

MicroRNA-218 regulates the chemo-sensitivity of cervical cancer cells through targeting survivin

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Background: Cervical cancer is one of the most lethal malignancies among women in the world. Every year about 311,365 women die because of cervical cancer. Chemo-resistance is the main reason of the lethal malignancies, and the mechanism of chemo-resistance in cervical cancer still remains largely elusive.

Purpose: Previous studies reported that microRNAs played important biological roles in the chemo-resistance in many types of cancers, in the present study we tried to investigate the biological roles of microRNA-218 in chemo-resistance in cervical cancer cells.

Results: Real-time PCR results indicated microRNA-218 was downregulated in cisplatin-resistant HeLa/DDP and SiHa/DDP cells compared with the mock HeLa and SiHa cells. CCK-8 assay results showed upregulation of microRNA-218 enhanced the cisplatin sensitivity of cervical cancer cells; while downregulation of microRNA-218 decreased the cisplatin sensitivity of cervical cancer cells. Dual-luciferase assay indicated survivin was a direct target of microRNA-218. Western blotting and PCR results indicated the expression of survivin in HeLa/DDP and SiHa/DDP cells was significantly increased compared with HeLa and SiHa cells. Further study indicated induction of microRNA-218 decreased the expression of survivin while inhibition of microRNA-218 increased the expression of survivin in cervical cancer cells. Cell apoptosis results indicated induction of microRNA-218 induced the cell apoptosis in cervical cancer cells.

Conclusion: Our data revealed microRNA-218 enhanced the cisplatin sensitivity in cervical cancer cells through regulation of cell growth and cell apoptosis, which could potentially benefit to the cervical cancer treatment in the future.

Keywords: miR-218, cervical cancer, cisplatin resistant, apoptosis, survivin

Introduction

Cervical cancer (CC) is the fourth most common malignancy cancer amongst women worldwide, and CC is the fourth leading cause of cancer-related death in women.¹ It was showed about 569,847 new CC patients were diagnosed every year, and about 311,365 women were died because of CC.¹ An important reason of the high mortality is that there were no obvious symptoms until cancer has been progressed to an advanced stage.² In the current stage, the clinical treatments of CC were mainly including: surgery therapy, chemotherapy, radiotherapy, and the combination therapy.³ For advanced CC patient, chemotherapy was recommended as a standard therapy, however the clinical drug did not show strong therapeutic efficacy.⁴⁻⁶ Like other cancers, the chemo-resistant greatly impacted the therapeutic efficacy of CC patients. Therefore, investigating of the mechanism of the chemo-resistant in CC has the great significance to explore the effective therapy for CC.

MicroRNAs (miRNAs) are a series of small, conserved, and non-coding short RNAs. They are formed by 18–25 nucleotides in length.⁷ Previous studies indicated that miRNAs were dysregulated in human cancers, and they bind to the UTR of messenger RNAs (mRNAs) to regulate the gene expression, which could regulate the human cancer cell growth and differentiation.^{8–12} Studies also indicated miRNAs played important biological roles in the chemo-resistance of human cancer.^{13,14} A growing body of evidence suggested miR-218 was significantly downregulated in human cancer tissues compared with the adjacent non-cancerous tissues,^{15–20} therefore miR-218 played as a tumor-suppressive miRNA in human cancer. Studies also indicated miR-218 inhibited cancer cell growth and invasion through regulation of oncogenic genes,^{21–25} and miR-218 increased the chemosensitivity of many types of human cancer cells, including esophageal cancer, colorectal cancer, breast cancer, and gastric cancer.^{26–29} Recently, studies indicated miR-218 was downregulated in CC and miR-218 regulated CC cell growth,^{30,31} however whether miR-218 regulates cisplatin (DDP) resistance in CC still remains largely elusive.

In the current study, we reported miR-218 was significantly downregulated in DDP-resistant human CC cells compared with their mock CC cells, and upregulation of miR-218 enhanced the DDP sensitivity of human CC cells through cell growth and cell apoptosis regulation by targeting survivin gene. These findings could potentially benefit to the CC treatment in the future.

