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Original Article

MicroRNA-146a improves sensitivity of cervical cancer cells to cisplatin by inhibiting proliferation

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Abstract: *Objective:* MicroRNA-146a has been proven to have a role in regulating many physiological processes in malignant cancers. In this study, we aimed to identify the biological function of microRNA-146a and its auxiliary sensitive effect on cisplatin treated cervical cells and its potential mechanism. *Methods:* Expression of miR-146a and EGFR was detected in 30 cervical carcinoma and normal cervix tissues by real-time PCR. The role of miR-146a in regulating cervical cell proliferative ability was identified by CCK8 assay. Luciferase assay and RT-PCR were then used to determine the regulatory activity of miR-146a target to gene EGFR. After Hela cells were transfected with miR-146a mimics or EGFR overexpression plasmid and treated with cisplatin, CCK8 assay was used to detect the viability of cervical cells. *Results:* The miR-146a expression level was reduced in cervical carcinoma tissues compared with normal cervix tissues. EGFR was the target gene of miR-146a and its expression level was increased in cervical carcinoma tissues. The cell viability assay showed that combined treatment of miR-146a and cisplatin decreased the viability of cervical cancer cells compared with the cisplatin group. The IC_{50} value of cisplatin changed from 9.574 μM/L to 7.042 μM/L. Finally, overexpression of EGFR in cisplatin stimulated Hela cells reduced viability. *Conclusions:* miR-146a could regulate the proliferative ability of cervical cancer cells by targeting EGFR. Furthermore, the sensitizing effect of miR-146a to cisplatin treatment was caused by decreased proliferative ability of Hela cells. The combination of miR-146a and cisplatin might be used in clinical cervical cancer treatment.

Keywords: Cervical cancer, MiR-146a, cisplatin, proliferation

Introduction

Cervical carcinoma is a common gynecological malignancy with a high incidence and mortality rate [1]. China remains one of the world's highest incidence of cervical cancer. Although HPV infection has been identified as the main cause of cervical cancer, due to the lack of effective clinical early diagnosis and tumor molecular markers, most cervical cancer patients are diagnosed at a late stage, and this therefore leads to poor prognosis [2]. For the treatment of cervical cancer, the main clinical treatment is surgery supplemented with radiotherapy and chemotherapy [3]. To improve the efficacy of radiotherapy and chemotherapy, development of new drugs and improvement of the sensitivity of tumor cells to drugs and radiation are the main methods.

MicroRNA (miRNA) plays an important role in the carcinogenesis, invasion, metastasis, and recurrence of cervical cancer, and is closely related to its prognosis [4, 5]. It can be stable in tissue, serum, and plasma samples, which can promote the apoptosis of cervical cancer cells by regulating target genes and change the sensitivity of cervical cancer cells to drugs and irradiation, which makes miRNA expected to be a potential target for cervical cancer [6, 7]. Previous research has reported many examples of abnormal expression of miRNAs in cervical cancer which promoted the sensitivity of chemotherapy [8, 9]. It was found that the increase

of miR-224 in SiHa cells significantly increased increases cell sensitivity to paclitaxel, and the IC_{50} value was significantly decreased than control group [10]. Huang et al. [11] reported that upregulation of miR-15a and miR-16 significantly enhances the autophagy and apoptosis of HeLa cells induced by camptothecin. The mechanism was shown to be due to overexpression of miR-15a/16 inhibiting the proliferative ability of cervical cancer cells by enhancing autophagy and enhancing the effect of CPT chemotherapy. Wang et al. [12] found that upregulation of miR-218 reduced radiation resistance of cervical cancer cells in both *in vivo* and *in vitro* experiments. It has also been suggested that miR-218 can be used as a predictor of radiotherapy sensitivity of cervical cancers and can be used as a new target for combined treatment of cervical cancer. miR-146a is one of those abnormal expressed miRNAs in cervical cancer. In this report, decreased expression of miR-146a was associated with high-risk human papillomavirus infection in penile squamous cell carcinoma [13]. However, the function of miR-146a in cervical cancer treatment has not been reported.

