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MicroRNA-708 inhibits the proliferation and chemoresistance of pancreatic cancer cells

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Abstract: Pancreatic cancer is one of the most aggressive malignancies with poor prognosis and high mortality. Recent studies showed that microRNAs are dysregulated and involved in the initiation and progression of pancreatic cancer. In this study, we found that miR-708 was significantly downregulated in pancreatic cancer tissues and cell lines. Lentivirus-mediated overexpression of miR-708 could significantly inhibit the proliferation and invasion, while enhanced chemosensitivity to gemcitabine in both Panc-1 and SW1990 cells. Luciferase reporter assay showed that miR-708 bound the 3'-untranslated region of survivin and suppressed the expression of survivin in pancreatic cancer cells. In pancreatic cancer tissues, survivin protein was highly expressed and negatively correlated with miR-708 expression. Furthermore, the restoration of survivin expression could partially antagonize proliferation inhibition and apoptosis induction by miR-708 in pancreatic cancer cells. The Panc-1 cells with overexpression of miR-708 also showed decreased proliferation capability in nude mouse model compared with parental cells. In conclusion, our results suggest that miR-708 inhibits pancreatic cancer and could be a novel potential candidate to treat pancreatic cancer.

Introduction

As one of the most common aggressive malignancies in the world, pancreatic cancer is the fourth major cause of cancer-related deaths with high mortality (Jin *et al*., 2018). Despite the increased surgical resection rate in recent decades, the 5-year survival rate has not been substantially improved for pancreatic cancer (Siegel *et al*., 2017). The poor prognosis is mainly attributed to the advanced stage when pancreatic cancer is diagnosed, and inherent cancer cell resistance to chemotherapeutic drugs (Zhang *et al*., 2019). While medicinal plants have been recently explored to screen effective agents to overcome cancer chemoresistance, the progress is still limited (Orozco Montes *et al*., 2019; Villa-Hernández *et al*., 2018).

Further understanding of oncogenesis and chemotherapy resistance mechanisms is pivotal to promote therapeutic effects for pancreatic cancer. Until now, the identification of new sensitive biomarkers and novel pharmacological targets remains an urgent task.

MicroRNAs (miRNAs) are endogenous small non-coding RNA molecules as long as approximately 22 nucleotides. MiRNAs can suppress the translation of target genes by binding to the 3' untranslated region (3'-UTR) and regulate cellular proliferation, apoptosis, and differentiation (Abreu *et al*., 2017). Multiple miRNAs have been involved in the development and progression of pancreatic ductal adenocarcinoma (PDAC) by modulating diverse cell signaling pathways (Zhang *et al*., 2018). Lin *et al.* (2018) reported that miR-1179 was downregulated in pancreatic cancer, and the restoration of miR-1179 expression could inhibit tumor proliferation both in vitro and *in vivo* by suppressing the expression of target gene E2F5. Recently, increasing evidence has suggested that aberrant miRNA expression was associated with the resistance to anticancer drugs (Cagle *et al*., 2019; Si *et al*., 2019). Our team previously reported that miR-17-5p inhibitor could enhance chemosensitivity to gemcitabine by the upregulation of Bim expression in pancreatic cancer cells (Yan *et al*., 2012). Lower miR-708 expression was reported recently in the late stage of ovarian cancer, and patients with higher miR-708 would have the potential of a better survival rate (Lin *et al*., 2015). miR-708 expression was also found to be decreased in gastric cancer tissues, and ectopic expression of miR-708 could inhibit proliferation and invasion of gastric cancer cells in vitro (Li *et al*., 2018). However, the role of miR-708 in the chemoresistance of pancreatic cancer remains elusive. Therefore, this study aimed to investigate the function of miR-708 in pancreatic cancer. We found that miR-708 expression was significantly decreased in pancreatic cancer tissues and cell lines. Furthermore, lentivirus-mediated overexpression of microRNA-708 could inhibit the proliferation, invasion, and chemoresistance to gemcitabine in pancreatic cancer cells. Our results provide novel insights into the crucial role of miR-708 in

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the tumorigenesis and progression of pancreatic cancer.

Materials and Methods

Tissue samples and cell lines

The matched 52 pancreatic carcinoma tissues and adjacent normal pancreatic tissues were obtained from patients at the Department of Hepatobiliary Surgery, the Third Affiliated Hospital of Soochow University, from 2014 to 2017. This study was approved by the Ethics Committee of the Third Affiliated Hospital of Soochow University. (Approval No. 2013038, October 2013). The samples were confirmed histologically, and all patients provided informed consent. The immortalized human pancreatic ductal epithelial cell line H6C7 was kindly provided by Dr. M.S. Tsao (Ontario Cancer Institute, University of Toronto, Canada), and was maintained in serum-free medium containing keratinocyte (Invitrogen, Carlsbad, CA, USA). The Panc-1 and SW1990 cell lines of pancreatic carcinoma were provided by the Culture Center of Wuhan University (Wuhan, China) and cultured in DMEM medium containing 10% fetal bovine serum (FBS). All the cells were routinely grown in a humidified atmosphere with 5% CO_2 at 37°C.