Materials and methods

Cells culture

Human CC cell line HeLa, HeLa/DDP, SiHa and SiHa/DDP were purchased from Fengbio Co., Ltd (Hunan, People's Republic of China). The cells were cultured in DMEM (Life Technologies, CA, USA) supplemented with 10% FBS, 1% penicillin and 1% streptomycin (Gibco, NY, USA) in a humidified incubator of 5% CO₂ at 37°C. DDP was added to evaluate the chemo-resistant capability of CC cells. DDP was purchased from Sigma (Shanghai, People's Republic of China).

Transfection

MiR-NC, miR-218 mimic, anti-miR-NC, and anti-miR-218 were purchased from Ambion (Austin, TX, USA). Small interfering RNA-218 (siRNA-218) targeting survivin and the siRNA control were purchased from

GenePharma (Shanghai, People's Republic of China). The miR-218 mimic, inhibitor, or control were diluted in Opti-MEM medium (Life Technologies, CA, USA) at room temperature (RT) for 15 mins, then transfected human CC cells with miR-218 mimic or inhibitor and cultured for 48 hrs. The expression of miR-218 was examined by qRT-PCR assay. siRNA transfection was performed with Lipofectamine RNAiMAX reagent (Invitrogen, CA, USA) following the manufacturer's instructions. The expression of survivin was examined by qRT-PCR and western assay.

RNA extraction and real-time PCR

Total RNA was isolated from mock or transfected CC cells by using TRIzol reagent (Invitrogen). MiR Complementary DNA (cDNA) was converted from total RNA by using PrimeScript miRNA cDNA Synthesis Kit (TaKaRa, Tokyo, Japan) and survivin cDNA was synthesized from total RNA using the PrimeScript RT Master Mix Kit (TaKaRa). MiR-218 quantification was performed with specific primers and probes using TaqMan MicroRNA Assays (Applied Biosystems, CA, USA), RNA U6 was used as the internal control. The survivin mRNA expression was measured by using qPCR assay (SYBR Green; Bio-Rad, USA) according to the manufacturer's instructions; GAPDH was used as the internal control. Relative mRNA expression was calculated by using the $2^{-\Delta\Delta CT}$ method. The sequences of the specific primers for mRNA amplification were as follows: survivin forward primer, 5'-GGACCACCGCATCTCTACAT-3' and reverse primer, 5'-GACAGAAAGGAAAGCGCAAC-3'; GAPDH forward primer, 5'-TCGACAGTCAGCCGCATCTTCTTT-3' and reverse primer, 5'-ACCAAATCCGTTGACTCCGACCTT-3'. All experiments were performed in triplicate.

miRNA target predictions

To further investigate the potential target of miR-218, potential genes were predicted by searching from targetscan (<http://www.targetscan.org>) and mirbase targets (<http://microRNA.sanger.ac.uk/cgi-bin/targets/v5/search.pl>). Survivin was predicted as a target of miR-218.

Cell viability and apoptosis assays

Cell viability was measured by Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay according to the manufacturer's protocol. Briefly, mock or transfected human CC cells were seeded into the 96-well plate and allowed to attach overnight. Then fresh prepared DDP,

mimic, inhibitor, or siRNAs were added into the wells as planned and cultured for 72 hrs in the incubator. At last, CCK-8 solution was added to the 96-well plate, and incubated for another 2 hrs at 37°C. The 450 nm absorbance was measured by using a microplate reader (Molecular Devices, USA). The cell viability was equal to the ratio of treatment group to the control group. The IC₅₀ was calculated as the DDP concentration of 50% reduction on cell viability. The CC cell apoptosis was measured by using the Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega, Beijing, People's Republic of China) according to the manufacturer's instructions. CellTiter-Blue (Promega) was used to measure the cell number. The relative Caspase-3/7 activity was calculated by the ratio of Apo-ONE and CellTiter-Blue signals. All experiments were performed in triplicate.

