Expression of miR-106 in endometrial carcinoma RL95-2 cells and effect on proliferation and invasion of cancer cells

XINGJUN LI[1], XIANGHUA YI[2], CHUANDING BIE[3] and ZHEMIN WANG[4]

[1]Department of Clinical Laboratory, Chongming Branch Hospital, Affiliated Xinhua Hospital, School of Medicine, Shanghai Jiaotong University; [2]Department of Pathology, Tongji Hospital Affiliated to Tongji University, Shanghai 202150; [3]Department of Pathology, Suizhou Maternal and Child Health-Care Hospital, Suizhou, Hubei 441300, [4]Department of Orthopaedics, Chongming Branch Hospital, Affiliated Xinhua Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 202150, P.R. China

Abstract. Expression of miR-106 in endometrial carcinoma RL95-2 cell line and its effect on proliferation and invasion of cancer cells was investigated. miR-106 expression vector was constructed and transiently transfected into *in vitro* cultured RL95-2 cells of human endometrial carcinoma. Cells were divided into three groups including blank control cells (MOCK group), miR-106 transfection group (miR-106 group) and negative control group (siNC group). Reverse-transcription quantitative PCR (RT-qPCR) was used to detect the expression of miR-106. Proliferation and *in vitro* migration of RL95-2 cells were detected by MTT and scratch assay, and cell apoptosis was detected by flow cytometry. Compared with MOCK and siNC group, cell apoptosis rate was significantly decreased but cell proliferation rate was significantly increased in miR-106 group ($p < 0.05$). In addition, cell migration and invasion ability was significantly increased in miR-106 group ($p < 0.05$). Overexpression of miR-106 can promote proliferation and inhibit apoptosis of endometrial cancer RL95-2 cells, and miR-106 may serve as a new target for the treatment of endometrial cancer in the future.

Introduction

Endometrial cancer is one of the three major malignant tumors in females and can occur in females of all age groups (1). Amant *et al* (2) showed that in 2016, ~1.2 million new cases of endometrial cancer were reported worldwide, and endometrial cancers are the third most common malignancy that threatens the life and health of women. In recent years, incidence of endometrial cancer showed an increasing trend. In some European and American countries, incidence of endometrial cancer is the highest among all gynecologic cancers (3).

Due to the high incidence, clinical treatment of endometrial cancer has attracted increasing attention. Breakthroughs have been made in the treatment of this disease, and the majority of patients achieve promising prognosis after surgery, radiotherapy and chemotherapy (4). Plante *et al* (5) showed that the 5-year survival rate of patients with endometrial cancer is ~85%. However, tumor metastasis may also occur in some patients due to the lack of timely or improper treatment, seriously affecting patients' prognosis (6).

At present, the pathogenesis, migration and invasion of endometrial carcinoma are not clear. In recent years, miRNAs have become popular research objects. As a class of non-coding endogenous RNAs with a length of 17-23 nt, miRNAs can directly react with the 3'-UTR region of mRNAs to regulate the expression of target genes. miRNAs may show upregulated expression pattern to play oncogenic roles or show downregulated expression pattern to play a role as tumor suppressor genes (7-9). However, Zheng *et al* (10) showed that miR-106 promoted cancer cell proliferation in endometrial cancer. Therefore, our study aimed to further investigate the regulatory role of miR-106 in endometrial cancer with an expectation of providing references for the targeted therapy of this disease.

Materials and methods

Main materials. Human cervical cancer cell line RL95-2 was provided by the Department of Pathophysiology, Anhui Medical University. RT-PCR reverse transcription kit and miRNA isolation kit were purchased from Qiagen, Inc. (Valencia, CA, USA). Fetal bovine serum (FBS) was purchased from Gibco (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Tetramethyl azoline blue (MTT) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (Sigma-Aldrich; Merck KGaA, St. Louis, MO, USA). Lipofectamine™ 2000 transfection reagent was purchased from Invitrogen; Thermo Fisher Scientific, Inc.

Correspondence to: Dr Zhemin Wang, Department of Orthopaedics, Chongming Branch Hospital, Affiliated Xinhua Hospital, School of Medicine, Shanghai Jiaotong University, 25 South Gate Road, Shanghai 202150, P.R. China
E-mail: wzr7bq@163.com

Table I. Primer sequences.

Primers	mir-106	U6
Forward	5'-CGGCTAAAGTGCTGACAGTGC-3'	5'-GCTTCGGCAGCACATATACTAAAAT-3'
Reverse	5'-GTGCAGGGTCCGAGGT-3'	5'-CGCTTCACGAATTTGCGTGTCAT-3'

Annexin V-FITC/PI apoptosis kit was purchased from BestBio (Shanghai, China). The study was approved by the Ethics Committee of Chongming Branch Hospital, Affiliated Xinhua Hospital, School of Medicine, Shanghai Jiaotong University (Suizhou, China). Signed informed consents were obtained from the patients or the guardians.

