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Original Research Article

Mir-23b down-regulates the expression of target gene of acetaldehyde dehydrogenase 1a1 and increases the sensitivity of cervical cancer stem cells to cisplatin

Yongbing Tao[1], Fuyun Mao[2], Weihong Gu[2], Ling Wu[2], Jing Guo[2], Ping Xu[2]*
[1]Huai'an Second People's Hospital and The Affiliated Huaian Hospital of Xuzhou Medical University, [2]Department of Gynaecology and Obstetrics, Huai'an Maternal and Child Health Care Center, Huai'an 223002, PR China

For correspondence:* ***Email:* *itgq01@163.com*

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Abstract

Purpose: *To study the effect of miR-23b on the expression of the target gene of acetaldehyde dehydrogenase 1A1 (ALDH1A1), and cisplatin (CDDP) susceptibility of cervical carcinoma stem cells.*
Methods: *Human cervical cancer cell line Hela cells were cultured in vitro, and miR-23b mimic and negative control were transfected into the cells using lipofectamine method. The growth of the two groups of cells was determined using growth curve method, and their proliferation measured using plate clone formation. The influence of treatments on the sensitivity of the cells to CDDP was assayed using MTT method. The mRNA expression of ALDH1A1 in Hela cells was assayed using real-time quantitative polymerase hain reation (PCR), while its protein expression was assayed by Western blot.*
Results: *The levels of expressions of ALDH1A1 protein and mRNA in the miR-23b overexpression group were significantly lower than those in the control group ($p < 0.05$). The sensitivities of Hela cells to CDDP in the ALDH1A1 inhibition group and the control group were dose-dependent to some extent, but cell inhibition in ALDH1A1 inhibition group markedly increased, relative to control when the CDDP dose was 0.1 ppc ($p < 0.01$).*
Conclusion: *Up-regulating the expression of miR-23b significantly inhibits the growth and proliferation of cervical cancer cells, and increases their sensitivity to CDDP via down-regulation of the expression of the target gene for ALDH1A1. Therefore, during cervical carcinoma treatment, increasing the level of miR-23b may produce a chemotherapeutic effect.*

Keywords: *MiR-23b, Acetaldehyde dehydrogenase 1A1, Cervical cancer, Cisplatin, Sensitivity*

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INTRODUCTION

Cancer of the cervix (CC) accounts for a majority of malignant gynecological neoplasms. Data show that the incidence of cervical cancer is 11.7 % worldwide, with most cases in developing countries. Recently, cervical cancer incidents have been increasing in the younger Chinese populations [1]. Cervical cancer (CC) patients show no obvious symptoms or signs in the early stage. However, with time, symptoms such as vaginal bleeding and discharge may occur. In the advanced stage, systemic failures such as anemia and cachexia may occur, leading to

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serious threat to women's health [2]. The main predisposing factor for CC is HPV infection.

Chemotherapy is one of the treatment methods for cervical cancer. However, the clinical efficacy of chemotherapy is not very satisfactory, and the 5-year survival of advanced cervical cancer patients is less than 40 % due to resistance to chemotherapy drugs and the poor constitution of patients in advanced stages [3]. Therefore, studies on the mechanism involved in the acquisition of drug resistance by tumor cells have become very popular amongst researchers. MicroRNAs (miRNAs) participate in various biological processes such as growth and development, cell proliferation and apoptosis. In recent years, studies have found that the abnormal expression of miRNA plays an important role in the pathogenesis of tumors [4,5].

Previous studies found that miR-23b is markedly downregulated in CC tissues, relative to healthy cervical tissues, implying that it may play a role as a tumor suppressor gene in cervical cancer [6]. Therefore, this study was aimed at investigating the effect of transfection of miR-23b on CC stem cell susceptibility to CDDP), and unraveling the underlying mechanism.

