MiR-30a Decreases Multidrug Resistance (MDR) of Gastric Cancer Cells

Chunying Li*  
Jinhai Zou*  
Guoqi Zheng  
Jiankun Chu

* Chunying Li and Jinhai Zou contributed equally to this study

Corresponding Author: Jinhai Zou, e-mail: jinhaizou@outlook.com
Source of support: Departmental sources

Background: The effectiveness of chemotherapy for gastric cancer is largely limited by either intrinsic or acquired drug resistance. In this study, we aimed to explore the association between miR-30a dysregulation and multidrug resistance (MDR) in gastric cancer cells.

Material/Methods: We recruited 20 patients with advanced gastric cancer. Chemosensitivity was assessed after completion of the chemotherapy. SGC-7901 and SGC-7901/DDP cells were transfected for miR-30a overexpression or knockdown. Then, MTT assay was performed to assess the IC50 of DDP and 5-FU in SGC-7901 and SGC-7901/DDP cells. Flow cytometry analysis was used to detect DPP- and 5-FU-induced cell apoptosis. Western blot analysis and immunofluorescence staining were used to assess EMT of the cells.

Results: MiR-30a was significantly downregulated in the chemoresistant tissues. In both SGC-7901 and SGC-7901/DDP cells, miR-30a overexpression decreased the expression of P-gp, a MDR-related protein. MTT assay and flow cytometry analysis showed that miR-30a inhibition increased chemoresistance, while miR-30a overexpression decreased chemoresistance in gastric cancer cells. Both Western blot analysis and immunofluorescence staining confirmed that miR-30a inhibition decreased E-cadherin but increased N-cadherin in SGC-7901 cells, while miR-30a overexpression increased E-cadherin but decreased N-cadherin in SGC-7901 cells.

Conclusions: MiR-30a can decrease multidrug resistance (MDR) of gastric cancer cells. It is also an important miRNA modulating EMT of the cancer cells.

MeSH Keywords: Cisplatin • Epithelial-Mesenchymal Transition • MicroRNAs • Stomach Neoplasms

Full-text PDF: http://www.medscimonit.com/abstract/index/idArt/898415
Background

Gastric cancer is one of the most aggressive and malignant cancers, and is the leading cause of cancer-related deaths globally [1, 2]. Currently, physical resection is the primary and also the most effective therapy for the patients with resectable tumors. However, due to low rates of early detection and diagnosis, many gastric cancer patients in China are diagnosed at an advanced and unresectable clinical stage [3]. For the patients with resectable tumors, chemotherapy is usually used as an adjuvant therapy following the surgery, and for the patients with unresectable tumors, chemotherapy is offered as the primary therapy [4]. However, the effectiveness of chemotherapy is largely limited by either intrinsic or acquired drug resistance, especially multidrug resistance (MDR) [1]. The mechanisms underlying MDR are quite complex. Earlier studies have revealed that multiple mechanisms, such as drug efflux, alteration of drug targets, enhanced DNA repair and cell proliferation, cell cycle change, and inactivation apoptotic pathways, all take part in chemoresistance development [5–7].

Material and Methods

Patient tissue collection

This study design was approved by the Ethics Committee of Hebei Medical University. We recruited 20 patients with histopathological confirmed advanced gastric cancer who received 2 cycles of platinum-based chemotherapy, from the First Hospital of Hebei Medical University. After completion of the chemotherapy, the responses of the patients were assessed by 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. Tumor tissues were obtained from gastroscopy biopsy.

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% CO2 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, antagoniR-30a (anti-miR-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 antagoniR-30a for knockdown. Transfection was performed using LipoFectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol.

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 instructions. 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). The mature miR-30a level was detected using the TaqMan MicroRNA Assays Kit (Applied Biosystems) according to the manufacturer’s instruction and quantified using the 2-ΔΔCT method.