Cell culture. Endometrial cancer cells RL95-2 were cultured in RPMI-1640 medium containing 10% FBS at 37°C with 5% CO_2.

Grouping. miR-106 primers were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) (Table I). High-fidelity DNA polymerase was used to amplify miR-106, and the product was subjected to *Bam*HI and *Hind*III digestion and was inserted into pcDNA3.1(-). Cells were divided into three groups including blank control cells (MOCK group), miR-106 transfection group (miR-106 group) and negative control group (siNC group).

Methods
Cell transfection. Digestion of cells using trypsin was performed and cells were counted. RL95-2 cells were seeded into 6-well plates with $2x10^5$ cells/well, followed by cell culture for 24 h. Lipofectamine™ 2000 was used for transfection according to the manufacturer's instructions when cell confluence reached 80%. Expression of miR-106 was detected 48 h after transfection.

Reverse transcription-quantitative PCR (RT-qPCR). Tissues were mixed with TRIzol reagent and kept at room temperature for 30 min to extract total RNA according to the manufacturer's instructions. The total RNA was then reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using cDNA synthesis kit (Biomiga Inc, San Diego, CA, USA). An ultraviolet spectrophotometer (Hitachi, Tokyo, Japan) and 1% denaturing agarose gel electrophoresis were used to test RNA quality. miR-106 reverse transcription was performed according to the manufacturer's instructions. miR-106 primers were designed and synthesized by Shanghai GenePharma Co., Ltd. (Table I). PCR reaction systems were prepared according to the manufacturer's instructions. PCR reaction conditions: 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec, 65°C for 30 sec and 72°C for 30 sec. U6 was as an endogenous control, and data were processed using the $2^{-\Delta\Delta Cq}$ method (11).

MTT assay. Density of RL95-2 cells was adjusted to $1x10^4$/ml and cultured in 96-well plates (37°C, 5% CO_2) for 24 h. P-miR-106 and empty plasmid were transfected into cells using Lipofectamine™ 2000, and three replicate wells were set. After 72 h, 20 μl of MTT reagent (5 mg/ml) was added to each well, followed by incubation for 2 h. Supernatant was discarded with a pipette and 100 ml of DMSO was added and shaken for 15 min. A microplate reader (Bio-Rad, Hercules, CA, USA) was used to measure the OD values at 450 nm and cell growth curves were plotted.

Flow cytometry. Cells in plates were rinsed with 2 ml PBS solution, followed by incubation with 0.5 ml 0.25% trypsin (without EDTA). After digestion, cells were resuspended to a density of $1x10^6$ cells/ml, followed by incubation with apoptosis detection solution Annexin V-FITC at room temperature for 15 min. After centrifugation at a speed of 375 x g for 5 min at 4°C, supernatant was removed, and cells were resuspended in 1X buffer. Finally, 10 μl PI was added and flow cytometry was performed. The experiment was repeated 3 times.

Cell scratch assay. Cells were inoculated into 6-well plates 24 h after transfection, and three replicate wells were set. A 20 μl tip was used to scratch cells when 90% confluence was reached. After washing with PBS 3 times, cells were cultured in 1% FBS in DMEM. This experiment was repeated 3 times.

Statistical analysis. SPSS 22.0 (IBM Corp., Armonk, NY, USA) statistical software was used for data analysis. The data are expressed as mean ± SD. Comparison between groups was done using one-way ANOVA test followed by post hoc test (Least Significant Difference). Paired t-test was used for comparison between two groups. $P<0.05$ was considered to be statistically significant.

Results

Expression of miR-106 after transfection. RT-qPCR results showed that the expression of miR-106 in miR-106 group was significantly higher than that in siNC and MOCK group (F=24.34, p<0.01). There was no significant difference between siNC and MOCK group (t=0.19, p=0.86, Fig. 1).

Cell apoptosis. Flow cytometry was used to detect apoptosis of transfected cells. Apoptosis rate of miR-106 group after transfection was 3.08±0.74%, which was significantly lower than that of the siNC group (14.83±1.02%) and MOCK group (13.17±0.94%), (F=147.20, p<0.01). There was no significant difference between siNC and MOCK group (t=0.03, p=0.97, Fig. 2).

Cell proliferation. After miR-106 was transfected into RL95-2 cells, results of MTT assay showed that there was no significant difference in cell proliferation rate between the two groups at 12-24 h (p>0.05). After 48 h, cell proliferation rate
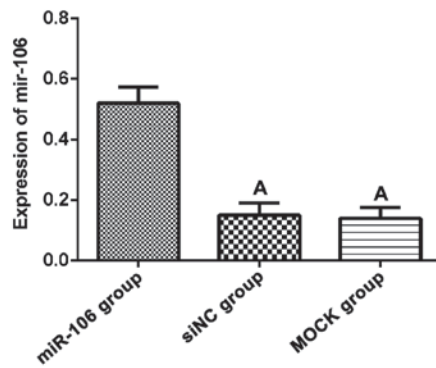
Figure 1. Expression of miR-106 in each group of cells. RT-qPCR results showed that the expression level of miR-106 in miR-106 group was significantly higher than that in siNC and MOCK group (p<0.05), but there was no significant differences between siNC and MOCK group (p>0.05). [A]P<0.05, compared with miR-106 group. RT-qPCR, reverse transcription-quantitative PCR.

