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MicroRNA-136-5p regulates gemcitabine resistance in pancreatic cancer *via* down-regulating ZNF32

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Abstract. – OBJECTIVE: We explored the regulation of microRNA-136-5p on gemcitabine resistance of pancreatic cancer (PCa) and further highlighted the crosstalk between microRNA-136-5p and ZNF32, so as to provide an effective theoretical basis for target treatment of PCa.

PATIENTS AND METHODS: MicroRNA-136-5p and ZNF32 levels in tumor specimens of 48 patients with PCa were examined by reverse transcriptase-polymerase chain reaction (RT-PCR) method, and the interplay between microRNA-136-5p and clinicopathological parameters, as well as prognosis of those patients was also analyzed. Meanwhile, in gemcitabine-resistant PCa cell lines PANC-1 and CFPAC-1, microRNA-136-5p overexpression model was constructed, and cell counting kit-8 (CCK-8), transwell, as well as cell wound healing assays were carried out to assess the impact of microRNA-136-5p on PCa cell functions. Finally, Luciferase assay and recovery experiments were conducted to specify the precise underlying mechanism.

RESULTS: qRT-PCR results revealed a significant low expression of microRNA-136-5p both in tumor tissues of PCa patients and in PCa cell lines compared to the normal control groups. In addition, microRNA-136-5p mimics markedly attenuated the proliferation and migration abilities of the gemcitabine-resistant PCa cell lines. The Luciferase assay verified certain binding sites between microRNA-136-5p and ZNF32, while qPCR results indicated a negative correlation between the two in PCa tissues. Moreover, recovery experiments demonstrated that ZNF32 overexpression reversed the inhibitory effect on the malignant progression of gemcitabine-resistant PCa cells induced by microRNA-136-5p.

CONCLUSIONS: MicroRNA-136-5p can reduce the proliferation rate and metastasis ability of PCa cells *via* regulating ZNF32, and thus alleviates gemcitabine resistance in PCa cells.

Key Words:
MicroRNA-136-5p, ZNF32, PCa, Malignant progression.

Introduction

Pancreatic cancer is one of the most malignant tumors with a poor prognosis and a 5-year survival rate less than 5%[1-3]. One of the main factors leading to the poor prognosis is the lack of accurate early diagnosis. Most patients are diagnosed at advanced stage and are prone to undergo metastasis. Surgical resection does not improve survival rates, even the postoperative adjuvant chemotherapy cannot effectively prolong patient survival time, due to the extensive chemotherapy drug resistance of PCa[3-5]. For the early stage of PCa, there is no accurate and effective screening method, besides, reliable diagnostic measures are also lacking. In view of this, it is necessary to improve the early diagnostic rate of PCa, and it is of great significance to find specific genes or proteins closely related to the development of PCa[6-8]. Gemcitabine (GEM) is a pyrimidine drug for the treatment of advanced or metastatic PCa, which can be used alone or in combination with other chemotherapy drugs. Gemcitabine resistance of PCa is an important factor for patients with low survival rate[9,10]. Therefore, in-depth understanding of the molecular mechanism of gemcitabine

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resistance in PCa and identification of new markers of gemcitabine resistance in PCa are of great significance for overcoming the poor prognosis of PCa[10,11].

MicroRNA is a kind of endogenous non-coding small RNA newly discovered in recent years. Through incomplete complementary pairing with the 3 'untranslated region (3' UTR) of target gene mRNA, it mediates post-transcriptional gene regulation[12,13]. MicroRNA is highly conserved in evolution, which is encoded by genomic DNA and transcribed under the action of RNA polymerase II[14,15]. In recent years, the role of miRNA in the occurrence and development of tumors has received increasing attention[14-16]. Research has shown that the miRNAs have important roles in regulating cell proliferation, differentiation and apoptosis, etc., and their expressions are significantly different in normal tissues and tumor tissues. They play a role as tumor suppressor genes or oncogenes in the development and progression of human tumors[16,17]. By comparing the miRNA expression profiles of human breast, lung, prostate, colon, thyroid, pancreatic and bladder cancers with the expression profiles of normal tissues, it has been found that the specific miRNA expression levels in each tumor have changed, suggesting that miRNAs may serve as biomarkers for tumor diagnosis[18-20]. By referring to articles and databases, we found that microRNA-136-5p expression level was closely linked to the pathological grade and prognosis of various tumors. However, its involvement in the progression of PCa has not been reported[21-23].

In this study, we collected tissue samples from PCa patients to assess the relationship between microRNA-136-5p level and the clinical characteristics of PCa patients, and further studied the regulatory mechanism of microRNA-136-5p's involvement in gemcitabine resistance of PCa.

Patients and Methods

Patients and PCa Samples

PCa tissues were obtained from 48 pairs of pancreatic surgical resection specimens in this hospital. All patients had not been treated with drugs and/or radiation before surgery. The excised experimental specimens were immediately immersed in an Eppendorf (EP; Hamburg, Germany) tube containing the preservation solution and stored at -80°C. All tissue samples used in the experiment were clearly diagnosed by two experienced pathologists. In this study, the diagnosis and selection of pancreatic cancer patients was based on the Guidelines proposed by the Union for International Cancer Control (UICC). Patients and their families had signed informed consent. This investigation was approved by the Ethics Committee of Linyi Central Hospital.

