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Original Paper

circZMYM2 Competed Endogenously with miR-335-5p to Regulate JMJD2C in Pancreatic Cancer

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Key Words
Pancreatic cancer • circRNA-ZMYM2 • miR-335-5p • JMJD2C

Abstract
Background/Aims: We aimed to study the involvement of circZMYM2 (hsa_circ_0099999) in pancreatic cancer (PC) cell proliferation, apoptosis and invasion and to figured out the underlying mechanism of circZMYM2 regulating miR-335-5p and *JMJD2C*. ***Methods:*** CircRNA differential expressions in twenty PC samples and paired normal tissue samples were analyzed using Arraystar Human CircRNA microarray V1. CircZMYM2 expression level was determined via qRT-PCR. The effects of circZMYM2 inhibition and overexpression on cell proliferation, cell apoptosis and cell invasion were investigated by CCK-8 assays, Flow cytometry assays and Transwell assays. An animal experiment on nude mice was put forward to test the influence of circZMYM2 knockdown on tumor growth. The relationship between circZMYM2, miR-335 and JMJD2C was verified by RNA pull down, dual-luciferase reporter assays and rescue experiment. The effect of circZMYM2 and miR-335-5p on the expression of JMJD2C protein was detected by western blot. ***Results:*** CircZMYM2 overexpression was observed in both PC tissues and cells. Knockdown of circZMYM2 inhibited proliferation, induced apoptosis, and weakened invasion ability of cancer cells. Tumor growth was restrained *in vivo*. CircZMYM2 repressed the expression of its target miR-335-5p. MiR-335-5p attenuated pancreatic cancer development via inhibition of *JMJD2C*. ***Conclusion:*** Our study demonstrated that circZMYM2 promoted PC progression. CircZMYM2 had a sponge effect on miR-335-5p and modulated the downstream oncogene *JMJD2C*.

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Introduction

Pancreatic cancer (PC) ranks among the top deadly cancers in that it generates threatening malignancies and is affecting an increasing number of victims [1]. Being fatal yet asymptomatic, PC is a prevalent cause of cancer-associated mortality [2]. It is often featured by resistance to chemotherapy treatment, which blocked satisfactory treatment of this malignancy [3]. It has been reported that quite a few microRNAs (miRNAs) are potently PC-inhibitory, implicating the possibility of therapeutic miRNA-application [1]. Thus, this study intends to unearth the underlying molecular mechanism in pancreatic cancer.

Circular RNAs (circRNAs) as a novel type of noncoding RNAs (ncRNAs) are widely studied in the development of various diseases, including human cancers [4]. Previous documentations have indicated that circRNAs might be potential tumor targets. The circRNA expression signatures of pancreatic ductal adenocarcinoma (PDAC) are dysregulated, so that circRNAs are hypothetically involved in the oncogenesis and development of PDAC [5]. Clusters of circRNAs are aberrantly expressed in PDAC tissues in contrast to normal tissue samples, which opens up opportunities of new PDAC treatments and unprecedented paths into PDAC biology [6]. Yet circRNAs' expression in PC retained unrevealed to us. It is high time we elucidate the biochemical mechanism of circRNAs underlying PC.

MiRNAs are short, single stranded noncoding RNAs that functioned to modulate post-transcriptional human gene expression levels, fulfilled through binding to the target messenger RNAs (mRNAs) at the latter's 3′ untranslated region (3′ UTR). This binding consequently leads to mRNA translational repression or degradation [7]. Emerging studies have described anomal expressions of miRNAs in PC, which tend to interact with the proliferation, apoptosis and cell cycle phases of PC cells by manipulating different molecules or signaling pathways [8]. For instance, the co-expression of miR-126 and miR-34a as introduced by oncolytic adenovirus is prospecting in PC target therapy [1]. However, the potential involvement of miR-355 has not been studied in pancreatic cancer.

The JMJ proteins have raised great interest due to their obvious significance in a wide range of cancer progressions. As part of JMJ family, the members belonging to its subset JMJD2/KDM4 are associated with pancreatic cancer development, tumor cell development and proliferation, as well as progression of abnormal cells to an invasive and highly metastatic form by regulating their migratory and invasive capacities [9]. Many studies have put forward that JMJD2C is associated with lung cancer [10], breast cancer (triple-negative) [9], colon cancer [11], and so on. The role of JMJD2C in pancreatic cancer mechanism could also be explored.

In our present approach, we identified circZMYM2 by microarray analysis and qRT-PCR to be significantly overexpressed in both human PC tissues samples and cell lines. We observed that upregulated circZMYM2 obviously promoted growth, invasion and metastasis of PC cells, through sponging miR-335 to suppress the expression level of JMJD2C. Our research may enlighten future pancreatic cancer treatment.

Materials and Methods

Human tissues and cell culture

PC and their counterpart adjacent tissue samples were obtained from 106 patients confirmed by pathological and cytological diagnose in the Third Affiliated Hospital of SooChow University. The Third Affiliated Hospital of SooChow University approved our protocol and the participants provided informed consent. Tumor samples and paired normal pancreas samples (> 5 cm away from tumor edge) obtained as surgical specimens were frozen immediately in liquid nitrogen (NL). Normal human pancreatic cell line HPDE6-C7 and PC cell line CFPAC-1 and PANC-1 were products of American Type Culture Collection (ATCC, Manassas, VA, USA). High-glucose Dulbecco's Modified Eagle Medium (DMEM) was applied as culture

medium for HPDE6-C7 and PANC-1. CFPAC-1 was maintained in Iscove's Modified Dulbecco's Medium (IMEM) (Invitrogen, Carlsbad, CA, USA). Both media contained 10% fetal bovine serum (FBS), and were maintained in a five-percent CO_2 atmosphere at 37°C.

Microarray analysis

The microarray datasets of GSE79634 were analyzed by GPL19978 to screen differentially expressed circRNAs. In total 40 samples were screened, including 20 PC and their paired para-carcinoma normal samples. CircRNAs exhibiting fold changes ≥ 2.0 and *P*-values ≤ 0.05 were selected as significantly differentially expressed circRNAs.

Quantitative real-time PCR (qRT-PCR)

Total RNA of PC were separated from tissue samples and tumor cells along with their normal counterparts through TRIzol reagent (Invitrogen, Carlsbad, CA, USA), strictly observing the protocols of the manufacturer. For each specimen, total RNA was quantified to 200 ng as assured by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). RNA reverse-transcription was conducted using ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) with reference to instructions provided by the manufacturer. Employing THUNDERBIRD SYBR® qPCR Mix (Toyobo), three groups of circRNAs were scaled by qRT-PCR, with the reaction conditions and procedures described as followed: 2 min at 94°C and 30 cycles of 10 s at 94°C, 30 s at 56°C, and 1 min at 72°C, and then 10 min at 72°C. Human β-actin and U6 were introduced here as the internal controls. The entire quantitative PCR was repeated in triplicate. mRNA and miRNA's relative quantification value was computed by $2^{-\Delta\Delta Ct}$. All the divergent primers used in qRT-PCR were shown in Table 1.