Real-time PCR

Total RNA was extracted from clinical tissues or cultured cells using TRIzol reagent (Invitrogen) following the manufacturer's instructions. cDNA was synthesized using TaqMan MicroRNA Reverse Transcription kit, and real-time PCR was performed using SYBR Green qPCR Master Mix Kit (Takara, Dalian, China) on a 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The primer sequences were as follows: miR-708, forward: 5'-CGC GGA TCC GAC TTC ATT CCC CTA ACC C-3' and reverse: 5'-CCG GAA TTC TGG CAC GCA GGA GAC AGT-3'; and U6, forward: 5'-CGC TTC GGC AGC ACA TAT AC-3' and reverse: 5'-AAA TAT GGA ACG CTT CAC GA-3'. The expression level of miR-708 was normalized to U6 and calculated according to the $2^{-\Delta\Delta Ct}$ method. The primers of survivin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed and synthesized by GenePharma (Shanghai, China). GAPDH was served as the internal reference for survivin mRNA.

Construction of vectors

To overexpress miR-708, a fragment of 88 bp containing the miR-708 precursor and the flanking sequence was synthesized and cloned into BamHI and EcoRI sites of lentiviral vector pHBLV-U6-ZsGreen-Puro (Promega, Madison, WI, USA), and recombinant lentivirus was constructed by co-transfection of miR-708 lentiviral vector and packaging plasmids to 293T cells using Lipofectamine 2000 (Invitrogen). Panc-1 and SW1990 cells were infected with lentivirus for 8 h in the presence of 8 mg/mL polybrene. Forty-eight hours after infection, 2 μg/mL puromycin was added to the culture medium to select the infected cells for one week. The two cell lines stably expressing miR-708 were established successfully.

To overexpress survivin, a 426 bp fragment of survivin cDNA was cloned into plasmid pcDNA3.1, and the recombinant plasmid was subsequently generated and transfected to cancer cells for 24 h using Lipofectamine 2000.

Cell viability assay

Cancer cells were inoculated in 96-well plates with a density of 3×10^3 cells/well overnight in triplicate. The cells were then incubated with 500 nmol/L of gemcitabine (Lilly France SA) at 37°C for 24 h. Cell viability was detected by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The absorbance at 570 nm was measured on a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell invasion assay

Invasion assay was performed using Matrigel-coated Transwell chamber (BD Biosciences, San Jose, CA, USA). Cancer cells were starved in serum-free medium overnight and were harvest and resuspended in FBS-free DMEM. A total of 5×10^4 cells in 100 μL DMEM were plated into the upper chambers. Then the lower chamber was covered with DMEM supplemented with 20% FBS. After incubation for 24 h at 37°C, non-invasive cells on the upper surface of the membrane were gently removed by cotton swabs, and the invaded cells were fixed with 4% formaldehyde and stained by 0.5% crystal violet. The stained cells were counted under an Olympus inverted microscope in five random visual fields.

Western blot analysis

Total protein was extracted after the cells were lysed in lysis buffer, then the concentration of total protein was measured by BCA assay. Equal proteins (40 μg/lane) were separated by 15% sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Sigma). After blocking by 5% skimmed milk, the membranes were incubated with primary antibodies for survivin, p-AKT, NF-kB P65, E-cadherin, and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. After extensive washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The protein bands were finally visualized using the SuperSignal enhanced chemiluminescence kit (Pierce, Rockford, IL, USA). GAPDH expression was detected as the internal control.

Luciferase reporter assay

The potential binding site in survivin 3'-UTR to miR-708 was predicted by TargetScan [http://www.targetscan.org/vert_71/]. The target sequence and mutation sequence were synthesized and then inserted into the XbaI site of pGL3 vector (Promega) and verified by sequencing. 2×10^5 Panc-1 cells were plated in a 6-well plate overnight, and then transfected with 800 ng of reconstructed pGL3-survivin-3'-UTR reporter using Lipofectamine 2000. Meanwhile, either 50 nM of miR-708 mimics (5'-AAG GAG CUU ACA AUC UAG CUG GG-3') or scramble (GenePharma) were co-transfected for 48 h. After the cells were lysed, luciferase activity was detected using the Dual-Luciferase Assay System (Promega).