Luciferase reporter assay

A fragment from the 3'UTR of survivin gene containing the predicted binding site of miR-218 was amplified by PCR from genomic DNA. The amplified fragment was cloned into the UTR downstream of the luciferase gene in the pMIR-reporter luciferase vector (Ambion, USA). A corresponding mutant construct was used as the control. Human CC cells were co-transfected with the testing firefly luciferase reporter plasmid together with a Renilla luciferase plasmid. Then, we harvested the cells and measured the dual-luciferase activities by using Dual-Glo Luciferase Assay System (Promega). Renilla signal was used as an internal control for normalization. All experiments were performed in triplicate.

Western blot

Total protein was extracted from mock or transfected human CC cells by using RIPA lysis buffer (Beyotime, Shanghai, People's Republic of China) according to the manufacturer's protocol. BCA assay (Beyotime) was performed to determine the protein concentration. First proteins were separated on a SDS-PAGE gel (10%), and then transfer the separated protein onto a PVDF membrane (GE, USA). The PVDF membrane was blocked with 5% BSA for 2 hrs at RT and further incubated with antibodies against survivin or GAPDH for 2 hrs at RT. The PVDF membrane was washed with PBST for three times and further probed with second antibody for 2 hrs at RT. The bands were detected by using the Novex ECL HRP chemiluminescent substrate reagent kit (Invitrogen). Alpha Innotech imaging software was used to take the band image (San Leandro, CA, USA). All experiments were performed in triplicate.

Statistical analysis

A one-way analysis of variance was performed to analyze the statistical difference between groups by using SPSS v13.0 software. The data were expressed as mean±SD. $P<0.05$ indicated a statistically significant difference. All experiments were performed in triplicate.

Results

miR-218 was associated with DDP chemosensitivity in CC

To investigate the potential biological roles of miR-218 on the DDP chemoresistance of CC cells, the expression level of miR-218 was measured in both DDP resistant and mock CC cells. First, we check the DDP resistance capability of the HeLa/DDP and SiHa/DDP cells, the HeLa, HeLa/DDP, SiHa, and SiHa/DDP cells were exposed to different concentrations of DDP ranging from 0 to 100 μ M and cultured for 72 hrs, the cell viability was measured by using CCK-8 assay. As shown in Figure 1A, the IC₅₀ of HeLa/DDP and SiHa/DDP cells were much higher than the IC₅₀ of mock HeLa and SiHa cells. The qPCR results indicated the expression of miR-218 was significantly downregulated in DDP resistant HeLa/DDP and SiHa/DDP cells compared to their mock cells (Figure 1B, $P<0.05$). To further confirm the effect of miR-218 on DDP chemosensitivity, we measured the cell viability of CC cells transfected with miR-218 or anti-miR-218 in the presence of DDP. The results indicated that suppression of miR-218 by anti-miR-218 transfection markedly decreased the DDP chemosensitivity of HeLa and SiHa cells (Figure 1C, $P<0.05$); while induction of miR-218 by miR-218 mimic transfection markedly improved the DDP chemosensitivity of HeLa/DDP and SiHa/DDP cells (Figure 1D, $P<0.05$). Our results indicated that deregulation of miR-218 is critically involved in DDP-based chemosensitivity in CC.

Survivin is a direct target of miR-218

In order to determine the molecular basis of the effect of miR-218 in CC, we used a prediction program to identify putative target genes of miR-218. By using the prediction program, we identified survivin is a potential target of miR-218 (Figure 2A). Further results showed that the expression of survivin was significantly increased in DDP resistant CC cells both in protein and mRNA level (Figure 2C). To confirm