Cell transfection and cultivation

PcDNA3.1-ZMYM2, pcDNA3.1-si-ZMYM2, pcDNA3.1-si-JMJD2C (cDNA vectors with silent mutations), pcDNA3.1 vectors, si-ZMYM2, si-JMJD2C, scramble siRNA, miR-335-5p mimics or negative control (NC) mimics and miR-335-5p inhibitor or NC inhibitors were produced by GenePharma in Shanghai, China. 24 h before transfection, CFPAC-1 and PANC-1 cells were 0.25%-trypsin digested and seeded in 6-well plates (1×10^6 cells/well). Cells were kept for 18-24 h at 37°C in a 5%-CO_2 environment. After the cells reached 80%-90% confluence, serum-and-antibiotics-free fresh medium substituted for the initial medium. 48-h Cell transfection with vectors was performed using Lipofectamine 2000 of Life Technologies Corporation (Gaithersburg, MD, USA) at 37°C and 5% CO_2. In Table 2 siRNA sequences were presented.

Cell Counting Kit-8 assay (CCK-8)

CCK-8 assay was performed post-transfection (0, 24, 48, or 72 h). CCK-8 solution (10 μL, Dojindo, Kumamoto, Japan) was added in each well and incubated at 37°C with the cells in a 5% CO_2 humidified chamber for 2 h. The absorbance at 450 nm was determined through a microplate reader (Bio-Rad, Hercules, CA, USA). For each cell group the absorbance was taken as the mean of six wells and the experiment was repeated in triplicate.

Flow cytometry analysis

To detect cell apoptosis PE Annexin V (BD Biosciences, San Jose, CA, USA) was applied, keeping to instructions of the manufacturer's. An FACS Calibur FCM (BD Biosciences) was used to observe cell apoptosis. Experiments in triplicate helped to reduce errors. FACS Diva software was adopted at data analysis.

Table 1. Primer sequence for qRT-PCR

Gene	Primer	Sequence
CircZMYM2	forward	5'-TTACCACCTGTTTTTGGCGA-3'
	reverse	5'-CTGGGATATACACAGGCACAGG-3'
miR-335-5p	forward	5'-CGTCCTCAAGAGCAATAACGAA-3'
	reverse	5'-GAATACCTCGGACCCTGC-3'
JMJD2C	forward	5'-AGCTCGATTTTCCACAGCCT-3'
	reverse	5'-AAACCTGGAGCTCAGCACTC-3'
β-actin	Forward	5'-GGACTTCGAGCAAGAGATGG-3'
	Reverse	5'-AGCACTGTGTTGGCGTACAG-3'
U6	Forward	5'-CTCGCTTCGGCAGCACATA-3'
	Reverse	5'-AACGATTCACGAATTTGCGT-3'

Table 2. siRNA sequence of circZMYM2 and JMJD2C

Gene	Sequence
si-circZMYM2	5'-AAAAAGATGATACTTGGAGGA-3'
NC-1	5'-CCCACCAGTTTGAGACTCCACAT-3'
si-JMJD2C	5'-GCCTAAGGCTGATGAGGAA-3'
NC-2	5'-GCCGACGGTTAAGGTAGAA-3'

Transwell assay

Each chamber was precoated with Matrigel (50 μL, BD Biosciences) before being placed in a 37°C incubator until the matrix gelled. After 2 h, 200 μL of serum-free medium hydrated matrigel were added. The transfected cells and control cells were trypsinized into cell suspension and counted. 5×10^3 cells (200 μL) in serum-free media were put into the top chamber. The bottom chamber was infused with DMEM (500 μL, with 10% FBS). After 24-h cultivation, cells failed to migrate were discarded. After PBS wash in duplicate, the membrane was fixed for 20 min in 4% methanol before crystal-violet staining (0.1%, 30 min). Migrated cells were PBS-washed twice and photographed under a microscope view. Cell migration was detected through the observed cell number.

Tumor xenograft assay

Twelve 6-week-old BALB/c nude mice were obtained from Japan SLC (Hamamatsu, Japan), and were randomly and equally assigned to two groups. The serum-free suspension (2×10^6 cells per mL) of PANC-1 cells pre-transfected with si-ZMYM2 or si-NC was subcutaneously injected into each mouse (200 μL). When the tumors grew to about 100-200 mm^3, the volume of each was calculated via $1/2\times L^2\times W$, where L was the length (mm) and W was the width (mm) of the tumor. Every 3 days the volume of each tumor was recorded as an average of 3 measurements. At the 25^{th} day of the experiment, all mice were sacrificed to excise the tumor for measuring the average volume and weight. The tumor tissues were used to perform qRT-PCR analysis of circZMYM2 expression.

Dual-luciferase reporter assay

The 3'UTR of wild-type JMJD2C (JMJD2C-WT) and mutated JMJD2C (JMJD2C-MUT) cDNA fragments were amplified by inserting into the XbaI and FseI binding sites of pGL3 vectors (Promega, Madison, WI, USA) to synthesize luciferase reporter vectors. HEK293T cells were injected into a 24-well plate for co-transfection with the above vectors and miR-543 or control mimics. The transfection was conducted by means of Lipofectamine 3000 (Life Technologies Corporation). The Firefly luminescence was normalized to the Renilla luminescence via Renilla luciferase vector pRL-SV50 (Promega) 48 h post-tranfection. A Dual-Luciferase Reporter Assay System (Promega) was adopted to analyze the resulting relative luciferase activity.

Western blot

After cell lysis in RAPI protein lysate (Beyotime, Shanghai, China) to collect total protein, quantification of the total protein was accomplished via Pierce BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Proteins-separation was conducted by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 200 mA, 120 min), and the proteins were electro-blotted onto a PVDF membrane (Merck Millipore, Billerica, MA, USA). Then the membrane was immersed in Tris Buffered Saline Tween (TBST) with 5% skim milk for an hour at room temperature. The mixture was cultivated overnight with primary antibodies, including anti-*JMJD2C* (1:200, Abcam, Cambridge, MA, USA) and anti-Actin (1:1000, Abcam) at 4 °C. After washed three times with TBST buffer, the HRP-linked antibody goat anti-rabbit IgG (1:1000, ab6702, Abcam) was added, followed by incubation at room temperature on a shaker for 1.5 h. After TBST-wash in triplicate, the blots were finally visualized under the ECL chemiluminescence system (Life Technologies Corporation), with their integral optical density analyzed using Lab Works4.5 software. β-actin was regarded as the control for JMJD2C protein.