Apoptosis assay by flow cytometry

Cells were harvested and washed in cold PBS and stained

with annexin V and PI using the annexin V-FITC Apoptosis Detection Kit (BD Biosciences). After incubation in the dark at room temperature for 20 min, the fluorescence intensity of each group of cells was immediately detected by flow cytometry (Coulter Biosciences, Brea, CA, USA).

Nude mouse model

Female BABL/c nude mice (4 weeks old) were purchased from the animal center of the Chinese Academy of Science (Shanghai, China). The animal experiments were approved by the Institutional Animal Care and Treatment Committee of Third Affiliated Hospital of Soochow University. 2×10^6 of tumor cells in a volume of 100 mL medium were injected subcutaneously into the right flank of each nude mouse (6 mice per group). After implantation for 7 days, tumor volume was measured every 7 days, using the following formula: volume = length (mm) × width^2 (mm)/2. All the mice were euthanized after 28 days.

Statistical analysis

Statistical analyses were carried out by SPSS 17.0 software (SPSS Inc., Chicago, IL, USA), and the comparison of numerical data among multiple groups was performed using the one-way ANOVA test. $p < 0.05$ was considered to be statistically significant.

Results

miR-708 expression was downregulated in pancreatic cancer tissues and cell lines

To explore the role of miR-708 in pancreatic cancer, the miR-708 expression level in human pancreatic cancer tissues and matched adjacent normal regions were detected by real-time PCR. We found that the expression of miR-708 was significantly downregulated in pancreatic cancer tissues compared with the normal adjacent tissues (Fig. 1(A), $p < 0.01$). We further confirmed that the miR-708 level was notably decreased in four pancreatic cancer cell lines, including Bxpc-3, Panc-1, Capan-1, and SW1990 compared to immortalized ductal epithelial cell line H6C7 (Fig. 1(B), $p < 0.05$). Among these cancer cell lines, Panc-1 and SW1990 showed relatively lower miR-708 level and were selected for subsequent experiments.

miR-708 overexpression suppressed proliferation, invasion and chemoresistance of pancreatic cancer cells in vitro

To reveal the function of miR-708 on the development of pancreatic cancer, the Panc-1 and SW1990 cell lines were infected with LV-miR-708 or negative control miRNA. RT-qPCR showed enhanced expression of miR-708 about 20-fold higher than control after infection of LV-miR-708 in both cell lines (Fig. 2(A), $p < 0.01$). We found a significant decrease in proliferation capability in cancer cells overexpressing miR-708 compared with control cells in a time-dependent manner, and the maximum inhibitory rate was 56.02 ± 3.98% and 53.24 ± 3.85% for Panc-1 and SW1990 on day 8, respectively (Fig. 2(B), $p < 0.05$).

Transwell invasion assay showed that Panc-1 and SW1990 infected by LV-miR-708 had significantly reduced invasive ability, as compared with control cells. The invasion rate was decreased to 38.51 ± 4.86% and 37.79 ± 4.95% in each cell line, respectively (Fig. 2(C), $p < 0.05$).

We also investigated the effect of miR-708 on gemcitabine chemosensitivity in pancreatic cancer cells in vitro. MTT assay showed that ectopic expression of miR-708 led to 18.13 ± 3.34 and 19.33 ± 3.55 loss of viability in Panc-1 and SW190 cells, respectively. Moreover, ectopic expression of miR-708 combined with gemcitabine could even cause 60.50 ± 6.14% and 61.33 ± 6.49% loss of viability (Fig. 2(D), $p < 0.01$).

MicroRNA-708 targeted survivin

To illustrate the molecular mechanisms of miR-708 in regulating malignant phenotypes of pancreatic cancer cells, bioinformatics analysis by TargetScan was carried out to predict the potential targets of miR-708. Survivin was predicted as a target of miR-708 (Fig. 3(A)). To further clarify whether the 3'-UTR of survivin is the functional target of miR-708, luciferase reporters harboring the miR-708 binding sites in survivin 3'-UTR and the mutant were reconstructed (Fig. 3(B)). After the reporters were co-transfected with miR-708 mimics into Panc-1 cells, miR-708 mimics significantly decreased luciferase activity of wild-type reporter but not the mutant reporter ($p < 0.05$; Fig. 3(C)). In addition, the survivin protein level was reduced in a dose-dependent manner when Panc-1 cells were transfected with 50–200 nM of miR-708 mimics (Fig. 3(D)). Taken together, these results indicate that miR-708 negatively regulated survivin expression by binding to its 3'-UTR, and survivin is a direct target of miR-708.

