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Original Article

Mechanism of miRNA-26a on the Proliferation of Pancreatic Tumor Cells by Regulating the Expression of Cycline2

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Abstract

Background: qRT-PCR was used to measure the expression of miRNA-26a in pancreatic epithelial cells (HPDE) and human pancreatic cancer cell lines PANC-1 and MIA PaCa-2.

Methods: PANC-1 and MIA PaCa-2 cell lines were infected with lentiviruses to construct PANC-miR-26a and MIA-miR-26a, and RT-PCR was used to detect the infection efficiency. The cell proliferation ability of PANC-miR-26a and MIA-miR-26a were examined by CCK-8 assay, and apoptosis was detected by flow cytometry. Western blotting was used to detect the expressions of CyclinE2 protein and mitochondria-associated apoptotic proteins.

Results: miR-26a was expressed in human normal pancreatic epithelial cells (HPDE), and not detected in PANC-1 and MIA PaCa-2; miR-26a was highly expressed in the cell lines PANC-miR-26a and MIA-miR-26a infected by the virus particles. The absorbance values of PANC-miR-26a and MIA-miR-26a were lower than those of NC1 and PANC-1 in control group. The apoptosis rates of PANC-miR-26a and MIA-miR-26a were substantially higher than those of the control group. The overexpression of miR-26a inhibited the expression of the target protein CyclinE2 in PANC-miR-26a and MIA-miR-26a. The expression of the anti-apoptotic protein Bcl-2 was decreased in PANC-miR-26a and MIA-miR-26a, while the expression of the pro-apoptotic protein Bax was increased. Compared with HPDE, miR-26a was down-regulated in PANC-1 and MIA PaCa-2. After overexpression of miR-26a, the proliferation of PANC-1 and MIA PaCa-2 cell lines was weakened.

Conclusion: Molecular mechanism is the negative regulation of CyclinE2 by miR-26a as well as the expressions of downstream mitochondrial apoptosis proteins Bcl-2 and Bax.

Keywords: Cycline2; miRNA-26a; Pancreatic cancer

Introduction

Pancreatic cancer, as one of malignant tumors, is common in the digestive system (1). It has a series of characteristics, including strong invasiveness, low early diagnosis rate, etc. (2). The progression of pancreatic cancer is a multi-stage, multi-factor and multi-gene interaction complex process (3).

The discovery of specific molecular markers and alternative molecular targeted therapies for early diagnosis is in urgent need (4, 5). MicroRNAs have approximately 18-22 nucleotides in length (6). The discovery and research of microRNAs has been selected as a technological breakthrough by Science and Nature. Endogenous small molecule RNA has been shown to bind by partially/completely pairing with the 3' untranslated region of the target protein mRNA. Thereby, the degradation of the target protein mRNA is pro-

moted, and the translation of the target protein is inhibited. The regulated expression of the related genes is achieved after transcription to affect various cell (7, 8).

Since the discovery of microRNAs for the first time in 1993, more and more microRNAs and their targets and corresponding functions have been studied (9). MicroRNAs were found to contribute to the process of tumor cell proliferation, invasion and metastasis and apoptosis (10, 11). MiRNA-26a is a microRNA located at the 3P21.3 site on human chromosome 3. The place of its location is a chromosomal instability site (12). MiRNA-26a has different expression levels in various tumor tissues, and it acts as a tumor suppressor gene in chronic lymphocytic leukemia (13). In glioma, it serves as a proto-oncogene (14). Studies on miRNA-26a include cancer survival, sensitivity to drugs, precancerous lesions, and changes in transcriptional patterns (15, 16). The involvement of miRNA-26a in the progression of pancreatic cancer and its functions in pancreatic cancer remain to be further studied.

We aimed to provide a theoretical basis for the in-depth understanding of the molecular mechanisms and targeted molecular therapies of pancreatic cancer.