Cell Lines and Reagents

The human PCa cell lines AsPC-1, PANC-1, MIA PaCa-2, CFPAC-1, BxPC-3 and the normal pancreatic ductal epithelial cell line HPNE [American Type Culture Collection (ATCC; Manassas, VA, USA)] were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose medium (Gibco, Rockville, MD, USA) containing 10% fetal calf serum (FCF; Gibco, Rockville, MD, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) in a 37°C, 5% CO_2 incubator. When the cells reached 80%-90% confluence, they were digested with 1×trypsin+ ethylenediaminetetraacetic acid (EDTA; Thermo Fisher Scientific, Waltham, MA, USA).

Transfection

For transient transfection, Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was mixed with NC mimic and microRNA-136-5p mimic (GenePharma, Shanghai, China).

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded in 96-well plates ($2*10^3$ cells/well) in 100 μL culture medium. CCK-8 assay (Dojindo Molecular Technologies, Kumamoto, Japan) was performed according to the manufacturer's protocol.

Transwell Assay

48 h after transfection, cells were prepared into cell suspensions and seeded in the upper chamber (50,000 cells/well) in serum-free medium, and then 10% fetal bovine serum (FBS) medium (Gibco, Rockville, MD, USA) was added to the lower compartment of the chamber. The migrated cells were counted after staining with crystal violet after washing and observed under a microscope.

Cell Wound Healing Test

Cells were digested, centrifuged, and resuspended in culture medium without FBS to adjust the density to 5×10^5 cells/mL. The density of the plated cells was determined according to the size of the cells (the majority of the number of cells plated was set to 50000 cells/well), and the confluency of the cells reached 90% or more the next day. After the stroke, cells were rinsed

Table I. Association of miR-136-5p expression with clinicopathologic characteristics of pancreatic cancer.

	Number of cases	miR-136-5p expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.939
<60	18	11	7	
≥60	30	18	12	
Gender				0.676
Male	16	9	7	
Female	32	20	12	
T stage				0.594
T1-T2	30	19	11	
T3-T4	18	10	8	
Lymph node metastasis				0.036
No	29	21	8	
Yes	19	8	11	
Distance metastasis				0.015
No	28	21	7	
Yes	20	8	12	

gently with phosphate-buffered saline (PBS) for 2-3 times and observed again after incubation in low-concentration serum medium for 24 h.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The extracted total RNA was reverse transcribed into complementary deoxyribose nucleic acids (cDNAs) using a reverse transcription kit (TaKaRa, Otsu, Shiga, Japan). Both mRNA and miRNA quantification were performed by using the SYBR Green PCR Master Mix (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal standards for mRNAs and miRNAs, respectively. The microRNA-136-5p: F: 5'-ACACTCCAGCTGGGACTCCATTTGTTTT-3', R: 5'-CCAGTGCAGGGTCCGAGGT-3'; U6: F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCG-3'; ZNF32: F: 5'-AGAATGTAGCGTTCTTCAATGTG-3', reverse: 5'-CCTGTA GTGTCTTCGAATCTGG-3'; GAPDH: F: 5'-AATTCCATGGCACCGTCAAG-3', R: 5'-TGGACTCCACGACGTACTC-3'.

Western Blot

Protein extraction was performed using a radioimmunoprecipitation assay (RIPA) kit (Beyotime, Shanghai, China). The protein samples were separated through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Primary antibody was then incubated on the membrane to determine protein levels in the cells. After incubation of the corresponding secondary antibody, Image J software (NIH, Bethesda, MD, USA) was used for protein quantification. All antibodies were provided by Cell Signaling Technology (Danvers, MA, USA).

Luciferase Assay

The relative fluorescence value after microRNA mimic or plasmid transfection was measured by a standardized method (Promega, Madison, WI, USA). The same experiment was repeated 3 times, and the average value was taken for statistical analysis.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 5 V5.01 software (La Jolla, CA, USA). Student's *t*-test was used to compare differences between samples analyzed. Data are expressed as mean ± standard deviation. *p*-value <0.05 was considered statistically significant.

Results

MiR-136-5p Was Lowly Expressed In PCa

48 pairs of tumor tissues and paracancerous tissues of patients with PCa were collected. The results of qRT-PCR revealed that microRNA-136-5p level in tumor tissues of patients with PCa was lower than that in adjacent tissues (Figure 1A), suggesting that miR-136 -5p may play an oncogene role in PCa. At the same time, *in vitro* cell lines showed a similar tendency (Figure 1B).