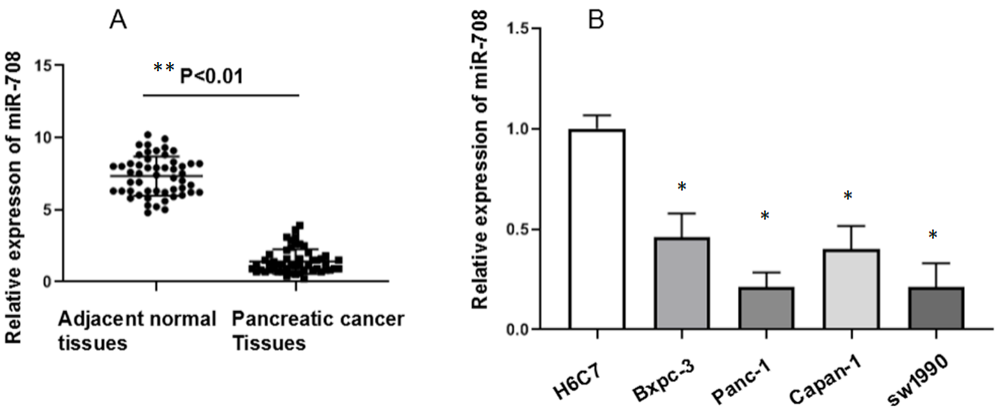

FIGURE 1. MiR-708 was downregulated in pancreatic cancer tissues. (A) miR-708 levels in 52 pancreatic cancer tissues and adjacent normal tissues were measured by real-time PCR. ** $p < 0.01$ (n = 52). (B) miR-708 levels in human pancreatic cancer cell lines and immortalized pancreatic ductal epithelial cell line H6C7 were measured by real-time PCR.* $p < 0.05$ *vs.* H6C7 cells (n = 3).