Statistical analysis

All data from three or more independent repetitions were represented as mean ± standard deviation (SD). The analysis was done within GraphPad Prism 6.0. Difference analysis was conducted by t test between two sets of data. For three groups or above, difference analysis was conducted by one-way ANOVAs. $P < 0.05$ was accepted as the scale for statistical significance.

Results

CircZMYM2 was highly expressed in PC tissues and cell lines

Based on microarray analyses, we filtered out 10 highly expressed circRNAs and 10 lowly expressed circRNAs in the tumor samples and visualized them in a heat-map (Fig. 1A). Through biological information analysis and literature search, the research object was identified as circZMYM2 (has_circ_0099999) with a full length of 5099 bp and a spliced length of 509 bp, located on human chromosome 13 (chr13: 20633586-20638685). Total RNA was extracted from pancreatic cancer and adjacent tissues of 106 patients, of whom the clinical characteristics were listed in Table 3. We eliminated the interference of linear RNA by adding RNase before examining the expression of circZMYM2 using qRT-PCR. CircZMYM2 expression level in PC was significantly increased comparing adjacent normal samples (Fig. 1B, $P < 0.01$). Likewise, its expression levels in CFPAC-1 and PANC-1 cell lines were both significantly higher than in contrast with normal pancreatic cells HPDE6-C7 as indicated by qRT-PCR results in cell lines (Fig. 1C). In a word, circZMYM2 was overexpressed in both PC tissues samples and cell lines.

CircZMYM2 promoted proliferation and invasion while attenuated apoptosis of PC cells

We performed qRT-PCR to examine the transfection efficiency of pcDNA3.1-circZMYM2 and circZMYM2 siRNA in CFPAC-1 and PANC-1 cell lines. CircZMYM2 expression in both cell lines were significantly decreased after transfection with si-circZMYM2, and significantly increased with pcDNA3.1-circZMYM2 (Fig. 2A-B, $P < 0.01$). Furthermore, rescue experiment was performed to demonstrate the specificity of si-circZMYM2. As shown in Fig. 2C-D, the transfection of pcDNA3.1 with silent mutations rescued the effect of si-circZMYM2, compared with si-circZMYM2 group, co-transfection group showed a higher circZMYM2 expression.

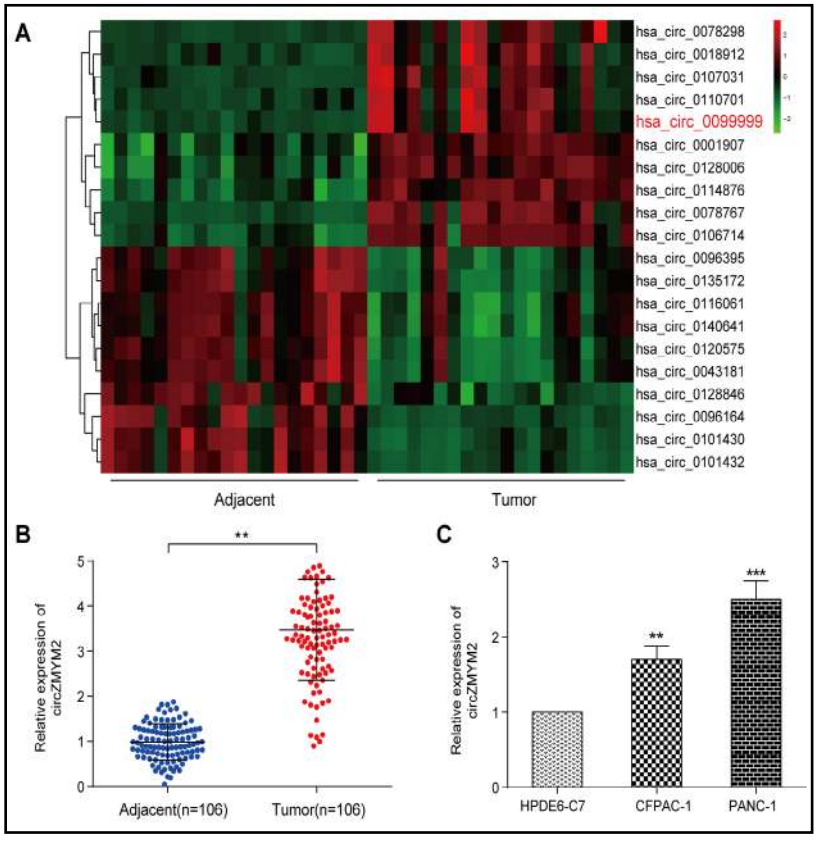

Fig. 1. CircZMYM2 expression was higher in pancreatic cancer tissues and cell lines. (A) Differentially expressed 20 circRNAs (10 upregulated and 10 downregulated) in pancreatic cancer tissue samples comparing para-cancerous tissues samples screened out by microarray dataset analyses was visualized in a heat-map. (B) The expression of circZMYM2 in pancreatic cancer tissues was higher than in adjacent tissues as was detected by qRT-PCR. (C) The expression of circZMYM2 in both pancreatic cancer cell line CFPAC-1 and PANC-1 was significantly higher than pancreatic cell line HPDE6-C7 based on qRT-PCR results. ** P<0.01, *** P<0.001 compared with HPDE6-C7.

Knockdown of circZMYM2 weakened the proliferation of CFPAC-1 and PANC-1 cells while circZMYM2 overexpression enhanced proliferation. Meanwhile, co-transfection of pcDNA3.1-si-circZMYM2 with si-circZMYM2 rescued the effect of si-circZMYM2 on cell viability, which further indicated the specificity of si-circZMYM2 (Fig. 2E-F, $P < 0.01$). The apoptosis rate of CFPAC-1 and PANC-1 cells in circZMYM2-downregulated group was significantly increased, while overexpression of circZMYM2 inhibited apoptosis (Fig. 2G, $P < 0.05$). The invasion rate of siRNA-transfected cell lines CFPAC-1 and PANC-1 was significantly lower comparing NC groups, while overexpression of circZMYM2 always significantly increased it (Fig. 2H, $P < 0.01$). In short, upregulated circZMYM2 prompted cell proliferation and invasion, and also inhibited apoptosis in PC.