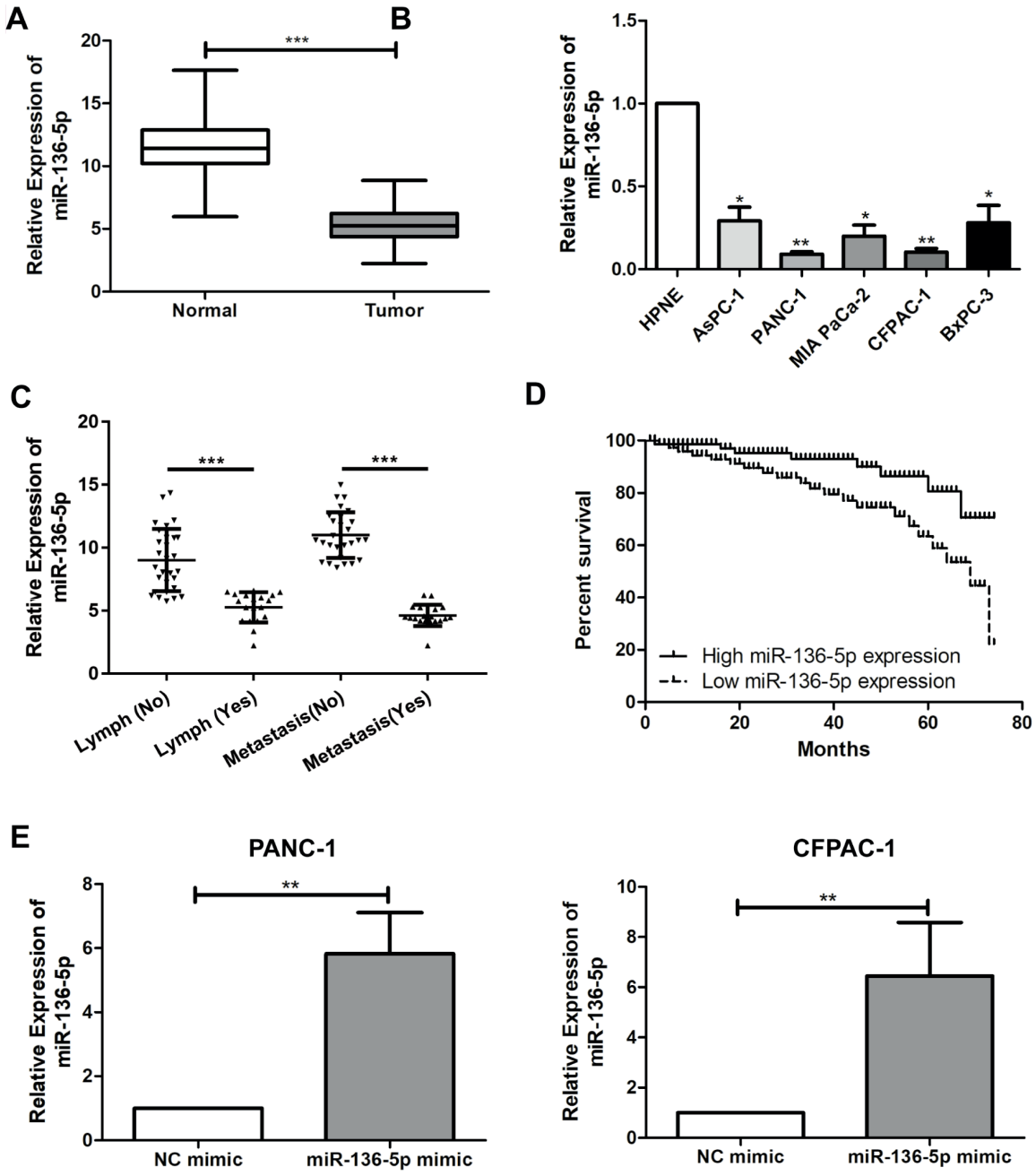

Figure 1. MiR-136-5p is underexpressed in pancreatic cancer tissues and cell lines. **A,** qRT-PCR was used to detect the difference in expression of miR-136-5p in tumor tissues and adjacent non-tumor tissues of patients with pancreatic cancer. **B,** qRT-PCR was used to detect the expression level of miR-136-5p in pancreatic cancer cell lines. **C,** qRT-PCR was used to detect the expression level of miR-136-5p in pancreatic cancer tissues with or without lymph node metastasis. **D,** Kaplan-Meier survival curve of pancreatic cancer patients was plotted based on miR-136-5p expression. **E,** qRT-PCR verified the transfection efficiency of the transfected miR-136-5p overexpression vector in PANC-1 and CFPAC-1 pancreatic cancer cell lines. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

MicroRNA-136-5p Was Closely Linked to Prognosis of PCa Patients

As shown in Table I, microRNA-136-5p low expression was positively relevant to the rate of lymph node or distant metastasis of patients with PCa (Figure 1C), but not with age, gender, or pathological stage. In addition, Kaplan-Meier survival curve revealed that high expression of microRNA-136-5p induces a poor prognosis of PCa ($p<0.05$; Figure 1D). To specify the functional changes of miR-136-5p against gemcitabine-resistant PCa cell lines, a microRNA-136-5p overexpression model was constructed (Figure 1E).

MicroRNA-136-5p Was Found Lowly Expressed In Gemcitabine Resistant PCa Cells

CCK-8 result revealed that the IC50 of PANC-1 and CFPAC-1 parental cells was lower than the

