of Biotechnology). Protein levels were quantified using bicinchoninic acid assay (Beyotime Institute of Biotechnology). Western blot analysis was performed, as previously described (17,18). The primary antibodies for CYLD (cat. no. 8462), P65 (cat. no. 8242), livin (cat. no. 5471), H3 (cat. no. 4499) and survivin (cat. no. 2808) were obtained from Cell Signaling Technology, Inc. The blots were incubated with the indicated primary antibodies or GAPDH antibody (cat. no. AP0063; Bioworld Technology, Inc., St. Louis Park, MN, USA) overnight at 4°C, then with goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (cat. no. sc-2030; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for 1 h. The protein bands were visualized using an Immobilon Western Chemiluminescent HRP Substrate kit (Beyotime Institute of Biotechnology,) and a ChemiDoc XRS+ Imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Grey value analysis of protein bands was performed for quantification of the protein levels using Image J software (version 1; National Institutes of Health, Bethesda, MD, USA). The levels of total protein were normalized to total GAPDH, and nuclear protein to H3. The fold changes were calculated.

Apoptosis assay. The cells were transfected with miR-20a mimic or inhibitor, respectively. After 24 h, the cells were treated with DDP at a final concentration of 10 μg/ml. After 48 h, apoptosis was assessed via the counting of annexin V-fluorescein isothiocyanate-positive and propidium iodide-negative cells using flow cytometry, as described previously (17,18).

Statistical analysis. Data are presented as the mean ± standard deviation from at least three separate experiments. Two-tailed Student's t-tests were performed for comparisons using SPSS (version 15.0; SPSS, Inc., Chicago, IL, USA) software. Spearman's non-parametric correlation test was performed to analyze the correlation between the expression levels of miR-186 and Twist1 by GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-20a is significantly upregulated in GC plasma samples and GC tissue samples. To determine the basal expression of miR-20a in GC, the present study first performed RT-qPCR analysis to examine the plasma levels of miR-20a in 28 patients with preoperative GC and 28 healthy subjects. The expression of miR-20a was significantly increased in the GC group, compared with the normal group (Fig. 1A). Furthermore, it was found that the expression levels of miR-20a were significantly higher in the 28 GC FFPE tissue samples, compared with those in the 28 samples of normal gastric tissue (Fig. 1B).
miR-20a is involved in GC chemotherapeutic resistance. To further investigate the potential role of miR-20a in GC chemoresistance, 30 plasma samples and 35 FFPE sections were collected from patients with advanced GC who had undergone two cycles of platinum-based chemotherapy. The response to chemotherapy was evaluated using standard WHO criteria, of CR, PR, SD and PD. According to these criteria, the plasma samples showed that 10 patients had PR, nine patients had SD and 11 patients had PD, with no patients in CR. In the tissue specimens, 12 had PR, 11 had SD and 12 had PD, with no cases of CR. The expression of plasma miR-20a in the PD+SD group were significantly higher, compared with that in the PR group (Fig. 2A). The upregulation of miR-20a was also detected in the FFPE tissues of the PD+SD group, compared with the PR group (Fig. 2B). The results of the RT-qPCR analysis revealed that the expression of miR-20a was also significantly increased in the SGC7901/DDP cells, compared with the parental SGC7901 cell line (Fig. 2C), which was consistent with the results obtained in the clinical GC samples. These results revealed that the overexpression of miR-20a was significantly associated with GC chemoresistance.

miR-20a regulates resistance to DDP in the SGC7901/DDP cell line. To examine the functional role of miR-20a in the chemoresistance of GC, the miR-20a mimic or inhibitor were transfected into the SGC7901 cells or SGC7901/DDP cells respectively. Prior to the miRNA transfection, the significant resistance of the SGC7901/DDP cells to DDP, compared with the parental SGC7901 cells was verified. The MTT assay revealed reduced sensitivity of the SGC7901 cells transfected with the miR-20a mimic to DDP, compared with that of the cells transfected with the mimic control (Fig. 3A). By contrast, transfection with the miR-20a inhibitor markedly enhanced the sensitivity of the SGC7901/DDP cells to DDP, compared with the control (Fig. 3B). Taken together, these results indicated that miR-20a modulated the resistance of the SGC7901/DDP cells to DDP.