EXPERIMENTAL

Reagents and equipment

The reagents and instruments used, and their sources (in brackets) were: human cervical cancer cell line Hela cell (Wuhan Yipu Biotechnology Co. Ltd); RPMI1640 culture medium (Wuhan Purity Biotechnology Co. Ltd); fetal bovine serum (Wuhan Punuosai Life Technology Co. Ltd.); Trizol extraction reagent and RIPA lysis solution (Shanghai Yuanye Biotechnology Co. Ltd); cisplatin (Beijing Kairuiji Biotechnology Co. Ltd); Lipofectamine 2000 Reagent (Shanghai Kemin Biotechnology Co. Ltd), and PCR kit (Annolun Biotechnology Co. Ltd). The others were quantitative primer sequence (Shanghai Shenggong Bioengineering Co. Ltd); rabbit anti-human ALDH1A1 monoclonal antibody and GAPDH polyclonal antibody (Shanghai Hengfei Biotechnology Co. Ltd); CO_2 incubator (Guangzhou Haohan Instrument Co. Ltd); inverted microscope (Beijing Jinda Sunshine Technology Co. Ltd); gel imaging analysis system (Shanghai Yihui Biotechnology Co. Ltd); vertical electrophoresis device (Shanghai Yihui Biotechnology Co. Ltd; ultraviolet spectrophotometer (Shanghai Kemin Biological Technology Co. Ltd); desktop centrifuge (Sichuan Shuke Instrument Co. Ltd); low-temperature high-speed centrifuge (Wuhan Yipu Biological Technology Co. Ltd); transfer electrophoresis (Shanghai Yihui Biological Technology Co. Ltd), and Thermal Cycler (Beijing Zeping Technology Co. Ltd).

Cell culture and transfection

Human cervical cancer cells (Hela cells) were put in RPMI1640 medium containing 10 % fetal bovine serum, penicillin (100 IU/mL) and streptomycin (100μg/mL), and were cultured at 37 °C and in a 5 % CO_2 incubator. The culture medium was changed routinely. When the cell fusion reached about 70 %, the cells were subjected to digestion using 0.25 % trypsin. Then, the cells in the logarithmic growth phase were placed in a 96-well plate and cultured in fresh serum-free medium for 12 h. Thereafter, the cells were randomly divided into miR-23b overexpression and control groups. Then, miR-23b mimic (ALDH1A1 mimic) and negative control were transfected into Hela cells using Lipofectamine method, and the relevant tests were performed following culturing for 48 h.

Determination of cell growth

Growth was measured in the two groups of cells using the growth curve method. Cells in logarithmic growth phase were seeded into a 24-well plate at a density of 5000 cells per well. From the second day after seeding, cell counting was done in 3 wells every day (4 times per well), and the mean count was recorded every day for 8 consecutive days. The medium was refreshed every 3rd day. A growth curve was drawn, with culture time as the horizontal axis and the cell number as the vertical axis.

Determination of cell proliferation

Cell proliferation was measured in the two groups with plate clone formation method. Cells at logarithmic growth phase were seeded in a 24-well plate at a density of 80 cells per well, with 3 wells in each group, and the growth medium was changed at 3- to 5-day intervals. After 14 days of culture, by which time the cells had formed clones (cell groups with more than 50 cells/group), 2ml of 10 % paraformaldehyde was added to the fixed cells, and the cells were stained with 2 % crystal violet. The cells were photographed with a digital camera, and the clones in each well were counted and average values were recorded. Cell clone formation was calculated as shown in Eq 1.

CC (%) = (Nccf/Nci)100 …….. (1)

where CC is cell clone formation, Nccf is th number of cell clones formed and Nci is the number of cells inoculated.

Assessment of sensitivity of cells to CDDP

The effect of the various treatments on the sensitivity of the cells to CDPP was determined with MTT method. A stock concentration of CDDP (100 ppc) was prepared in PBS. Cells at logarithmic growth phase were inoculated in a 96-well plate (8000 cells/well) at 37 °C and 5 % CO_2. The stock solution of CDDP was diluted to concentrations of 0.02, 0.1, 0.5, 2.5, 12.5 ppc using complete medium, and the different concentrations of diluted CDDP were added separately to the cells after the cells adhered to the wall. Three replicate wells were set up for each CDPP concentration, as well as a corresponding control group.

