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MiR-223 regulates CDDP resistance in pancreatic cancer *via* targeting FoxO3a

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Abstract. – OBJECTIVE: FoxO3a is a well-defined tumor suppressor gene in the forkhead transcription factor O subfamily (FoxO), and its reduction is related to the occurrence of various tumors. It was found that miR-223 expression is abnormally elevated in pancreatic cancer tissues. Bioinformatics analysis revealed a targeted complementary binding relationship between miR-223 and FoxO3a. This study explored whether miR-155 regulates the expression of FoxO3a and affects the proliferation, apoptosis, and cisplatin (CDDP) resistance of oral cancer cells.

MATERIALS AND METHODS: Dual-Luciferase reporter gene assay validated the targeted relationship between miR-223 and FoxO3a. The CDDP-resistant pancreatic cancer cell line BXPC3/CDDP was established, and the expressions of miR-223 and FoxO3a were compared. BXPC3/CDDP cells were divided into miR-NC group and miR-223 inhibitor group. QRT-PCR was adopted to test miR-223 and FoxO3a mRNA expressions. Western blot was performed to determine FoxO3a protein expression. Cell apoptosis was detected by flow cytometry and cell proliferation was detected by EdU staining.

RESULTS: There was a targeted regulatory relationship between miR-223 and FoxO3a mRNA. The expression of miR-223 was significantly higher, while the expression of FoxO3a mRNA and protein was significantly lower in BXPC3/CDDP cells than that in BXPC3 cells. Cell Counting Kit-8 (CCK-8) experiments showed that the same concentration of CDDP exhibited significantly lower proliferation inhibition in BXPC3/CDDP cells than BXPC3 cells. Compared with miR-NC group, transfection of miR-223 inhibitor significantly increased the expression of FoxO3a in BXPC3/CDDP cells, which significantly attenuated cell proliferation and enhanced apoptosis in CDDP-treated cells.

CONCLUSIONS: Increased expression of miR-233 was associated with CDDP resistance in pancreatic cancer cells. Inhibition of miR-223 expression upregulated FoxO3a expression, restrained pancreatic cancer cell proliferation, promoted cell apoptosis, and enhanced CDDP sensitivity in pancreatic cancer cells.

Key Words:
Pancreatic cancer, CDDP, Resistance, MiR-223, FoxO3a.

Introduction

Pancreatic cancer (PC) is a digestive system malignant tumor with high degree of malignancy. The treatment effect is not satisfactory and the prognosis is poor[1,2]. Chemotherapy is an important method in the treatment of pancreatic cancer, whereas drug resistance is a limiting factor affecting the efficacy of chemotherapy[3-5].

The forkhead transcription factor O subfamily (FoxO) is a class of evolutionarily highly conserved transcription factors that are widely involved in the regulation of various biological processes, such as embryonic development, cell proliferation, cell cycle, and apoptosis. FoxO3a is a tumor suppressor gene in the FoxO transcription factor family[6-8]. Several studies[9-12] indicated that decreased expression and functional activity of FoxO3a is associated with the development, progression, and drug resistance of various tumors. The decreased expression and impaired functional activity of FoxO3a has also been confirmed to be closely related to the occurrence and progression of pancreatic cancer[13-15].

MicroRNA is an endogenous non-coding small-molecule single-stranded RNA of eukaryotes with a length of about 22-25 nucleotides, which complementarily binds to the 3'-UTR of the target gene mRNA to degrade or inhibit the translation. MicroRNAs, which account for 1% of human genes, regulate the expression of more than 30% of human genes[16]. MicroRNA abnormalities and altered expression of target gene expression play a crucial role in tumorigenesis[17,18]. It was shown that elevated expression of miR-223 is associated with the onset and resistance

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of pancreatic cancer[19-21]. Bioinformatics analysis revealed a targeted complementary binding site between miR-223 and FoxO3a. This study explored whether miR-155 regulates the expression of FoxO3a and affects the proliferation, apoptosis, and cisplatin (CDDP) resistance of oral cancer cells.