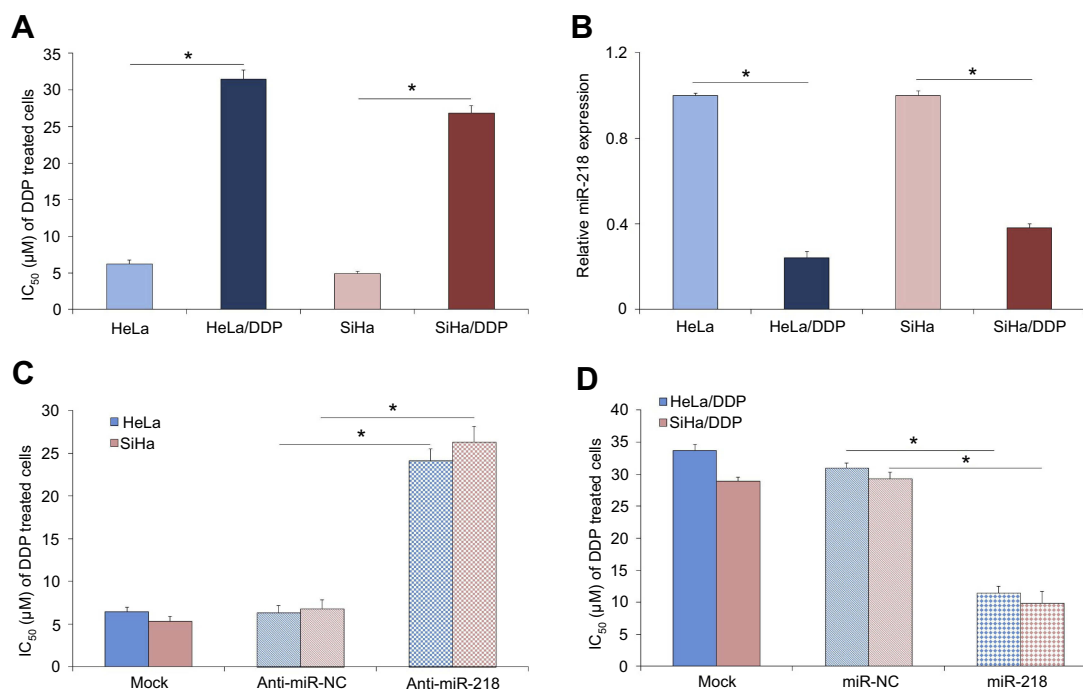


Figure 1 The effect of miR-218 on the DDP sensitivity in CC cells. **(A)** The IC₅₀ of DDP in CC cells, the cell viability was measured by CCK-8 assay in the presence of DDP. **(B)** The expression of miR-218 in CC cells, the expression was measured by qRT-PCR assay. **(C)** Inhibition of miR-218 decreased the sensitivity of CC cell to DDP. **(D)** Induction of miR-218 enhanced the sensitivity of CC cell to DDP. The data were shown as the mean±SD. **P*<0.05.

whether survivin is a direct target of miR-218 in CC cells, we co-transfected miR-218 and luciferase reporter plasmids with wild type (WT) or mutant type (Mut) of 3'-UTRs of survivin into CC cells. Then, we measured the luciferase values. As shown in Figure 2B, miR-218 remarkably decreased the luciferase activity in WT groups in HeLa/DDP and SiHa/DDP cells and miR-218 did not affect the luciferase activity in Mut groups. These results suggested survivin is a direct target of miR-218 in CC cells.

Survivin contributes to DDP chemoresistance in CC

To investigate whether survivin was associated with the DDP chemoresistance in CC, loss of function and gain of function assays were performed. Our results indicated that survivin siRNA transfection significantly decreased the expression of survivin in HeLa/DDP and SiHa/DDP cells (Figure 3A), and survivin expression inhibition increased the DDP sensitivity of CC cells (Figure 3B, *P*<0.05). In HeLa and SiHa cells, survivin mimic transfection increased the expression of survivin (Figure 3C), and survivin expression induction decreased the DDP sensitivity of CC cells

(Figure 3D, *P*<0.05). These results suggested survivin functionally regulated the DDP chemosensitivity in CC cells.

