Effects of miR-940 on Proliferation, Migration and Cell Cycle Behavior in Human Cervical Cancer Cells Hela and Its Mechanism

Haibo Fan, Shanxi Provincial Cancer Hospital, Shanxi, China

Abstract:
Objective: To investigate the effect of miR-940 on proliferation and migration in human cervical cancer cells Hela and its mechanism, miRNA microarray data were obtained from the GEO database, and miR-940 was screened from the GEO database. The expression of miR-940 in 26 cervical carcinoma tissue and Hela cells was detected by real-time PCR. After the overexpression of miR-940 in Hela, the proliferation, migration and cell cycle of Hela cells were detected by MTT, transwell and flow cytometry. The dual-luciferase reporter system was used to identify the miR-940 target gene. The target gene was detected by Western Blot in Hela cells. The expression of miR-940 in cervical cancer tissues and human cervical cancer cells was significantly down-regulated. The overexpression of miR-940 significantly inhibited the proliferation and migration of Hela cells and prolonged the cell cycle. ZNF24 acts as a miR-940 target gene and exhibits low expression in cervical cancer cells overexpressing miR-940. The expression of miR-940 in cervical cancer can inhibit the proliferation and migration of human cervical cancer cells and may play a role in regulating the ZNF24 gene. It may provide the theoretical basis for understanding the mechanism of cervical cancer.

keywords: human cervical cancer; cell proliferation; cell migration; cell cycle

As a common female malignancy, cervical cancer has been showing an upward trend in recent years due to its high incidence, easy invasion and metastasis, and low cure rate (Lee B et al., 2016; Mezei AK et al., 2017). Although the treatment of cervical cancer continues to progress, cervical cancer metastasis remains the leading cause of death in cervical cancer patients, and it has been among the top of female cancer mortality (Ramlov A et al., 2017). Therefore, from the molecular level to find the potential markers in the development of cervical cancer, in-depth understanding of cervical cancer metastasis mechanism for the diagnosis and treatment of cervical cancer to provide more targets urgently need to be solved (Yang Y et al., 2016). MiRNA, as a class of highly conserved, noncoding, single-stranded small RNA, have been shown to play an important role in multiple species by post-transcriptional regulation involving almost all physiological and pathological processes, and many miRNA are also involved in the development of cervical cancer, but most miRNA don’t have a clear effect in regulating cervical cancer metastasis (Lai XJ et al., 2016; Huang P et al., 2016; Fang H et al., 2016). In this study, we investigated the effect of miR-940 on the proliferation and migration of Hela in human cervical cancer cells and its mechanism.

1. Results and analysis
1.1 Screening of differentially expressed miRNAs in cervical cancer tissues

In this study, miRNA chip data of cervical cancer were downloaded and obtained from the National Center for Biotechnology Information (NCBI) Gene Expression Database (GEO). Six cases of cervical cancer tissue samples and 3 cases of adjacent tissues were numbered
According to the set numerical filtering criteria, we screened 150 differential miRNAs and stratified cluster analysis of differential miRNAs (Figure 1). The miR-940 was further studied by data analysis and preliminary laboratory studies.

1.2 The differential expression of miR-940 in cervical cancer tissues and human cervical cancer cells Hela

MiR-940 decreased about 35% in cervical cancer tissues compared with adjacent tissues and decreased by about 40% in human cervical cancer cells Hela, and the difference was statistically significant (P <0.05, Fig. 2), indicating that miR-940 is involved in the development of cervical cancer process, and may play an important role.

1.3 MiR-940 inhibits Hela proliferation in human cervical cancer cells

The expression of miR-940 was significantly up-regulated in miR-940 mimics and NC and stable culture in human cervical cancer cell Hela (P <0.05, Fig. 3A). MTT assay showed that miR-940 mimic had a significant reduction in cell proliferation and a significant inhibitory effect at 72 h after transfection (Fig. 3B), indicating that miR-940 significantly inhibited Hela proliferation in human cervical cancer cells.
1.4 MiR-940 inhibits the migration of Hela in human cervical cancer cells

Hela cells transfected with miR-940 mimic 48 hours later, compared with the control group transfected with NC, the number of cell migration was significantly lower than the control group (P <0.05, Figure 4).

1.5 Overexpression of miR-940 prolongs the cell cycle of human cervical cancer cell Hela

Hela cells were transfected with miR-940 mimic for 48 hours and the cell cycle was detected by flow cytometry. The results showed that the Hela cell cycle was prolonged (Fig. 5).