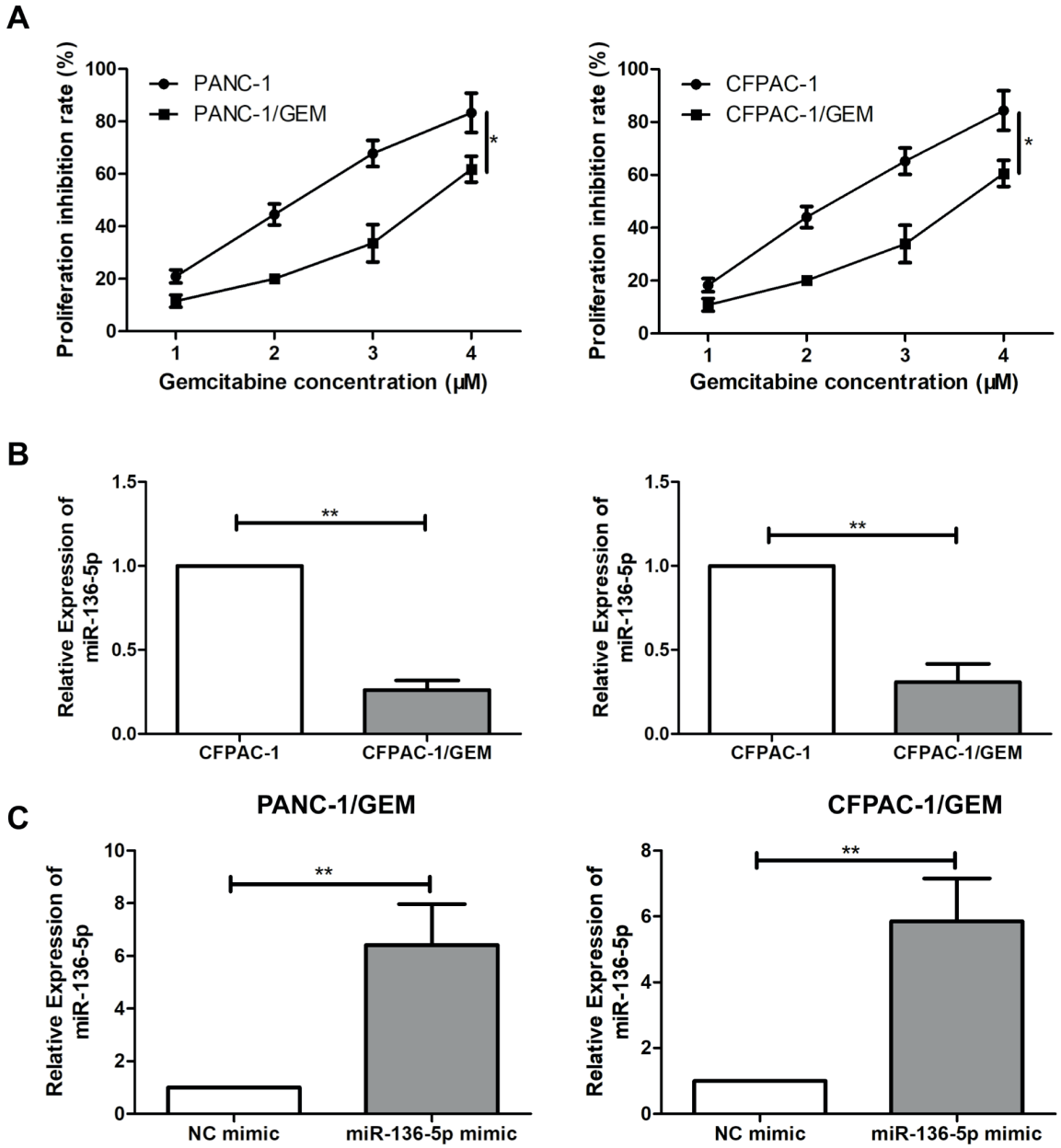

Figure 2. Identification of gemcitabine resistance in pancreatic cancer and expression of miR-136-5p in PANC-1/GEM and CFPAC-1/GEM. **A,** The CCK-8 method was performed to identify the gemcitabine resistance in the parental PANC-1 and CFPAC-1, PANC-1/GEM and CFPAC-1/GEM cell lines. **B,** qRT-PCR was used to show the expression of miR-136-5p in the parental PANC-1 and CFPAC-1, PANC-1/GEM and CFPAC-1/GEM cell lines. **C,** qRT-PCR was used to disclose the transfection efficiency of miR-136-5p in PANC-1/GEM and CFPAC-1/GEM cell lines. Data are mean ± SD, *$p<0.05$, **$p<0.01$.