Table 3. Correlation between circZMYM2 expression and clinicopathologic characteristics in pancreatic cancer patients. Note: **P<0.01

Clinicopathologic parameters	Number	circZMYM2 expression level		
		Low	High	P
Total cases	106	33	73	
Gender				0.656
Male	58	17	41	
Female	48	16	32	
Age				0.463
＜60	47	15	44	
≥60	59	18	39	
Differentiation				0.531
Well	12	4	10	
Moderate	76	24	42	
Poor	18	5	16	
Lymph-node metastasis				0.016**
Negative	46	20	26	
Positive	60	13	47	
Perineural invasion				0.110
Negative	52	20	32	
Positive	54	13	41	

Knockdown of circZMYM2 inhibited tumorigenesis of pancreatic cancer cells in vivo

PANC-1 cells transfected with siRNA of circZMYM2 (si-circZMYM2) and negative control (NC) were injected subcutaneously into nude mice. During 25 days of observation, silencing of circZMYM2 slowed down the formation of tumors (Fig. 3A-B, $P < 0.01$). On the 25th day, the average tumor weight of the si-circZMYM2-transfected mice was significantly lower relative to NC mice (Fig. 3C, $P < 0.01$). CircZMYM2 in tumor from si-circZMYM2 group expressed in significantly lower level than in NC group based on qRT-PCR results (Fig. 3D, $P < 0.01$). Therefore, circZMYM2 downregulation attenuated tumorigenesis of pancreatic cancer *in vivo*.

Targeting relationship was detected between circZMYM2 and miR-335-5p

Potential circZMYM2-targeted miRNAs were predicted by circular RNA interactome (https://circinteractome.nia.nih.gov), with 12 out of all 26 predictions visualized in Fig. 4A. The matching probability (context + score percentile) of over 95 points was set as the screening criteria. In total 7 miRNAs were selected, including miR-335-5p, miR-593, miR-587, miR-326, miR-330-5p, miR-873 and miR-1205. The prediction of sequences was shown in Table 4. MiR-335-5p was highly likely to be matched with circZMYM2 and was therefore adopted in further analysis. We performed an RNA pull down assay to clarify if circZMYM2 targeted miR-335-5p. In brief, the circZMYM2 probe was incubated with cytoplasmic extract of both CFPAC-1 and PANC-1 cells to form RNA-protein complexes. Specific magnetic beads were mixed with the solution. After separation of beads, we found a large amount of circZMYM2 and miR-335-5p comparing control probe (Fig. 4B-C). qRT-PCR results showed that in both cell lines, circZMYM2 knockdown significantly increased miR-335-5p expression while high expression of circZMYM2 significantly reduced miR-335-5p expression (Fig. 4D, $P < 0.01$). However, the expression of miR-335-5p did not affect that of circZMYM2 (Fig. 4E). In summary, circZMYM2 targeted miR-335-5p.

CircZMYM2 affected JMJD2C expression via miR-335-5p

Genes with complementary sequences of miR-335-5p was predicted by TargetScan, among which *JMJD2C* was found overexpressed in pancreatic cancer (Fig. S1 - for all supplemental material see www.karger.com/ 10.1159/000495868/). The sequence of *JMJD2C*-wt with the region predicted to interact with miR-335-5p and the corresponding sequence of *JMJD2C*-mut were inserted into pmirGLO plasmids and transfected into PANC-1 cells. While the relative luciferase activity of *JMJD2C*-wt group with miR-335-5p mimics