Materials and Methods

Main Reagents and Materials

Human normal pancreatic epithelial cell HPDE6-C7, pancreatic cancer cell BXPC3, and HEK293T cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM), Opti-MEM medium, and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). TRIzol and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA). QuantiTect SYBR Green RT-PCR Kit was purchased from Qiagen (Germantown, MD, USA). MiR-223 mimic, miR-223 inhibitor, and miR-NC were designed and synthesized by Ribobio (Guangzhou, China). Rabbit anti-human FoxO3a monoclonal antibody was purchased from Abcam (Cambridge, MA, USA). Rabbit anti-human β-actin polyclonal antibody and goat anti-rabbit HRP-conjugated secondary antibody were purchased from Sangon (Shanghai, China). Annexin V/PI apoptosis detection kit and EdU cell proliferation detection reagent were purchased from Beyotime (Nantong, China). Dual-Glo Luciferase Assay System, pGL3 vector was purchased from Promega (Madison, WI, USA). CDDP was purchased from Medchemexpress (Monmouth Junction, NJ, USA). CCK-8 was purchased from Dojindo Laboratories (Shanghai, China).

Cell Culture

HPDE6-C7 and BXPC3 cells were inoculated in DMEM medium containing 10% FBS and 1% streptomycin at 5% CO_2 and 37°C. The cells were passaged at 1:4 or 1:5 and used for experiments at logarithmic growth phase.

CDDP Drug Resistant Cell Model Establishment

BXPC3 cells in logarithmic phase were treated by CDDP from 0.2 μg/mL for 2 weeks. Then, cells kept growing and were treated by increased concentration of CDDP up to 1.6 μg/mL when the cells can stably grow in CDDP. At last, the cells can be stably passaged in CDDP to obtain CDDP resistant BXPC3/CDDP cell line.

BXPC3 and BXPC3/CDDP cells were treated by different concentrations (0, 0.01, 0.1, 1, 10, 100, and 1000 μg/mL) of CDDP for 48 h. Next, the cells were added with CCK-8 to measure the absorbance value (A450). Inhibition rate = (1-A450 in drug group)/A450 in control × 100%. IC50 was calculated using SPSS software. Resistance index (RI) = IC50 of drug resistant cell/IC50 of parent cell.

Dual-Luciferase Reporter Gene Assay

The PCR product of the FoxO3a 3'-UTR full-length fragment or mutant fragment was double-digested and then ligated into the pGL3 vector. After sequencing, the plasmid was designated as pGL3-FoxO3a-WT and pGL3-FoxO3a-MUT. The HEK293T cells were transfected with pGL3-FoxO3a-WT (or pGL3-FoxO3a-MUT) together with miR-223 mimic (or miR-NC) by Lipo 2000. After incubated for 48 h, luciferase activity was detected by Dual-Glo Luciferase Assay System kit according to the manual instructions.

Cell Transfection and Grouping

BXPC3/CDDP cells were divided into miR-NC group and miR-223 inhibitor group. The cells were seeded in 6-well plate and treated with 3.2 μg/mL CDDP for 48 h. Next, the cells were tested on Beckmann FC500 MCL flow cytometry to evaluate cell apoptosis. The cells were digested and incubated with 10 μM EdU for 2 h. Next, the cells were treated with 3.2 μg/mL CDDP for 48 h. Then, the cell proliferation was determined by EdU staining kit according to the manual.

QRT-PCR

TRIzol reagent was used to extract RNA and reversely transcribe RNA to cDNA. The qRT-PCR system contained 10.0 μL 2×QuantiTect SYBR Green RT-PCR Master Mix, 1.0 μL 0.5 μm/L primers, 2 μg template RNA, 0.5 μL QuantiTect RT Mix, and ddH_2O. The reaction was performed on Bio-Rad CFX96 with conditions: 45° C for 5 min, 94° C for 30 s, followed by 40 cycles of 95° C for 5 s, and 60° C for 30 s. The primer sequence was miR-223: Forward-5'-GTGCAGGGTCCGAGGT-3′ and Reverse-5′-CGGGCTGTCAGTTTGTCA-3′; FoxO3a: Forward-5'-TTCAAGGATAAGGGCGACAGCAAC-3′ and Reverse-5′-CTGCCAGGCCACTTGGAGAG-3′.

Western Blot

Total protein was extracted from the cells by Radioimmunoprecipitation Assay (RIPA). After quantified by the bicinchoninic acid (BCA) method, a total of 50 μg protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane at 250 mA for 100 min. After blocked with 5% skim milk at room temperature, the membrane was incubated with primary antibody at 4° C overnight (FoxO3a 1:1000 and β-actin 1:8000). After washed by Phosphate-Buffered Saline and Tween (PBST), the membrane was further incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 60 min (1:10000). At last, the membrane was treated by enhanced chemiluminescence (ECL) reagent and developed.