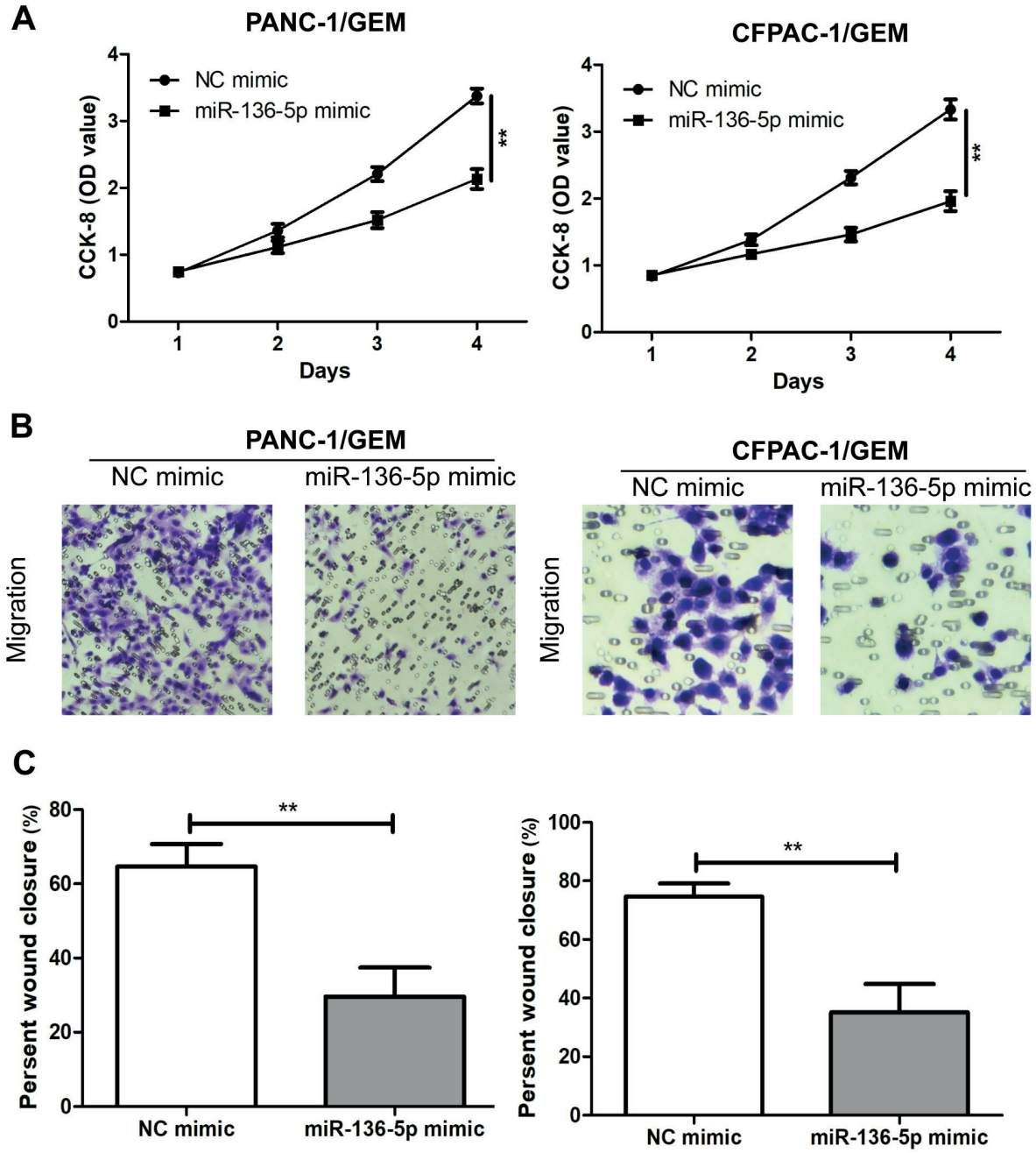

Figure 3. Overexpression of miR-136-5p inhibits the proliferation and metastasis of gemcitabine-resistant pancreatic cancer cells. **A,** The CCK-8 assay was performed to detect the effect of miR-136-5p overexpression on pancreatic cancer cell proliferation in the gemcitabine-resistant pancreatic cancer cell lines PANC-1 and CFPAC-1. **B,** Transwell migration assay was used to detect the migration of pancreatic cancer cells after transfection of miR-136-5p overexpression vector in gemcitabine-resistant pancreatic cancer cell lines PANC-1 and CFPAC-1 (magnification: 40×). **C,** The cell scratch assay examined the effect of pancreatic cancer cell crawling ability after transfection of the miR-136-5p overexpression vector in the gemcitabine-resistant pancreatic cancer cell lines PANC-1 and CFPAC-1 (magnification: 40×). Data are average ± SD, $^{**}p<0.01$.

gemcitabine resistant cell lines (Figure 2A). In addition, qRT-PCR analysis of differential expression of microRNA-136-5p suggested that microRNA-136-5p level is lower in PANC-1/GEM and CFPAC-1/GEM cells than in parental PANC-1 and CFPAC-1 (both $p<0.05$) (Figure 2B), suggesting that microRNA-136-5p could affect gemcitabine resistance of PCa cells. Subsequently,

the microRNA-136-5p overexpression vector was constructed in the gemcitabine-resistant cell lines PANC-1/GEM and CFPAC-1/GEM, which was confirmed by qPCR asssy (Figure 2C).

MicroRNA-136-5p Mimic Reduced Cell Proliferation Rate and Suppressed Metastasis of Gemcitabine Resistant PCa Cells

After transfecting the microRNA-136-5p overexpression vector in the gemcitabine-resistant PANC-1 and CFPAC-1 cell lines, CCK-8 assay was used to detect the growth curve and proliferation characteristics of PCa cells. The results revealed that microRNA-136-5p mimic remarkably weakened the proliferation ability of PCa cells ($p<0.05$; Figure 3A). In addition, transwell assay (Figure 3B) and cell scratch assay (Figure 3C) in PCa cell lines PANC-1 and CFPAC-1 transfected with microRNA-136-5p overexpression vector revealed that the migration and invasion ability of gemcitabine-resistant PCa cells was remarkably decreased after overexpression of microRNA-136-5p. It was concluded that microRNA-136-5p can be involved in gemcitabine resistance by suppressing the proliferative ability and metastasis of PCa cells.

MicroRNA-136-5p Was Bound to ZNF32

The microRNA-136-5p and the mutation microRNA-136-5p sequence were cloned into the Luciferase reporter plasmid pmirGLO. The results of the Luciferase reporter gene assay indicated that microRNA-136-5p can be targeted by ZNF32 through specific binding sites (Figure 4A). Furthermore, qRT-PCR experiments showed that the expression levels of ZNF32 were remarkably decreased in PANC-1 and CFPAC-1 transfected with the microRNA-136-5p overexpression vector (Figure 4B). Furthermore, qRT-PCR detection revealed a significant decrease in microRNA-136-5p expression after ZNF32 was overexpressed in the gemcitabine-resistant PANC-1 and CFPAC-1 cell lines (Figure 4C).