Western blot analysis

Conventional Western blot analysis was performed following the methods described in a previous study [20]. The primary
antibodies used included anti-Snail (1: 500, ab82846, Abcam, Cambridge, UK), anti-Vimentin (1: 2000, ab92547, Abcam) anti-E-cadherin (1: 1000, ab77287, Abcam), anti-slug (1: 1000, ab27568, Abcam), anti-P-gp (1: 2000, ab129450, Abcam), and anti-β-actin (1: 2000, ab8227, Abcam). The second antibody 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). The relative gray-scale intensity was quantified using ImageJ software (v1.45).

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) or 5-FU (15 μg/mL) for 48 h. Then, the ratio of apoptotic cells was determined using the Annexin V-FITC Apoptosis Detection Kit (V13241, Invitrogen) according to manufacturer's instructions, in a FACS Calibur device (BD Biosciences, Franklin Lakes, NJ). Data acquisition was done using CellQuest 3.2 software (BD Biosciences). Each test was performed with at least 3 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 (5×10³ cells/well) and incubated at 37°C in a humidified 5% CO₂ atmosphere for 24 h. Then, DDP was added with the final concentrations of 0.02, 0.2, 1, 2, 10, and 20 μg/mL to the culture medium. 5-FU was added with the final concentrations of 0.2, 1, 5, 10, 20, and 50 μg/mL to the culture medium. At 48 h after DDP or 5-FU administration, cell viability was assessed using a MTT assay. Three independent experiments were performed in triplicate.

Fluorescence microscopy

SGC-7901 with or without transfection of anti-miR-30a and SGC-7901/DDP cells with or without transfection of miR-30a mimics were grown on coverslips. Then, the cells were fixed in methanol, permeabilized in 0.1% Triton X-100, and blocked in 1% BSA. To detect the expression of E-cadherin and N-cadherin, the coverslips were probed with primary antibodies against E-cadherin (1: 500, ab40772, Abcam) and N-cadherin (1: 100, ab76011, Abcam), respectively, at 4°C overnight. After the incubation, the coverslips were washed and further incubated with secondary Alexa Fluor®-conjugated donkey anti-rabbit IgG H&L (1: 500, ab15074, Abcam) and Alexa Fluor®-conjugated donkey anti-rabbit polyclonal antibody (1: 500, ab15073, Abcam), respectively, for 1 h at room temperature. Nuclei were stained with Gold Antifade Reagent with DAPI (Invitrogen). Digital immunofluorescent images were obtained using an Eclipse Ti-S inverted phase/fluorescent microscope (Nikon, Tochigi, Japan).

Statistical analysis

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

Results

Chemoresistant gastric cancer is associated with decreased miR-30a expression and enhanced EMT

The involvement of miR-30a in chemosensitivity regulation was observed in several types of cancer, such as renal cell carcinoma cells, ovarian cancer, and osteosarcoma [21–23]. After completion of the chemotheraphy, there were 7 chemoresistant patients (with PD) and 13 chemosensitive patients (8 patients with PR and 5 cases with SD). Then, by performing qRT-PCR analysis, we quantified miR-30a levels in the gastric cancerous tissue samples from the patients. Generally, miR-30a expression was significantly lower in the tissues from chemoresistant patients than in that from chemosensitive patients (Figure 1A). Then, we decided to investigate the role of miR-30a in regulation of chemosensitivity by using in vitro gastric cancer cell line SGC-7901 and SGC-7901/DDP. Consistent with gastric cancer tissue data, miR-30a expression was also significantly lower in SGC-7901/DDP cells than in SGC-7901 cells (Figure 1B). Previous studies reported that EMT is also an important physiological change affecting chemosensitivity. The results of Western blot analysis showed the expressions of Snail, Vimentin, and Slug were significantly higher in SGC-7901/DDP cells than in SGC-7901 cells, while the expression of E-cadherin was significantly lower in SGC-7901/DDP cells than in SGC-7901 cells (Figure 1C). Then, we performed immunofluorescence staining to detect the expression of E-cadherin and N-cadherin in SGC-7901/DDP cells and in SGC-7901 cells. The results showed that SGC-7901 cells had higher E-cadherin expression, while SGC-7901/DDP cells had higher N-cadherin expression (Figure 1D).