Methods

Cell culture

Pancreatic epithelial cells HPDE, human pancreatic cancer cell line PANC-1 and MIA PaCa-2 were placed in DMEM medium containing 10% fetal calf serum in a 37 °C, 5% CO_2 incubator. Cells were digested with 0.25% trypsin when grown to 70%-80% confluency.

qRT-PCR detection of miRNA-26a expression in each cell line

The total RNA of each cell strain was extracted using the TRIzol RNA extraction kit, and the total RNA concentration of each cell line was determined. A sample of A260/A280 of 1.8-2.0 was selected for subsequent experiments. Based on the instructions of the reverse transcription kit, the cDNA (Table 1, Primers synthesized by Sangon Biotech) was reversely transcribed. The reverse transcription system was subjected to a bath at 25 °C for 5 min, then was changed to 37 °C for 120 min and to 85 °C for 5 min, and then stored on ice for cooling. U6 acts as an internal reference and the expression of miRNA-26a in each group of cell lines were detected in accordance with the qRT-PCR kit instructions. The reaction conditions were: pre-denaturation at 95 °C for 1 min, 95 °C for 1 min, 60 °C for 1 min, 40 cycles; 95 °C for 15 s and 60 °C 30 s. The chain dissolution curve of the amplified product was detected at 90 °C for 15 s, and the 2-△△Ct was used to calculate the relative expression.

Table 1: Primers for reverse transcription and Real-time PCR

	Forward primer (5'-3')	*Reverse primer (5'-3')*
U6 (PCR)	CTTCGGCAGCACATATAC	GAACGCTTCACGAATTTGC
U6 (RT-CR)	GAACGCTTCACGAATTTGC	
miR-26a (PCR)	CTGTCAACGATACGCTAC	GTAATCCAGGATAGGCTG
miR-26a (RT-PCR)	GCTGTCAACGATACGCTACCTAACGGCATGACAGTGTCAGCCTA	

Lentivirus infection and RT-PCR detection of infection efficiency

When the cell lines PANC-1 and MIA PaCa-2 were cultured in an adherent manner to 70%-80%, the subculture was performed with 0.05% trypsin. Cells in each group were plated into 24 well plates at 1 x 10^5/well (3 replicate wells per kind of cell). After 24 h of culture, the original medium was replaced with 2 mL of fresh medium containing 6 ug/mL polybrene, and an appropriate amount of virus suspension was added (miRNA-26a overexpression lentiviral vector and

control vector were packaged and constructed by Open Biosystems, USA). After incubating for 24 h at 37 °C, the virus-containing medium was replaced with fresh medium. After 48 h, puromycin 2 μg/mL was added to screen the cell strain stably expressing the virus. After the cells were overgrown, the infection efficiency was detected by RT-PCR after regular communication. PANC-1 and MIA PaCa-2 cell lines infected with viral particles overexpressing miR-26a are expressed as PANC-miR-26a and MIA-miR-26a. The PANC-1 and MIA PaCa-2 cell lines contaminated with empty vector virus particles were designated as NC1 and NC2, respectively.

CCK-8 assay to detect cell proliferation

The cells of the lentivirus-infected cells and the control cells were trypsinized and then seeded in a 96-well plate at 2×10^3 cells/well (3 replicate wells per cell). After incubation for 24 hours, 10 uL/well of CCK-8 reagent was added, and incubation was continued for 2 hours. The OD value was detected by a microplate reader (measuring wavelength: 450 nm, reference wavelength: 650 nm). The detection was performed every 4 h for 4 consecutive days, and then the cell proliferation curve was plotted.

Flow cytometry to detect apoptosis

After the lentivirus-infected cells and the control cells were cultured to logarithmic growth phase, the number was adjusted to about 5×10^6 cells. The cells were harvested with trypsin (without EDTA) and harvested in a 10 ml centrifuge tube. After the addition of pre-cooled PBS buffer without calcium and magnesium ions, centrifuge at 1000 rpm for 5 min. After washing twice, the supernatant was discarded. Add 100 μL of binding buffer, incubate for 15 min in the dark, and add 5 μL of LAnnexin V-FITC and 10 μL of PI stain (MA0220, Meilun Bio, China). After 20 min in the dark, flow cytometry was used to detect.

