Downregulation of miR-374b-5p promotes chemotherapeutic resistance in pancreatic cancer by upregulating multiple anti-apoptotic proteins

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Abstract. Resistance to first-line chemotherapeutic drugs such as gemcitabine contributes to the poor prognosis of patients with pancreatic cancer. MicroRNAs (miRNA) regulate chemoresistance in pancreatic cancer. By analyzing the miRNA sequencing dataset of pancreatic cancer from The Cancer Genome Atlas, it was demonstrated that miR-374b-5p expression was dramatically reduced in pancreatic cancer tissues compared with adjacent normal tissues, as well as decreased in chemoresistant compared with chemosensitive pancreatic carcinoma tissues. The decreased expression of miR-374-5p was associated with poor overall and progression-free survival in patients with pancreatic cancer. Furthermore, increased expression of miR-374b-5p abrogated, while the silencing miR-374b-5p increased the chemoresistance of pancreatic cancer cells to gemcitabine *in vitro*. Importantly, the upregulation of miR-374b-5p ameliorated the chemoresistance of pancreatic cancer cells to gemcitabine *in vivo*. It was also demonstrated that miR-374b-5p targeted several anti-apoptotic proteins, including B-cell lymphoma 2, Baculoviral IAP Repeat Containing 3 and X-linked inhibitor of apoptosis in pancreatic cancer cells, which further attenuated chemoresistance in pancreatic cancer. Therefore, the results of the current study indicate that miR-374b-5p serves as a potential diagnostic marker. It also suggests that miR-374b-5p sensitizes cells to chemotherapy and may be used in combination with chemotherapeutic agents such as gemcitabine to treat patients with pancreatic cancer.

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Introduction

Pancreatic cancer is one of the most devastating malignancies of the gastrointestinal tract as well as one of the most common causes of cancer-associated mortality worldwide (1). Surgery is the optimal method of treating patients with pancreatic cancer; however, the application of surgical therapy in patients with pancreatic cancer is limited, as the majority of patients are at an advanced stage of the disease at diagnosis. Indeed, ≤20% of patients with pancreatic cancer are suitable to undergo such surgery (2). For the majority of patients with pancreatic cancer, the primary method of treatment is chemotherapy and gemcitabine is the most common chemotherapeutic drug used to treat patients with inoperable pancreatic cancer. Although progress has been made in the treatment of pancreatic cancer over the past few decades, the median survival time of such patients remains <1 year (3,4). The failure of chemotherapy is attributed to the complete resistance against chemotherapeutic drugs and chemotherapy resistance presents a major clinical challenge in the treatment of cancer. Therefore, it is important to understand the molecular mechanisms underlying the development of chemoresistance in pancreatic cancer in order to develop effective therapeutic targets.

The resistance of cancer cells to a wide spectrum of chemotherapeutic drugs may be associated with the intrinsic resistance of cancer cells and/or acquired resistance following several cycles of chemotherapy (5). The most common mechanism by which chemoresistance arises is the development of efflux pumps on the surface of tumor cells that eject anticancer drugs from inside tumor cells to the outside environment (6). Other anti-drug mechanisms include the upregulation of DNA repair enzymes, insensitivity to drug-induced apoptosis, stromal proliferation, induction of drug-detoxifying mechanism and reduced angiogenesis, all of which serve a crucial role in the development of drug resistance of cancer cells (7-11). Therefore, it is crucial to develop comprehensive understanding of the specific mechanism by which chemoresistance arises in pancreatic cancer cells in order to identify a novel therapeutic strategy to overcome chemoresistance

and improve the clinical therapeutic response of patients with pancreatic cancer.

Micro (mi)RNAs post-transcriptionally regulate a variety of target genes by binding to the 3'-untranslated region (3'-UTR) of their target genes (12). The aberrant expression of miRNAs serves either oncogenic or tumor-suppressive roles in the tumorigenesis, progression and metastasis of various types of cancer (13-19). Previous studies have demonstrated that miRNAs are important mediators of chemoresistance in pancreatic cancer. Chaudhary *et al* (20) reported that miR-205-5p was differentially downregulated in pancreatic cancer cells and tissues. It was also demonstrated that the ectopic expression of miR-205-3p in combination with gemcitabine significantly reduced the proliferation and tumor growth of pancreatic cancer cells in mouse models (20). Furthermore the oncogenic miRNA miR-181c is dramatically elevated in pancreatic cancer tissues and this high expression of miR-181c induces chemoresistance in pancreatic cancer by inactivating the Hippo signaling pathway (21). These results indicate that the dysregulation of miRNAs serves an important role in the development of chemoresistance in pancreatic cancer.