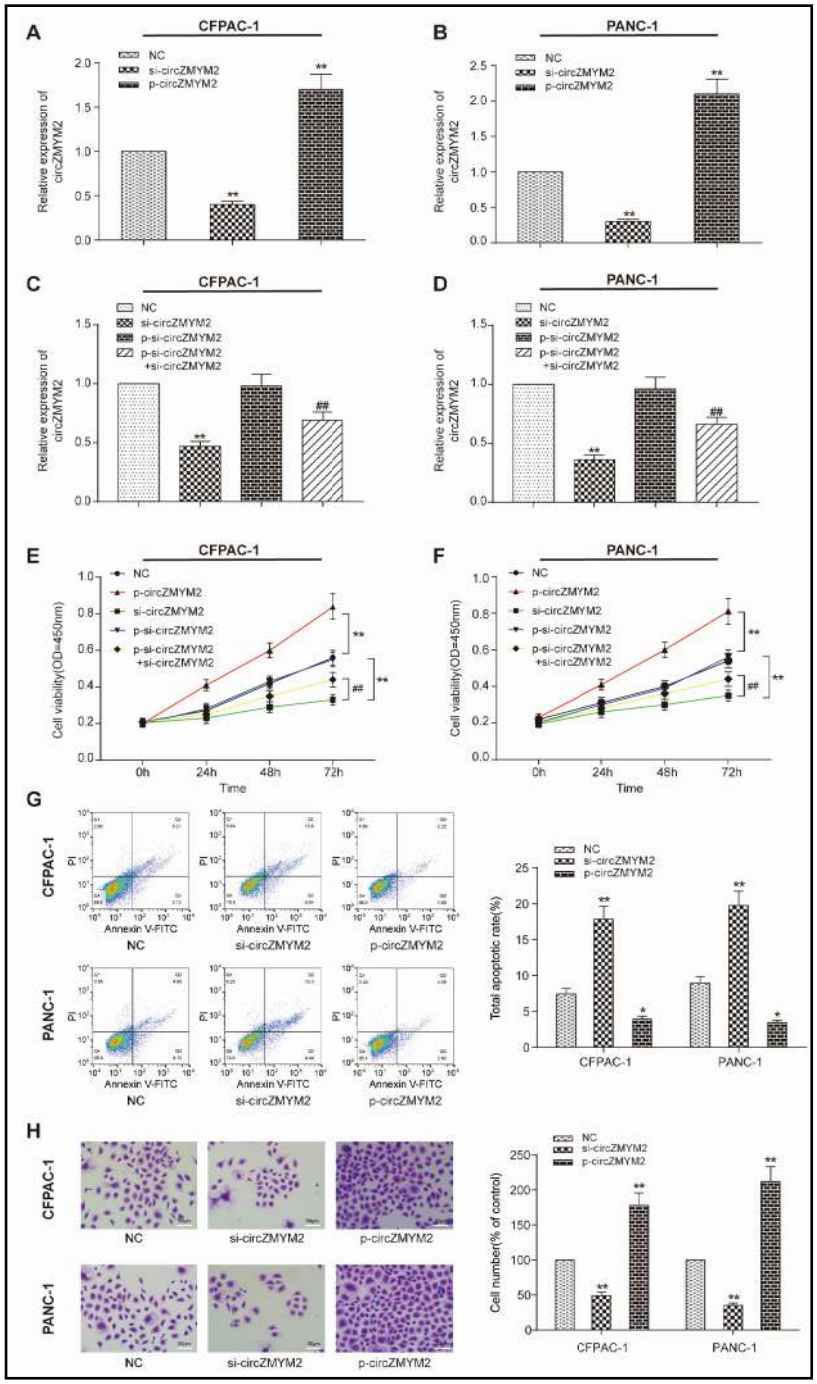

Fig. 2. Effects of circZMYM2 on proliferation, apoptosis and invasion of pancreatic cancer cells. CFPAC-1 and PANC-1 cells were randomly assigned to three groups: NC group, which meant negative control, si-circZMYM2 group, indicating transfection with si-circZMYM2, and p-circZMYM2 group, indicating transfection with pcDNA3.1-ZMYM2 vectors. p-si-circZMYM2 group, indicating transfection with pcDNA3.1-si-ZMYM2 vectors. (A-B) The relative expressions of circZMYM2 in both cell lines were detected by qRT-PCR. Transfection with si-circZMYM2 refrained circZMYM2 expression while transfection with pcDNA3.1-ZMYM2 accelerated pcDNA3.1-ZMYM2 expression. Both indicated successful transfection. (C-D) The specificity of si-circZMYM2 was detected by qRT-PCR. Transfection with si-circZMYM2 refrained circZMYM2 expression while co-transfection with pcDNA3.1-si-ZMYM2 and si-circZMYM2 rescued circZMYM2 expression. Which excluded the possibility of off-target effect and verified the specificity of si-circZMYM2. (E-F) The effect of knockdown or overexpression of circZMYM2 on the proliferation of CFPAC-1 and PANC-1 cells was examined by CCK-8. Knockdown of circZMYM2 result in lowered cell viability while overexpressed circZMYM2 prompted cell proliferation. Overexpression of si-circZMYM2 with si-circZMYM2 rescued the effect of knockdown of circZMYM2. (G) The effects of circZMYM2 on apoptosis rate of pancreatic cancer cells CFPAC-1 and PANC-1 were detected by flow cytometry. Knockdown of circZMYM2 induced cell apoptosis while overexpressed circZMYM2 inhibited apoptosis. (H) Effect of circZMYM2 on invasion ability of pancreatic cancer cells CFPAC-1 and PANC-1 was investigated using a Transwell assay. Knockdown of circZMYM2 reduced the number of invaded cells while overexpressed circZMYM2 increased it. ** P<0.01 compared with NC group. ##P<0.01 compared with si-circZMYM2 group.

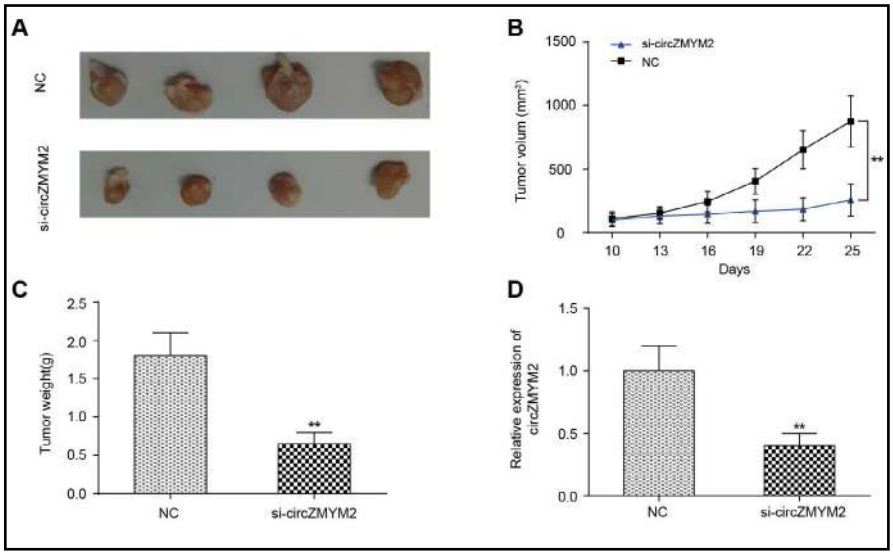

Fig. 3. Knockdown of circZMYM2 attenuated pancreatic tumor formation in vivo. (A) Tumors were removed from nude mice 25 days after injection. Picture of mice tumors illustrated that knockdown of circZMYM2 significantly reduced tumor size. (B) Tumor volumes of nude mice were measured every 3 days from the 10th day after injection. Silencing of circZMYM2 significantly slowed down pancreatic tumor growth. (C) On the 25th day, tumor weight of the si-circZMYM2 group was significantly lower than that of the NC group as shown in a bar graph. (D) The expression of circZMYM2 in si-circZMYM2 group was significantly lower than that in NC group as revealed by qRT-PCR results. ** P<0.01 compared with NC group.

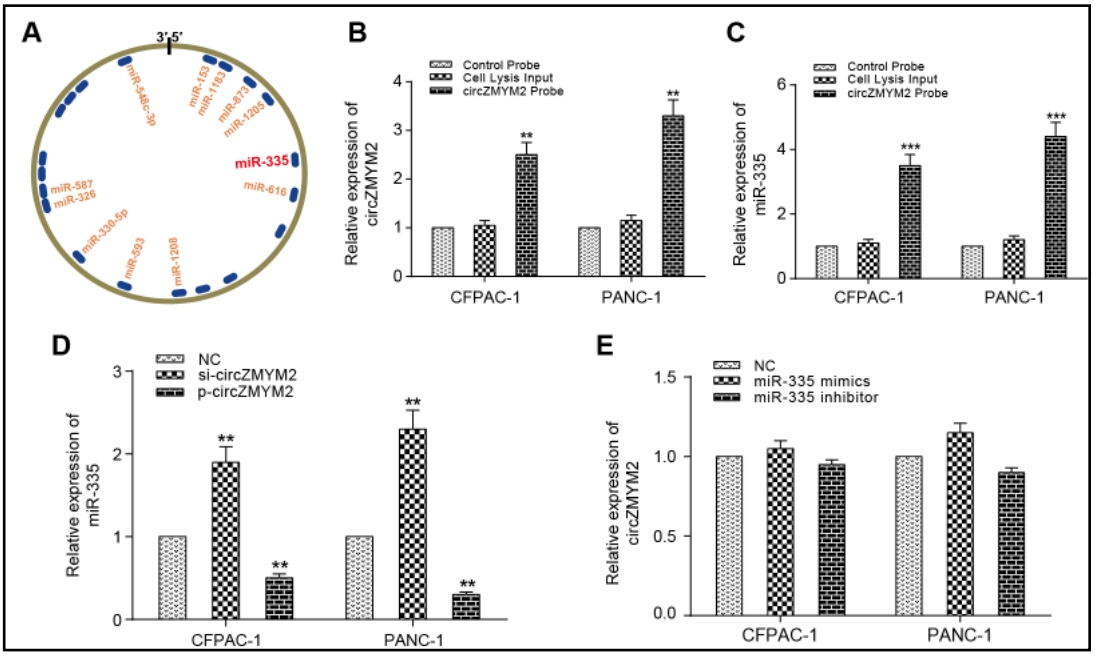

Fig. 4. Targeting relationship was identified between circZMYM2 and miR-335-5p. (A) Potential target miRNAs of circZMYM2 with binding sites were visualized. (B-C) An RNA pull down experiment was performed in CFPAC-1 and PANC-1 cells. High levels of circZMYM2 and miR-335-5p were detected using qRT-PCR. ** P＜0.01, *** P＜0.001, compared with control probe. (D) QRT-PCR results showed that low expression of circZMYM2 significantly increased the expression level of miR-335-5p, while high expression of circZMYM2 significantly reduced the expression level of miR-335-5p. ** P<0.01 compared with NC group. (E) Overexpression or low expression of miR-335-5p did not affect the expression level of circZMYM2.