Cell Apoptosis Detection

The cells were digested by trypsin and collected. After resuspended in 100 μl binding buffer, the cells were added with 5 μl Annexin V-FITC and 5 μl Propidium Iodide (PI) at room temperature avoid of light for 15 min. Then, the cells were tested on flow cytometry.

Cell Proliferation Detection

The cells were added with EdU solution at 10 μM in logarithmic phase. After incubated for 2 h, the cells were seeded for 48 h and digested by trypsin. After fixed in paraformaldehyde, the cells were incubated with 100 μL TritonX-100 at room temperature and in 500 μL reaction fluid at room temperature avoid of light for 30 min. At last, the cell was tested on flow cytometry.

Statistical Analysis

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was applied for data analysis. Measurement data were presented as mean ± standard deviation and compared by *t*-test or one-way ANOVA with Bonferroni post-hoc analysis. $p < 0.05$ was depicted as significant difference.

Results

The Targeted Regulatory Relationship Between MiR-223 and FoxO3a mRNA

Bioinformatics analysis showed the complementary binding site between miR-223 and the 3'-UTR of FoxO3a mRNA (Figure 1A). Dual-Luciferase reporter gene assay exhibited that miR-223 mimic transfection significantly reduced the relative luciferase activity in HEK293T cells transfected by pGL3-FoxO3a-WT but not by pGL3-FoxO3a-MUT, showing that FoxO3a was the target gene of miR-223 (Figure 1B).

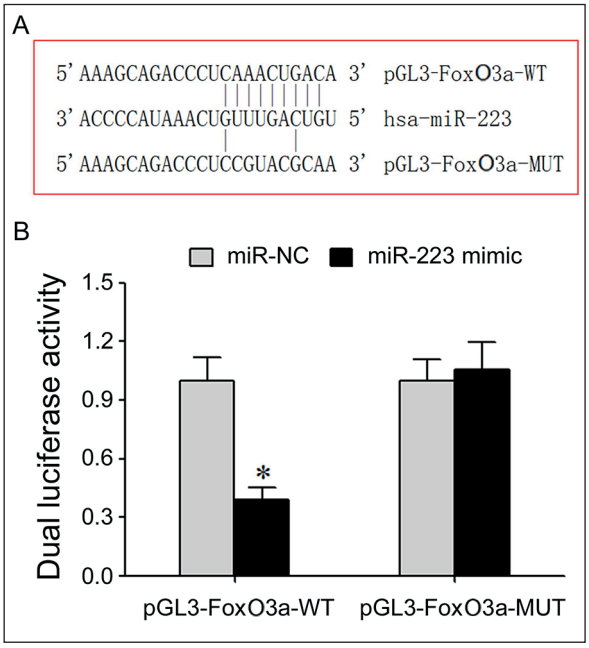

Figure 1. The targeted regulatory relationship between miR-223 and FoxO3a. **A,** Complementary binding site between miR-223 and the 3'-UTR of FoxO3a mRNA. **B,** Dual-Luciferase reporter assay. *$p < 0.05$, compared with miR-NC.

BXPC3/CDDP Cells Exhibited Strong Proliferation and Drug Resistance

CCK-8 assay detected that the proliferative activities of parental BXPC3 cells were significantly lower than those of BXPC3/CDDP cells under the same dose treatment of CDDP (Figure 2). The IC_{50} of BXPC3 cells was 1.95 ± 0.18 μg/mL, while it was 24.26 ± 2.13 μg/mL in the drug-resistant BXPC3/CDDP cells, leading the RI of BXPC3/CDDP cells at 12.44 (Table I).

MiR-223 Upregulated, While FoxO3a Decreased in Drug Resistant Cell

QRT-PCR demonstrated that compared with human normal pancreatic epithelial cells, miR-223 expression was significantly elevated in BXPC3 cells, and its level was significantly higher in BXPC3/CDDP cells than that of parental BX-