Flow cytometry to detect cell cycle

Cells in logarithmic growth phase (about 1×10^7 cells) were digested with trypsin -EDTA (ethylene diamine tetraacetic acid) and blown into single-cell suspension. The pre-cooled PBS buffer without calcium and magnesium ions was added and centrifuged at 1000 rpm for 5 min. The cells were washed for 3 times and the supernatant was discarded. 75% ethanol precooled at -20°C (diluted with 0.01 mol/L PBS) was added, sealed immediately after mixed well, and stored in a refrigerator at 4°C. After centrifugation at 1000 rpm for 5 min, the cells were washed twice with PBS buffer, resuspended with 100 uL PBS, and added with 0.5% PI dye. The cells were incubated at 4°C for 20 min in dark and filtered with 300 mesh nylon strainer. The cell cycle was detected by flow cytometer and PI was calculated. PI=(S phase+G_2 phase)/(G_1 phase+S phase+G_2 phase)×100%.

CyclinE2 protein extraction refers to western blotting detection

After the cells of the respective groups infected with the lentivirus and the control cells were cultured and expanded in a 6-well plate, the culture solution was discarded. Wash three times with 1 mL of PBS. 200 μL of protein lysate (100:1 protease inhibitor) was added and incubated for 2 h at 37 °C. The supernatant (without cells) was pipetted into an EP tube and centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatant is the protein extract. The protein extracted from each group of cells was quantified according to the BCA protein quantification kit, and the total protein of each group was adjusted to 10 μg. After the addition, the cells were electrophoresed on a 10% SDS-PAGE, transfected onto a PVDF membrane by a wet transfer method, and sealed with 1×PBS containing 5% skim milk powder for 4 h at room temperature. The blocking solution was diluted at 1:1000 with the corresponding antibody (CyclinE2 antibody, YM-Y5281R, Yuanmu Bio, China, and Actin antibody, LS-B11095, LSBio, USA), and the membrane was placed in the primary antibody dilution and incubated overnight at 4 °C. The membrane was washed 5 min x 3 times with TBST. We set the membrane in a secondary antibody dilution (1:3000 diluted mouse anti-rabbit IgG, LS-C60914, LSBio, USA) for 2 h at room tempera-

ture. After the membrane was washed with TBST for 5 min × 3 times, develop with ECL (Amersham).

Extraction of mitochondria-associated apoptotic proteins and detection by western blotting

After the cells of the respective groups infected with the lentivirus and the control cells were cultured and expanded in a 6-well plate, the cells of each group were collected and centrifuged at 600 g for 4 minutes at 4 °C, and the supernatant was discarded. After resuspending 10 mL of 4 °C pre-cooled PBS, the supernatant was discarded. We resuspended the cells in 1 mL of cytosolic buffer and placed on ice for 10 min. After grinding in a pre-cooled grinder for 10-20 min, transfer the liquid to a centrifuge tube and centrifuge at 600 g for 10 min at 4 °C. The supernatant was centrifugated at 10000 g 4 °C for 35 min. The supernatant was the cytosolic protein. After the collected precipitate was dissolved in 100 uL of mitochondrial buffer solution, the mitochondrial protein solution was obtained by blowing the tip of the gun. Western blotting was used to detect the mitochondrial protein expression in each group. The corresponding antibodies were diluted at 1:1500 in blocking solution (rabbit anti-human Bcl-2, LS-B6548, LSBio, USA; rabbit anti-human Bax, LS-C210311, LSBio, USA and rabbit anti-human Actin antibody). The secondary antibody was diluted in blocking solution (1:3000, murine anti-rabbit IgG, LS-C60914, LS Bio, USA).

Detection and analysis of dual-luciferase reporter system

Cells in logarithmic growth phase (about 1×10^7 cells) were washed with PBS and digested with 0.25% trypsin. After the cells became round and lost their ability to attach to the wall, they were blown into single-cell suspension and counted. 2×10^4 cells per well were inoculated in 12-well culture plates, thoroughly mixed, and cultured in an incubator at 37°C for 24 h. When the degree of cell fusion reached 90%, there were 3 duplicate tuples in each group, PANC-1+miR-26a mimics, PANC-1 +miR-26a mimics NC, MIA-miR-26a +miR-26a mimics and MIA-miR-26a+miR-26a mimics NC. After transfection for 48 h, the cells were washed twice with PBS, added with cell lysate (80 ul/well), and standing at room temperature for 15 min. After centrifuged at 12000 g for 5 min, the lysate was placed into the EP_tube. The lysate was placed in 1.5 ml EP tube, added with 40 uL LARII and 5 uL sample, fully mixed for 10 s, and then put into a microporous plate luminometer through a multi-functional microplate reader Tecan Infinite F200/M200. Luciferase Reporter Assay System (Promega) was used to detect fluorescence intensity, after recording relevant data, the EP tube was removed, 40 uL Stop&Glo substrate was added and fully mixed for 10 s. Fluorescence intensity was detected and the ratio of the two was standardized.