Cisplatin, as a first-line chemotherapy for the treatment of solid cancer, is used in clinical treatment of ovarian, cervical, bladder, head and neck, and lung cancers [14-16]. However, the role of cisplatin in the cervical cancer treatment at the same time will cause serious side effects such as myelosuppression, granulocytopenia, thrombocytopenia, nephrotoxicity, and neurotoxic anemia. In view of the above blood system toxicity and acquired drug resistance which limit the cisplatin based chemotherapy in cervical cancer treatment. Therefore improving the low dose of cisplatin chemotherapy is one of the most effective methods of clinical treatment of cervical cancer.

In this report, we focused on the decreased expression of miR-146a in cervical carcinoma tissues and its potential mechanism. We found miR-146a could regulate the proliferative ability of cervical cancer cells by targeting EGFR. Further studies have shown that the sensitizing effect of miR-146a to cisplatin treatment was caused by the decreased proliferative ability of Hela cells. Lastly, the sensitizing effect of miR-146a was caused by the decreased protein level of EGFR.

Materials and methods

Clinical samples

Thirty cervical cancer tissues and normal cervix tissues used in this research were collected from patients for surgery or cervical cancer diagnosis. Normal cervix tissues were from tissues collected by uterectomy because of hysteromyoma. Each surgical specimen of normal cervix tissues and cervical cancer tumor tissues was instantly frozen in liquid nitrogen until use. All the procedures involved in these patients were performed according to the standard procedure of the Ethics Committee. The Ethics Committee of the People's Hospital of SND approved this study. The analysis was performed in accordance with the ethical standards of the hospital and the tenets of the Declaration of Helsinki/Declaration of Istanbul. The patient reported in this study provided written consent.

Cell line and cell culture

Hela cells (human cervical cancer cell line) were purchased from American Type Culture Collection. Hela cells were maintained in a Dulbecco's Modified Eagle's Media supplemented with 10% fetal bovine serum. All cultured cells were maintained at 37°C in a humidified 5% CO_2 incubator.

RNA isolation and real-time PCR

After Hela cells were stimulated with cisplatin or transfected with miR-146a inhibitor and mimics, cells were collected by centrifugation, the supernatant was discarded and extracted RNA with Trizol reagent. Extraction procedures were carried out according to the operating instructions. All the tissues were grinded for RNA isolation. The protocol was the same as the cultured cells. RNA quantification and purity was confirmed by UV spectrophotometer and agarose gel electrophoresis. According to the manufacturer's instructions of ABI reverse transcription kit, 1 μg of total RNA was taken for reverse transcription. The reverse transcription reaction program was as follows: 25°C, 10 min; 37°C, 120 min and 85°C, 5 min. Real-time PCR amplification conditions were processed according to Roche transcription instructions. The primer sequence of EGFR was: (Forward) AGG-

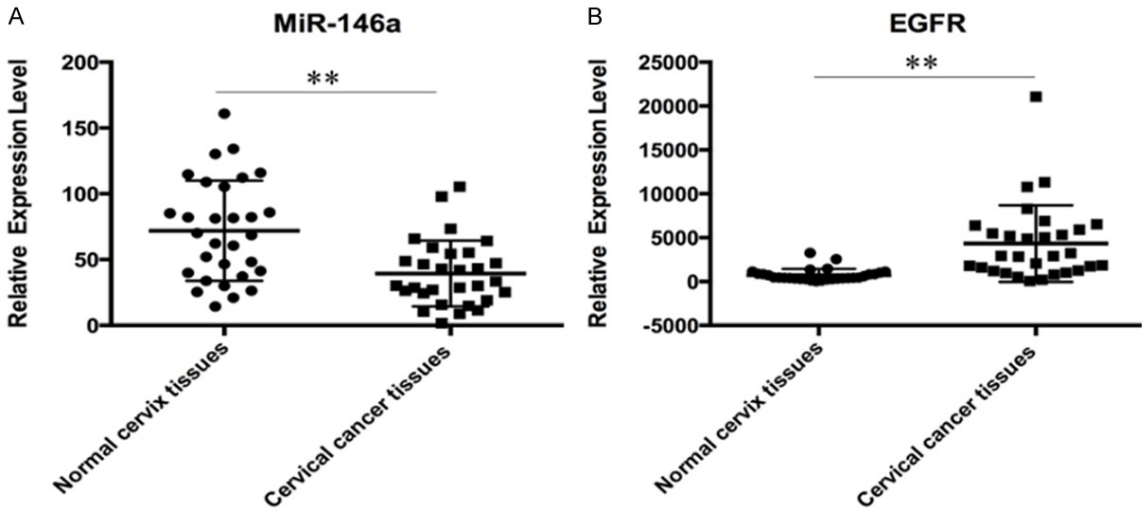

Figure 1. Expression level of miR146a in cervical cancer and normal cervix tissues. The 30 cervical cancer tissues and 30 normal cervix tissues were collected for RNA isolation. A. Expression level of miR-146a was analyzed in cervical cancer tissues compared with normal cervix tissues. B. Expression level of EGFR was analyzed in cervical cancer tissues compared with normal cervix tissues. (**P<0.01).