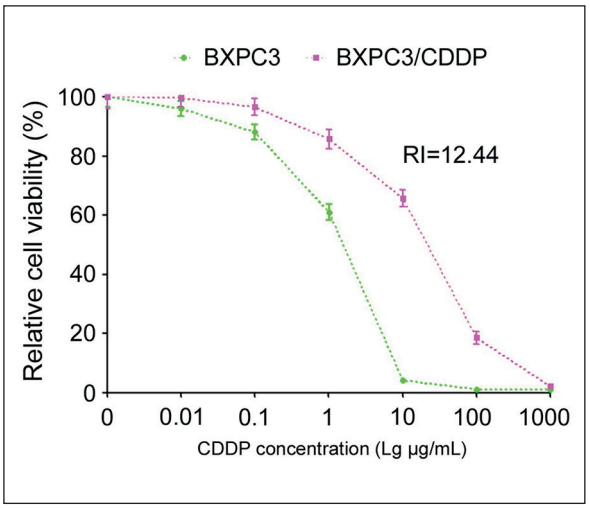

Figure 2. CCK-8 detection of BXPC3 and BXPC3/CDDP cell viabilities.

Table I. RI comparison.

Cell	IC50	RI
SKOV3	1.95	12.44
SKOV3/CDDP	24.26	

PC3 cells (Figure 3A). QRT-PCR revealed that the FoxO3a mRNA expression in BXPC3 cells was significantly lower than that of normal oral epithelial cells, and it was apparently lower in BXPC3/CDDP cells compared with the parental BXPC3 cells (Figure 3B). Western blot exhibited that compared with normal pancreatic epithelial cells, FoxO3a protein expression in BXPC3 cells was significantly downregulated, and its level in BXPC3/CDDP cells was significantly attenuated (Figure 3C).

MiR-223 Downregulation Promoted BXPC3/CDDP Cell Apoptosis and Enhanced CDDP Sensitivity

QRT-PCR showed that miR-223 inhibitor transfection significantly downregulated miR-223 expression and elevated FoxO3a mRNA level in BXPC3/CDDP cells compared with miR-NC group (Figure 4A). Western blot demonstrated that miR-223 inhibitor transfection significantly elevated FoxO3a protein level in BXPC3/CDDP cells (Figure 4B). Flow cytometry revealed that transfection of miR-223 inhibitor significantly enhanced cell apoptosis, while inhibited cell proliferation in BXPC3/CDDP cells (Figures 4C and 4D).

Discussion

Pancreatic cancer is characterized by high malignancy, high mortality, and poor prognosis. Its 5-year survival rate is less than 3%, and the average survival time is only about half a year, thus is one of the malignant tumors with worst prognosis[22,23]. Chemotherapy is an important method in the treatment of pancreatic cancer, whereas drug resistance is an unfavorable factor affecting the effect of chemotherapy[3-5]. Therefore, exploring the mechanism of chemotherapy drug resistance in pancreatic cancer has important clinical significance for increasing the effect of chemotherapy, guiding individualized treatment, and improving the prognosis of patients.

FoxO3a is the core member of the FoxO transcription factor family. It can regulate the expression of various genes related to cell proliferation, cell cycle, and apoptosis, such as BIM, p27Kip1, and cyclin D1[24]. A number of studies[9-12]

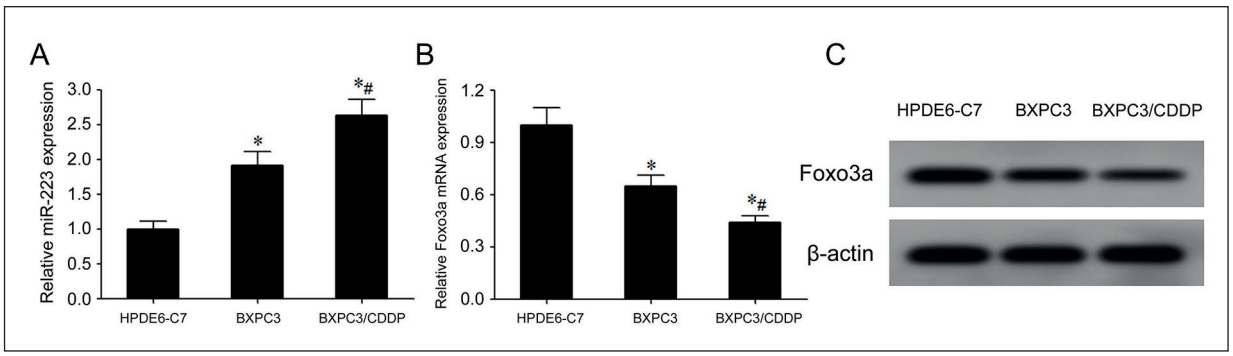

Figure 3. MiR-223 elevated, while FoxO3a downregulated in drug resistant cells. **A,** QRT-PCR detection of miR-223 expression. **B,** QRT-PCR detection of FoxO3a expression. **C,** Western blot detection of FoxO3a protein expression; * $p < 0.05$, compared with HPDE6-C7 cells; # $p < 0.05$, compared with BXPC3 cells.