The present study measured miR-374b-5p expression by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and evaluated whether there was an association between miR-374b-5p levels, clinicopathological characteristics and overall and progression-free survival of patients with pancreatic cancer. The effects of miR-374b-5p expression on chemoresistance were examined by performing gain or loss of function assays *in vitro* and *in vivo* and the potential targets of miR-374b-5p were identified using bioinformatics analysis, RT-qPCR, western blotting, luciferase reporter and RNA immunoprecipitation assays. The results revealed that the decreased expression of miR-374b-5p promoted the resistance of pancreatic cancer cells to the chemotherapeutic reagent gemcitabine by enhancing the expression of several anti-apoptotic proteins, including B-cell lymphoma 2 (BCL2), Baculoviral IAP Repeat Containing 3 (BIRC3) and X-linked inhibitor of apoptosis (XIAP). These results suggest that the delivery of miR-374b-5p may be developed as a novel therapeutic strategy to treat patients with chemotherapy resistant pancreatic cancer.

Materials and methods

Cell lines and cell culture. The human pancreatic cancer cell lines BxPC-3, PANC-1, AsPC-1, SW1990, Capan-1, Capan-2, CFPAC-1 and MIA PaCa-2 were all obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). All human pancreatic cancer cell lines were maintained in RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare, Logan, UT, USA) and 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained under a humidified atmosphere of 5% CO_2 at 37˚C.

Patients and tumor tissues. A total of 87 pancreatic cancer tissues and 8 adjacent normal tissues were obtained from 87 patients (51 males and 36 females) with a median age of 65 years old, undergoing surgery at the First Hospital of Jilin University (Changchun, China) between January 2007 and December 2015. Adjacent normal tissues were taken from 8 of these patients (5 males and 3 females), with median age of 60. Information regarding the response of patients to chemotherapy was obtained for only 63 patients with pancreatic cancer. Patients were diagnosed with pancreatic cancer based on clinicopathological evidence. Specimens were immediately snap-frozen and stored in liquid nitrogen tanks. Written informed consent from each patient enrolled in the study was obtained and ethical approval for the use of human tissues was obtained from the Institutional Research Ethics Committee at the First Hospital of Jilin University (ethics no. 2005-001). Clinicopathological information regarding the patients is presented in Tables I and II. Tumors were graded and classified according to the AJCC Cancer Staging Manual (22). The present study examined the expression of miR-374b-5p in the 87 pancreatic tissues and 8 adjacent normal tissues. Levels of miR-374b-5p expression in all pancreatic cancer tissues were normalized to that in the pancreatic cancer tissue with the lowest level of miR-374b-5p expression. Subsequently, the median miR-374b-5p expression in the pancreatic cancer tissues was used as the cutoff to distinguish between tissues exhibiting high and low expression of miR-374b-5p.

RT-qPCR. Total RNA from tissues or cells was extracted using an RNA Isolation kit (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's protocol. mRNA and miRNA were reverse transcribed using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Reverse transcription was performed at 42˚C for 60 min and 70˚C for 10 min. qPCR was performed using LightCycler® 480 SYBR-Green I Master (Roche Diagnostics, Basel, Switzerland). And was performed at 50˚C for 3 min, 95˚C for 10 min, followed by 45 cycles of 95˚C for 10 sec, 60˚C for 20 sec and 70˚C for 10 sec and then increased from 66˚C to 95˚C to obtain the melting curve. Each sample was analyzed in triplicate. qPCR was performed according to a standard method, as previously described (23). The following primers were used in the qPCR reaction: BCL2, forward, 5'-TCCGCATCAGGAAGGCTAGA-3' and reverse, 5'-AGGACCAGGCCTCCAAGCT-3'; BIRC3, forward, 5'-TTTCCGTGGCTCTTATTCAAACT-3' and reverse, 5'-GCACAGTGGTAGGAACTTCTCAT-3'; XIAP, forward, 5'-AATAGTGCCACGCAGTCTACA-3' and reverse, 5'-CAGATGGCCTGTCTAAGGCAA-3'; GAPDH, forward, 5'-ACAACTTTGGTATCGTGGAAGG-3' and reverse, 5'-GCCATCACGCCACAGTTTC-3'. Primers for U6 (patent no. MQP-0202; http://www.ribobio.com/sitecn/product_info.aspx?id=51) and miR-374b-5p (patent no. miRQ0004955-1-2, http://www.ribobio.com/sitecn/product_info.aspx?id=203689) were synthesized and purified by Guangzhou RiboBio (Guangzhou, China). U6 or GAPDH were used as endogenous controls for miRNA and mRNA, respectively. Relative fold expressions were calculated using the comparative threshold cycle ($2^{-\Delta\Delta Cq}$) method (24).