miR-218 modulated DDP chemosensitivity of CC cells through targeting survivin

To investigate the effect of miR-218 on DDP chemosensitivity in CC cells, CCK-8 assay was performed. The results indicated that upregulation of miR-218 significantly decreased the IC₅₀ of DDP in HeLa/DDP and SiHa/DDP cells; while downregulation of miR-218 significantly enhanced the IC₅₀ of DDP in HeLa and SiHa cells (Figure 4A, *P*<0.05). Therefore, miR-218 modulated the DDP chemosensitivity of CC cells in vitro. Then, we performed western and qPCR assays to test the effect of miR-218 on the expression of survivin in CC cells. The results indicated that upregulation of miR-218 decreased the expression of survivin both in mRNA (Figure 4C) and protein (Figure 4B) level in CC cells; while downregulation of miR-218 increased the expression of survivin both in mRNA (Figure 4C) and protein (Figure 4B) level in CC cells. These results suggested that miR-218 modulated DDP chemosensitivity of CC cells through targeting survivin.

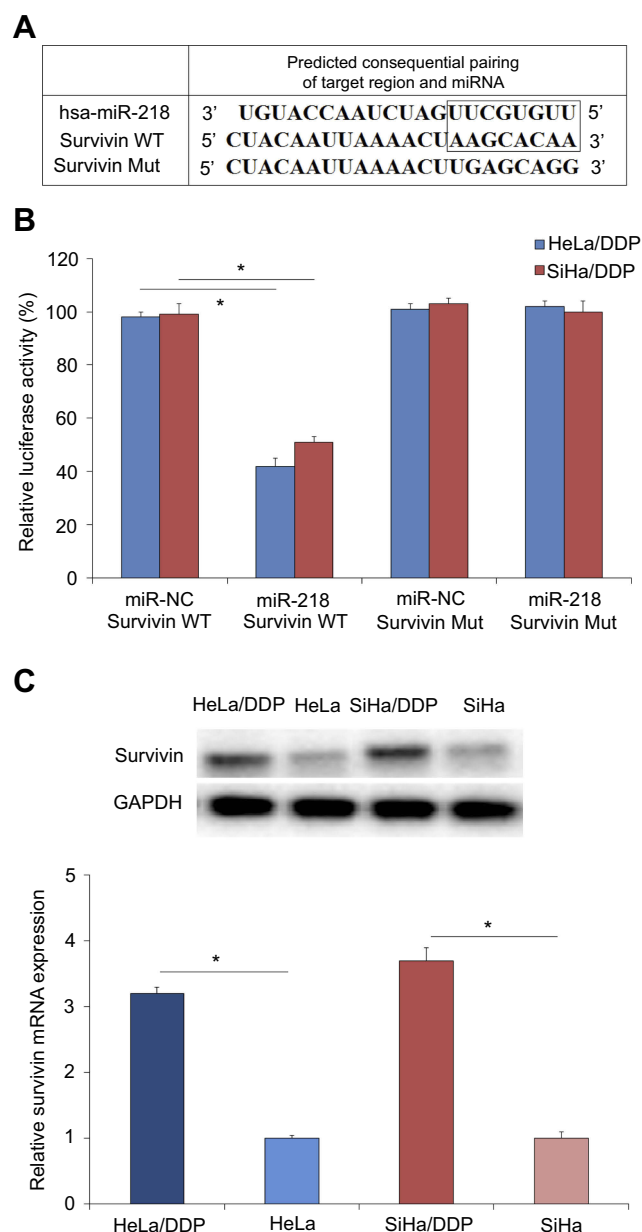


Figure 2 MiR-218 directly targets survivin in CC cells. (A) The wild type and mutant of miR-218 targeting sequences in survivin mRNA. (B) The luciferase activity in the CC cells. (C) The expression of survivin in CC cells. Western and qRT-PCR assays were performed. The data were shown as the mean±SD. * $P<0.05$.