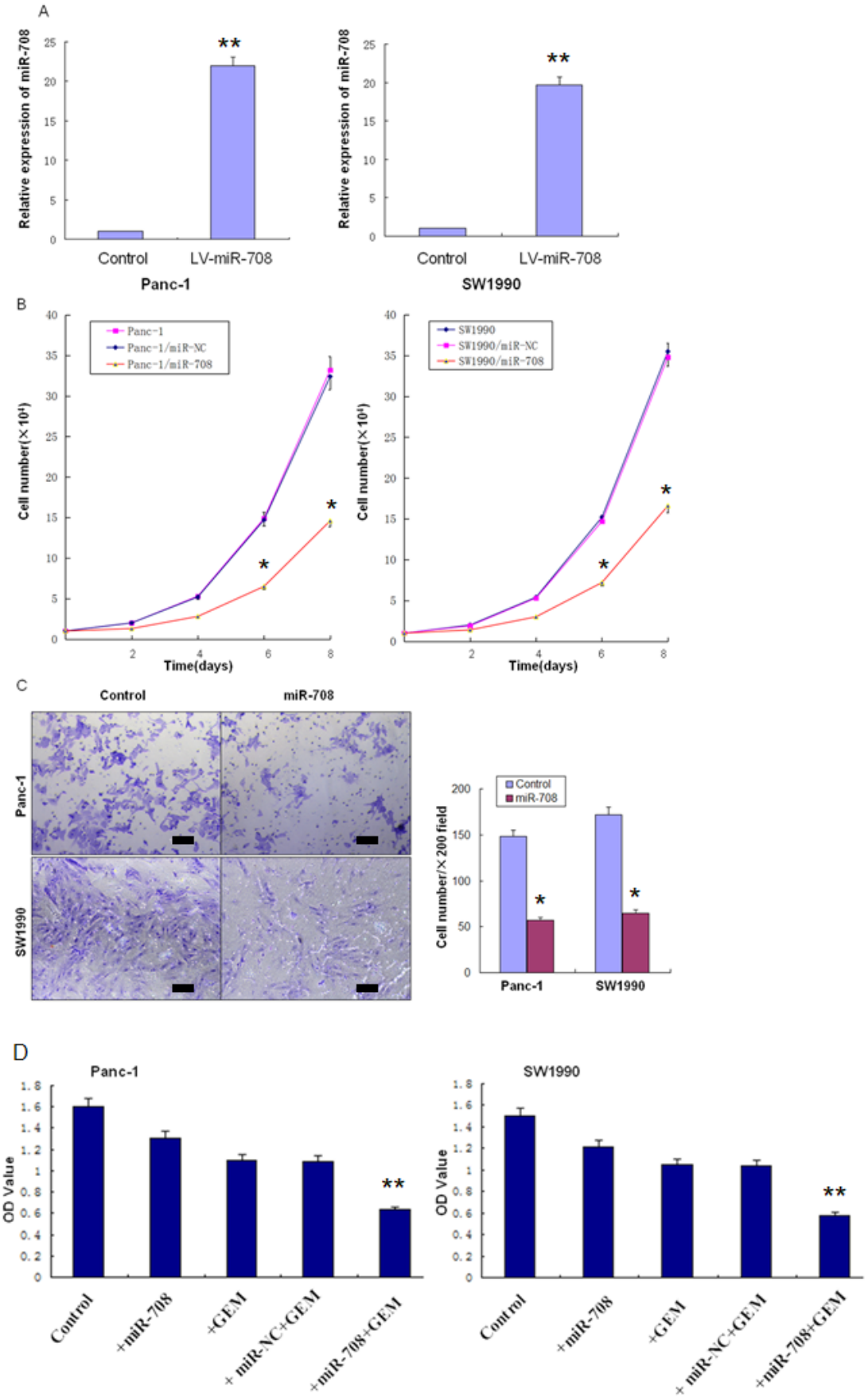

FIGURE 2. Restoration of miR-708 inhibited the proliferation and invasion of pancreatic cancer cells. (A) The upregulation of miR-708 was validated by real-time PCR in Panc-1 and SW1990 cells infected with miR-708 lentiviral vector. ** $p < 0.01$ *vs.* Control (n = 3). (B) The proliferation of Panc-1 and SW1990 cells was significantly suppressed in a time-dependent manner with the overexpression of miR-708. * $p < 0.05$ *vs.* Control (n = 3). (C) Overexpression of miR-708 decreased the invasion capacity of pancreatic cancer cells. Scale bar: 100 μm. * $p < 0.05$ *vs.* Control (n = 3). (D) Overexpression of miR-708 inhibited the resistance of both Panc-1 and SW1990 cells to gemcitabine. ** $p < 0.01$ *vs.* Control (n = 3).

Furthermore, both NF-κB and p-Akt levels decreased while E-cadherin, cleaved caspase-3, and cleaved caspase-7 levels increased when survivin expression was suppressed by miR-708 (Fig. 3(E)). These results suggest a proapoptotic role of miR-708.

Survivin was overexpressed in pancreatic cancer tissues and negatively correlated with miR-708 expression

To further explore the association between miR-708 and survivin, we detected the abundance of survivin in pancreatic cancer tissues and paired adjacent normal tissue. Compared with normal tissues, survivin mRNA levels significantly increased in pancreatic cancer tissues (Fig. 4(A)), The correlation between miR-708 expression and survivin level was analyzed by the Spearman's test, and miR-708 had significant inverse association with survivin mRNA expression in pancreatic cancer tissues (Fig. 4(B)).

Survivin antagonized miR-708 on pancreatic cancer cell growth and apoptosis

To explore whether survivin mediates the biological function of miR-708 in regulating the proliferation and apoptosis of pancreatic cancer cells, both Panc-1 and SW1990 cells were transfected with survivin construct to overexpress survivin (Fig. 5(A)). Flow cytometry assay showed that survivin overexpression partially inhibited the apoptosis induced by miR-708 in each cell line (Fig. 5(B)). The change of proliferation capacity of Panc-1 and SW1990 cells expressing both miR-708 and survivin indicated that survivin overexpression significantly rescued the inhibitory effect of miR-708 on the growth of pancreatic cancer cells (Fig. 5(C)).