Table 4. MiRNA predictions of circZMYM2

CircRNA and mirbase ID	CircRNA (top) - miRNA (bottom) pairing	Site type	Context+ score percentile
circZMYM2 hsa-miR-335	5'-AAGGUUUCUUCAGAUGCUCUUGA-3' 3'-UGUAAAAAGCAAUAACGAGAACU-5'	8mer-1a	99
circZMYM2 hsa-miR-593	5'-AAGACGAGGGGAAAACAGAGACA-3' 3'-UCUUUGGGGUCGUCUCUGU-5'	8mer-1a	99
circZMYM2 hsa-miR-587	5'-UGCUGAGGAGCUUGAUAUGGAAA-3' 3'-CACUGAGUAGUGGAUACCUUU-5'	8mer-1a	99
circZMYM2 hsa-miR-326	5'-UGCUGAGGAGCUUGAUAUGGAAA-3' 3'-CACUGAGUAGUGGAUACCUUU-5'	8mer-1a	98
circZMYM2 hsa-miR-330-5p	5'-AUCGAAAUAGAUUUUCCCAGAGC-3' 3'-CGGAUUCUGUGUCCGGGUCUCU-5'	7mer-m8	96
circZMYM2 hsa-miR-873	5'-CCUGUUCCUACUACAGUUCCUGU-3' 3'-UCCUCUGAGUGUUCAAGGACG-5'	7mer-m8	96
circZMYM2 hsa-miR-1205	5'-AGCAGUGAGAAGAUUCCUGCAGC-3' 3'-GAGUUUCGUUUGGGACGUCU-5'	7mer-m8	96

was significantly lower comparing its NC group, that of *JMJD2C*-mut group with miR-335-5p mimics did not diverge significantly from NC group case (Fig. 5A-B, $P < 0.01$). We concluded that miR-335-5p targets *JMJD2C*. The changes of JMJD2C protein expression level after circZMYM2 knockdown or miR-335-5p over-expression were explored by western blot. MiR-335-5p overexpression decreased the level of JMJD2C protein, while si-circZMYM2 downregulated JMJD2C protein level through the sponge effect on miR-335-5p (Fig. 5C, $P < 0.01$). CircZMYM2 regulated downstream gene *JMJD2C* by modulating miR-335-5p (Fig. 5D).

MiR-335-5p inhibits PC progression by inhibiting JMJD2C

Rescue experiment was performed to demonstrate the specificity of si-*JMJD2C*. Si-*JMJD2C* significantly inhibited the expression of *JMJD2C* in CFPAC-1 and PANC-1 cells, and the co-transfection of pcDNA3.1-si-*JMJD2C* with si-*JMJD2C* rescued the effect of RNAi phenotypes (Fig. 6A-B, $P<0.01$). After transfection of CFPAC-1 and PANC-1 cells with si-*JMJD2C* or miR-335-5p inhibitors respectively, they were divided into seven transfection groups including NC, si-*JMJD2C*, miR-335-5p inhibitor, si-*JMJD2C*+miR-335-5p inhibitor group, si-*JMJD2C*+p-circZMYM2 group, p-si-JMJD2C group and p-si-JMJD2C+si-JMJD2C group. CCK-8 experiments showed that knockdown of *JMJD2C* suppressed pancreatic cancer cell proliferation, whereas miR-335-5p inhibitors promoted the proliferation of cells and abolished the suppressive-role of si-*JMJD2C* on cell proliferation, which restored the viability of cancer cells. Furthermore, JMJD2C overexpression abrogate the effects of si-circZMYM2 as well. Similarly, co-transfection of pcDNA3.1-si-JMJD2C with si-JMJD2C rescued the effect of si-JMJD2C, indicating the specificity of si-JMJD2C. (Fig. 6C-D, $P < 0.01$). Observation under a flow cytometer showed that low expression of *JMJD2C* promoted apoptosis of cancer cells while miR-335-5p inhibitor inhibited apoptosis. MiR-335-5p inhibitor restrained the effect of si-*JMJD2C* on cells and reduced tumor cell apoptosis rate. JMJD2C overexpression abrogate the effects of si-circZMYM2 on cell apoptosis (Fig. 6E, $P < 0.01$). Transwell cell invasion experiments yielded the same results as indicated by invaded cell numbers (Fig. 6F, $P < 0.01$). Therefore, we concluded that miR-335-5p suppressed PC by inhibiting *JMJD2C* expression.

