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Original Article

Regulation of miR-181 on chemotherapy-resistant cervical cancer cells

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Abstract: MiR-181 was highly expressed in lung cancer cells of cisplatin-resistance. In this study, we sought to explore the connection of miR-181 expression with cell proliferation, invasion and apoptosis in cervical cancer cells. Real-time polymerase chain reaction and Western blot were performed to determine the expression level of miR-181 at mRNA and protein level. Then MTT and flow cytometry were respectively used to explore the proliferation, apoptosis in cervical cancer cells, lactic dehydrogenase assay was performed to test the cytotoxicity. The results suggested that overexpression of miR-181 significantly increased the proliferation and decreased apoptosis of cytotoxicity cervical cancer cells compared with the control group. Moreover, LDH activity of miR-181-overexpressed cells was significantly decreased, suggesting reduction of the cytotoxicity and the increase of drugs-resistance. These results proved that miR-181 could be tightly involved in protection of cervical cancer. This study revealed the key roles of miR-181 and its potential in treating cervical cancer.

Keywords: MiR-181, cervical cancer, cell apoptosis, cell proliferation, cytotoxicity Introduction

Introduction

Cervical cancer (CC), is the third most common cancer in developed countries but is the second leading cause of cancer morbidity and mortality for women around the world, despite the existence of effective screening methods [1-3]. It is the term for a malignant neoplasm arising from the cervix due to the uncontrolled growth of cells that have the capability to attack or spread to other parts of the body [4, 5]. Commonly, cisplatin, 5-FU and other chemotherapy drugs are the main treatments for advanced cervical cancer. However, growing resistance often cause the failure of chemotherapy, and the lifetime shortening and life quality falling of the patient survival.

In recent years, studies on molecular biology mechanism of tumor cells to chemotherapy resistance have become a new hot spot. MicroRNAs are noncoding RNA molecules of 18-25 nucleotides in length and function at the transcriptional or post-transcriptional level in diversity of biological processes by targeting the 3'-UTR of mRNAs [6]. Recent data revealed that microRNA played key roles in the resistance of tumor cell [7-11]. For example, Li G, et al., suggested that miR-101 inhibited the proliferation and promoted DDP-induced apoptosis of DDP-resistant gastric cancer cells [12]. Wang T found that hsa-miR-503 regulates cisplatin resistance of human gastric cancer cells partly by targeting IGF1R and BCL2 [13]. Zhuang M, et al., has investigated the possible role of miR-143 in the development of cisplatin resistance in human gastric cancer cell line and proved that hsa-miR-143 could modulate cisplatin resistance of human gastric cancer cell line at least in part by targeting IGF1R and BCL2 [14]. All these studies have illustrated that the drug resistance of cancer cells can be modulated by abnormal expression of microRNAs.

Cisplatin is a most active drug for the treatment of different cancers, however, acquired cisplatin resistance is easily seen in cancer patients, such as ovarian cancer, cervical cancer and lung cancer and so on [15-17]. Cisplatin resistance in cancer cells is due to a pleiotropic phenotype transition allowing cells to resist cell death and is recognized as a major obstacle to cancer therapy [17]. MiR-181, was found to exist overexpressed in cisplatin-resistant lung

cancer lines [18]. However, the role of miR-181 on the chemotherapy drug resistance in cervical cancers remains unclear.

Keeping in view this fact, the objective of the current study was to preliminarily investigate the biological function and its possible mechanism of miR-181 of resistance to chemotherapy in cervical cancer HeLa cell line. MiR-181 expression was detected by quantitative real-time PCR. Cisplatin resistance changes of cells were tested via MTT assay. Western blot, Lactic dehydrogenase assay, flow cytometry, cell proliferation, and apoptosis assay were used to elucidate the mechanism of miR-181 in cisplatin resistance formation. To further investigate the mechanism by which the combination of regulatory protein cyclin and apoptosis protein caspase-3 with cisplatin resistance of HeLa, western blot was constructed to monitor the protein expression and expression changes of cyclin and caspase-3 were proved to be infected by the cisplatin treatment.

The present study also provides a new understanding of the cisplatin resistance mechanism of cervical cancer and proved miR-181 could modulate cisplatin resistance of human cervical cancer cell line. Above on, targeting miR-181 may provide a novel strategy for the treatment of cervical cancer.