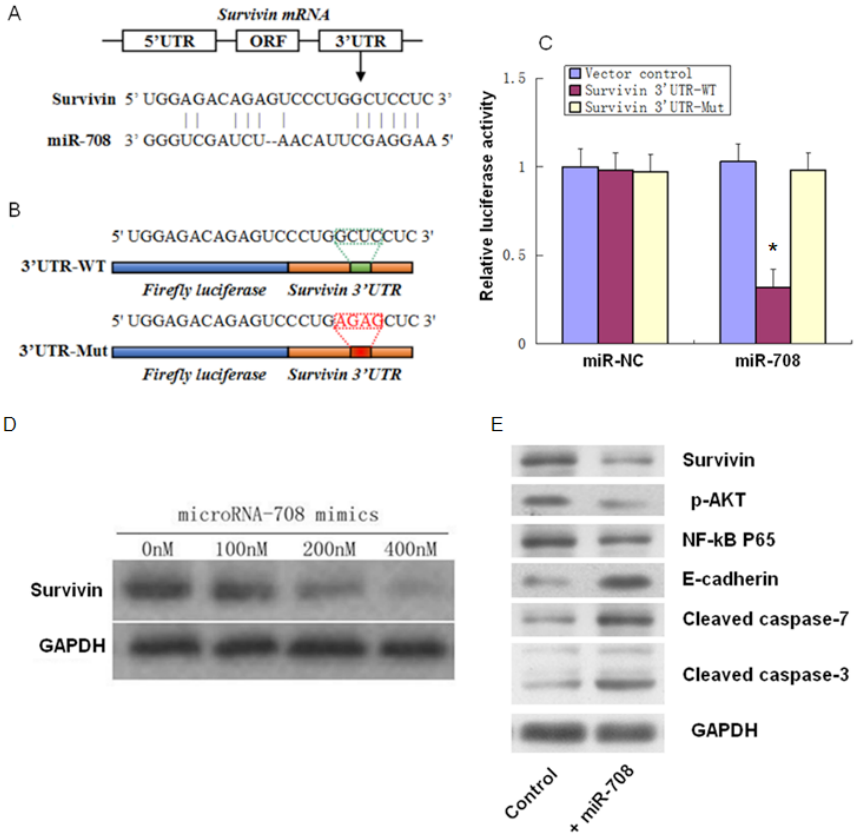

FIGURE 3. Survivin was the target gene of miR-708. (A) The putative binding site of miR-708 in survivin 3'UTR region (556-579 bp). (B) The mutant base sequence in survivin 3'UTR region in mutant reporter. (C) miR-708 mimics significantly decreased luciferase activity of wild-type reporter but not mutant reporter. * $p < 0.05$ *vs.* Control (n = 3). (D) Survivin protein level after 48 h of transfection with 50-200 nM miR-708 mimics. (E) In Panc-1 cells treated with infection by miR-708, Western blot analysis showed lower protein levels of survivin, p-AKT and NF-kB, and higher protein levels of E-cadherin, cleaved caspase-7 and cleaved caspase-3.

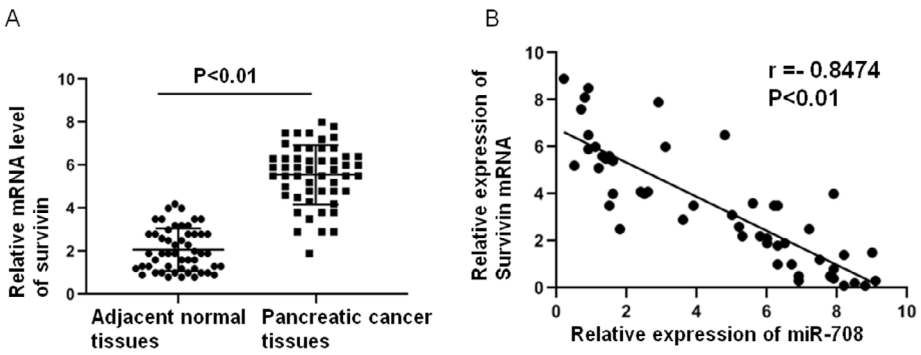

FIGURE 4. The expression of survivin mRNA in 52 cases of pancreatic cancer tissues and matched normal tissues. (A) Survivin mRNA level was significantly increased in pancreatic cancer tissues ($p < 0.01$, n = 52). (B) The negative correlation between the expression of miR-708 and survivin mRNA was verified by Spearman test.

