European Review for Medical and Pharmacological Sciences 2016; 20: 1733-1739

MiR-30a increases cisplatin sensitivity of gastric cancer cells through suppressing epithelial-to-mesenchymal transition (EMT)

L.-L. WANG[1], X.-H. ZHANG[2], X. ZHANG[3], J.-K. CHU[4]

[1]Clinical Laboratory, the Central Hospital of Yishui, Linyi, Shandong, China
[2]Department of Cardiology, People's Hospital of Zhangqiu City, Zhangqiu, Shandong, China
3Department of General Surgery, Taian City Central Hospital, Taian, Shandong, China
[4]Department of Gastroenterology, The First Hospital of Hebei Medical University, Shijiazhuang, Hebei, China

Lili Wang and *Xinhong Zhang* contributed equally to this study

Abstract. – OBJECTIVE: MiR-30a can target multiple proteins involved in epithelial-to-mesenchymal transition (EMT). In this study, we investigated the association between miR-30a and cisplatin (DDP) sensitivity in gastric cancer. In addition, the regulation of miR-30a in EMT in SGC-7901 cells and SGC-7901/DDP cells and their involvement in cisplatin sensitivity were further investigated.

PATIENTS AND METHODS: 20 advanced gastric cancer patients who received platinum-based chemotherapy were recruited. Chemosensitivity was assessed after completion of the therapy. MiR-30a expression was quantified and compared between chemosensitive and chemoresistant groups. SGC-7901 cells and SGC-7901/DDP cells were further used for the in-vitro gain-and-loss study to investigate the effect of miR-30a on EMT and cisplatin sensitivity.

RESULTS: Chemosensitive patients had significantly higher miR-30a expression than the chemoresistant counterparts. SGC-7901 cells had significantly higher miR-30a expression than SGC-7901/DDP cells. Knockdown of endogenous miR-30a promoted the elongated fibroblast-like morphologic alteration of SGC-7901 cells and also enhanced Snail and Vimentin expression. MiR-30a overexpression induced morphological changes from an extended, fibroblast-like morphology to more epithelial-like morphology in SGC-7901/DDP cells and decreased Snail and Vimentin level. The cancer cells with miR-30a overexpression had significantly higher DDP sensitivity, while the cells with miR-30a knockdown had decreased sensitivity.

CONCLUSIONS: EMT is associated with cisplatin resistance in gastric cancer. MiR-30a is an important miRNA modulating EMT and cisplatin sensitivity of SGC-7901 and SGC-7901/DDP cells.

Key Words:
Gastric cancer, EMT, miR-30a, Cisplatin sensitivity.

Introduction

Gastric cancer (GC) is one of the most common malignancies and has been the leading cause of cancer-related deaths across the world[1]. Although tumor resection is the most effective therapeutic strategy, a large proportion of the patients in China often diagnosed at an advanced and unresectable clinical stage[2]. Therefore, chemotherapy is still one of treatment methods for the patients as either primary therapy or the adjuvant therapy after surgery[3]. However, the effectiveness of chemotherapy highly depends on drug sensitivity of the tumor. The intrinsic or acquired drug resistance, especially multidrug resistance (MDR) is a major clinical obstacle for successful chemotherapy. Although previous studies revealed that the mechanisms such as enhanced DNA repair, weakened cell apoptosis, mutation of drug targets, drug efflux and alterations in drug metabolism were involved[4-6], the mechanisms of MDR are quite complex and have not been fully revealed[7,8].

Previous studies[9,10] found that miRNAs are also involved in regulation of MDR of gastric cancer through multiple pathways. For instance, miR-15b and miR-16 modulate multidrug resistance by targeting BCL2[11]; miR-106a can induce multidrug resistance by targeting RUNX3[12]; and miR-1284 modulates multidrug resistance by targeting EIF4A1[13]. Recent studies[14,15] reported that epithe-

Corresponding Author: Jiankun Chu, MD; e-mail: jiankunchu@outlook.com

lial-mesenchymal transition (EMT), which contribute to cancer metastasis and characterized by loss of E-cadherin expression is also involved in MDR development. miRNAs can also modulate MDR through affecting EMT[16]. For example, one recent study[17] found the PDGF-D/miR-106a/Twist1 pathway orchestrates EMT in gemcitabine resistance hepatoma cells. MiR-203 can promote EMT via targeting Snail2 and enhance chemoresistance in human glioblastoma[18]. MiR-30a is an important miRNA that can directly target multiple critical modulators of EMT, such as Snai1[19,20] and Vimentin[21] and, thereby, inhibiting EMT.