ZNF32 Was Highly Expressed In PCa Tissues and Cell Lines

qRT-PCR revealed that ZNF32 expression was increased in PCa tissues in comparison to adjacent tissues (Figure 4D). Moreover, we found that ZNF32 and microRNA-136-5p expression levels were negative relevant in PCa tissue samples (Figure 4E).

MicroRNA-136-5p Modulated ZNF32 in Gemcitabine Resistant PCa Cells

Western blotting and qRT-PCR verified an enhanced expression of microRNA-136-5p and ZNF32 levels after co-transfection of microRNA-136-5p mimic and ZNF32 overexpression plasmid (Figure 5A, 5B). Subsequently, transwell migration assay (Figure 5C) and cell scratch assay (Figure 5D) were performed, which suggested that co-transfection of microRNA-136-5p and ZNF32 reversed the inhibition of cell proliferation and metastasis induced by microRNA-136-5p alone. In sum, microRNA-136-5p modulated ZNF32 in gemcitabine resistant PCa cells.

Discussion

Pancreatic cancer is characterized by fast progression, poor prognosis and easy recurrence. It has a short median survival and is not sensitive to current comprehensive treatment methods such as chemoradiotherapy, with it 5-year survival rate less than 5%[1-3]. At present, the molecular mechanism of the regulation of PCa has not been fully clarified. Therefore, clarifying the molecular mechanism of PCa is conducive to the development of new clinical treatment methods and could provide important molecular therapeutic targets[5-8]. Chemotherapy resistance is the main reason limiting the overall survival rate of PCa patients[4,5]. The fact that some tumors do not respond to chemotherapy, and others rapidly recur and are often refractory to further therapy with the same or similar agents suggests that within the heterogeneous primary tumor there are cells that are intrinsically resistant to therapy[4]. In addition, almost all agents may have chemotherapy resistance[5]. Gemcitabine is a standard chemotherapy drug for adjuvant treatment of advanced PCa, but its progression-free survival is only 0.9 to 4.2 months, mainly due to chemotherapy resistance[9,10]. Therefore, an in-depth study of the mechanism of gemcitabine resistance in PCa will help develop new therapeutic strategies for PCa[11].

Current studies[12,13] have shown that miRNAs play an important role in the occurrence and development of tumors, and there are great changes in the miRNA expression profiles between tumor tissues and surrounding normal tissues. These differentially expressed miRNAs may play a decisive role in the occurrence and development of tumors as oncogenes or tumor suppressor genes[12-15]. MiRNA can also be used for the diagnosis and