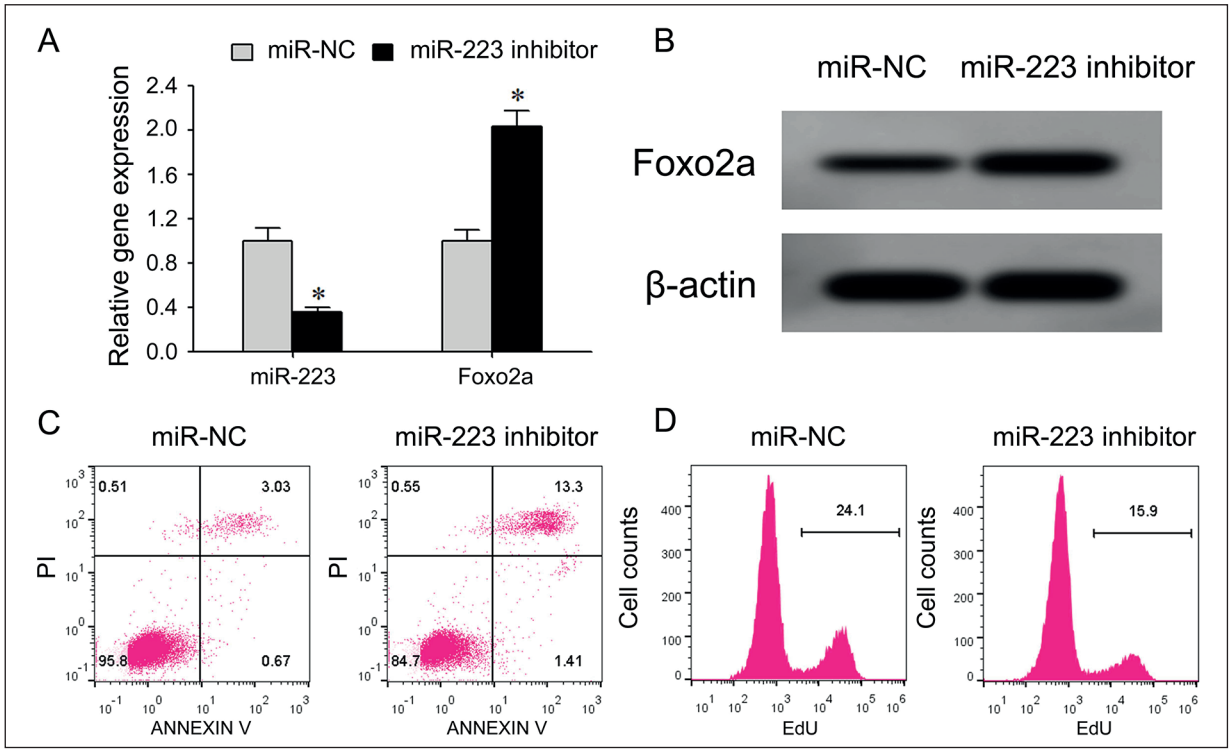

Figure 4. Downregulation of miR-223 enhanced BXPC3/CDDP cell apoptosis and CDDP sensitivity. **A,** QRT-PCR detection of miR-223 expression. **B,** Western blot detection of FoxO3a protein expression. **C,** Flow cytometry detection of cell apoptosis. **D,** Flow cytometry detection of cell proliferation; *$p < 0.05$, compared with miR-NC.

confirmed that FoxO3a is associated with the occurrence, progression, and drug resistance of various tumors, such as prostate cancer, colorectal cancer, breast cancer.

MiR-223 is a well-researched microRNA that plays an important role in the development, progression and drug resistance of lung cancer[25], intestinal cancer[26], gallbladder cancer[27], and other tumors. It was found that elevated expression of miR-223 is associated with the occurrence and resistance of pancreatic cancer[19-21]. This study explored whether miR-155 regulates the expression of FoxO3a and affects the proliferation, apoptosis, and cisplatin (CDDP) resistance of oral cancer cells.