1.6 MiR-940 acts on target human gene ZNF24 in human cervical cancer cell Hela

The miR-940 target gene was predicted using the miRecords integration database, and the candidate genes such as ZNF24 were selected from the predicted results. The relative activity analysis of miR-940 mimics and reporter gene vector pLUC-ZNF24 was carried out by dual luciferase reporter assay system. The relative activity analysis of firefly luciferase showed that miR-940 could down-regulate the hRluc expression of ZNF24 gene 3’UTR (P <0.05). After the mutation of the binding site, this regulatory relationship disappeared, indicating that miR-940 could regulate the expression of hRluc through the binding site (Fig. 6), indicating that in the human cervical cancer cell Hela, there is a relationship of targeted regulation between ZNF24 and miR-940.

1.7 ZNF24 expression was decreased in overexpressing miR-940 human cervical cancer cell Hela

The expression of ZNF24 protein in the experimental
The gray value of the experimental group showed a significant decrease ($P < 0.05$, Fig. 7).

2. Discussion

With miRNA as the representative of non-coding RNA in the field of cancer research deepening, more and more cervical cancer-related miRNA was found to be deeply involved in its development and treatment process (Yin XZ et al., 2016; Zhang X et al., 2016; Li Lianxiang, 2014). The expression of miR-373 in cervical cancer was significantly lower than that in adjacent normal breast tissue. MiR-373 could inhibit the expression of B cell lymphoma factor (Bcl)-6 in colorectal cancer tissues (Qu YH et al., 2017) The apoptotic rate of miR-373 analogue group was significantly higher than that of normal control group. The cell proliferation rate, invasion ability and migration ability were significantly lower than those of normal control group. The results showed that miR-373 expression could inhibit the proliferation and invasion of cervical cancer cells. The mechanism may be that low expression of miR-373 promotes Bcl-6 up-regulation and promotes the proliferation of cervical cancer cells. Shen Y et al. (Shen et al., 2017) found that miR-660-5p could inhibit the proliferation of cervical cancer cells by blocking the G1 phase of MDA-MB-231 cell cycle by binding to target gene TFCP2; He XH (He XH et al., 2016) found that miR-155 was negatively correlated with ErbB2 expression in ErbB2-positive cervical cancer by analyzing the clinical cervical cancer samples. MiR-155 inhibits ErbB2 transcription to activate HDAC2 by targeting and direct targeting of binding ErbB2 mRNA coding regions, and MiR-155 inhibits ErbB2 expression during transcription and after transcription. Functional studies have shown that miR-155 inhibits ErbB2-induced malignant transformation of human mammary gland epithelial cells. These results reveal the important regulatory role of miR-155: ErbB2 regulatory axis in the malignant transformation of mammary epithelial cells, and reveals a new functional pattern of miR-155 in cervical cancer.

Figure 5 Overexpression of miR-940 prolongs the cell cycle of human cervical carcinoma cell line Hela

Figure 6 Dual luciferase assay verified the working target gene of miR-940 in human cervical carcinoma cell line Hela is ZNF24
In the early stage of this study, we analyzed the data of GEO cervical cancer chip, and got a total of 271 miRNAs in cervical cancer. Through successful transfection of mimic, we conducted a study of miR-940 regulation of cervical cancer cell growth and migration mechanisms. First, miR-940 expression was significantly reduced by about 40% in clinical cervical cancer specimens and human cervical cancer Hela, suggesting that miR-940 may play an important regulatory role in the development of cervical cancer. Secondly, miR-940 could significantly inhibit the growth and migration of cervical cancer cells. The results showed that miR-940 could significantly inhibit the growth and migration of cervical cancer cells. Finally, taking into account the miRNA as an endogenous non-coding RNA, its functional mode is mainly through the complete or partial complementation of the target gene 3 affecting the target mRNA stability, and even degradation of mRNA, in order to achieve post-transcriptional level adjustment target Gene expression. Bioinformatics predicts ZNF24 is one of the miR-940 potential target genes. It is possible that the miR-940 may be regulated by ZNF24 in the cervix cancer.