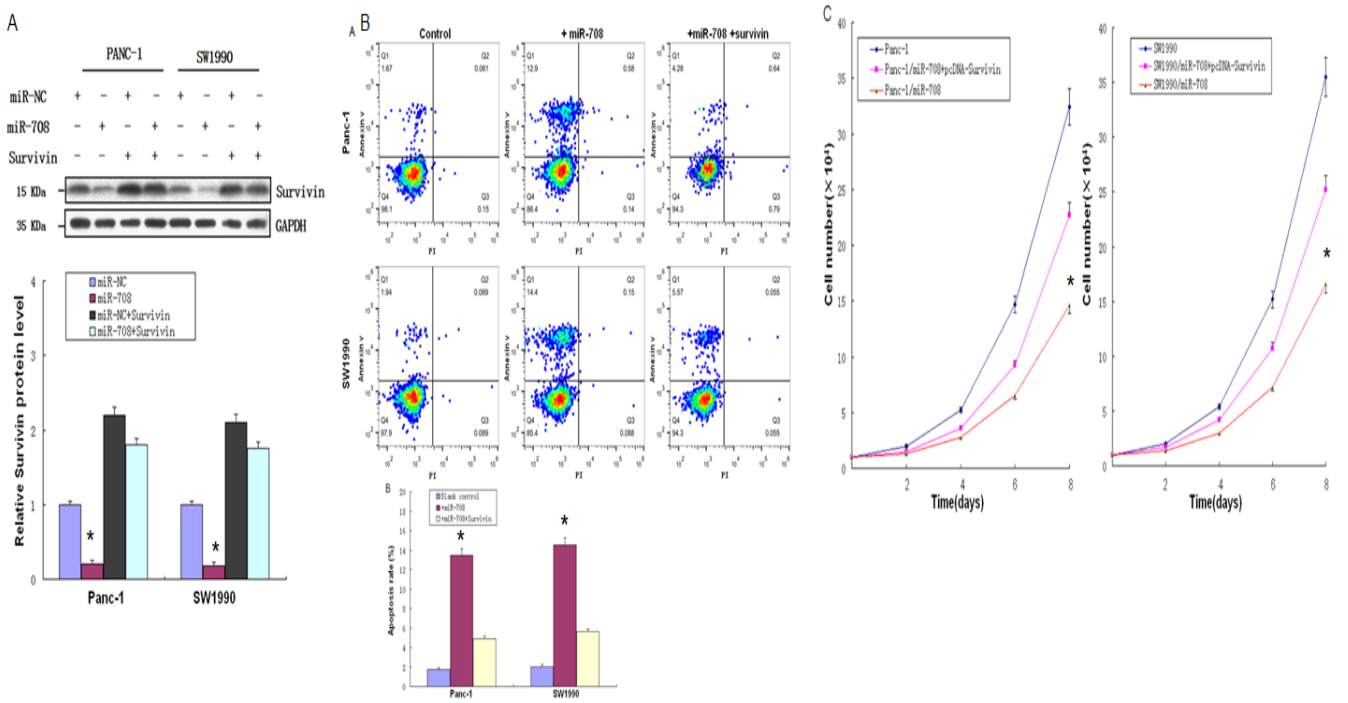

FIGURE 5. Survivin reversed pancreatic cancer cell growth inhibition and apoptosis induction by miR-708. (A) The upregulation of survivin in Panc-1 and SW1990 cells transfected with survivin vector. * $p < 0.05$ *vs.* other groups (n = 3). (B) Apoptosis analysis by flow cytometry (apoptotic rate = Q1 + Q2) in Panc-1 and SW1990 cells. * $p < 0.05$ *vs.* other groups (n = 3). (C) Restoration of survivin partially reversed the inhibitory effect of miR-708 on Panc-1 and SW1990 cell proliferation. * $p < 0.05$ *vs.* other groups (n = 3).

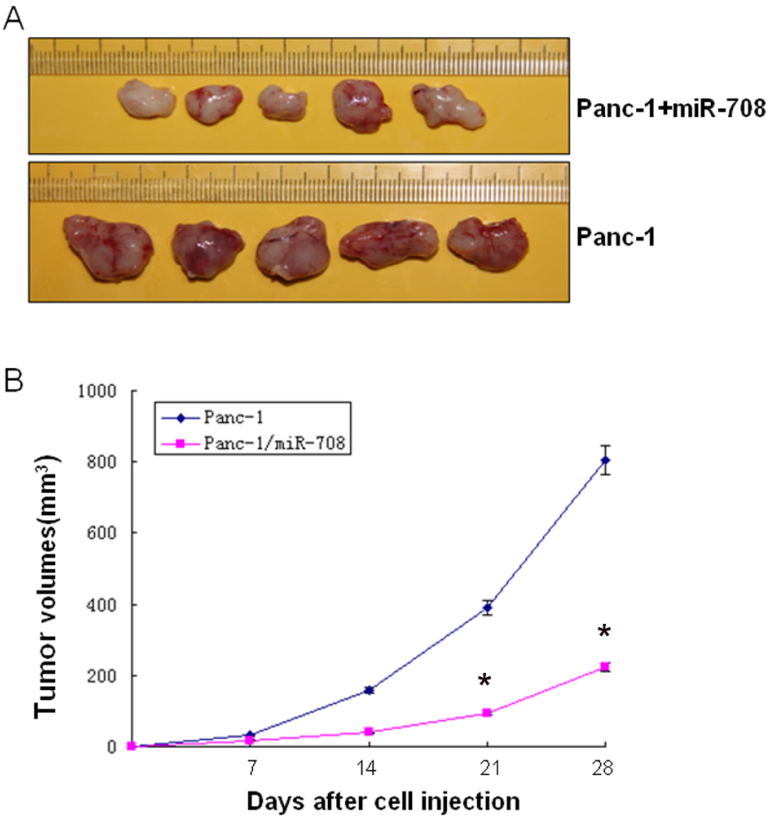

FIGURE 6. Anti-tumor effect of miR-708 in xenograft model. (A) Panc-1 cells were subcutaneously injected into nude mice. Mice were euthanized after 28 days and xenograft tumors were resected. (B) The volume of xenograft tumors administrated with miR-708 significantly decreased. * $p < 0.05$ *vs.* Control (n = 5).

Overexpression of miR-708 inhibited pancreatic cancer growth in xenograft model

Panc-1 cells infected with LV-miR-708 and control cells were injected into mice to evaluate the growth capability of xenograft tumors *in vivo*. The volume of xenograft tumors resulting from the injection of Panc-1 cells overexpressing miR-708 was significantly reduced compared with the control group (Fig. 6, $p < 0.05$).