In this study, we investigated the association between miR-30a and cisplatin (DDP) sensitivity in gastric cancer. In addition, the regulation of miR-30a in EMT in SGC-7901 cells and SGC-7901/DDP cells and their involvement in cisplatin sensitivity were further investigated.

Patients and Methods

Clinical Samples Collection

All the procedures were approved by Institutional Review Boards of Hebei Medical University. Written informed consent was obtained from each participant before the study. 20 histopathological confirmed advanced gastric cancer patients who received 2 cycles of platinum-based chemotherapy were recruited from the First Hospital of Hebei Medical University. Tumor tissues were obtained from gastroscope biopsy. Response to chemotherapy was evaluated after completion of the 2 cycles of chemotherapy according to the criteria defined by the World Health Organization, which defines the responses as complete remission (CR), partial remission (PR), stable disease (SD), and progressive disease (PD). The patients with CR, PR or SD are considered as chemosensitive, while the patients with PD are considered as chemoresistant.

Cell Culture

The human gastric cancer cell line SGC-7901 and the cisplatin-resistant variant SGC-7901/DDP were all obtained from KeyGEN Biotechnology Company (Nanjing, China). The cells were maintained in RPMI-1640 medium supplemented with 10% FBS in a humidified atmosphere with 5% CO_2 at 37°C. To maintain the cisplatin-resistant phenotype, the medium for SGC-7901/DDP was additionally supplemented with 1 μg/mL DDP.

Cell Transfection

MiR-30a mimics, antagomiR-30a and the scramble negative controls were synthesized by GenePharma (Shanghai, China). SGC-7901 or SGC-7901 cells were transfected with 100 nM miR-30a for overexpression or 100 nM antogamiR-30a for the knockdown. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The morphological phenotype of the cells after transfection was captured using a CCD camera (PixeLINK, Ottawa, ON, USA) in a light microscopy.

QRT-PCR Analysis of miR-30a Expression

Firstly, total RNA was extracted from the tissue or cell samples by using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. Then, miRNA-specific cDNA was obtained by reverse transcription (RT) using the stem-loop primers and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The mature miR-30a level was detected using the TaqMan MicroRNA Assays Kit (Applied Biosystems) according to manufacturer's instruction and quantified by using the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

Conventional western blot analysis was performing following the methods described in one previous study. The primary antibodies used include anti-Snail (1:500, ab82846, Abcam, Cambridge, UK) anti-Vimentin (1:2000, ab92547, Abcam, Cambridge, UK) anti-E-cadherin (1:1000, ab77287, Abcam) and anti-β-actin (1: 2000, ab8227, Abcam). The second antibody used was HRP conjugated Goat Anti-Rabbit IgG H&L (1:5000, ab6721, Abcam). The signal intensity of the protein bands was visualized using the ECL Western blotting substrate (Promega, Madison, WI, USA).

Flow Cytometry Analysis

SGC-7901 with miR-30a knockdown and SGC-7901/DDP cells with miR-30a overexpression were treated with DDP (10 μg/mL) for 48 hours. Then, the ratio of apoptotic cells were determined using the Annexin V-FITC Apoptosis Detection Kit (V13241, Invitrogen) according to manufacturer's instruction in a FACSCaliber (BD Biosciences, Franklin Lakes, NJ, USA). Data acquisition was done using CellQuest 3.2 software (BD Biosciences). Each test was performed with at least three repeats.

In vitro Drug Sensitivity Assay

SGC-7901 cells and SGC-7901/DDP cells with miR-30a overexpression or knockdown were seeded in 96-well plates (5×10^3 cells/well) and incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 hours. Then, DDP was added with the final concentrations of 0.02, 0.2 2 and 20 μg/mL to the culture medium. 48 hours later after DDP administration, cell viability was assessed using a MTT assay. Three independent experiments were performed in triplicate.

Statistical Analysis

Quantitative data were presented as mean ± SD. The statistical difference between groups was assessed using *t*-test (Mann-Whitney rank sum test). $p < 0.05$ was considered as statistically significant.