Materials and methods

Cell lines and transfection

Human cervical cancer HeLa cell line was cultured in Dulbecco Modified Eagle Medium supplemented with 10% Fetal Bovine Serum and 1% penicillin/streptomycin (Vega Pharma Limited, Zhejiang, China) at 37°C in a humidified incubator containing 5% CO_2. HeLa cells were selected and maintained in DMEM complete medium containing 1 mg/mL G418 (Invitrogen) after transfection with pCR-miR-181 or pCR-miR-NC (control). After 2-4 weeks selection, the remaining cells were stably overexpressed with miR-181 or miR-NC [1].

Cisplatin treatment

Cisplatin (Sigma-Aldrich, Johannesburg, South Africa) was prepared before each treatment period by dissolving the powder in 0.9% NaCl solution to obtain a 0.001 M stock solution. It was then added to sub-confluent cells to reach final working concentration of 15 μM and incubated for a period of 24 h. Cisplatin dose-response curves and non-toxic validation of the above mentioned working concentration is described in previous work [19].

MTT assay

The cell survival rate was determined by a standard spectrophotometric 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells in different groups were incubated for 4 h with the MTT solution (5 mg/mL) and the supernatant was discarded. The precipitate was dissolved in 200 mL of dimethylsulfoxide for 10 min and the absorbance values at 490 nm (A490 nm) were measured. The cell survival ratio of each group was calculated as follows: survival ratio-A490 nm of experiment group/A490 nm of control group [20].

Lactic dehydrogenase

Leakage of enzymes such as LDH into the culture medium is a well-known indicator of damage or injury to the cell membrane. Briefly, 1×10^5 cells/well of HeLa cells was transferred to 96-well plates. The plates were incubated overnight at 37°C to allow the cells to attach and proliferate. On the next day, 300 μL of fresh medium containing drug concentrations (myricetin, methyl eugenol and cisplatin or their combinations) were added to each well, and the plates were incubated at 37°C in 5% CO_2. All drug concentrations were tested at least in triplicate wells and the assays were repeated independently three times. After 48 h, the plates were removed from the incubator and then 100 μL of medium from each well was carefully transferred to new plates. 100 μL of LDH substrate prepared according to the manufacturer's direction (Cytotoxicity Detection Kit, Roche Chemical Co.) was added to each well. After 20 min shaking at room temperature, the enzymatic reaction was arrested by adding 50 μL of 1 M hydrochloric acid.

Lactate dehydrogenase activity was determined by change in absorbance at 490 nm. For the purpose of calculating percent cytotoxicity values, background LDH release from culture cells was considered as low control and triton-X 100 (0.01%) treated cells as high control [21].

Leakage (%) = [A490 (sample)-A490 (low control)/A490 (high control)-A490 (low control)] × 100%

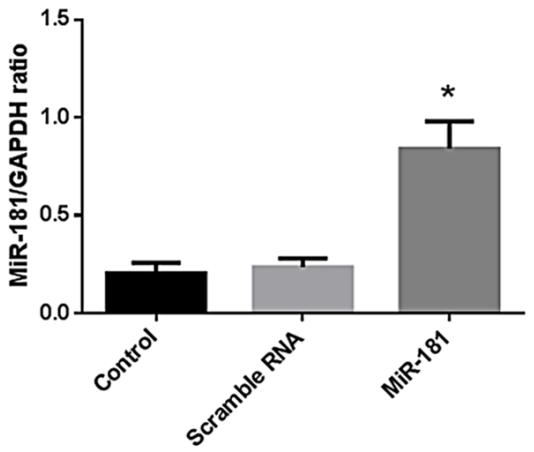

Figure 1. The relative expression level of miR-181. The expression level ratio of microRNA-181and GAPDH. *means P<0.05 compared with controlled group.

Flow cytometry

Cervical cancer cells (HeLa) (1×10^5) in a 60 mm dish were subjected to cisplatin (1 μM), myricetin (60 μM) and methyl eugenol (60 μM) (Sigma Chemical Co. USA) or their combinations for 48 hours. The cells were collected by trypsinization and washed twice with PBS (Sigma Chemical Co.). Cells were incubated in 50% ethanol at -20°C overnight and then treated with 40 μg/mL RNase A (Guangzhou Geneshun Biotech Ltd. China), then stained with 10 μg/mL of propidium iodide (PI) (Guangzhou Geneshun Biotech Ltd. China). Finally, the stained cells were analyzed by using FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) [21].