CYLD is a direct target of miR-20a. Using TargetScan software for miRNA target prediction, the present study found CYLD as a potential direct target gene of miR-20a. To assess the relevance of miR-20a/CYLD, immunohistochemistry was performed to examine the expression of CYLD in 28 GC tissue samples. The expression of miR-20a was also analyzed using RT-qPCR analysis in the same specimens. Among these 28 specimens, using the median expression value of miR-20a as a cutoff point, the cohort was divided into miR-20a-high or miR-20a-low tumors. The protein expression levels of CYLD in the miR-20a-high group were significantly lower, compared with those in the miR-20a-low group (Fig. 4A). An inverse correlation (R²=0.4574; P<0.001) between the expression of miR-20a and the protein levels of CYLD was observed using Spearman's correlation analysis (Fig. 4B). In addition, western blot analysis revealed that CYLD was significantly decreased at the protein level in the SGC7901/DDP cells, compared with the parental SGC7901 cell line (Fig. 4C).

To further confirm whether CYLD was a target for miR-20a, luciferase reporter plasmids containing the 3'UTR of CYLD were constructed and co-transfected with miR-20a mimic in the HEK293T human embryonic kidney cell line. The results showed that luciferase activity was significantly decreased in the cells transfected with the miR-20a mimic, compared with the vector control (Fig. 4D). These results showed that miR-20a may negatively regulate the expression of CYLD by directly targeting its 3'UTR.

miR-20a represses the expression of CYLD, leading to NFκB activation and upregulation of the downstream targets, survivin and livin. In the present study, the upregulation of miR-20a in the SGC7901/DDP cells was concurrent with the downregulation of CYLD, compared with the parental SGC7901 cells. In addition, the NFκB pathway-associated proteins, p65, livin and survivin, showed significantly higher levels of expression in the SGC7901/DDP cells, compared with the SGC7901 cells (Fig. 5A). The present study verified that CYLD was a direct
target of miR-20a, and also a negative regulator of the NFκB pathway. Thus, it was hypothesized that miR-20a may activate the NFκB pathway by repressing the expression of CYLD. To confirm this, the SGC7901/DDP cells and SGC7901 cells were transfected with miR-20a inhibitor or mimic, respectively, and western blot analysis was performed to examine the expression of these proteins. In the SGC7901/DDP cells, at 72 h post-transfection, the results demonstrated that the reduced level of miR-20a increased the expression of CYLD, but decreased the expression of p65, survivin and livin (Fig. 5B). By contrast, the expression of CYLD in the SGC7901 cells transfected with the miR-20a mimic was downregulated, whereas the expression levels of p65, survivin and livin were upregulated (Fig. 5C). These results suggested that miR-20a repressed the expression of CYLD, leading to activation of the NFκB pathway and upregulation of the downstream targets, livin and survivin.

**Inhibition of miR-20a sensitizes SGC7901/DDP cells to DDP-induced apoptosis.** The overexpression of anti-apoptotic proteins, including livin and survivin, has been reported to contribute to resistance to drug-induced apoptosis in several types of cancer (17,18,22). In the present study, it was
demonstrated that the overexpression of miR-20a may enhance the resistance of GC cells to DDP, at least in part, via the activation of NFκB and its downstream targets, livin and survivin. Thus, it was hypothesized that miR-20a may also be associated with DDP resistance by regulating the apoptosis of GC cells. To further confirm this, the SGC7901/DDP and SGC7901 cells were transfected with the inhibitor or mimic of miR-20a, respectively, followed by the analysis of DDP-induced apoptosis. The
results indicated that there was a higher proportion of apoptotic cells in the SGC7901/DDP cells transfected with miR-20a inhibitor, compared with those transfected with the inhibitor control following DDP treatment (Fig. 6A). By contrast, the ectopic expression of miR-20a resulted in a decrease in the apoptosis induced by DDP in the SGC7901 cells (Fig. 6B).

Discussion

miR-20a is a member of the miR-17-92 cluster and acts as an oncogene. It has been demonstrated that miR-20a is upregulated, and promotes tumorigenesis and cancer progression in diverse cancer subtypes, including cervical cancer (23), ovarian cancer (24), osteosarcoma (25), anaplastic thyroid cancer (26) and nasopharyngeal carcinoma (27). A study by Li et al (28) revealed that miR-20a was markedly increased in GC tissues and cell lines, and that the ectopic expression of miR-20a promoted the proliferation, migration and invasion of GC cells. A study by Chai et al (29) suggested that miR-20a targeted BNIP2 and contributed to chemotherapeutic resistance in colorectal adenocarcinoma cell lines. In the present study, it was confirmed that miR-20a was significantly upregulated in SGC7901/DDP cells and was associated with GC chemoresistance. In addition, miR-20a regulated the resistance of the SGC7901/DDP cell line to DDP.