Results

Decreased miR-30a Expression and EMT is Associated with Cisplatin-Resistance of Gastric Cancer Cells

MiR-30a is usually downregulated in gastric cancer. In addition, it has also been reported as a miRNA related to chemoresistance in some types of cancer[22-24]. Based on 20 tissue samples obtained from gastric cancer patients, we firstly investigate the expression of miR-30a in chemo-sensitive and resistant tumor tissues. Among the 20 patients, there were 0 case of CR, 8 cases of PR, 5 cases of SD and 7 cases of PD. The results showed the chemosensitive tissues generally had significantly higher expression of miR-30a than the chemoresistant tissues (Figure 1A). By performing qRT-PCR analysis, we confirmed that the cisplatin-sensitive SGC-7901 cells had significantly higher miR-30a expression than the cisplatin-resistant SGC-7901/DDP cells (Figure 1B). We observed that SGC-7901 cells showed typical epithelial cell morphology, while SGC-7901/DDP cells presented more elongated fibroblastoid-like morphology (Figure 1C). Therefore, we further investigate whether there is an EMT in these two cell lines. By performing Western blot analysis, we observed SGC-7901/DDP cells had higher expressions of Snail and Vimentin, two mesenchymal markers, while SGC-7901 cells had higher a level of E-Cadherin, an epithelial marker (Figure 1D). These results suggest that decreased miR-30a expression and EMT is associated with cisplatin-resistance of gastric cancer cells.

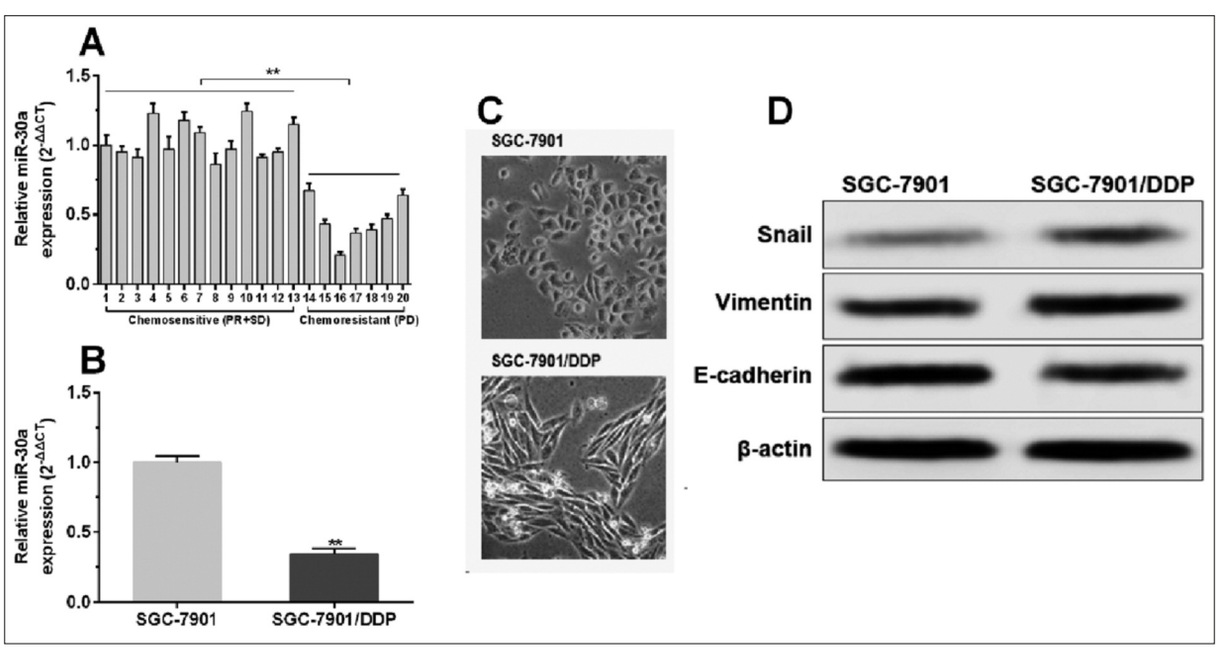

Figure 1. Decreased miR-30a expression and EMT is associated with cisplatin-resistance of gastric cancer cells. ***A,*** QRT-PCR analysis of miR-30a expression in 20 cases of gastric cancer tissues, among which there were 13 chemosensitive cases (0 cases of CR, 8 cases of PR and 5 cases of SD) and 7 chemoresistant cases (PD). ***B,*** QRT-PCR analysis of miR-30a expression in SGC-7901 cells and SGC-7901/DDP cells. ***C,*** Morphology of SGC-7901 cells and SGC-7901/DDP cells. ***D,*** Western blot analysis of Snail, Vimentin and E-cadherin expression in SGC-7901 cells and SGC-7901/DDP cells. $^{**}p < 0.01$.