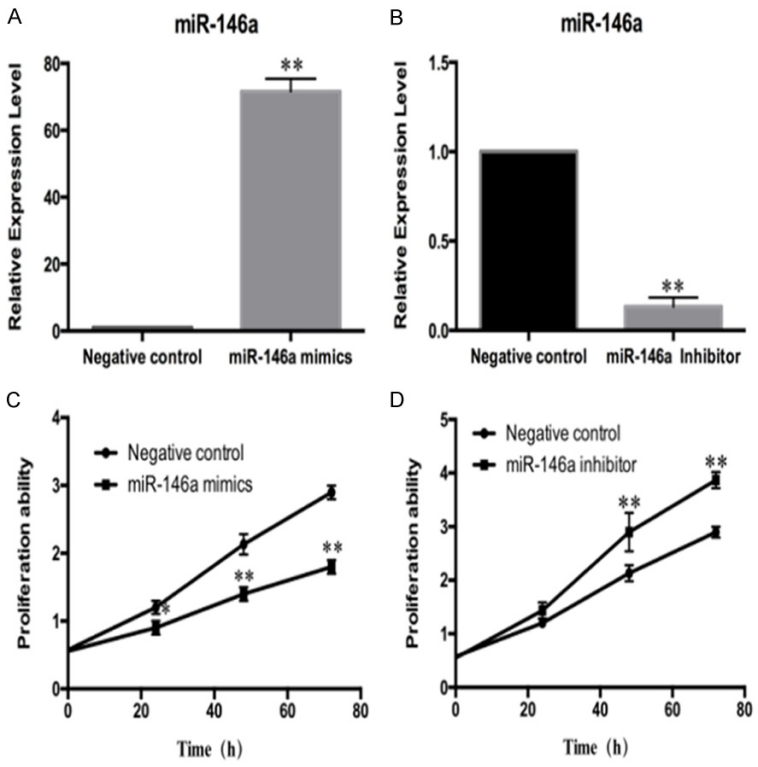

Figure 2. miR-146a inhibits the proliferation ability of Hela cells. Hela cells were transfected with miR-146a mimics and inhibitor, expression level of miR-146a was analyzed by real-time PCR. A. The effect of miR-146a mimics was evaluated by real-time PCR. B. The proliferative ability of miR-146a mimics transfected Hela cells were analyzed by CCK-8 assay. C. The effect of miR-146a inhibitor was evaluated by real-time PCR. D. The proliferative ability of miR-146a inhibitor transfected Hela cells were analyzed by CCK-8 assay. (**P<0.01).

CACGAGTAACAAGCTCAC; (Reverse) ATGAGGACATAACCAGCCACC).

Western blot assay

After Hela cells were transfected with EGFR overexpression plasmid, cells were collected by centrifugation, the supernatant was discarded. Cell pellets was suspended in cell lysis buffer containing protease inhibitors. The supernatant of cell suspension was separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels after boiled for 5 min in SDS-loading buffer. Subsequent transfer of the protein from the gel to PVDF membranes was performed. In order to block nonspecific protein-protein interactions, PVDF membranes were blocked in TBST buffer containing 5% non-fat milk at room temperature. After diluting the antibodies (EGFR, 1:4000; β-Actin, 1:5000) in TBST buffer, the PVDF membranes was gently shaken in the diluted antibodies overnight at 4°C. The next