Statistical processing

All data were analyzed by SPSS 23.0 (Chicago, IL, USA), and the significance test was performed using independent sample t-test and ANOVA. $P < 0.05$ was considered to be a significant difference.

Results

qRT-PCR detection of miRNA-26a expression

The expression of miR-26a was significantly lower in human pancreatic cancer cell lines PANC-1 and MIA PaCa-2 lower than that in the HPDE cell line ($P<0.05$) (Fig. 1).

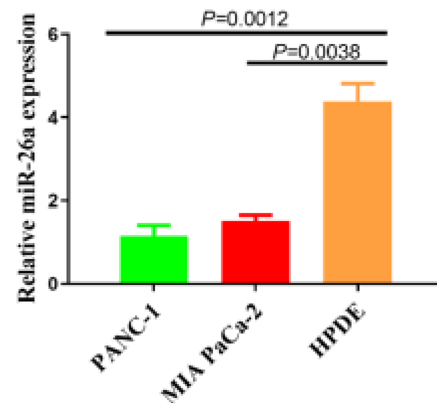


Fig. 1: Expression of miR-26a in each cell line

RT-PCR detection of lentivirus infection efficiency

According to Fig. 2A and Fig. 2B, miR-26a was highly expressed in PANC-miR-26a and MIA-miR-26a, and the expression level of miR-26a in NC1, NC2 and blank control cell lines (PANC-1 and MIA PaCa-2) were low (P <0.05).

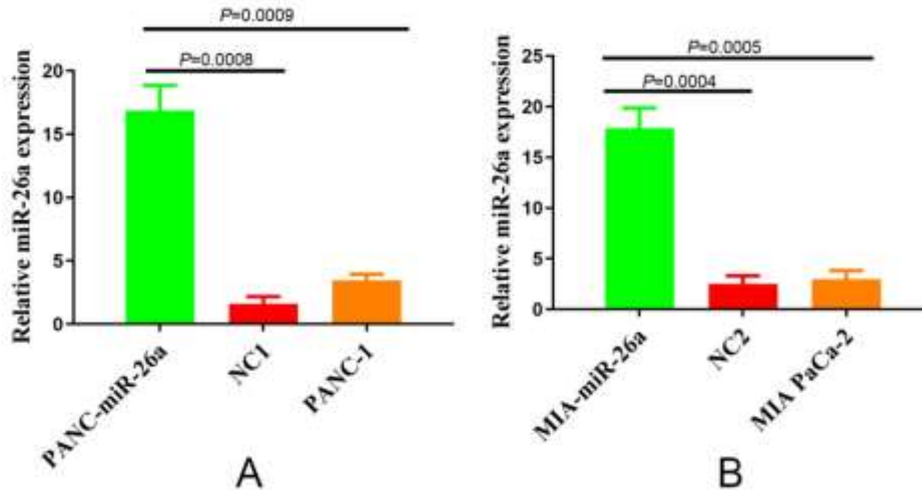


Fig. 2: Expression of miR-26a in each cell line after lentivirus infection. **A:** PANC-1 cell line; **B:** MIA PaCa-2 cell line

CCK-8 assay to detect cell proliferation

At 72 h and 96 h, the absorbance of PANC-miR-26a was substantially lower than that of controls NC1 and PANC-1 (Fig. 3A). At 96 h, the absorbance of MIA-miR-26a was also radically lower than that of NC2 and MIA PaCa-2 (Fig. 3B). Therefore, it can be concluded that the cell lines PANC-1 and MIA PaCa-2 overexpressing miR-26a grow slowly. The growth of the two pancreatic cancer cells was inhibited, and the growth cycle was slowed down.