Complete cell cultivation medium (100 µl) was added to each well, and the wells were maintained as before for 48 h. Thereafter, MTT assay was used for determination of cell viability. The absorbance of each well was measured in a microplate reader, and cell inhibition (C) was computed as shown in Eq 2.

C (%) = {(Ac – Ae)/Ac - Ab)}100 …….. (2)

where Ac, Ae and Ab are the absorbance of control, experimental and blank groups, respectively.

$$c\,(\%) = \frac{(ODc) - ODe) \times 100}{(ODc) - (ODb)}$$

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from **c**ells in logarithmic growth phase (5 - 10×10^6 cells) using 1 ml of Trizol reagent, and the concentration of RNA was determined using UV spectrophotometer. Then, cDNA was synthesized with RT kit in accordance with the manufacturer's instructions. Then, PCR amplification was done according to the fluorescence quantitative PCR kit instructions. The PCR conditions were: prior-denaturation at 94 °C for 4 min, 94 °C for 30 sec, and annealing at 50 °C for 30 sec, extension at 72 °C for 30 sec, and a total of 45 cycles. The expression level of ALDH1A1 mRNA was calculated with $2^{-\triangle\triangle CT}$ formula.

Assay of ALDH1A1 protein expression

Western blotting was used to assay ALDH1A1 protein expression. Total protein was extracted from Hela cells in logarithmic growth phase using RIPA protein lysing buffer, and BCA kit was used to determine the protein content of the lysate. Then, 50 µg protein from each group was resolved via SDS-PAGE, followed by transfer to PVDF membrane which was blocked with 5 % skimmed milk powder prior to incubation with diluted ALDH1A1 primary antibody overnight at 4°C. This was followed with incubation with secondary antibody for 1h at room temperature. The bands were subjected to chemiluminescence analysis. Imaging scanning analysis system was used to scan, develop, and quantify the protein bands.

Table 1: Primer sequence

Gene		Primer sequence
miR-23b	Forward primer	5'-TGTTAGCTGATGCCGACTTC-3 '
	Reverse primer	5'-TTCTTAGCCCGCTCAACACT-3'
ALDH1A1	Forward primer	5'-TGGTTCGACGCATTAAGGAA-3'
	Reverse primer	5'-TCTCTGCAGCACCCTATCAG-3'
β-Actin	Forward primer	5'-GGCGGAACTCTGAGCAAA-3'
	Reverse primer	5'-ACATCTGCTGGAAGGTGGAC-3'

Statistical analysis

Measurement data are expressed as mean ± standard deviation (SD). The data were statistically analyzed with SPSS21.0 software package. Two-group comparisons were done with *t*-test. Values of $p < 0.05$ were regarded as indicative of statistically significant differences.

RESULTS

Influence of overexpressed miR-23b on the growth and proliferative capacity of Hela cells

The growth of cervical cancer Hela cells in the miR-23b overexpression group was significantly lower than that in the control group ($p < 0.05$), as shown in Figure 1. Results from plate clone formation experiment showed that the number of cervical cancer Hela cell clones in the miR-23b overexpression cells was significantly decreased, relative to control (Table 2).

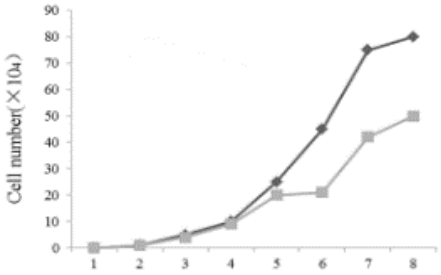

Figure 1: Growth curves of Hela cells in each group. ■ *Control* ◆ *miR-23b over-expression*

Table 2: Number of Hela cell clones

Group	Cell number
miR-23b over-expression	10.23 ± 3.67
Control	35.47 ± 5.88
t	8.920
P-value	< 0.001