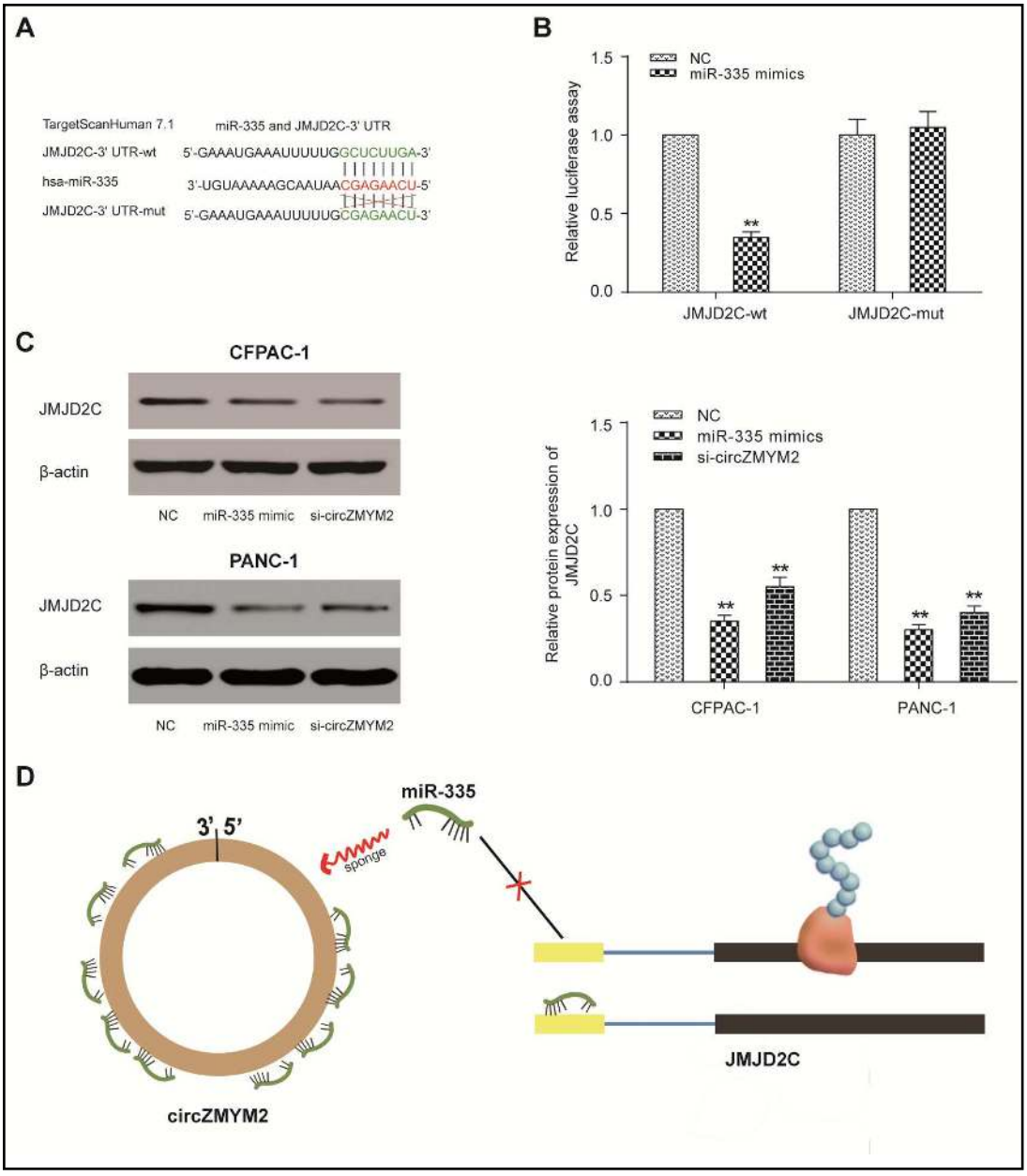

Fig. 5. Effect of circZMYM2 and miR-335-5p on JMJD2C Expression. (A) The sequences of circZMYM2-wt, miR-335-5p containing the binding site of circZMYM2-wt and circZMYM2-mut were given. (B) Targeting relationship between miR-335-5p and JMJD2C was verified by dual-luciferase reporter assay. Co-transfection of circZMYM2-wt and miR-335-5p mimics cut down luciferase activity of PANC-1 cells. Transfection with circZMYM2-mut plasmids didn't influence luciferase activity. (C) The effect of circZMYM2 and miR-335-5p on the expression of JMJD2C was detected by western blot. In both CFPAC-1 and PANC-1 cell lines, overexpression of miR-335-5p and knockdown of circZMYM2 reduced JMJD2C expression. (D) The regulation of circZMYM2 was diagramed. CircZMYM2 adsorbed miR-335-5p to prevent miR-335-5p from inhibiting JMJD2C expression, and therefore result in the up-regulation of oncogene JMJD2C. ** P <0.01 compared with NC group.