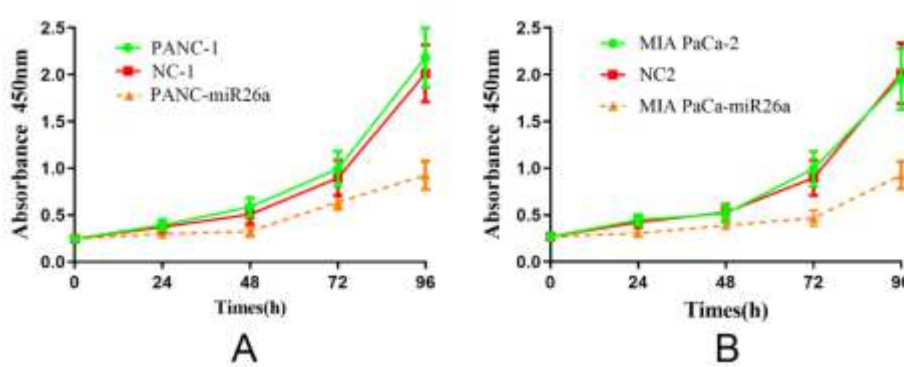


Fig. 3: Proliferation of individual cell lines after lentivirus infection. **A:** PANC-1 cell line; **B:** MIA PaCa-2 cell line

Flow cytometry to detect apoptosis

Compared with PANC-1 and NC1, the apoptotic rate of pancreatic cancer cell PANC-miR-26a overexpressing miR-26a was substantially increased (P<0.05) (Fig. 4B). The gene miR-26a can promote the apoptosis of pancreatic cancer

cell PANC. Compared with NC2 and MIA PaCa-2, the apoptotic rate of pancreatic cancer cell MIA-miR-26a overexpressing miR-26a was radically increased (*P*<0.05) (Fig. 4C).

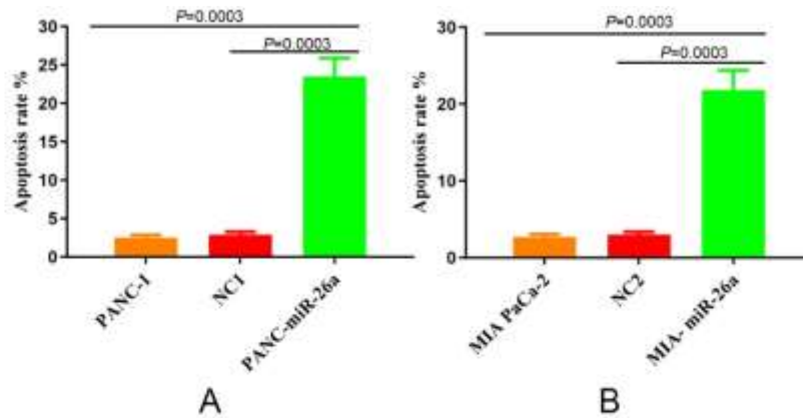


Fig. 4: Apoptosis rate of each cell line after lentivirus infection. **A:** PANC-1 cell line; **B:** MIA PaCa-2 cell line

Flow cytometry to detect cell cycle

As shown in Figure 5, the cell cycle of cell line overexpressing miR-26a changed compared with the control group, and the cells were mainly blocked in G1 phase, with the proportion of G1 phase increased by about 10% compared with the control group, indicating that miR-26a inhibited the cycle of pancreatic cancer cells, and the cells were blocked in G1 phase.