miR-218 modulated cell apoptosis of CC cells

Previous study indicated that survivin expression was associated with cell apoptosis, in the present study we suppose miR-218 regulated the CC cells growth through the cell apoptosis regulation. To confirm our hypothesis we measured the effect of miR-218 on CC cell apoptosis. As shown in Figure 5, upregulation of miR-218 in DDP-resistant CC cells significantly induced the cell apoptosis, while downregulation of miR-218 in mock CC cells

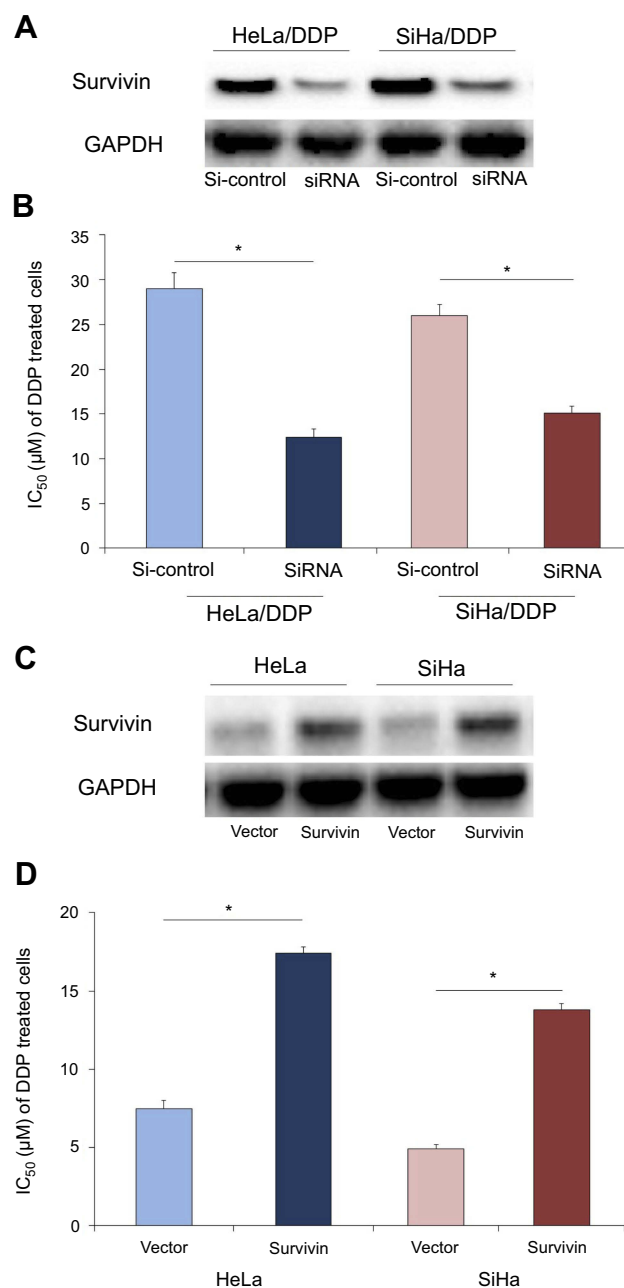


Figure 3 The effect of survivin on the DDP sensitivity in CC cells. (A) The expression of survivin in treated CC cells. (B) Inhibition of survivin enhanced the DDP sensitivity on CC cells. (C) The expression of survivin in treated CC cells. (D) Induction of survivin decreased the DDP sensitivity on CC cells. The data were shown as the mean±SD. * $P<0.05$.

significantly decreased the cell apoptosis. These findings suggested miR-218 modulated DDP-based chemosensitivity of CC cells might attribute to the cell apoptosis regulation.

Discussion

DDP is one of the broadest used anticancer drugs in the treatment of many types of cancers in clinical.³² Chemo-resistance is the major impediment to effective cancer therapy in clinical.