The human zinc finger protein gene ZNF24 is located at 18q 12.1 and consists of 4 exons encoding a 368 amino acid class Krüppel protein. It has been shown that there are deletions of ZNF24 loci in various human tumors such as invasive cervical cancer, colon cancer, gastric cardia adenocarcinoma, testicular germ cell tumor and hematopoietic malignancy, especially in colon cancer, cervical cancer and ovarian malignant tumor tissue, The expression of ZNF24 mRNA was significantly up-regulated (Jia D et al., 2015). These results suggest that the development of tumors such as cervical cancer and colon cancer may be related to the regulation of ZNF24. A large number of studies have shown that a variety of tumors are associated with abnormal expression of VEGF and PDGFR-β. It is speculated that ZNF24 regulates the development or progression of cervical cancer and colon cancer by regulating the expression of PDGFR-β and VEGF.

In this study, we examined the expression of miR-940 in human cervical carcinoma Hela, and explored the mechanism of miR-940 expression in cervical cancer, which may have an important prognostic value, and possibly provide new ideas for cancer diagnosis and treatment.

3. Materials and methods
3.1 Major instruments and reagents
Total RNA Extraction Reagent Trizol was purchased from Invitrogen (California, USA), miR-940mimics / NC from Shanghai GenePharma Co. Ltd. (Shanghai, China), Reverse Transcription Kit Reverse Transcription System
purchased from Takara Corporation (Japan), SYBR® PrimeScript™ RT-PCR Kit was purchased from Takara Corporation (Japan), mRNA SYBR Green Fluorescence Quantitative PCR reagent was purchased from Takara Corporation (Japan), miR-940 real-time PCR primers were purchased from Ruibo Biology (Guangzhou, China), Propidium Iodide / propidium iodide, RNase A, MTT Cell Proliferation and Cytotoxicity Assay Kit were purchased from Beyotime Biotechnology (Jiangsu, China), FBS, streptomycin, 0.25% trypsin, PRMI 1640 were purchased from Gibco (USA), Cultured human cervical cancer cells Hela complete medium formulating as medium + 8% DMSO + 20% FBS.

3.2 Real-time PCR detection
MiR-940 expression amount Trizol method was used to extract cervical cancer tissue or cell total RNA, take 10μL RNA for reverse transcription, U6 as the internal reference for quantitative analysis, miR-940 quantitative reaction system follows 12.5 μL SYBR Premix Ex Taq, 1 μL PCR Forward Primer, 1 μL Uni-miR qPCR Primer, 2 μL cDNA template, 8.5 μL ddH2O, total volume of 25 μL, and 3 parallel wells per sample. The procedure was as follows: pre-denaturation 95 ℃ 30 s; 95 ℃ 5 s, 60 ℃ 20 s, 40 cycles; using 2-ΔΔCt method to calculate the relative expression of miR-940.

3.3 MTT assay for the proliferation of cervical cancer cells
The overexpressed miR-940 cervical cancer cells were stably cultured in 96-well plates and cultured in complete medium. MTT assay was performed at 24 h, 36 h, 48 h and 72 h, respectively. 4 replicates per group. Add 20 μL of MTT (5 mg / mL PBS dissolved) to each well of the 96 wells, incubate 4 h and discard the supernatant. Add 150 μL of Formazan solution to each well and shake 10 min to dissolve the crystals well. The absorbance was measured at 490 nm.

3.4 Cell migration experiments
When the Hela cell confluency reached 80%, the cells were washed twice with preheated PBS. Digestion of cells with trypsin, transferred to the centrifuge tube, 1100rpm centrifugation 5min, to the supernatant. The cells were resuspended in complete medium and washed and counted for 5 * 104 cells / mL. Make sure the stopper of the OrisTM Tissue Culture Treated Plate firmly closes the bottom of the plate. Add 100 μL of suspended cells to each well and add along the pore walls. Gently pat the plate, so that cell suspension evenly distributed in each cell culture wells. Placed in the incubator, cultured overnight, until the cells adhere to the wall. NC and miR-940 mimic were transfected into cells by Lipofectamine2000 standard procedure. The final concentration was 50nM, and three wells were set in each group. Another reference hole was set up. Only Lipo2000 was added but no mimics was added. The Stopper was removed until the end of the experiment. After 6 h transfection, the transfected NC group and the stopper of the miR-940 mimic group were removed with the OrisTMstopper tool. The Stopper of the reference hole was removed until the end of the experiment. The medium was blotted off and the cells were gently washed with 100 μL sterile PBS to remove the non-adherent cells. 100 μL complete medium was added to each well and the OrisTM Tissue Culture Treated Plate was placed in an incubator for 48 h. According to the dye specification, cell staining was performed. Use Acumen ex3 to capture images and get data. Data acquisition: Move the mouse to the center of the reference hole to get the coordinates (X, Y) of the center. According to the center coordinates (X, Y) to find the center of the other experimental hole, counting the number of cells 0-0.96mm away from the center as the number of migratory cells.