Discussion

As the most aggressive malignancy of the digestive system, pancreatic cancer still leads to high mortality of the patients. Illustrating the molecular mechanisms that contribute to the tumorigenesis and progression of pancreatic cancer is crucial to the development of novel therapeutic strategies. In the present study, we found that miR-708 expression was significantly downregulated in pancreatic cancer tissues and cells.

Lentivirus-mediated overexpression of miR-708 could suppress the proliferation, invasion, and chemoresistance to gemcitabine in pancreatic cancer cells. MiR-708 negatively regulated survivin protein expression at a post-transcriptional level. Restoration of survivin expression could partially rescue proliferation inhibition and apoptosis induction by miR-708. Moreover, Panc-1 cells stably overexpressing miR-708 showed decreased proliferation capability in a nude mouse model in vivo. These results reveal the important role of the miR-708/survivin axis in modulating the development and progression of pancreatic cancer.

Recent evidence showed that aberrant miRNA expression was involved in the development and progression of malignant tumors, including pancreatic cancer (Zhu *et al*., 2018; Liu *et al*., 2018). miR-708 was reported to be highly expressed in the brain as well as the eyes and play a role in endoplasmic reticulum stress (Jang *et al*., 2012). Previous studies have reported the different expression levels of miR-708 in several cancer cells, which indicated that miR-708 may play different roles in a cancer type-dependent manner. For example, Li *et al*. (2015) found that miR-708 was downregulated in hepatocellular carcinoma (HCC) tissues and enforced expression of miR-708 suppressed the invasion and migration of HCC cell lines HepG2 and SMMC-7721. Qin *et al*. (2017) reported that restoration of miR-708 could sensitize ovarian cancer cells to cisplatin. However, Lei *et al*. (2014) reported that miR-708 was highly expressed in colorectal cancer (CRC) tissues, and inhibition of miR-708 suppressed cell proliferation and invasion in vitro. In this study, we found that miR-708 expression was significantly downregulated in pancreatic cancer cells. Our results confirmed that the restoration of miR-708 expression could significantly suppress the proliferation, induce apoptosis, and enhance chemosensitivity to gemcitabine in both Panc-1 and SW1990 cells.

As the smallest member of inhibitors of apoptosis protein (IAP) family, survivin participates in multiple biological processes, including restraining apoptosis and regulating cell division (Wu *et al*., 2018; Bi *et al*., 2018). Aberrantly high expression of survivin was associated with cancer progression and poor prognosis (Han *et al*., 2018). Survivin blocks apoptosis downstream components of both mitochondrial pathway and death receptor pathways by directly suppressing the activity of caspase-3, caspase-7, and caspase-9 (Li *et al*., 2018). These findings indicate that survivin may be a promising therapeutic target for cancer.

In this study, miR-708 was found to bind the 3′-UTR of survivin and negatively regulate the expression of survivin protein in pancreatic cancer cells. Moreover, miR-708 expression was negatively correlated with the survivin mRNA level in pancreatic cancer tissues. Restoration of survivin could partially reverse apoptosis and proliferation inhibition induced by miR-708 in both pancreatic cell lines of Panc-1 and SW1990. These data confirmed miR-708 as a novel upstream regulator of survivin.

Survivin was reported to activate NF-κB p65 via upregulating the transcriptional activity of IKKβ promoter in esophageal squamous cell carcinoma (Zeng *et al*., 2016). EMT is a critical process for epithelial-derived malignant tumor cells to acquire the ability of migration and invasion. Loss of E-cadherin as the tumor-suppressor protein is the central event for the development of malignant melanoma (Spangler *et al*., 2011). Activation of Akt, a serine/threonine kinase, has been found in multiple cancers. Akt activation promoted the progression of melanoma via upregulating NF-κB and inhibiting apoptosis (Zhao *et al*., 2010). AKT activation has been reported to be associated with the overexpression of survivin in melanoma cells (Simonetti *et al*., 2015). Our data showed that both NF-κB and p-Akt levels decreased when survivin was suppressed by miR-708, while E-cadherin was increased. We also confirmed that miR-708 overexpression led to increased levels of cleaved caspase-3 and caspase-7, further supporting the proapoptotic function of miR-708.

In summary, our results confirmed that miR-708 was downregulated in pancreatic cancer tissues and cells. Restoration of miR-708 significantly inhibited the proliferation, invasion, and chemoresistance of pancreatic cancer cells via directly targeting survivin. Therefore, the upregulation of miR-708 could be utilized as a novel approach for the treatment of pancreatic cancer.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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