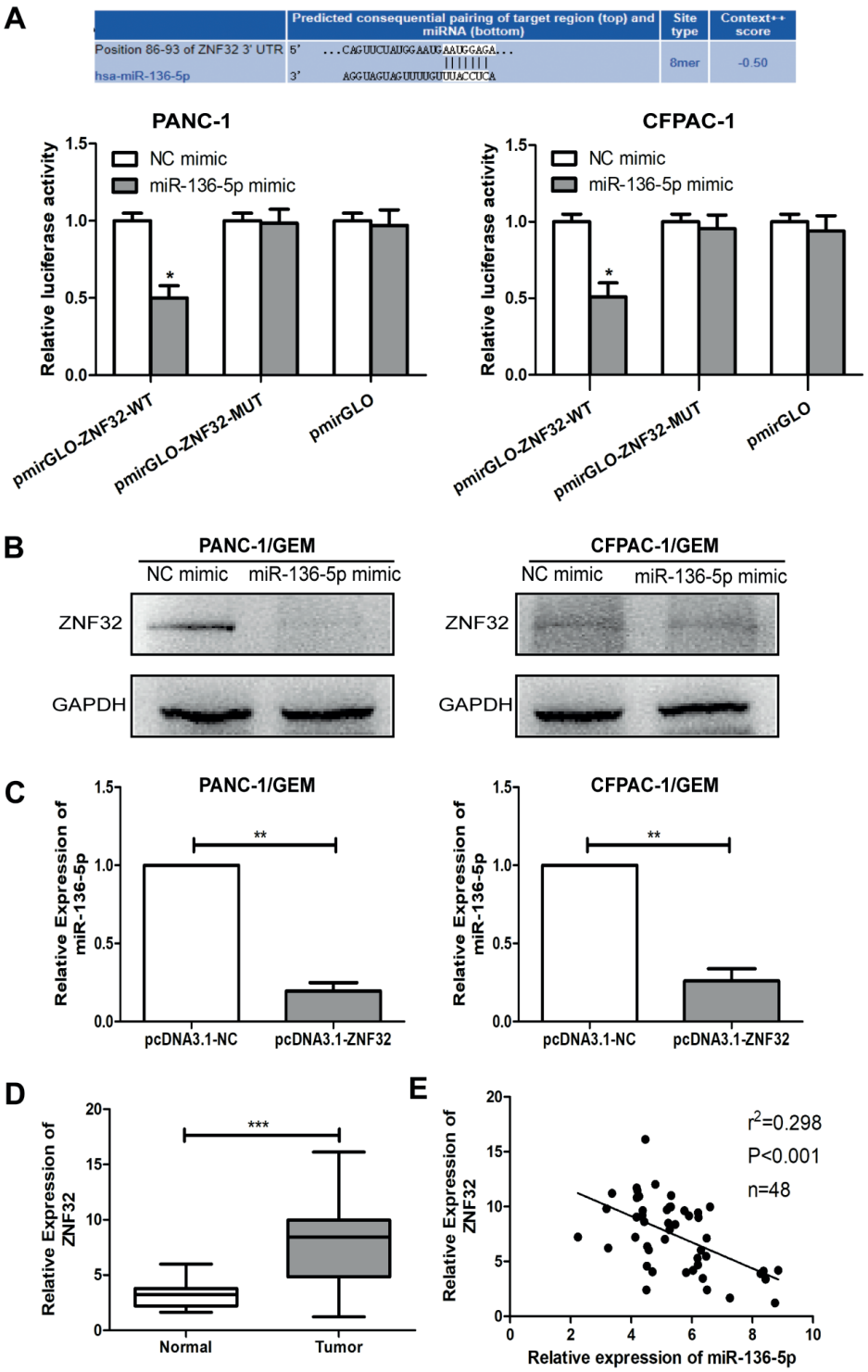

Figure 4. Direct targeting of miR-136-5p to ZNF32. **A,** The results of the Dual-Luciferase reporter assay in pancreatic cancer cell lines PANC-1 and CFPAC-1 indicated that miR-136-5p can be targeted by ZNF32 through specific binding sites. **B,** Western blotting was used to detect the expression level of ZNF32 after overexpression of miR-136-5p in gemcitabine-resistant pancreatic cancer cell lines PANC-1 and CFPAC-1. **C,** qRT-PCR was used to reveal the expression level of miR-136-5p after overexpression of ZNF32 in gemcitabine-resistant pancreatic cancer cell lines PANC-1 and CFPAC-1. **D,** qRT-PCR was used to uncover the differential expression of ZNF32 in pancreatic cancer tumor tissues and adjacent non-tumor tissues. **E,** qRT-PCR showed a significant negative correlation between miR-136-5p and ZNF32 expression in pancreatic cancer tissues. Data are mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