RT-PCR

Total RNA was isolated from cervical squamous cell carcinoma tissues and their adjacent normal tissues using the RNA Extraction Kit (Invitrogen). cDNA were synthesized with Invitrogen's SuperScript One-Step RT-PCR Kit; each reaction contained 2 μg total RNA, 2 μL Oligo (dT) (500 μg/mL), and 7.5 μL DEPC water. Reactions were heated for denaturation at 65°C for 5 min, and then quenched on ice for 5 min. The following reagents were then added to each reaction: 4 μL 5×1st Buffer, 2 μL 0.1 M DTT, 1 μL dNTPs (10 mM each), 0.5 μL RNase Inhibitor (40 U/μL), 1 μL M-MLV (200 U/μL); the total volume of each reaction was 20 μL. The reactions were kept at 25°C for 10 min, 37°C 1 h, and then 70°C for 10 min to terminate the reaction. MiR-181 level was determined by real-time RT-PCR using a Light Cycler 480 (Roche Diagnostics) with the forward primer, 5'-AACACGAAGCACGATCAGTCC-3', and the reverse primer, 5'-CTCATTTTGGCAAGTATCCGA-3'. To normalize the amount of cDNA in each sample, the housekeeping gene aldehyde-3-phosphate dehydrogenase (GAPDH) was quantified on the control of experiment with specific primers (forward: 5'-TGTTGCCATCAATGACCCCTT-3'; reverse: 5'-CTCCACGACGTACTCAGCG-3'); the amplicons were 202 bp. Each reaction contained cDNA 500 ng, 2×PCR buffer for EvaGreen 10 μL, 20× EvaGreen 0.6 μL, forward primer and reverse primer were 0.6 μL (10 μM) respectively, Cap Taq polymerase 0.3 μL (5 U/μL); add DEPC water to 20 μL. Reaction conditions were: initial denaturation for 5 min at 95°C; then 40 cycles of denaturation for 15 sec at 95°C, primer annealing for 15 sec at 55°C, extension for 20 sec at 72°C, and UPL fluorescence measurement for 3 sec at 76°C [22].

Western blotting

The cells were lysed with RIPA lysis buffer (Beyotime Biotechnology) on ice for 20 min and centrifuged at 12,000 rpm for 20 min at 4°C. The protein concentrations of the supernatants were then quantified by BCA kit (Beyotime Biotechnology). Equal proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.05% Tween 20 at pH 7.5 for 30 min, incubated with primary antibodies at 4°C overnight, and then conjugated with secondary antibodies for 1 h at room temperature. The signals of immunoblotted proteins were visualized with an enhanced chemiluminescence detection kit by Chemidoc XRS imaging system (Quantity One Quantitation software, Bio-Rad Laboratories, Hercules, CA, USA) [23].

Statistic analysis

All collected data were firstly tested for the normal distribution using one-sample K-S test. Enumeration data were analyzed by chi-square test or rank-sum test. Measurement data were tested by student t-test (for two groups) or analysis of variance (ANOVA, for more than three groups). Further between-group-comparison was then performed by post-hoc Tukey test. A

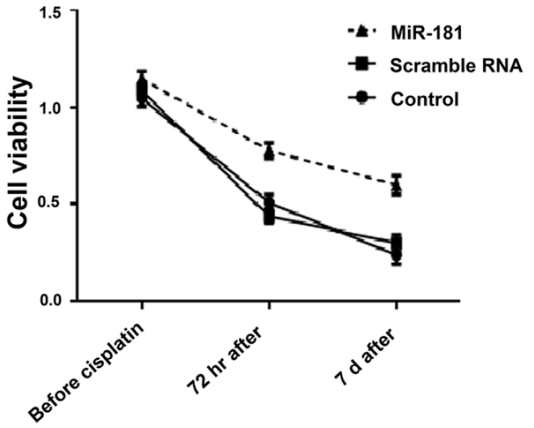

Figure 2. Cell proliferation curve of Hela. The controlled cell group of before cisplatin processing was identified as 1.0.

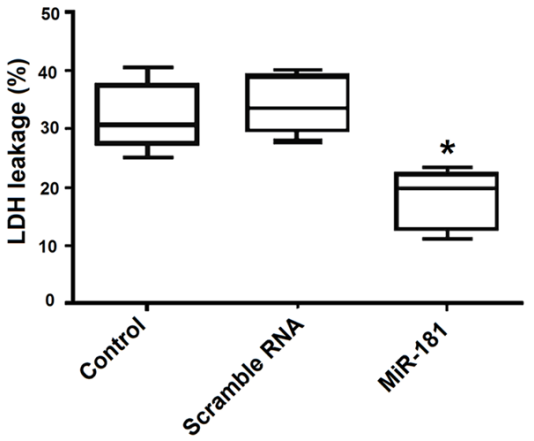

Figure 3. LDH of cell permeability. *indicates significant difference compared with control group (P<0.05).

statistical significance was defined when P< 0.05.