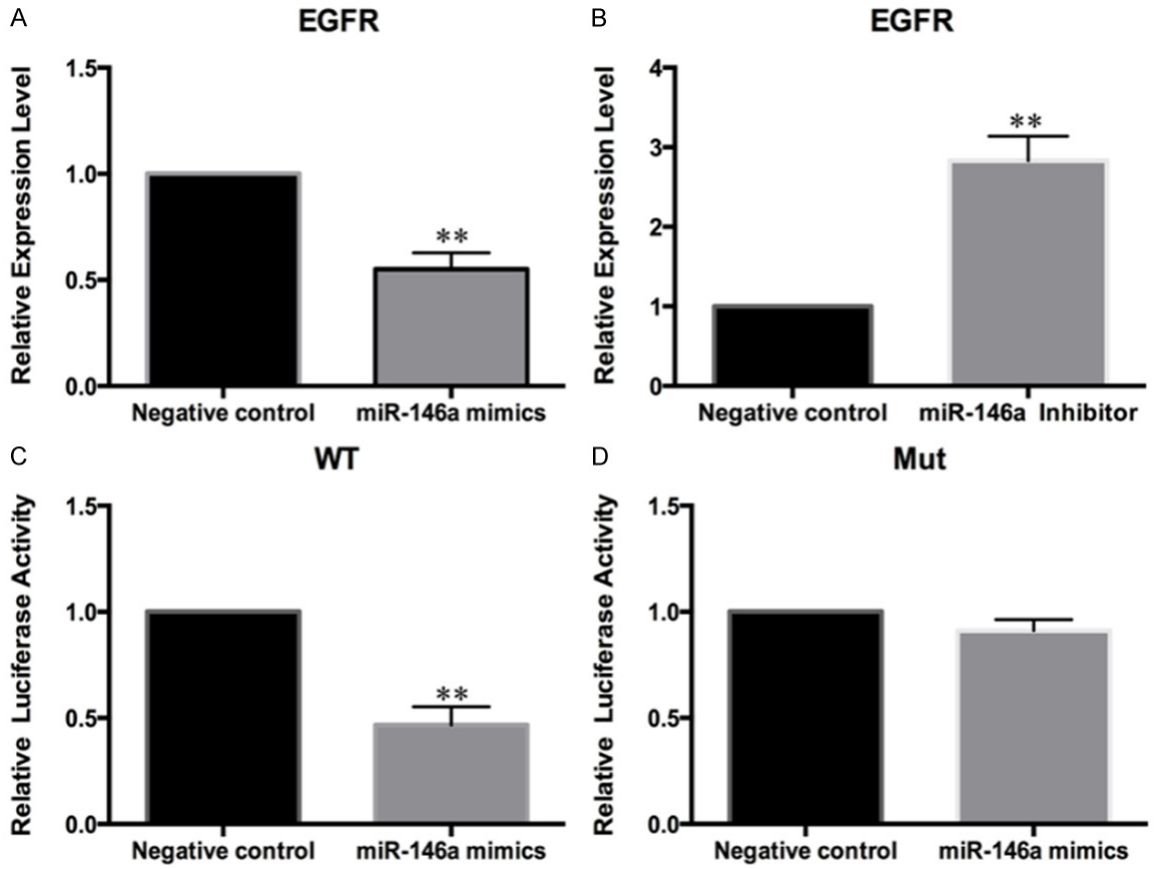

Figure 3. EGFR is a target gene of miR-146a. Hela cells were transfected with miR-146a mimics and inhibitor; the expression level of EGFR was analyzed by real-time PCR. A. The expression level of EGFR was decreased in miR-146a mimics transfected cells compared with control. B. The expression level of EGFR was increased in miR-146a inhibitor transfected cells compared with control. C. Luciferase plasmid of wild-type PGL3/EGFR-3'UTR 3'UTR was transfected with miR-146a. The luciferase activity was determined 24 hours after transfection. D. Luciferase plasmid of mutant PGL3/EGFR-3'UTR 3'UTR was transfected with miR-146a. The luciferase activity was determined 24 hours after transfection. (**P<0.01).