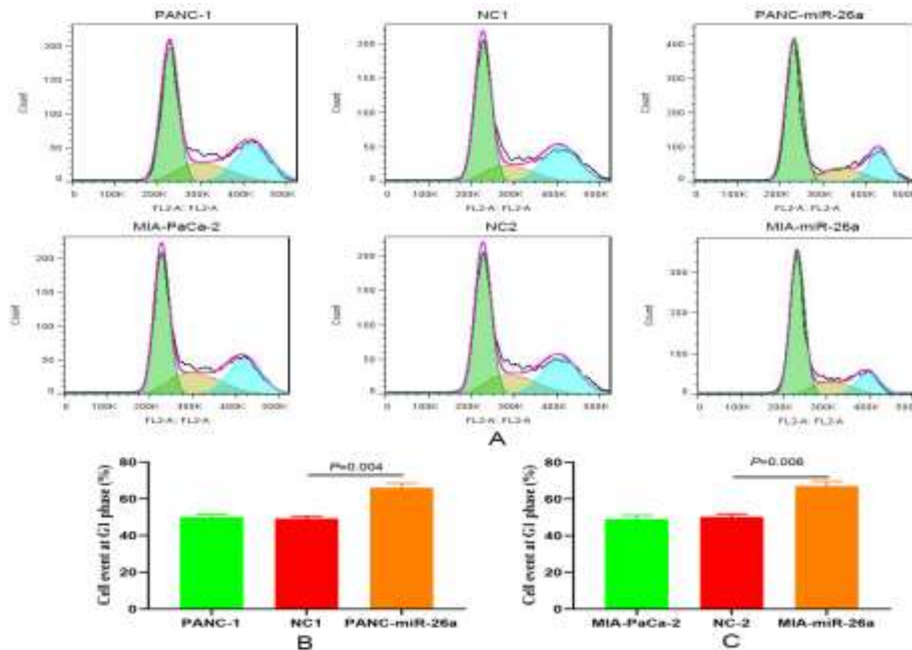
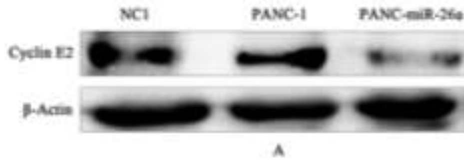


Fig. 5: Expression of CyclinE2 protein in cell cycle after lentivirus infection.
A: Representative results of cell cycle test; **B:** PANC-1 cell line; **C:** MIA PaCa-2 cell line; **D:** The expression of CyclinE2 protein in cells detected by Western blot

Effect of miR-26a overexpression on the expression of target protein CyclinE2

Western blotting results showed that pancreatic cancer cell line PANC-miR-26a overexpressing miR-26a inhibited CyclinE2 protein expression compared to PANC-1 and NC1 (Fig. 6A). The expression level of CyclinE2 protein in pancreatic cancer cell line MIA-miR-26a overexpressing miR-26a was also lower than that of NC2 and MIA PaCa-2 (Fig. 6B).



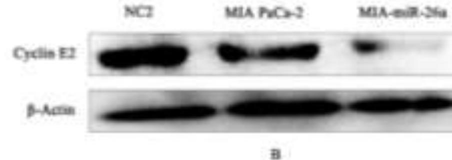
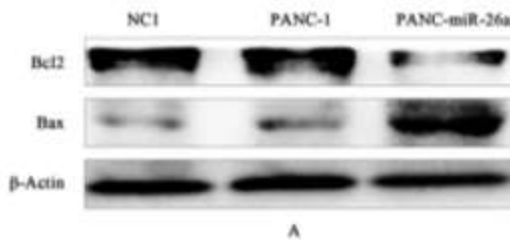


Fig. 6: Expression of CyclinE2 protein in each cell line after lentivirus infection. **A:** PANC-1 cell line; **B:** MIA PaCa-2 cell line

Effect of miR-26a overexpression on the expression of mitochondria-associated apoptotic proteins

Compared with PANC-1 and NC1, pancreatic cancer cell PANC-miR-26a overexpressing miR-26a inhibited the expression of anti-apoptotic protein Bcl-2 but promoted the expression of the pro-apoptotic protein Bax (Fig. 7A). Compared with NC2 and MIA PaCa-2, in pancreatic cancer cell line MIA-miR-26a overexpressing miR-26a, the expression level of the anti-apoptotic protein Bcl-2 was lower, and the expression level of the proapoptotic protein Bax was higher (Fig. 7B).



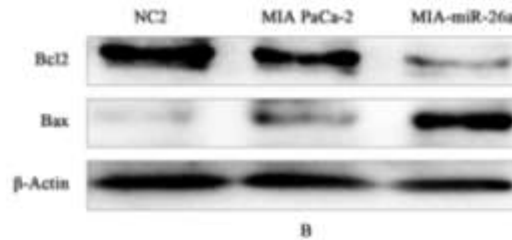


Fig. 7: Expression of related apoptotic proteins in each cell line after lentivirus infection. **A:** PANC-1 cell line; **B:** MIA PaCa-2 cell line

Discussion

Reports on miRNAs started in 1993, and since then researchers have continued in-depth and extensive miRNA research (6). MiRNAs was involved in the progression of various cancers (17, 18). MiR-26a was reported to involve in the metastasis and differentiation of cells of pancreatic cancer, glioma, and bladder cancer, and miR-26a has been confirmed to target Ezh2, Cyclin D2 and Cyclin E2 in tumors (19).