MiR-30a Modulates EMT in SGC-7901 and SGC-7901/DDP Cells

Then, we further investigated the association between miR-30a and EMT. SGC-7901 cells were firstly transfected with antagomiR-30a (Figure 2A), while SGC-7901/DDP cells were transfected miR-30a mimics (Figure 2B). Knockdown of endogenous miR-30a promoted the elongated fibroblast-like morphologic alteration of SGC-7901 cells (Figure 2C). In comparison, overexpression of miR-30a in SGC-7901/DDP cells induced morphological changes from an extended, fibroblast-like morphology to more epithelial-like morphology (Figure 2C). Also, we also observed that miR-30a inhibition increased Snail and Vimentin expression but decreased E-Cadherin expression in SGC-7901 cells (Figure 2D). MiR-30a overexpression decreased Snail and Vimentin level but increased E-Cadherin expression in SGC-7901/DDP cells (Figure 2D). These results suggest that miR-30a modulates EMT in SGC-7901 and SGC-7901/DDP cells.

MiR-30a Modulates Cisplatin Sensitivity of SGC-7901 and SGC-7901/DDP Cells

We further investigated the effect of miR-30a on cisplatin sensitivity of SGC-7901 and SGC-7901/DDP cells. SGC-7901 cells were also transfected with miR-30a for overexpression (Figure 3A) and SGC-7901/DDP were transfected with antagomiR-30a for knockdown (Figure 3B). MTT assay showed that both SGC-7901 and SGC-7901/DDP cells with miR-30a overexpression had increased sensitivity to cisplatin, while the cells treated with antagomiR-30a had substantially reduced cisplatin sensitivity (Figure 3C-D). In addition, we also found that SGC-7901 cells with miR-30a knockdown had a lower pro-