MiR-30a can modulate MDR of gastric cancer cells

Then, we overexpressed miR-30a and inhibited endogenous miR-30a in SGC-7901 and SGC-7901/DDP cells, respectively (Figure 2A, 2B). In both SGC-7901 and SGC-7901/DDP cells, miR-30a overexpression decreased the expression of P-gp, an MDR-related protein [24] (Figure 2C). Then, we performed MTT assay to assess the IC₅₀ of DPP and 5-FU in SGC-7901 and SGC-7901/DDP cells. The results showed that miR-30a overexpression decreased IC₅₀ of DPP and 5-FU in SGC-7901 and
SGC-7901/DDP cells, while miR-30a inhibition increased the IC50 of DPP and 5-FU in the cells (Figure 2D, 2E). Then, we performed flow cytometry analysis to detect DPP and 5-FU-induced cell apoptosis in both SGC-7901 and SGC-7901/DDP cells. The results showed that miR-30a inhibition decreased the DPP- and 5-FU-induced cell apoptosis in SGC-7901 cells (Figure 2F, 2H), while miR-30a overexpression increased DPP and 5-FU induced cell apoptosis in SGC-7901/DDP cells (Figure 2F, 2H). These results suggest that miR-30a can modulate MDR in gastric cancer cells.

**MiR-30a suppresses EMT in gastric cancer cells**

Then, we further investigated the regulative effect of miR-30a on EMT of the gastric cancer cells. By performing Western blot analysis, we observed that the miR-30a inhibition decreased E-cadherin, but increased N-cadherin in SGC-7901 cells (Figure 3A–3C), while miR-30a overexpression increased E-cadherin, but decreased N-cadherin in SGC-7901/DDP cells (Figure 3A–3C). Subsequent immunofluorescence staining confirmed the changes in E-cadherin and N-cadherin in the cells (Figure 3D).

**Discussion**

Previous studies suggest that miR-30a plays an important role in suppressing the development of gastric neoplasm and metastasis of gastric cancer via inhibiting EMT. In detail, miR-30a can also target HNF4gamma, which regulates the development
of intestinal metaplasia, a neoplastic precursor of gastric adenocarcinoma [25]. Overexpression of Runx-related transcription factor 3 (RUNX3) in gastric cancer cells leads to increased miR-30a level, which directly binds to the 3’ untranslated region of vimentin and decreased its protein level [17]. In other types of cancer, previous studies found that miR-30a can also modulate EMT by targeting multiple important modulators of EMT. In non-small cell lung cancer cells and hepatocellular carcinoma cells, miR-30a can directly target Snail1, which enhances EMT by transcriptional repressing of E-cadherin [19,26]. Decreased expression of miR-30a is associated with enhanced EMT and facilitated tumor cell migration and invasion [26]. In breast cancer, miR-30a can also target Vimentin and Slug, 2 master regulators of EMT in breast cancer [17,27,28].

In fact, the association between miR-30a and chemoresistance was also observed in some types of cancer. In ovarian cancer, miR-30a can decrease chemoresistance by inhibiting endothelin A receptor [29]. In osteosarcoma cells, miR-30a downregulation leads to chemoresistance via activating Beclin-1-mediated autophagy [23]. However, whether miR-30a is associated with MDR in gastric cancer is not clear. In this study, we firstly assessed whether miR-30a is associated with chemoresistance in gastric cancer. Based on cancerous tissues obtained from 20 gastric cancer tissues, we found that miR-30a was significantly downregulated in the chemoresistant tissues. Therefore, we decided to further explore the role of miR-30a in MDR of gastric cancer based on cisplatin-sensitive SGC-7901 cells and the cisplatin-resistant SGC-7901/DDP cells. In both SGC-7901 and SGC-7901/DDP cells, miR-30a overexpression decreased the expression of P-gp, an MDR-related protein [24]. Then, we