The association between the expression level of miR-26a and the progression of pancreatic cancer (20) and its potential for the treatment of pancreatic cancer (21, 22) were studied in this study. qRT-PCR (23) was used to confirm that the expression of miR-26a was down-regulated in pancreatic cancer cell lines PANC-1 and MIA PaCa-2. The results of RT-PCR indicated that miR-26a was stably overexpressed in the PANC-miR-26a and MIA-miR-26a cell lines, demonstrating that these two cell lines can be used in subsequent experiments. The results of CCK-8 assay showed that compared with the control group, their cell cycle was also blocked, which led to a decrease in proliferative ability. The apoptosis rate of these two cell lines was higher than that of the control group.

The results of cell cycle detection by flow cytometry demonstrated that the overexpression of miR-26a promoted the arrest of pancreatic cancer cell lines PANC-1 and MIA PaCa-2 in G1 phase and the cell cycle arrest of the two cell lines affects the proliferation of pancreatic cancer cells, suggesting that miR-26a may regulate cell cycle by acting on cell cycle factors and affect cell proliferation. Western blotting experiments showed that the expression of Cyclin E2 protein in pancreatic cancer cell lines PANC-miR-26a and MIA-miR-26a overexpressing miR-26a was decreased. Cyclin E2 is a key protein that determines the G1/S progression in the cell cycle (24). MiR-26a affects the proliferative capacity of pancreatic cancer cells PANC-1 and MIA PaCa-2 by negatively regulating CyclinE2, consistent with the previous report that the miR-26a overexpression vector can inhibit the expression of Cyclin E2 and Cyclin D2 in liver cancer cells (19).

There are approximately 100 targets per mi RNA, which form a regulatory network, regulating protein-coding genes (25). Therefore, cyclin E2 is most likely only one of the many regulated proteins of miR-26a. Western blotting of mitochondria-associated apoptotic proteins showed that compared with the control groups, in PANC-miR-26a and MIA-miR-26a, the expression levels of the anti-apoptotic protein Bcl-2 is lower, and the expression level of the pro-apoptotic protein Bax is higher (26, 27). This result demonstrates that pancreatic cancer cells overexpressing miR-26a affect the expression of mitochondrial apoptosis proteins Bcl-2 and Bax, and thus affect the proliferation of pancreatic cancer cells PANC-1 and MIA PaCa-2. Overexpression of miR-26a activates pancreatic cancer cells PANC-1 and MIA PaCa-2 to promote apoptosis through the mitochondrial apoptotic pathway (28). Studies have confirmed the lower expression of miR-26a in liver cancer, thyroid cancer, bladder cancer, oral squamous cell carcinoma, nasopharyngeal carcinoma, and breast cancer than in its normal tissues (29-34). Moreover, overexpression of miR-26a causes the decreased proliferation of the corresponding tumor cells, the increased apoptotic rate, and changes in the progression of the cell cycle.

MiR-26a could induce cell cycle arrest in liver cancer and inhibit the proliferation of liver cancer cells (35). According to the study on nasopharyngeal carcinoma (36), transfection of miR-26a can induce G1 arrest in nasopharyngeal carcinoma cells, inhibit proliferation and colony formation of nasopharyngeal carcinoma cells. In the transfection of miR-26a in lymphoma cells, Sander et al (37) found that overexpression of miR-26a leads to a decrease in my-induced lymphocyte proliferation and impaired cycle progression, consistent with the results of this study. The results confirmed that in these tumors, miR-26a could change the cell cycle, block cells, inhibit cell proliferation, and have great contributions in tumor suppressor genes in the progression of tumors.

Conclusion

Therefore, miR-26a also acts as a tumor suppressor gene in the progression of pancreatic cancer. However, due to its various targets and complex regulatory network, the role and molecular mechanism of miR-26a in pancreatic cancer still needs further research. As a specific molecular marker for early diagnosis of pancreatic cancer and a target for alternative molecular targeted therapy, further research is needed.

Ethical considerations

Ethical issues (including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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There is no financial source of this study.

Conflicts of interest

The authors declare that there is no conflict of interest.

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