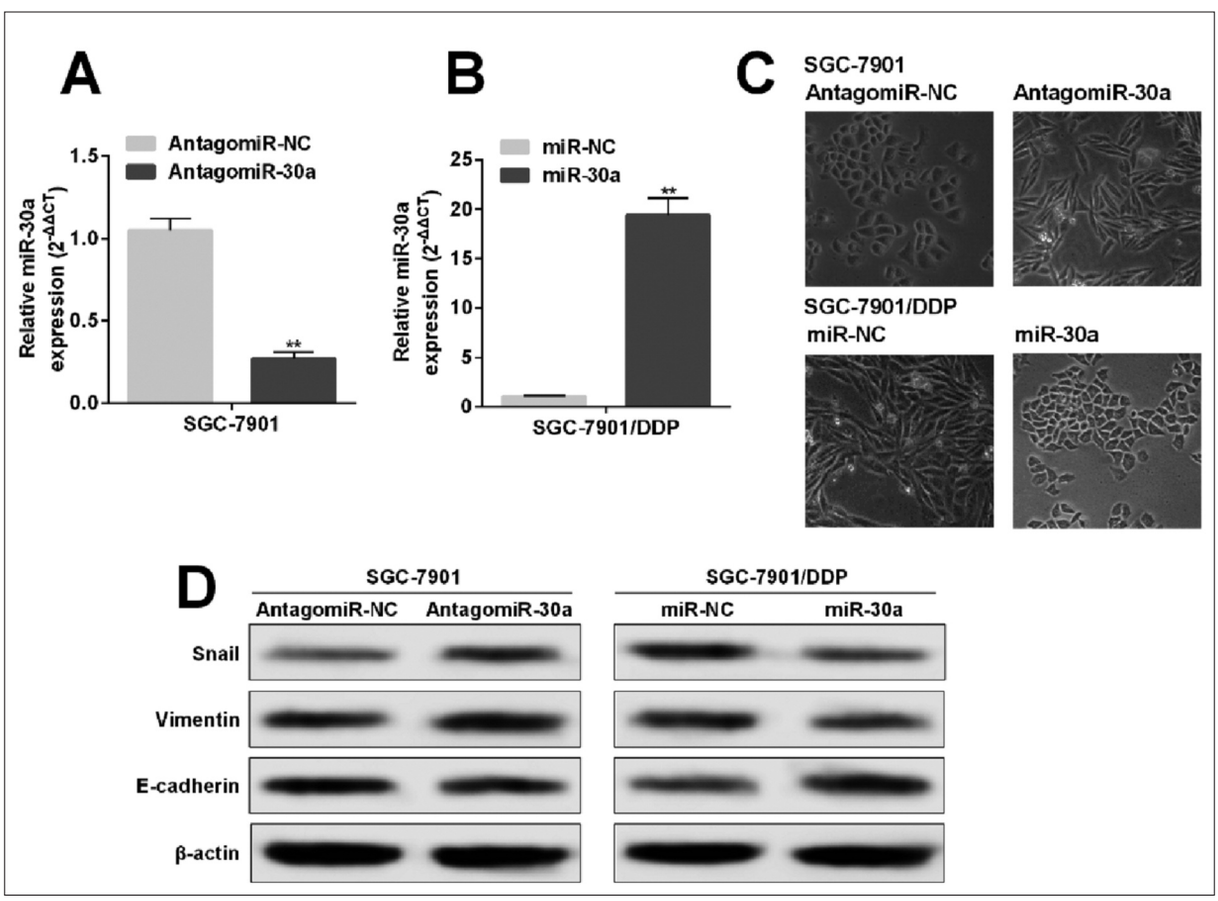

Figure 2. MiR-30a modulates EMT in SGC-7901 and SGC-7901/DDP cells. ***A,*** and ***B,*** QRT-PCR analysis of miR-30a expression in SGC-7901 cells transfected with 100 nM antagomiR-30a ***(A)*** and in SGC-7901/DDP cells transfected with 100 nM miR-30a mimics ***(B)***. ***C,*** Morphology of SGC-7901 cells with miR-30a knockdown and SGC-7901/DDP cells with miR-30a overexpression. ***D,*** Western blot analysis of Snail, Vimentin and E-cadherin expression in SGC-7901 cells with miR-30a knockdown and in SGC-7901/DDP cells with miR-30a overexpression. $^{**}p < 0.01$.

portion of apoptotic cells after cisplatin treatment (Figure 3E and G), while SGC-7901/DDP cells treated with miR-30a mimics had substantially increased the proportion of apoptotic cells after the treatment (Figure 3F and H). These results suggest that miR-30a can modulate cisplatin sensitivity of SGC-7901 and SGC-7901/DDP cells.

Discussion

Previous studies[25] observed that high miR-30a expression, together with other three miRNAs, including let-7a and miR-126 are protective miRNA signature of gastric cancer patients. These three miRNAs are significantly associated with better overall survival and relapse-free survival outcomes[25]. However, the molecular mechanisms underlying the protective effects are not clear. miR-30a is an important miRNA that modulates EMT in gastric cancer. MiR-30 directly targets HNF4γ, a transcription factor that facilitates transcription of metaplasia markers in the stomach[26]. In addition, in multiple types of cancer, the suppressive effect of miR-30a on EMT was also observed. For example, miR-30a directly targets Snai1, a known transcriptional repressor of E-cadherin and modulator of EMT and inhibits invasion and metastasis of non-small cell lung cancer cells[20]. Similar regulative effect of miR-30a was also observed in hepatocellular carcinoma (HCC). One recent study found that miR-30a downregulation was significantly associated with worse disease-free survival (DFS) of HCC patients[27]. Loss of miR-30a is associated with facilitated tumor cell migration, invasion and EMT[27]. Besides, previous investigations also observed that miR-30a can also target Vimentin and downregulate its expression in breast cancer[28] and gastric cancer[21]. In addition, miR-30a can also target Slug, one of the master regulators of EMT in breast cancer[29].

Based on patient tissues, we observed that the chemosensitive cases had significantly higher miR-30a expression than the chemoresistant counterparts. By performing qRT-PCR analysis, we observed that the cisplatin-sensitive SGC-7901 cells had significantly higher miR-30a expression than the cisplatin-resistant SGC-7901/DDP cells. Also, SGC-7901 cells presented epithelial cell morphology, while SGC-7901/DDP cells presented more elongated fibroblastoid-like morphology. SGC-7901/DDP cells also had higher expression of Snail and Vi-