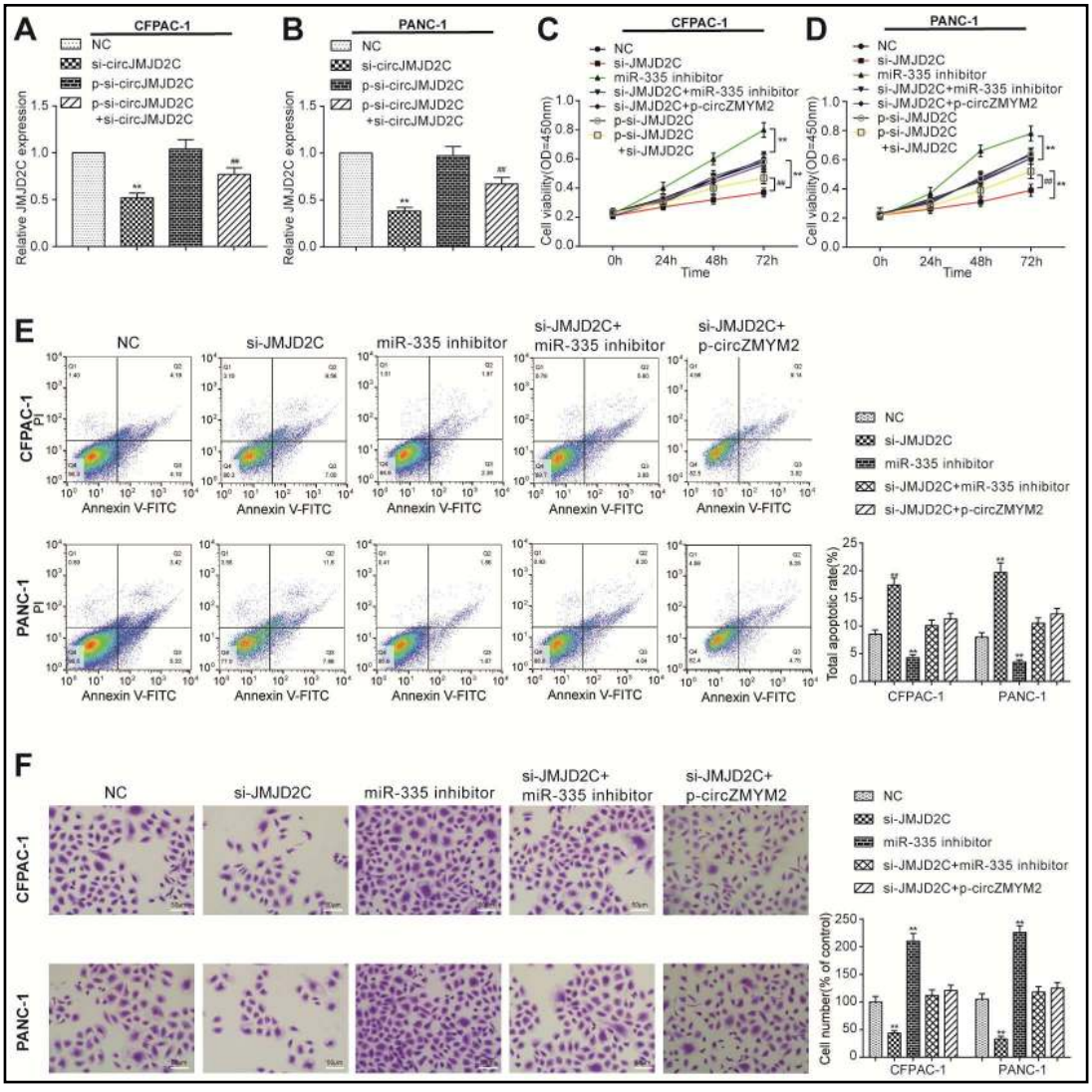

Fig. 6. MiR-335-5p repressed tumor growth by inhibiting JMJD2C. (A-B) The specificity of si-JMJD2C was detected by qRT-PCR. Transfection with si-JMJD2C refrained JMJD2C expression while co-transfection of pcDNA3.1-si-JMJD2C and si-JMJD2C rescued JMJD2C expression. Which excluded the possibility of off-target effect and verified the specificity of si-JMJD2C. (C-D) CFPAC-1 and PANC-1 cells were assigned into six groups including NC, si-JMJD2C, miR-335-5p inhibitor, si-JMJD2C+miR-335-5p inhibitor, si-JMJD2C+pcDNA3.1-ZMYM2, pcDNA3.1-si-JMJD2C group and pcDNA3.1-si-JMJD2C+ si-JMJD2C group, named after varied transfection. CCK-8 assay results showed that knockdown of JMJD2C inhibited the proliferation of pancreatic cancer cells CFPAC-1 and PANC-1, while miR-335-5p inhibitor increased proliferation rate and refrained the inhibition of si-JMJD2C on proliferation. JMJD2C overexpression abrogate the effects of si-circZMYM2. Overexpression of si-JMJD2C with si-JMJD2C rescued the effect of si-JMJD2C. (E) Flow cytometry assay results showed that JMJD2C knockdown significantly increased apoptosis rate of pancreatic cancer cells. MiR-335-5p inhibitor suppressed the apoptosis of cancer cells, and further refrained si-JMJD2C from inducing apoptosis. JMJD2C overexpression abrogate the effects of si-circZMYM2 on cell apoptosis. (F) Transwell assay results revealed in number of invaded cells that low expression of JMJD2C inhibited cancer cell invasion, and miR-335-5p inhibitor promoted cancer invasion. Co-transfection of miR-335-5p and si-JMJD2C reversed the latter induced invaded cell up-growth. JMJD2C overexpression abrogate the effects of si-circZMYM2 on cell invasion. ** P<0.01 compared with NC group.

Discussion

On presentation of PC, the vast majority of patients are suffering from incurably-advanced or metastatic tumor, in spite of advanced chemotherapy regimens and improved overall survival [12]. This study mainly investigated a circZMYM2/miR-335/JMJD2C axis in pancreatic cancer to figure out novel targets in PC prognosis. In brief, mediated by an increased expression of its target miR-335-5p, silencing of circZMYM2 inhibited *JMJD2C* expression and led to repressed cell proliferation, prompted apoptosis, and attenuated invasion ability of PC cells, along with reduced tumor growth rate.

CircRNAs are stable, diverse and conserved noncoding RNAs in mammalian cells, which are widely involved in human cancer initiation and development, including PC [5]. As was discovered by Yang *et al.*, circLDLRAD3 was upregulated and was significantly associated with cell invasion and metastasis in PC [13]. Chen *et al.* studied the circrna_100782 regulation through the IL6-STAT3 pathway on PC cell proliferation [14]. In the present study, circZMYM2 affects JMJD2C expression by miR-335. Our findings illustrated that circRNAs are anomaly expressed in PC cells and tissues comparing their normal counterparts, which developed potentials for novel treatment or underlying biology of PC.

Researchers have uncovered that miR-335 is widely dysregulated in human cancers and regulates oncogenesis and tumor progression [15]. Though lacking a broader-scale underling mechanism study, miR-335 has been revealed as a PC inhibitor by Gao *et al.*, targeting OCT4 [16]. Therefore, the mechanism was of great significance in pancreatic cancer studies. MiR-335 was also reported to mediate paclitaxel-resistance of breast cancer cells partly through regulation of Wnt/β-catenin signaling pathway [17] and then we suspected that miR-335 activates other signaling pathway. The level of DCLK1 and miR-195 showed an inverse correlation in PC tissues and cells, where higher DCLK1 level is associated with higher TNM (tumor, node, and metastasis) stage, higher rate of lymph node metastasis, and poor survival [18]. In the presented study, miR-335-5p inhibits the development of PC through the inhibition of JMJD2C.

We found that JMJD2C is overexpressed and acts as a key regulator in pancreatic cancer, which went in line with previous studies. In human lung cancer, the expression of JMJD2C was increased in the majority of cancer tissues compared with in adjacent tissues. Furthermore, JMJD2C expression was found to be higher in metastatic than in non-metastatic lung cancer tissues [10]. The histone demethylase activity of JMJD2C was essential for breast cancer progression given its role in the maintenance of chromosomal stability and cell growth, further highlighting its potential of becoming a therapeutic target [9]. Overexpression of JMJD2C also conferred a pro-growth effect on colon carcinoma cells. Hence inhibition of JMJD2C by curcuminoids or other small molecule was proposed as an adjuvant therapy [11]. With reference to our present findings on pancreatic cancer, JMJD2C upregulation could be reversed by miR-335-5p at downregulation of circZMYM2.

Nevertheless, limitations in this report are to be taken into consideration. For example, we identified circZMYM2 as "microRNA sponges" which up-regulated JMJD2C expression via miR-355-5p, but downstream mechanism was not explored. In addition, the relationship between miR-355-5p and the development of PC or survival time needs investigation by future experiments.

Pancreatic cancer is difficult to cure at present, and our study of pancreatic cancer-related genes allows us to better treat it. Despite that there remained a lot more to be learnt about the role of miR-355-5p, our current finding suggested miR-355-5p as a potent strategy for miRNA-based PC treatment.

Conclusion

In conclusion, we detected in PC tissues as well as cell lines highly-expressed circZMYM2. CircZMYM2 stimulated the proliferation and invasion process of PC cells, while obstructed the apoptosis. Besides, knockdown of circZMYM2 inhibited tumorigenesis *in vivo*. We found that circZMYM2 impose effects by sponging miR-335-5p expression, which negatively regulate JMJD2C expression. This finding may shed new light on future therapeutic target studies in pancreatic cancer.

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Disclosure Statement

The authors confirm that they have no potential conflict of interests.

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