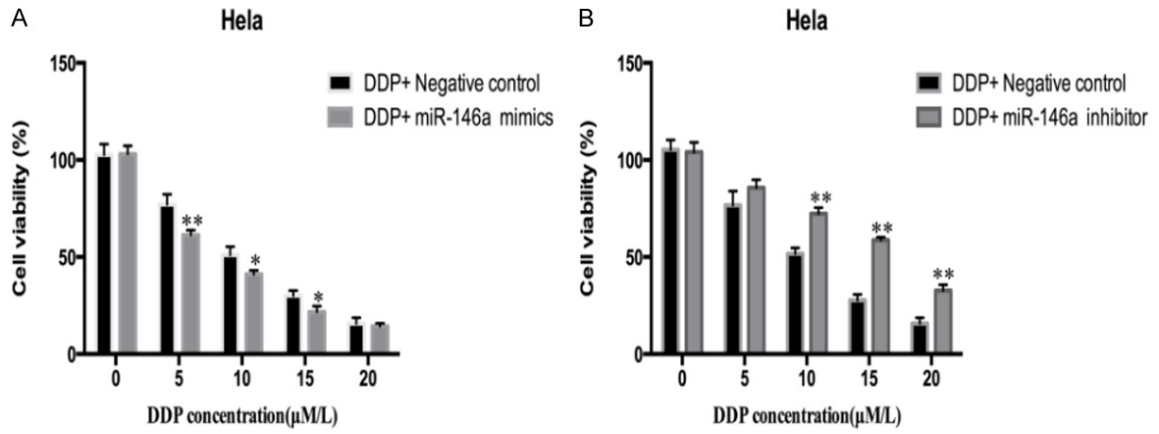

Figure 4. MicroRNA-146a increases sensitivity of cervical cells to cisplatin. A. Hela cells were transfected with miR-146a mimics and negative control before treated with DDP from the concentration of 5 μM/L to 20 μM/L, 48 h after stimulation cells were harvested proliferation detection. The proliferation of Hela cells was evaluated by CCK-8 assay. B. Hela cells were transfected with miR-146a inhibitor and negative control before treated with DDP from the concentration of 5 μM/L to 20 μM/L, 48 h after stimulation cells were harvested proliferation detection. The cell proliferation of Hela cells was evaluated by CCK-8 assay. (*P<0.05, **P<0.01).

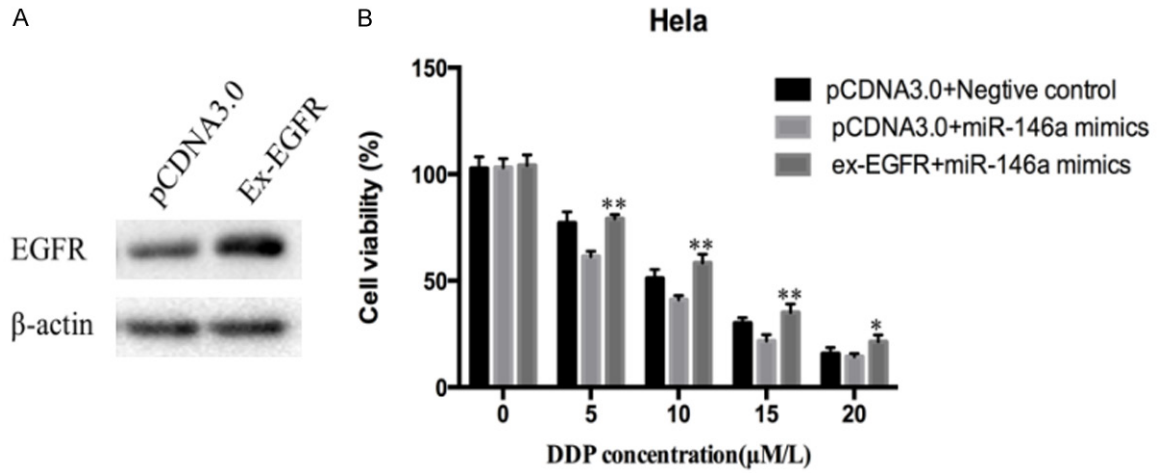

Figure 5. The reversion effect of overexpression EGFR to cisplatin treatment in microRNA-146a transfected Hela cells. A. Hela cells were transfected with EGFR overexpression plasmid and pCDNA3.0 plasmid. The overexpression effect of EGFR in Hela cells were confirmed by Western blot. B. Hela cells were divided in three groups according to the different transfection plasmid (pCDNA3.0 + Negative control, pCDNA3.0 + miR-146a mimics and ex-EGFR + miR-146a mimics), then all the cells were treated with cisplatin from the concentration of 5 μM/L to 20 μM/L, and 48h after stimulation the cells were harvested for proliferation evaluated by CCK-8 assay. (*P<0.05, **P<0.01).

day washed PVDF membranes with TBST buffer, then incubated with peroxidase-conjugated individual secondary antibodies shaking for 1 hour. Eventually, prepare ECL solution in the dark room, the exposure time was determined according to the fluorescence intensity.