3.5 Flow cytometry Hela cell cycle
The cells were cultured with trypsin digestion, add 1 ml of 75% pre-cooled ethanol was added. The cells were beaten evenly and fixed at 4 ℃ for more than 12 hours, and then added to the cells. PBS washed, centrifuged at 1000 rpm, 5 min, washed twice. The cells were resuspended in 0.5 ml PBS and PI and RNaseA were added to each well at a final concentration of 50 μg / ml for 30 min at 37 ℃. Flow cytometry was used to detect cell cycle.

3.6 The miR-940 target gene was verified by double luciferase vector method
(1) vector construction: Based on the software predicting miR-940 target gene is ZNF24, PCR primers were used to design the amplification primers according to ZNF24
(human) 3'UTR sequence information, and the ZNF24 gene was amplified by PCR in the treatment group. Of the 3'UTR sequence, which was cloned into the pLUC double luciferase reporter vector. The reported fluorescence of the vector was hRluc and the corrected fluorescence was hluc (for internal reference correction). Among them, the mutant ZNF24 gene 3'UTR sequence construction vector was designated pLUC-ZNF24-Mutant, and the unmutated vector was designated pLUC-ZNF24. 

(2) Transfection and fluorescence detection: 293T cells were routinely cultured at 37 °C and 5% CO2; 293T cells in logarithmic growth phase were inoculated into 96-well plates at 1.5 × 10^4 cells per well. The total volume of 100 μL per well was cultured in a 37 °C incubator for 24 h; (3) 10 μL of OPTI-MEM medium diluted miR-940mimics or Non-targetControl, 15 μL of OPTI-MEM medium diluted pLUC-ZNF24-Mutant vector or pLUC- ZNF24 vector, 25 μL OPTI-MEM medium diluted 0.25 μL LipofectamineTM2000 reagent, mixing after 5 min, a total of 50 μL, gently shake, put it aside for 20min; before the plasmid and mimics were added to the cell, each hole was first taken away 50 μL medium, then added to the above 50 μL mixture, resulting in a final volume of 100 μL per well. The concentration of mimics was 50nM, the concentration of the plasmid was 100ng / hole, and each group was filled with 3 wells. After 6h, add 100μl fresh medium. After the cell lysate was well mixed, 100 μL / well was added the report gene cell lysate, fully lysate the cells; fully cracked, 10000-15000g centrifugation for 3-5 minutes, take the supernatant for determination; add 100 μL firefly luciferase (hRluc) detection reagent, Rlu was measured after mixing with a gun or in other suitable ways. To complete the above-mentioned steps in the determination of the firefly luciferase, add 100 μL renan luciferase (hluc) working solution, mix with a gun or even mix it in other appropriate ways, then determine Luc.

3.7 Western blot was used to detect ZNF24 protein

BCA method was used to determine the protein concentration, the denaturation of boiling water, the preparation of 10% separation gel and 5% concentrated gel, SDS-PAGE electrophoresis. After the electrophoresis, 6 sheets of filter paper and 1 PVDF film were cut according to the separation area. PVDF membrane was activated by methanol for 10s, then put with the filter paper and separation gel into the transfer buffer for 10min. The separation gel, PVDF membrane and filter paper were sorted according to the “sandwich” (negative electrode - filter paper - separation gel - PVDF film – three-layer filter paper- positive electrode), making no bubble between filter paper and separation gel. The positive current is set to 48mA according to 1mA/ cm2, 40min semi-dry electric transfer method; after film transfer, remove the PVDF membrane, observe the pre-marker transfer, and accordingly determine the transfer of the target protein; the sealed liquid immersion PVDF membrane, closed after room temperature shaker reaction 1h, and TBS slightly washed several times; with the closed solution diluted with the corresponding primary resistance, Bed incubation 2h, and TBS wash the film 3 times, each time 15min; then added with the closure of the corresponding dilution of the corresponding two-body temperature shaking incubator 1h, and TBS washing film 3 times, each 15min; ECL color detection.

3.8 Statistical processing

SPSS17.0 software was used for data analysis. Perform homogeneity of variance test, normality test. The data of the measurement data are expressed . Univariate comparison between the two groups of data was made using t test; comparison between multiple groups of data using one-way analysis of variance; variance is not a non-parametric test. the difference of P <0.05 was statistically significant.

References