Figure 2. MiR-30a can modulate MDR of gastric cancer cells. (A, B) QRT-PCR analysis of miR-30a expression in SGC-7901 cells (A) and SGC-7901/DDP cells (B) transfected with 100 nM miR-30a mimics OR 100 nM antagomiR-30a (anti-miR-30a). (C) Western blot analysis of P-gp expression in SGC-7901 cells and SGC-7901/DDP cells with miR-30a overexpression. (D, E) MTT assay of DDP or 5-FU sensitivity of SGC-7901 cells (D) and SGC-7901 cells (E) with miR-30a overexpression or knockdown. (F) Representative images of flow cytometric analysis of apoptotic SGC-7901 cells with miR-30a knockdown and SGC-7901/DDP cells with miR-30a overexpression 48 h after treatment with 10 μg/ml DDP. (G, H) Quantification of the apoptotic cells 48 h after treatment with 10 μg/ml DDP (G) or 15 μg/ml 5-FU (H) for 48 h. *p<0.05, **p<0.01.
performed MTT assay to assess the IC50 of DPP and 5-FU in SGC-7901 and SGC-7901/DDP cells and also conducted flow cytometry analysis to assess DPP- and 5-FU-induced apoptosis after miR-30a overexpression or knockdown. The results showed that miR-30a inhibition increased chemoresistance, while miR-30a overexpression decreased chemoresistance in gastric cancer cells. A previous study found that high miR-30a expression, together with another 3 miRNAs, including let-7a and miR-126, are protective miRNA signatures of gastric cancer patients [30]. Relatively high expressions of the 3 miRNAs are significantly associated with better overall survival and relapse-free survival [30]. Based on the findings above, we infer that the contribution of high miR-30a to chemosensitivity might be one of the mechanisms associated with good prognosis.

Since previous studies suggest that EMT can affect MDR [14,15], we further performed studies to verify the effect of miR-30a on EMT of the gastric cancer cells. Both Western blot analysis and immunofluorescence staining confirmed that miR-30a inhibition decreased E-cadherin but increased N-cadherin in SGC-7901 cells, while miR-30a overexpression increased E-cadherin but decreased N-cadherin in SGC-7901 cells.

Conclusions

MiR-30a can decrease multidrug resistance (MDR) of gastric cancer cells. It is also an important miRNA modulating EMT of the cancer cells.

References:

1. Burris HA, III: Overcoming acquired resistance to anticancer therapy: Focus on the PI3K/AKT/mTOR pathway. Cancer Chemother Pharmacol, 2013; 71: 829–42
2. Siegel R, Ma J, Zou Z, Jemal A: Cancer statistics, 2014. Cancer J Clin, 2014; 64: 9–29
3. Hu Y, Ying M, Huang C et al., Chinese Laparoscopic Gastrointestinal Surgery Study Group: Oncologic outcomes of laparoscopy-assisted gastrectomy for advanced gastric cancer: A large-scale multicenter retrospective cohort study from China. Surg Endosc, 2014; 28: 2048–56
4. Dong CX, Fu JF, Ye XY et al: Surgical resection of advanced gastric cancer following trastuzumab/oxaliplatin/capcitabine combination therapy. World J Gastroenterol, 2014; 20: 12355–58
5. Zhang D, Fan D: New insights into the mechanisms of gastric cancer multidrug resistance and future perspectives. Future Oncol, 2010; 6: 527–37

6. Wang Y, Wu K, Yang Z et al: Multidrug-resistance related long non-coding RNA expression profile analysis of gastric cancer. PLoS One, 2015; 10: e0135461

7. Zhao W, Chen R, Zhao M et al: High glucose promotes gastric cancer chemoresistance in vivo and in vitro. Mol Med Rep, 2015; 12: 843–50

8. Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell, 2004; 116: 281–97

9. Xia L, Zhang D, Du R et al: miR-15b and miR-16 regulate multidrug resistance by targeting BCL2 in human gastric cancer cells. Int J Cancer, 2008; 123: 372–79

10. Zhu W, Shan X, Wang T et al: miR-181b regulates multidrug resistance by targeting BCL2 in human cancer cell lines. Int J Cancer, 2010; 127: 2520–29

11. Zhu W, Zhu D, Lu S et al: miR-497 modulates multidrug resistance of human cancer cell lines by targeting BCL2. Med Oncol, 2012; 29: 384–91