Cell proliferation and viability

Hela cells were suspended in complete medium to adjust cell concentration to 5×10^6 cells/mL, and cells were grown in 96-well cell culture plates for 100 μL each well. After treating with cisplatin, all the cells were harvested in medium 24 h and 48 h after normal culture for proliferation detection. Cell proliferative ability was detected by CCK8 assay kit according to the manufacturer's steps. Absorbance was detected using a microplate reader at the wavelength of 450 nm.

Luciferase reporter assay

All Hela cells were plated at 1×10^5 cells per well in 24-well plate and transfected 24 hours later using Lipofectamine 2000. Each transfection contained 0.8 μg of the vector DNA and 0.8 ng pRL-SV40 plasmids. The pGL3-promoter vector was used as negative control. Two days after transfection, luciferase assays were performed in accordance to the manufacturer's instructions and independent triplicate experiments were done for each plasmid construct.

Statistical analysis

Each experiment in this article was repeated at least three times. Results are shown as the mean value ± standard deviation (SD). Additionally, statistical analysis was carried out using Student's t-test. And P value less than 0.01 or 0.05 is thought to be with significance.

Results

MicroRNA-146a expression in cervical cancer and normal cervix tissues

Thirty cervical carcinoma tissues and 30 normal cervix tissues were collected and isolated for obtaining RNA. According to the real-time PCR results, the relative expression level of miR-146a was lower in cervical carcinoma tissues than in normal cervix tissues. miR-146a was down regulated in 73.3% (22/30) cervical carcinoma tissues compared with normal cervix tissues. In contrast, the expression level of EGFR was significantly increased in cervical carcinoma tissues. All result are shown in **Figure 1**.

MicroRNA-146a inhibits the proliferative ability of cervical cells

In order to evaluate the effect of miR-146a on cervical cancer cell's proliferative ability, we transfected miR-146a mimics in Hela cells, 24

h, 48 h, and 72 h after transfection the CCK-8 results showed that, the cell proliferative ability of the miR-146a mimics group was significantly decreased compared with negative controls (**Figure 2**). The IC_{50} value of cisplatin decreased from 9.574 μM/L to 7.042 μM/L. Then we transfected miR-146a inhibitor in Hela cells, the CCK-8 results showed that, compared with controls, the cell viability of miR-146a inhibitor group was significantly increased. The IC_{50} value of cisplatin increased from 9.479 μM/L to 15.735 μM/L.

MicroRNA-146a regulates the expression level of EGFR

To investigate the mechanisms by which miR-146a influences cervical cancer cell proliferative, we searched for putative protein coding gene targets of miR-146a. According to a previous study, EGFR was one of the target genes of miR-146a. EGFR was also an important gene in regulating proliferation signaling which was activated by its ligand EGF. In order to evaluate whether EGFR was regulated by miR-146a, we transfected Hela cells with miR-146a mimics and inhibitor and EGFR expression level was detected by Q-PCR. EGFR expression level changed contrary to miR-146a level (**Figure 3**). Furthermore, we constructed luciferase reporter plasmid of the 3'-UTR region of EGFR named PGL3/EGFR-3'UTR and a mutant plasmid of PGL3/EGFR-3'UTR. As shown in **Figure 3**, miR-146a mimics significantly suppressed the luciferase activity of PGL3/EGFR-3'UTR, but had no effect on PGL3/EGFR-3'UTR mutant plasmid. These results suggest that EGFR is a target gene of miR-146a in Hela cells.

MicroRNA-146a increases the sensitivity of cervical cells to cisplatin

Cisplatin was one the most common chemotherapy drugs in cancer therapy. In order to find the appropriate concentration of cisplatin to Hela cells, a series concentration of cisplatin was tested. Next, we transfected Hela cells with miR-146a mimics then treated cervical cells with cisplatin. 48 h after cisplatin treatment, CCK-8 results were applied to evaluate the cell proliferation and viability of Hela cells. Results showed that Hela cells viability was repressed by cisplatin treatment with dosage dependent effect (**Figure 4A**). Furthermore, cisplatin-induced repression of cell viability was enhanced by miR-146a overexpression while it was attenuated by miR-146a knock down (**Figure 4B**).