Results

MiR-181 expression level in vitro

The miR-181 expression in cells transfected after 2 weeks was tested by RT-PCR. As shown in **Figure 1**, the transfection of plasmid could effectively improve the expression of miR-181, telling the stable expression model was successfully established.

MiR-181 increased hela cell viability

Next, we further analyzed the proliferation of cisplatin treatment cell groups by MTT method. As shown in **Figure 2**, the proliferation of HeLa

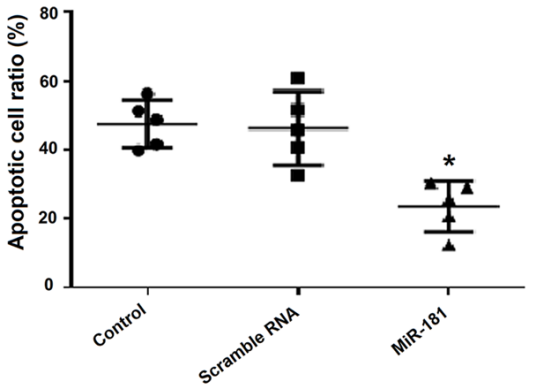

Figure 4. Cell apoptosis rate. Annexin V/PI double standard method of flow cytometry was constructed to calculate the cells apoptosis rate of cisplatin treatment 7 days later. *indicates significant difference compared with control group (P<0.05).

has increased dramatically after miR-181 overexpressed, indicating that miR-181 overexpression may up-regulate the resistance of tumor cells.

Lactate dehydrogenase activity

Lactate dehydrogenase (LDH) is an important index of cytotoxicity measuring. In this step of research, the contents of LDH of each cell groups were measured. **Figure 3** shown the LDH activity of cells treated with cisplatin. The results shown in **Figure 3** convinced us LDH activity of miR-181-overexpressed cells was significantly decreased, and this suggesting the cytotoxicity reduction and the drugs-resistance increase.

Cell apoptosis

Counting on the cytotoxicity decrease of overexpressed miR-181 cells by the LDH assay, we further observed cell apoptosis after cisplatin treatment through flow cytometry method. The results were shown in **Figure 4**. Compared with the controlled group, cells with miR-181 overexpression, shown significantly reduced apoptosis rate. The result above demonstrated that miR-181 could protect tumor cells.

Cell cycle and apoptosis related proteins

To preliminary explore the related mechanism of cisplatin resistance of HeLa, we tested the expression of cycle regulatory proteins cyclin and apoptosis protein caspase-3 by Western blotting assay. The results are shown as **Figure 5**. It can tell that for cells of miR-181 overex-

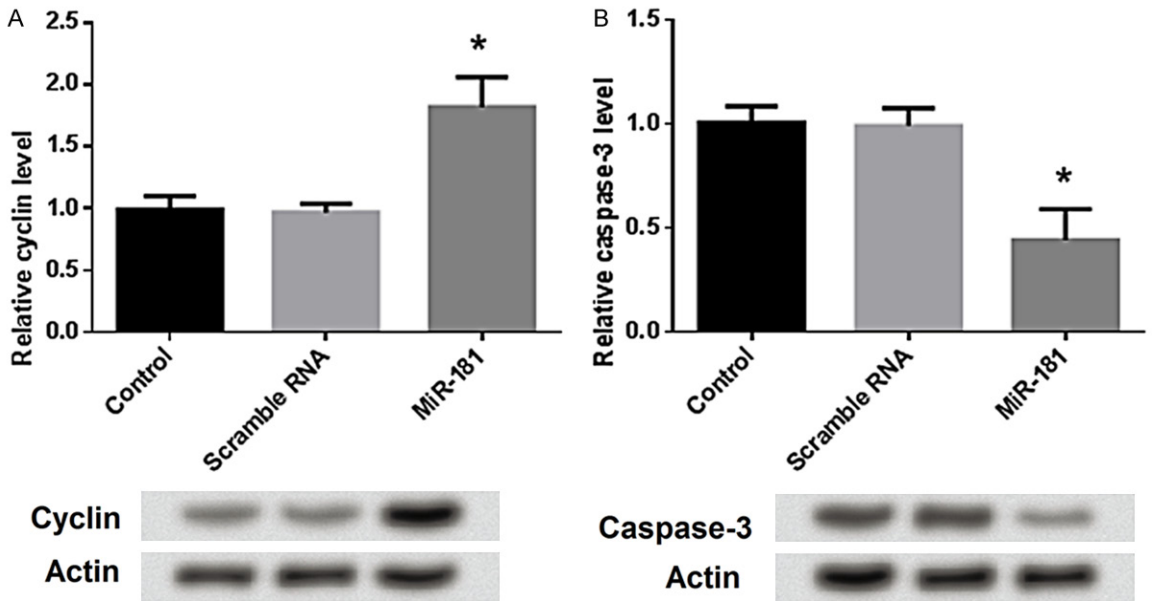

Figure 5. Cyclin (A) and Apoptin (B) expression. Calculating the relative levels affirm the control group as 1.0. *indicates significant difference compared with control group (P<0.05).

pressed, cyclin protein expression levels increased significantly (**Figure 5A**), while the expression of caspase 3 levels dropped significantly (P<0.05) (**Figure 5B**).