In this study, Dual-Luciferase reporter gene assay exhibited that miR-223 mimic transfection significantly reduced the relative Luciferase activity in HEK293T cells transfected by pGL3-FoxO3a-WT but not by pGL3-FoxO3a-MUT, detecting that FoxO3a was the target gene of miR-223. CCK-8 assay showed that the proliferative activities of parental BXPC3 cells were significantly lower than those of BXPC3/CDDP cells under the same dose treatment of CDDP, revealing the successful establishment of CDDP resistant cell line. MRNA and protein assays showed that the expression of miR-223 in BXPC3 cells was significantly higher, while the expression of FoxO3a was significantly lower than that in human normal pancreatic epithelial cell HPDE6-C7, and their levels changed more significantly in BXPC3/CDDP cells than that of parental BXPC3 cells. The results indicated that elevated expression of miR-223 plays a role in reducing the expression of FoxO3a, and is not only related to the pathogenesis of pancreatic cancer, but also involved in the regulation of CDDP resistance. Debernardi et al[28] reported that the expression of miR-223 in the urine of patients with pancreatic cancer was markedly higher than that of healthy controls (p=0.035). In addition, the detection of urine miR-223 expression can also be used to distinguish between early pancreatic cancer and chronic pancreatitis. He et al[29] demonstrated that the expression of miR-223 was significantly increased in tumor tissues of patients with pancreatic cancer compared with adjacent tissues, and was positively correlated with poor prognosis (p=0.03). Komatsu et al[30] revealed

that the expression of miR-223 was abnormally elevated in pancreatic cancer tumor tissues compared with normal pancreatic tissue. In addition, compared with healthy controls, the expression level of miR-223 was also significantly upregulated in the peripheral blood plasma pancreatic cancer patients. Rachagani et al[31] observed that the expression level of miR-223 was significantly elevated in the process of spontaneously developing pancreatic cancer in genetically edited mice compared with normal mice. In this study, the expression level of miR-223 was significantly increased, and it was associated with drug resistance in pancreatic cancer, which was consistent with studies by Debernardi et al[28], He et al[29], and Komatsu et al[30].

Currently, miR-223 has not been reported to regulate the drug resistance of pancreatic cancer cells. Therefore, this study further explores whether miR-223 affects CDDP resistance of pancreatic cancer cells by regulating FoxO3a expression. The results exhibited that transfection of miR-223 inhibitor significantly increased the expression of FoxO3a in drug-resistant pancreatic cancer BXPC3/CDDP cells, which significantly enhanced cell apoptosis, attenuated cell proliferation ability, and weakened resistance to CDDP. He et al[29] showed that the expression of miR-223 was significantly increased in pancreatic cancer cell lines Aspc-1, SW1990, PANC-1, BXPC3, Hs766T, and Capan-1 compared with normal pancreatic ductal epithelial cells. MiR-223 mimic transfection into pancreatic cancer PANC-1 and BXPC3 cells promoted proliferation, migration, and invasion by targeted inhibiting FBXW7 expression. On the contrary, transfection of anti-miR-223 elevated the expression of the tumor suppressor gene FBXW7 and attenuated the proliferation, migration, and invasion of pancreatic cancer cells. Ma et al[21] revealed that the plant-derived natural compound Genistein can reduce the expression of miR-223 to upregulate the expression of its target gene FBXW7, inhibit pancreatic cancer cell proliferation and invasion, and promote cell apoptosis. Ma et al[20] found that gemcitabine-resistant pancreatic cancer Aspc-1 and PANC-1 cells can obtain strong epithelial-to-mesenchymal transition (EMT) ability. Moreover, the expression of miR-223 was significantly increased, while the expression of the target gene FBXW7 was significantly declined in the drug-resistant cells. Down-regulation of miR-223 expression reversed the EMT process of pancreatic cancer cells and attenuated cell migration and invasion. This study revealed that elevated expression of miR-223 plays a role in inhibiting FoxO3a expression, promoting pancreatic cancer cell proliferation, reducing cell apoptosis, and enhancing CDDP resistance. However, the relationship between miR-223 and FoxO3a *in vivo* is still unclear. It is necessary to detect and compare the expression differences of miR-223 and FoxO3a in the tumor tissues of drug-resistant patients and non-resistant patients.

Conclusions

Increased expression of miR-233 was associated with CDDP resistance in pancreatic cancer cells. Inhibition of miR-223 expression upregulated FoxO3a expression, restrained pancreatic cancer cell proliferation, promoted cell apoptosis, and enhanced CDDP sensitivity in pancreatic cancer cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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