MicroRNA-146a increases the sensitivity of cervical cells to cisplatin by targeting EGFR

We validated that miR-146a overexpression could increase the sensitivity of Hela cells to cisplatin treatment. Next, we investigated the role of miR-146a in regulating Hela cells chemo-sensitivity to cisplatin. We transfected Hela cells with miR-146 mimics and EGFR overexpression plasmid, then treated cells with cisplatin. The IC_{50} value of control group, miR-146 mimics, and miR-146 mimics combined ex-EGFR group were 9.573 μM/L, 7.045 μM/L, 10.820 μM/L, respectively. The results showed that EGFR overexpression reversed the decreased cell viability caused by miR-146 mimics (**Figure 5**).

Discussion

Improve the sensitivity of chemotherapy drugs in cancer treatment was an effective way to decrease side effect of chemotherapy drugs. Studies have demonstrated microRNAs are associated with carcinogenesis and cancer progression. Moreover, the roles of deregulated microRNAs in the improvement of chemotherapy sensitivity have attracted increase interest in recent years. In this study, we found that microRNA-146a could improve sensitivity of cervical cells to cisplatin by inhibiting proliferation. The beneficial effect of miR-146a in cervical cancer treatment was achieved by regulating target gene EGFR expression which might be used as a novel strategy in cervical cancer treatment.

Accumulating evidence has confirmed that non-coding RNAs could regulate the sensitivity of cancer cells to chemotherapy. For example, an lncRNA named UCA1 could enhance 5-fluorouracil resistance by inhibiting the expression of miR-204-5p in colorectal cancer [17]. In another report, miR-218 was significantly increased in cisplatin-resistant oral cancer cells and promote cisplatin resistance via the PPP2R5A/Wnt signaling pathway [18]. High expression of EGFR increases the proliferative ability of cancer cells [19, 20], and also could indicate prognosis of non-small lung cancer. In our results, miR-146a was decreased in cervical cancer tis-

sues. Furthermore, we confirmed EGFR was the target gene of miR-146a in cervical cancer cells. Therefore, we speculated that high expression of EGFR was associated with the development of cervical cancer. In the following experiments, overexpression of EGFR in cisplatin stimulated cervical cancer cells, the sensitization effect of miR-146a was repressed. These result suggested that EGFR overexpression is a risk factor for cervical cancer cisplatin resistance.

The previous reports have found miR-146a participated in many biological processes such as differentiation, proliferation, and apoptosis [21-23]. The expression level of miR-146a was significantly decreased in cervical cancer tissues and gastric cancer tissues compared with adjacent normal tissues which suggested its potential role in tumor suppression [24, 25]. The biological function of miR-146a was achieved by regulating target genes. In the present study, miR-146a regulated the proliferation ability of cervical cancer cells by targeting EGFR. EGFR has been proved as the target gene of miR-146a in both lung cancer cells, pancreatic cancer cells, and bronchial smooth muscle cells [26-28]. According to a previous report, miR-146a has many target genes, such as Fbxl10, which was regulated by miR-146a in ischemic stroke [29]. Furthermore, the chemotherapeutic sensitivity and prognosis of advanced gastric cancer affected by miR-146a through regulating the expression of LIN52 [24]. Therefore, there might another signaling which involved in the proliferation regulated by miR-146a.

Cisplatin is a first-line chemotherapy for the treatment of cervical cancer. However, the emergence of resistance has limited the clinical use of chemical drugs. In this study, miR-146a could increase the sensitivity of cervical cancer cells to cisplatin. According to this result, reducing the dosage of chemical drugs means slowing down the occurrence of drug resistance. From now on, miRNAs affect the sensitivity of chemotherapy not only have been proved in cell and animal experiments, but some small sample clinical trials have investigated the effect of changes in miRNA expression on chemo sensitivity in patients with cancer. So finding differentially expressed miRNAs is a most critical precondition.

In conclusion, we found that miR-146a could increase the sensitivity of cervical cancers to cisplatin and the potential mechanism is miR-146a could regulate the proliferation ability of cervical cancer cells by targeting EGFR. The decreased expression level of miR-146a in cervical cancer tissues might be the reason of EGFR overexpression in cervical cancer. The combined effect of miR-146a and cisplatin to cervical cancer cells is a potential novel strategy in clinical cervical cancer treatment.

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Disclosure of conflict of interest

None.

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