12. Tang Y, Zhang Z, Liu Z et al: miR-308-5p regulates multidrug resistance of gastric cancer by targeting AKB1 and ZNRD1. Oncogene, 2014; 33: 3267–76

13. An Y, Zhang Z, Shang Y et al: miR-23b-3p regulates the chemoresistance of gastric cancer cells by targeting ATG12 and HMG2. Cell Death Dis, 2015; 6: e1766

14. Duran GE, Wang YC, Francisco EB et al: Mechanisms of resistance to cabazitaxel. Mol Cancer Ther, 2015; 14: 193–201

15. Mallini P, Lennard T, Kirby J, Meeson A: Epithelial-to-mesenchymal transition: What is the impact on breast cancer stem cells and drug resistance. Cancer Treat Rev, 2014; 40: 341–48

16. Li F, Mahato RI: MicroRNAs and drug resistance in prostate cancers. Mol Pharm, 2014; 11: 2539–52

17. Liu Z, Chen L, Zhang X et al: RUNX3 regulates vimentin expression via miR-30a during epithelial-mesenchymal transition in gastric cancer cells. J Cell Mol, Med, 2014; 18: 610–23

18. Zhou Q, Yang M, Lan H, Yu X: miR-30a negatively regulates TGF-beta1-induced epithelial-mesenchymal transition and peritoneal fibrosis by targeting Snai1. Am J Pathol, 2013; 183: 808–19

19. Kumarswamy R, Mudduluru G, Ceppi P et al: MicroRNA-30a inhibits epithelial-to-mesenchymal transition by targeting Snai1 and is downregulated in non-small cell lung cancer. Int J Cancer, 2012; 130: 2044–53

20. Kim Y, Kim H, Park H et al: miR-326-histone deacetylase-3 feedback loop regulates the invasion and tumorigenic and angiogenic response to anti-cancer drugs. J Biol Chem, 2014; 289: 28019–39

21. Zheng B, Zhu H, Gu D et al: MiRNA-30a-mediated autophagy inhibition sensitizes renal cell carcinoma cells to sorafenib. Biochem Biophys Res Commun, 2015; 459: 234–39

22. Sestito R, Cianfrocca R, Rosano L et al: miR-30a inhibits endothelin A receptor and chemoresistance in ovarian carcinoma. Oncotarget, 2016; 7(4): 4009–23

23. Xu R, Liu S, Chen H, Lao L: MicroRNA-30a downregulation contributes to chemoresistance of osteosarcoma cells through activating Beclin-1-mediated autophagy. Oncol Rep, 2016; 35: 1757–63

24. Diestra JE, Condom E, Del Muro XG et al: Expression of multidrug resistance proteins P-glycoprotein, multidrug resistance protein 1, breast cancer resistance protein and lung resistance related protein in locally advanced bladder cancer treated with neoadjuvant chemotherapy: biological and clinical implications. J Urol, 2003; 170: 1383–87

25. Sousa JF, Nam KT, Petersen CP et al: miR-30-HNF4gamma and miR-194-NR2F2 regulatory networks contribute to the upregulation of metaplasia markers in the stomach. Gut, 2015 [Epub ahead of print]

26. Liu Z, Tu K, Liu Q: Effects of microRNA-30a on migration, invasion and prognosis of hepatocellular carcinoma. FEBS Lett, 2014; 588: 3089–97

27. Cheng CW, Wang HW, Chang CW et al: MicroRNA-30a inhibits cell migration and invasion by downregulating vimentin expression and is a potential prognostic marker in breast cancer. Breast Cancer Res Treat, 2012; 134: 1081–93

28. Chang CW, Yu JC et al: MicroRNA-30a increases tight junction protein expression to suppress the epithelial-mesenchymal transition and metastasis by targeting Slug in breast cancer. Oncotarget, 2016 [Epub ahead of print]

29. Sestito R, Cianfrocca R, Rosano L et al: miR-30a inhibits endothelin A receptor and chemoresistance in ovarian carcinoma. Oncotarget, 2016; 7: 4009–23

30. Li X, Zhang Y, Zhang Y et al: Survival prediction of gastric cancer by a seven-microRNA signature. Gut, 2010; 59: 579–85