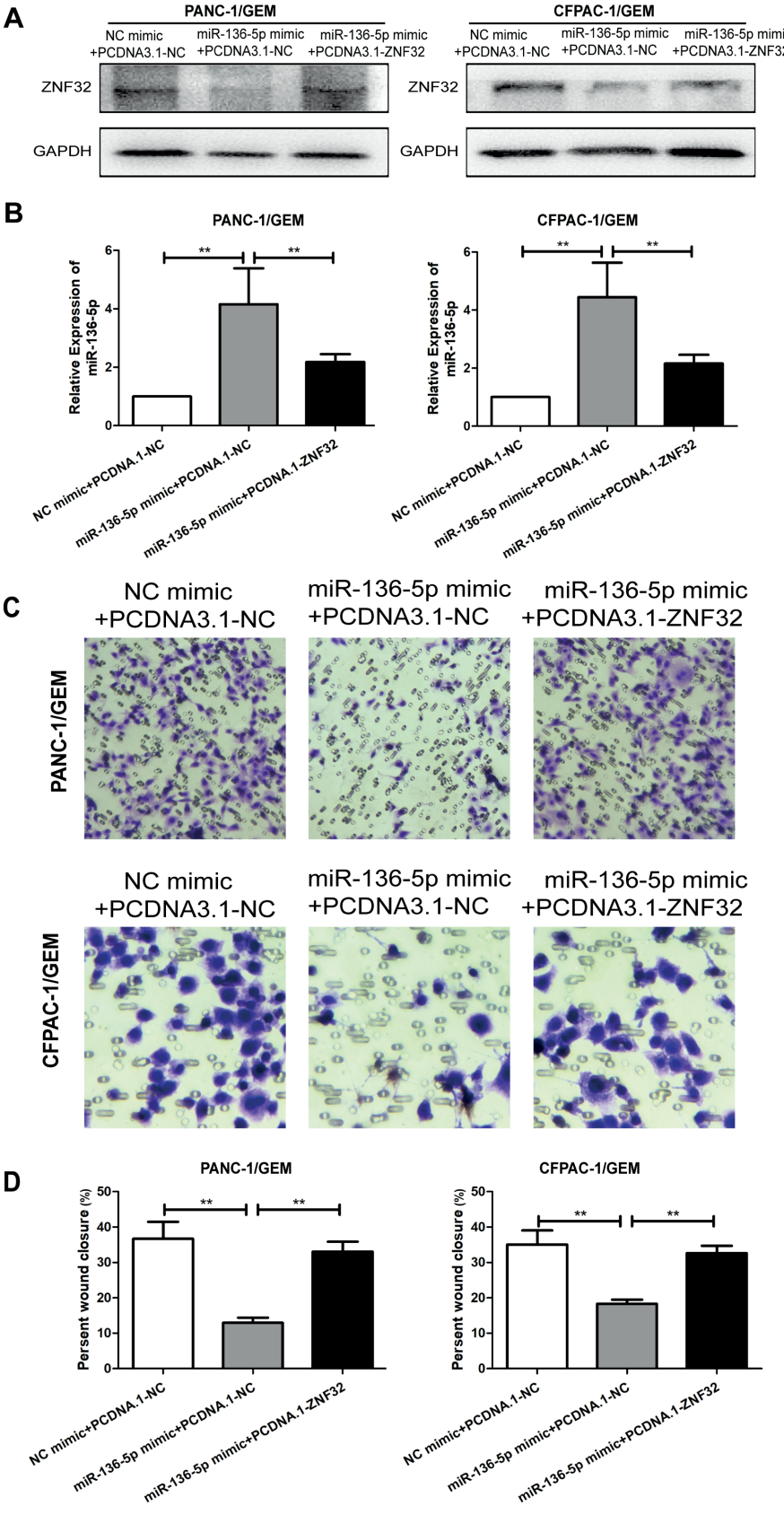

Figure 5. MiR-136-5p regulates the malignant progression of ZNF32 in pancreatic cancer. **A,** Western blotting was used to detect the transfection efficiency of ZNF32 after co-transfection of miR-136-5p and ZNF32 overexpression vectors in gemcitabine-resistant pancreatic cancer cell lines PANC-1 and CFPAC-1. **B,** qRT-PCR was used to observe the transfection efficiency of miR-136-5p after co-transfection of miR-136-5p and ZNF32 overexpression vectors in gemcitabine-resistant pancreatic cancer cell lines PANC-1 and CFPAC-1. **C,** The transwell migration assay was performed to disclose the effects of co-transfection of miR-136-5p and ZNF32 overexpression vectors on the migration of pancreatic cancer cells in the gemcitabine-resistant pancreatic cancer cell lines PANC-1 and CFPAC-1 (magnification: 40×). **D,** The cell scratch assay was performed to indicate the effect of co-transfection of miR-136-5p and ZNF32 overexpression vector in the gemcitabine-resistant pancreatic cancer cell lines PANC-1 and CFPAC-1. Data are average ± SD, $^{**}p<0.01$

prognosis of tumors. The difference in miRNA expression in tumor tissues and their corresponding paracancerous tissues is of great significance in determining whether the occurrence of miRNA is related to tumorigenesis[14-16]. To explore the role of miRNA in the malignant progression of PCa and to find an effective way to reverse the development of PCa, we performed preliminary work through high-throughput detection of micro-RNAs expression profiles in PCa tissues, and found the expression of miR-136-5p was significantly different between PCa tissues and normal adjacent tissues, so we further explored its effect on pancreatic malignant progression.

In this study, we found that the expression of miR-136-5p in tumor tissues of pancreatic cancer patients was significantly lower than that in adjacent tissues, and was positively correlated with lymph node metastasis, distant metastasis and poor prognosis of pancreatic cancer, so we believe that miR-136-5p may play a role in tumor suppression in pancreatic cancer. Subsequently, we established a microRNA-136-5p overexpression model in gemcitabine-resistant PCa cells, and proved by CCK-8, transwell and cell scratch experiments that microRNA-136-5p could inhibit the proliferation and metastasis of PCa cells. In addition, CCK-8 confirmed that the IC50 of PANC-1 and CFPAC-1 parental cells was lower than the gemcitabine-resistant cell lines PANC-1/GEM and CFPAC-1/GEM, while the expression of miR-136-5p was lower in PANC-1/GEM and CFPAC-1/GEM than that of parental PANC-1 and CFPAC-1, suggesting that miR-136-5p was associated with gemcitabine resistance in pancreatic cancer. These results suggested that microRNA-136-5p may be a key predictor of the malignant progression of PCa and may be involved in the development of gemcitabine resistance in pancreatic cancer cells.

MiRNAs can participate in the development of disease by regulating signaling pathways[24-26]. MiRNA-146 can negatively regulate TLR-NFκB signaling pathway and participate in the regulation of immune function. It is also reported that miRNAs can participate in embryonic development and tumor development by regulating Hedgehog pathway. Besides, miR-378 participates in the growth and angiogenesis of glioma through SUFU[24-26]. We used bioinformatics methods to predict the possible interaction between microRNA-136-5p and ZNF32. ZNF32 is a new member of the Kruppel like transcription factor family (KLF), which maintains mitochondrial membrane potential and enhances cellular defense against oxidative stress[27,28]. ZNF32 is also closely related to malignant progression of tumors, and can promote the proliferation, metastasis and differentiation of tumors[29,30]. In this study, we found that microRNA-136-5p sequence contained ZNF32 binding sites through bioinformatics analysis, and observed that microRNA-136-5p could directly bind to ZNF32 through qRT-PCR, Luciferase assay and other molecular biology experiments. In addition, to further explore whether microRNA-136-5p can play a regulatory role in PCa through ZNF32, we conducted cell recovery experiment and transwell migration and cell scratch experiment results. Results revealed that overexpression of ZNF32 could reduce the expression level of microRNA-136-5p and reverse the inhibition of overexpression of microRNA-136-5p on the migration ability of PCa cells, thus indicating that microRNA-136-5p affected the malignant progression of PCa cells *via* regulating ZNF32. For the first time, we elucidated the oncogenic role of microRNA-136-5p in the progression of PCa, and the regulation mechanism of microRNA-136-5p and ZNF32 was explored in depth. Therefore, microRNA-136-5p is expected to be the diagnosis and treatment biomarker of PCa.

Conclusions

Shortly, microRNA-136-5p inhibits the proliferation and metastasis of PCa cells *via* regulating ZNF32, and thus alleviates gemcitabine resistance in PCa cells. Thus, microRNA-136-5p represents a novel potential biomarker and therapeutic target of PCa.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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