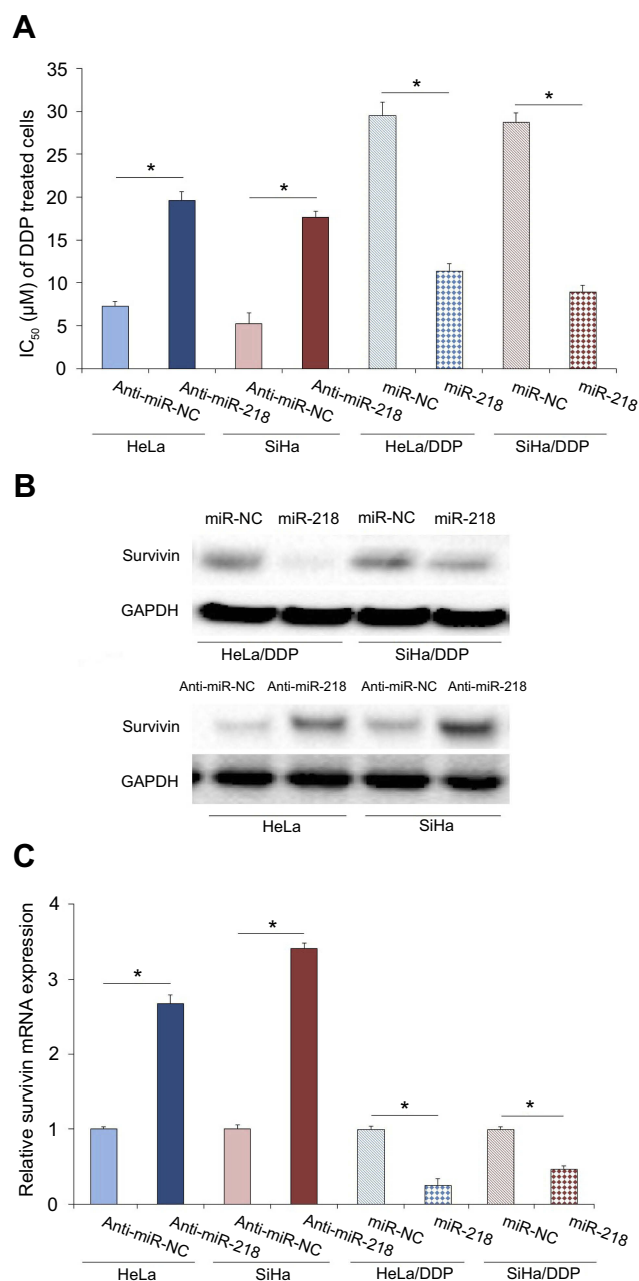


Figure 4 MiR-218 regulates the DDP sensitivity in CC cell by regulation of survivin. (A) The effect of miR-218 on the DDP sensitivity in CC cells. (B) The effect of miR-218 on the expression of survivin in CC cells in protein level. (C) The effect of miR-218 on the expression of survivin in CC cells in mRNA level. The data were shown as the mean±SD. *P<0.05.

It was reported a few genes and pathways were involved in the chemo-resistance of DDP by regulation of cell proliferation, cell apoptosis, drug efflux, and cancer angiogenesis.^{33,34} Besides continuous and multiple DDP treatment also caused lots of side effects,³⁵ therefore enhance the sensitivity of CC cells to DDP provided a new sight for resolving the challenge. However, the mechanism of chemo-resistance in the treatment of CC is still not clearly understood currently.

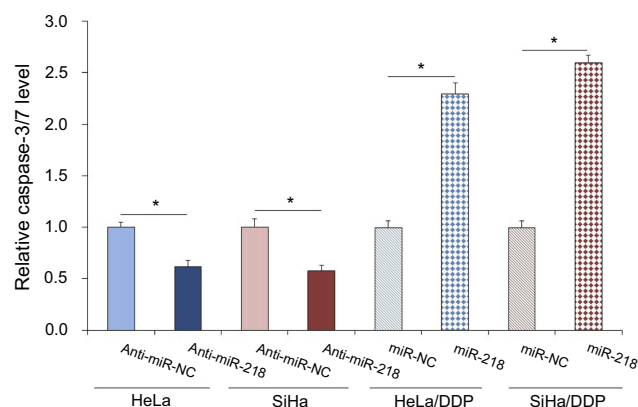


Figure 5 The caspase-3/7 activity CC cells with indicated treatment were measured in the presence of DDP. The data were shown as the mean±SD. *P<0.05.