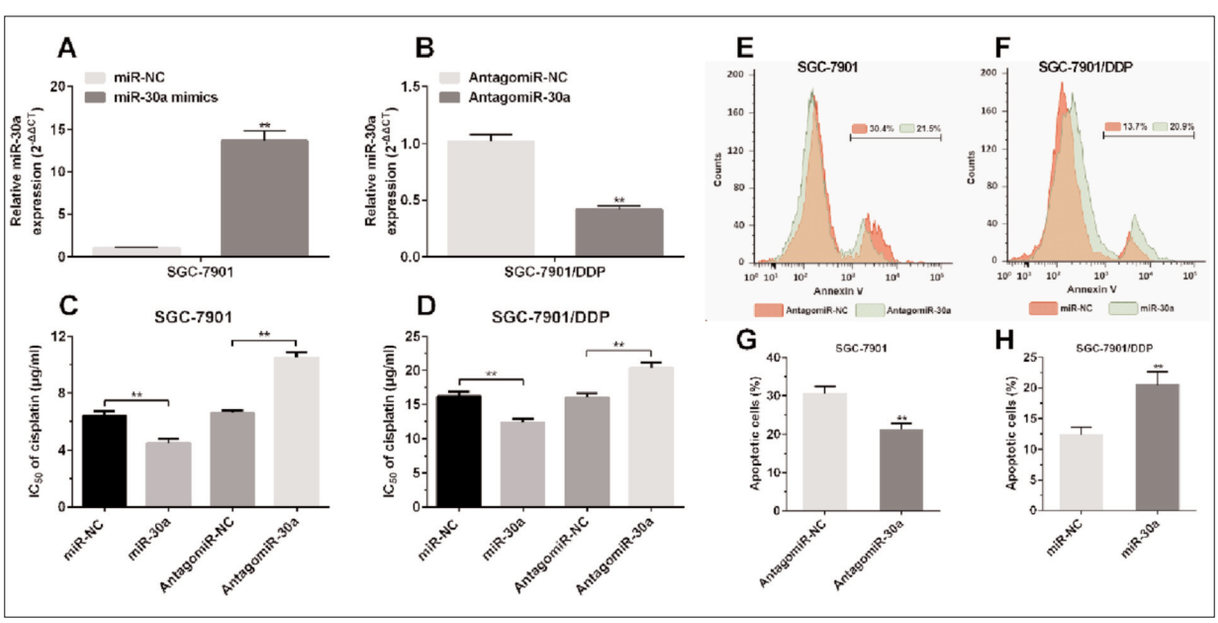

Figure 3. MiR-30a modulates cisplatin sensitivity of SGC-7901 and SGC-7901/DDP cells. ***A,*** and ***B,*** QRT-PCR analysis of miR-30a expression in SGC-7901 cells transfected with 100 nM miR-30a mimics ***(A)*** and in SGC-7901/DDP cells transfected with 100 nM antagomiR-30a ***(B)***. ***C,*** and ***D,*** MTT assay of DDP sensitivity of SGC-7901 cells ***(C)*** and SGC-7901 cells ***(D)*** with miR-30a overexpression or knockdown. E and F. Representative images of flow cytometric analysis of apoptotic SGC-7901 cells with miR-30a knockdown ***(E)*** and SGC-7901/DDP cells with miR-30a overexpression ***(F)*** 48 hours after treatment with 10 μg/mL DDP. ***G,*** and ***H,*** Quantification of the apoptotic cells showed in Figure ***E,*** and ***F.*** $^{**}p < 0.01$.

mentin than SGC-7901 cells. Therefore, we decided to further verify the role of miR-30a in EMT in SGC-7901 and SGC-7901/DDP cells. Knockdown of endogenous miR-30a promoted the elongated fibroblast-like morphologic alteration of SGC-7901 cells and also enhanced Snail and Vimentin expression. MiR-30a overexpression induced morphological changes from an extended, fibroblast-like morphology to more epithelial-like morphology in SGC-7901/DDP cells and decreased Snail and Vimentin levels. These results further confirmed that miR-30a is an important modulator of EMT in SGC-7901 and SGC-7901/DDP cells.

Increased EMT is associated with higher level of chemoresistance, even MDR[30,31]. High expression of Snail and Slug can enhance both radioresistance and chemoresistance via antagonizing p53-mediated apoptosis and acquire a stem-like phenotype[30]. Considering the strong regulative effect of miR-30a on EMT, we then studied effect of miR-30a on cisplatin sensitivity of SGC-7901 and SGC-7901/DDP cells. MTT assay of cell viability and flow cytometry analysis of cell apoptosis both showed that the cancer cells with miR-30a overexpression had significantly higher DDP sensitivity, while the cells with miR-30a knockdown had decreased sensitivity.

Conclusions

EMT is associated with cisplatin-resistance of gastric cancer. MiR-30a is an important miRNA modulating EMT and cisplatin sensitivity of SGC-7901 and SGC-7901/DDP cells.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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