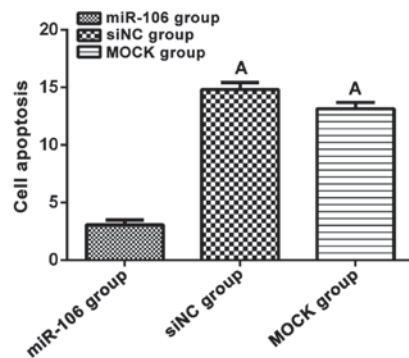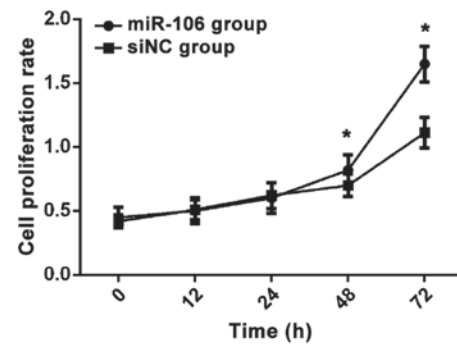Figure 3. MTT proliferation curve. Results of MTT assay showed that there was no significant difference in cell proliferation rate between the two groups at 12-24 h (p>0.05). After 48 h, cell proliferation rate in miR-106 group was significantly higher than that in siNC group. After 72 h, cell proliferation rate in miR-106 group was significantly higher than that in siNC group. [*]P<0.05.

Figure 2. Cell apoptosis. Flow cytometry was used to detect cell apoptosis of each group after transfection. Cell apoptosis of miR-106 group was significantly lower than that in the other two groups (p<0.05). [A]P<0.05, compared with miR-106 group.

in miR-106 group was significantly higher than that in siNC group (p<0.05, Fig. 3).

Scratch assay results. By observing the width of scratched wounds under an inverted light microscope, cell migration of miR-106 group was significantly accelerated compared with siNC group (p<0.05).

Discussion

Endometrial cancer accounts for ~10% of female malignancies, and onset age of this disease is becoming increasingly younger (12). Early stage of endometrial cancer has no obvious symptoms. Many patients were diagnosed at middle or advance stages and the best treatment timing was missed, which in turn leads to poor prognosis (13). Therefore, identification of gene targets and development of targeted therapeutic drugs for endometrial cancer is of great clinical significance. It has been proved that (14-16) proliferation, apoptosis, and invasion of cancer cells significantly affect the prognosis of patients. Proliferation, apoptosis and invasion of cancer cells is a multi-step and multi-factor involved biological process. In recent years, miRNAs have been shown to inhibit the translation of mRNA in almost all species (17,18). Jonas and

Izaurralde (19) demonstrated that miRNAs are involved in all biological processes in the body and have a strong regulatory role in normal cellular function (20). miRNAs participate in the occurrence and development of multiple tumors by regulating the proliferation, migration, apoptosis, and angiogenesis of tumor cells (20). Zheng *et al* (10) demonstrated that miR-106 promoted cancer cell proliferation in endometrial cancer. Therefore, we studied the effect of miR-106 on the proliferation and apoptosis of endometrial cancer RL95-2 cells with an expectation of providing references for targeted gene therapy.

miR-106 is located on chromosome Xq26.2 and consists of 23 nucleotides and is upmethyl thiazolyl tetrazoliumated in various tumors (21). In this study, we successfully constructed a miR-106 eukaryotic expression vector and successfully transfected it into endometrial cancer RL95-2 cells to detect its expression and biological functions. Expression of miR-106 in RL95-2 cells transfected with miR-106 was significantly higher than that in siNC and MOCK group (p<0.05). Wang *et al* (22) demonstrated that miR-106 enhances the self-renewal ability of glioma cells and the invasion ability of glioma stem cells by inhibiting the expression of matrix metalloproteinase-2 (TIMP-2). MMT assay and flow cytometry results also showed that miR-106 significantly affected apoptosis of RL95-2 cells, and miR-106 can effectively promote proliferation of endometrial cancer RL95-2 cells and inhibit cell apoptosis.

There are still deficiencies in this study. Due to the limited experimental conditions, in-depth investigation on the mechanism of the function of miR-106 in patients with endometrial cancer was not performed. Only *in vitro* experiments were performed and *in vivo* validation is lacking. Diagnostic and prognostic values of miR-106 for endometrial cancer were not evaluated.

In summary, miR-106 overexpression can promote the proliferation of endometrial cancer RL95-2 cells and inhibit cell apoptosis. miR-106 shows promise as a new target for the treatment of endometrial cancer.

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