In 1993, the first miRNA was identified. MiRNAs were noncoding, single-stranded RNAs, and widely present in eukaryotes.³⁶ Previous studies indicated that miRNAs were aberrantly expressed in many types of tumors. They regulated many biological processes, including cell proliferation, cell apoptosis, and stress response.^{37,38} Studies also have documented miRNAs played an important role in chemo-resistance in cancers.^{39,40,41} For example, researchers had reported that miR-21, miR-200b, and miR-15b regulated the chemo-resistance in human cancer cells.^{42–44} MiR-218 is an intronic miRNA, and it was encoded within intronic sequences of tumor suppressor gene SLIT2 and SLIT3.²⁵ It was reported miR-218 was downregulated in various human cancers, including non-small cell lung cancer, oral cancer, and CC.^{22,24,45–50} Studies also indicated miR-218 was associated in the chemo-resistant in cancer cells.^{51–53} However, the biological role of miR-218 in chemo-resistance in CC cells was not fully understood. In order to investigate the role of miR-218 in chemo-resistance in CC cells, we performed various assays in DDP resistant HeLa/DDP, SiHa/DDP and their mock HeLa, SiHa cells. We found miR-218 was downregulated in DDP resistant HeLa/DDP, SiHa/DDP cells compared with their mock cells, and upregulation of miR-218 significantly enhanced the DDP sensitivity of CC cells. By using miR target tools we found survivin is a target gene of miR-218, and dual-luciferase assay results indicated survivin is a direct target gene of miR-218.

Survivin is a member of the apoptosis inhibitor family, it is encoded by Baculoviral inhibitor of apoptosis repeat containing 5.⁵⁴ Survivin was being found dysregulated in many types of human cancers, including breast, lung cancer, prostate cancer, gastric cancer, and

colon cancer.⁵⁵ Previous study indicated survivin exhibited anti-apoptotic capability through the inhibition of caspase-9 activity.⁵⁶ Studies also indicated survivin played important biological roles in chemo-resistance in cancer cells, and survivin would be a biomarker for chemo-resistance in cancer treatment.^{56,57} In the present study, we reported the expression of survivin was significantly upregulated in DDP resistant CC cells, and knock-down of survivin enhanced the DDP sensitivity of CC cells. We also reported upregulation of miR-218 decreased the expression of survivin, while downregulation of miR-218 increased the expression of survivin. As mentioned below, survivin could inhibit apoptosis in cells. We supposed miR-218 regulated the CC cell apoptosis by the regulation of survivin. Our results indicated that upregulation of miR-218 significantly increased the CC cell apoptosis to DDP.

Taken it together, in the present study we reported miR-218 was an important mediator of the DDP chemo-resistance in CC. MiR-218 enhanced the DDP sensitivity by cell proliferation and cell apoptosis regulation, which might through the survivin regulation in CC. These findings suggested that combination treatment of DDP with miRNAs could potentially benefit to the CC treatment in the future. However, we should note that cervical tumorigenesis and chemo-resistance are complicated and complicated processes, and a lot of other factors were involved in the complicated process. Therefore, further studies are needed to investigate the precise mechanisms of DDP chemo-resistance in CC.

Conclusion

Our data suggested microRNA-218 enhanced the DDP sensitivity in CC cells through regulation of cell growth and cell apoptosis, which could potentially benefit to the CC treatment in the future.

Abbreviation list

DDP, cisplatin; CC, cervical cancer; miRNAs, microRNAs; mRNA, messenger RNA; siRNA, small interfering RNA; cDNA, complementary DNA; CCK-8, Cell Counting Kit-8; WT, wild type; Mut, mutant type; BIRC5, Baculoviral inhibitor of apoptosis repeat containing 5.

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Disclosure

The authors report no conflicts of interest in this work.

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