Discussion

Cervical cancer is one of the most common malignancies diagnosed in women during pregnancy, which is simultaneously a leading cause of cancer death in women worldwide [24-26]. Great advances but still insufficient achievements in the mechanism and treatment of cervical cancer have been obtained during last several years.

The platinum-derived drugs cisplatin is among the most commonly used chemotherapy drugs in cancer therapy, including cervical cancer [27]. However, various malignant tumors frequently acquire resistance to cisplatin, this incontrovertible a major clinical problem and the acquisition of resistance significantly undermines the curative potential of cisplatin against many cancers, Chemotherapy-resistance were proposed as the most commonly cause of the failure of chemotherapy [28]. Scientists had made great efforts to find mechanism and methods that could contributes to cisplatin resistance in cancer therapy, and inhibition of apoptosis, changes of cellular drug accumulation, detoxification of the drug, and repair of the DNA adducts were proposed as the mainly mechanism [28]. In the study of Sun R, et al., SOX4 was found to modulate cancer proliferation by regulating the cell cycle, and inhibiting cancer cell sensitivity to therapeutic drug. SOX4 was then confirmed as a target for cervical cancer chemotherapy [29]. Guo L, et al., evaluated the connection between genetic polymorphisms in the PI3K/Akt pathway and chemotherapeutic outcomes following platinum-based neoadjuvant chemotherapy NAC of patients with squamous cervical cancer, finding that genetic polymorphisms are associated with sensitivity to platinum-based chemotherapy in the PI3K/Akt pathway in SCC patients [30]. Thus, we can conclude from these investigations that, by regulation of cell cycle and cell apoptosis pathway, miRNAs involved in the resistance of tumor cells and drug sensitivity adjustment process [31-34].

MiR-181 was once verified high-expressed in lung cancer cells of cisplatin resistance [35], but there is no further research on the detailed mechanism so far. Accumulating evidence shows that activity of the miR-181 is closely related to tumorigenesis. Overexpression of miR-181 evidently inhibited cell proliferation, migration, and invasion and promotes cell apoptosis in lung cancer [36]. Moreover, in the study of prostate cancer, it was proved that miR-181 overexpression markedly promoted the proliferation of LNCaP cells, suggesting that miR-181 functions as a growth-suppres-

sive miRNA during prostate cancer development [37].

In this study, we preliminary discusses the role of miR-181 in cervical cancer chemoresistance. The expression profile of miR-181 and its contribution to cervical cancer cells proliferation, invasiveness and drug resistance were studied in this project. We sought to explore the function of overexpressed miR-181 in Hela cell line on the cell proliferation, and apoptosis in vitro. The results revealed that miR-181 can protect tumor cells by inhibiting cell apoptosis and enhancing the cell proliferation ability. This investigation confirmed the connection between the mir-181 and cervical cancer resistance.

Lactate dehydrogenase (LDH) is an important index of cytotoxicity measuring, so, next, we tested the LDH activity of miR-181-overexpressed cells and compared with the control groups, the results provides evidence that overexpression of miR-181 could notably reduce the cytotoxicity and thus increase the drug-resistance.

Finally, to giving clearer insights to the potential mechanisms of miR-181 contributions to drug-resistance, we further investigated the mechanism of the combination of regulatory protein cyclin and apoptosis protein caspase-3 with cisplatin resistance of HeLa. In this part of research, western blot was constructed to monitor the protein expression and expression changes of cyclin and caspase-3. As a result, miR-181 markedly up-regulates cyclin protein expression levels, while down-regulate the expression of caspase 3 levels. Both of them were proposed to be involved in the regulation process of cisplatin treatment. Thus, we can verdict that miR-181 may interfere in cisplatin-resistance process via regulating of apoptosis-related protein.

Taken together, these observations not only reveal a new function of miR-181 and its most possible mechanism in cervical cancer, but also suggest it as a potential target for developing therapeutics against the chemoresistance. Specific aspects of the follow-up mechanism referring to animal models, as well as signal pathway require further validation experiments.

Disclosure of conflict of interest

None.

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