Several studies have shown that constitutively activated NFκB signaling and upregulation of drug resistance-associated proteins, including B cell lymphoma-2, myeloid cell leukemia-1, multidrug resistance 1 and X-linked inhibitor of apoptosis protein, are critical in mediating resistance in various types of cancer (9,30,31). CYLD, a deubiquitination enzyme, acts as a tumor suppressor in different types of cancer. Currently, CYLD is known to function as a negative regulator of the NFκB signaling pathway, and is closely involved in regulating the apoptosis of cancer cells. The ubiquitination of key signaling molecules by E3 ubiquitin ligases is required in NFκB signaling transmission, whereas NFκB activation is limited by ubiquitin deconjugation induced by deubiquitinases, including CYLD (32-35). In addition, previous investigations have suggested that inhibition of the NFκB pathway sensitized cells to chemotherapeutic drugs in GC (36,37). In the present study, it was found that miR-20a directly targeted CYLD, demonstrating a novel mechanism of CYLD dysregulation in GC. Further investigation was performed to determine whether there was a correlation between miR-20a and the NFκB pathway, to clarify the effects of miR-20a on GC chemoresistance. It was found that the overexpression of miR-20a in the SGC7901/DDP cells was concurrent with the downregulation of CYLD and the upregulation of p65. The expression level of CYLD was upregulated and the expression of p65 was downregulated when the SGC7901/DDP cells were transfected with the miR-20a inhibitor. In the SGC7901 cells, the ectopic expression of miR-20a decreased the expression of CYLD and increased the expression of p65. The aforementioned results indicated that miR-20a was essential, at least in part, for activating the NFκB pathway in GC. Acting as an upstream regulator of the NFκB pathway, the downregulation of miR-20a may be vital in NFκB pathway suppression.

Figure 6. Inhibition of miR-20a sensitizes SGC7901/DDP cells to DDP-induced apoptosis. (A) SGC7901/DDP cells were transfected with miR-20a inhibitor or control following DDP treatment. An increased number of cells undergoing apoptosis were found in the cells with downregulated expression of miR-20a. (B) SGC7901 cells were transfected with the miR-20a mimic or control. There were fewer cells undergoing apoptosis in the cells overexpressing miR-20a. Representative flow cytometry results are shown adjacent to the graphs. Data are presented as the mean ± standard deviation. *P<0.01. miR/miRNA, microRNA; DDP, cisplatin; PI, propidium iodide; AV, avidin.
Livin and survivin, members of the inhibitor of apoptosis protein family, function as anti-apoptotic factors and are important in inhibiting apoptosis (38). The overexpression of inhibitor of apoptosis is correlated with cancer chemoresistance. The present study suggested that miR-20a regulated the expression levels of livin and survivin via NFκB signaling. The inhibition of miR-20a rendered the SGC7901/DDP cells more sensitive to DDP-induced apoptosis. It was hypothesized that miR-20a may also be involved in the development of DDP resistance by modulating the apoptosis of GC cells.

However, the mechanism of miR-20a upregulation was not exact. Using the UCSC database, the present study found that NFκB binding sites were present in the promoter regions of miR-20a, which indicated that NFκB may directly regulate the expression of miR-20a. A study by Zhou et al (39) supported this. Therefore, it was hypothesized that the positive feedback loop, miR-20a/CYLD/NFκB/miR-20a, was an important mechanism for the sustained activation of NFκB in drug-resistant GC cells. However, further investigation is required to confirm this.

In conclusion, the present study provided the first evidence, to the best of our knowledge, that miR-20a directly targeted CYLD, resulting in activation of the NFκB pathway and the downstream targets, livin and survivin, which potentially contributed to GC chemoresistance. These results may provide theoretical information for miR-20a as a key target to reverse chemotherapy resistance. However, the results of the present study were obtained from cell lines and do not necessarily reflect the actual surrogates for clinical tumors. Therefore, further investigation is required to assess the role of miR-20a in vivo and clinically.

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