Influence of overexpressed miR-23b on the sensitivity of Hela cells to CDDP

Results from the MTT assay showed that with increase in the dose of CDDP, the % inhibition of cells gradually increased. The sensitivity of Hela cells to CDDP in the miR-23b overexpression group and the control group was dose-dependent to a certain extent. The % cell inhibition in the miR-23b overexpression group was markedly raised, relative to control value, when the CDDP doses were 2.5 and 12.5 ppc ($p < 0.01$), and the half inhibitory concentration was as low as 3.68 µg/mL in the control group to 1.9 µg/mL. These results are shown in Figure 2.

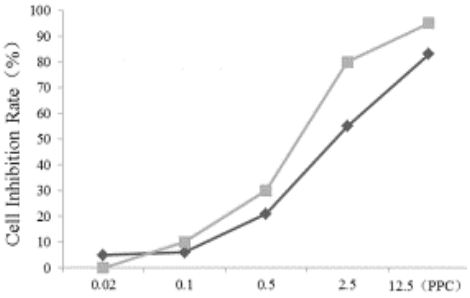

Figure 2: Influence of overexpressed miR-23b on the sensitivity of Hela cells to CDDP. ■ *Control* ◆ *miR-23b over-expression*

Effect of overexpression of miR-23b on the mRNA and protein expressions of ALDH1A1 in Hela cells

The expressions of ALDH1A1 protein and mRNA in the miR-23b overexpression group were markedly decreased, when compared with control values (Figure 3 and Table 3).

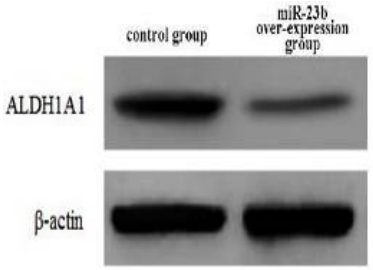

Figure 3: Effect of overexpression of miR-23b on the protein expression of ALDH1A1 in Hela cells

Table 3: Influence of overexpressed miR-23b on mRNA and protein expressions of ALDH1A1 in Hela cells (mean ± SD, n = 6)

Group	ALDH1A1 mRNA	ALDH1A1 protein
miR-23b over-expression	0.41 ± 0.05	0.12 ± 0.03
Control	1.02 ± 0.14	0.30 ± 0.06
t	10.051	6.573
P-value	< 0.001	< 0.001

Effect of down-regulation of ALDH1A1 expression on the sensitivity of Hela cells to CDDP

Results of MTT assay showed that with increase in dose of CDDP, the % cell inhibition gradually increased. The sensitivity of Hela cells to CDDP in the ALDH1A1 inhibition group and the control group was dose-dependent to a certain extent. The % cell inhibition in the ALDH1A1 inhibition group was markedly higher than the control value as from CDDP dose of 0.1 ppc ($p < 0.01$), and the half inhibitory concentration was significantly lower than that of the control group. These results are presented in Figure 4.

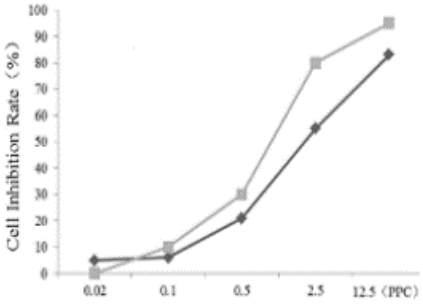

Figure 4: Effect of down-regulation of ALDH1A1 expression on the sensitivity of Hela cells to CDDP. ■ *Control* ◆ *miR-23b over-expression*

DISCUSSION

The incidence of cervical cancer ranks first place among the malignant tumors of female genital organs in China. In recent years, with advancements in medical science and technology, and improved screening of the disease, its incidence has been reduced to some extent, but its incidence is showing an increasing trend in younger populations. Young patients have higher requirements for good quality of life after treatment. Thus, the clinical treatment mode for cervical cancer needs further research and transformation [7]. In terms of incidence, cervical cancer is a complex process abnormally regulated by multiple steps and multiple genes.

Cervical cancer gradually develops from normal cervical epithelium to cervical intraepithelial neoplasia, and subsequently to invasive cervical cancer. The specific pathogenesis of cervical cancer is currently unclear. The clinical treatments for cervical cancer involve a combination of surgery, radiotherapy and chemotherapy. Chemotherapy drugs are essential for patients in advanced stages of cervical cancer. In particular, platinum-based chemotherapy drugs play an important role in improving the prognosis of patients with advanced cervical cancer [8,9].

Cancer stem cells exhibit primary drug resistance to chemotherapeutic drugs, leading to serious consequences on treatment efficacy. Previous studies have found that abnormal miRNA expression is closely related to exacerbation of cervical cancer and drug resistance [10]. Thus, clinical treatment efficacy and prognosis of patients may be improved by studying variations in miRNA levels in cancerous tissues and their relationship with cervical cancer.

MicroRNAs (miRNAs), endogenous non-coding single-stranded small RNAs with length of about 22 nucleotides, are important post-transcriptional regulators in organisms. They regulate the expression levels of target genes post-transcriptionally, and participate in regulation of almost all disease processes. They are highly conserved and tissue-specific, and they are closely related to human diseases such as tumors [11]. The miRNAs regulate mRNA and key proteins involved in cellular processes such as differentiation and proliferation, and are also involved as oncogenes in a variety of cancers [12].

In recent years, research has shown that the serum expression profile of circulating miRNA in cervical cancer patients is markedly different from that in normal healthy people, and it is related to the invasion of cervical cancer, in addition to its potential use as a new non-invasive marker for early screening and diagnosis of cervical cancer [13]. It is known that miR-23b is located on chromosome 9. Studies have demonstrated abnormal expression of miR-23b in pancreatic cancer, liver cancer, prostate cancer and other malignant tumors, and its expression may be high or low in different tumors [14].

The results obtained in this study demonstrate that the growth and the number of clones of cervical cancer Hela cells in the miR-23b overexpression group were significantly lower than those of the control group, suggesting that overexpression of miR-23b inhibits the growth and proliferation of the tumor cells. The results of MTT assay showed that with increase in CDDP dose, cell inhibition of the miR-23b overexpression group was significantly higher than that of the control group when the CDDP doses were 2.5 and 12.5 ppc, suggesting that miR-23b overexpression partially reverses cell resistance to CDDP.

A study has shown that ALDH1A1 has significant enhancing effect on the metabolism of chemotherapeutic drugs such as CDDP and cyclophosphamide, thereby leading to chemotherapy resistance of tumor cells [15]. The results of this study showed that the expressions of ALDH1A1 mRNA and protein in cervical cancer Hela cells in the miR-23b overexpression group were significantly lower than those in the control group. The results of the MTT test showed that the percentage cell inhibition in ALDH1A1 inhibition group was significantly higher than that of the control group when CDDP dose was at least 0.10 ppc. Therefore, it may be speculated that downregulation of miR-23b in cervical carcinoma increases the expression of ALDH1A1 gene, thereby promoting cell resistance to chemotherapy drugs.

CONCLUSION

The findings of this study have shown that miR-23b is lowly expressed in cervical carcinoma. Up-regulation of the expression of miR-23b significantly inhibits the growth and proliferation of cervical cancer cells and increases their sensitivity to CDDP via a mechanism related to down-regulation of the expression of the target gene ALDH1A1. Therefore, increasing the level of miR-23b may contribute to the chemotherapeutic management of cervical cancer.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

This study was done by the authors named in this article, and the authors accept all liabilities resulting from claims which relate to this article and its contents. The study was conceived and designed by Ping Xu; Yongbing Tao, Fuyun Mao, Weihong Gu, Ling Wu, Jing Guo, Ping Xu collected and analyzed the data; Yongbing Tao wrote the manuscript. All authors read and approved the manuscript for publication.

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