Plasmid, small interfering (si) RNA and transfection. The human miR-374b-5p expression plasmid was generated by cloning the genomic pre-miR-374b gene into the retroviral transfer plasmid pMSCV-puro (Takara Bio, Inc., Kusatsu, Japan) to generate the plasmid pMSCV-miR-374b-5p. pMSCV-miR-374b-5p was then cotransfected with the pIK packaging plasmid (cat no. 75483) into

Table I. Clinicopathological information of the 87 patients with pancreatic cancer for microRNA-374b-5p expression analysis.

	Cases (n)	Percentage (%)
Sex		
Male	51	58.6
Female	36	41.4
Age (years)		
≤60	28	32.2
>60	59	67.8
Histopathology		
Ductal adenocarcinoma	70	80.5
Other	17	19.5
Location		
Head of pancreas	71	81.6
Other	16	18.4
Grade		
G1	16	18.4
G2	47	54.0
G3	24	27.6
T classification		
T1	8	9.2
T2	12	13.8
T3	60	69.0
T4	7	8.0
N classification		
N0	25	28.7
N1	62	71.3
M classification		
M0	83	95.4
M1	4	4.6
Stage		
Stage I	13	14.9
Stage II	64	73.6
Stage III	6	6.9
Stage IV	4	4.6
Chemotherapeutic response		
Sensitivity	29	33.3
Resistance	34	39.1
Not available	24	27.6

Other histopathology includes: Adenocarcinoma not otherwise specified, mucinous carcinoma and neuroendocrine carcinoma. T, tumor volume; N, lymph node metastasis; M, distant organ metastasis.

Table II. Clinicopathological information of the 8 patients with pancreatic cancer that underwent microRNA-374b-5p expression analysis.

	Cases (n)	Percentage (%)
Sex		
Male	5	62.5
Female	3	37.5
Age (years)		
≤60	3	37.5
>60	5	62.5
Histopathology		
Ductal adenocarcinoma	6	75.0
Other	2	25.0
Location		
Head of pancreas	7	87.5
Other	1	12.5
Grade		
G1	1	12.5
G2	3	37.5
G3	4	50.0
Stage		
Stage I	2	25
Stage II	5	62.5
Stage III	1	12.5
Stage IV	0	0

Other histopathology includes: Adenocarcinoma not otherwise specified (1 case) and neuroendocrine carcinoma (1 case).

PCR clone reactions are listed in Table III. They were then cloned into a pmirGLO luciferase reporter vector (Promega Corporation, Madison, WI, USA). The miArrest plasmids for anti-miR-374b-5p and negative controls were constructed and cloned into phU6 plasmids (Shanghai GeneChem Co., Ltd., Shanghai, China). The sequence of the anti-miR-374b-5p is CACTTAGCAGGTTGTAT TATAT. siRNAs for BCL2 (patent no. stB0003729A-1-5, http://www.ribobio.com/sitecn/product_info.aspx?id=278148), BIRC3 (patent no. stB0001202A-1-5, http://www.ribobio.com/sitecn/product_info.aspx?id=275621) and XIAP (patent no. stB0002398A-1-5, http://www.ribobio.com/sitecn/product_info.aspx?id=276817) knockdown and siRNA negative control (part of the BCL2, BIRC2 and XIAP siRNA kits) were obtained from Guangzhou Ribobio. Transfection of siRNAs and plasmids was performed using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) in BxPC-3 and SW1990 cells following the manufacturer's protocol. The concentration of each siRNA used in transfection was 50 nmol/l, temperature was 37°C and the duration of transfection was 48 h. The period of time between transfection and subsequent experimentation was <7 days.

Western blotting. Western blotting was performed following a previously described method (26). Briefly, cells were seeded in 100-mm tissue culture dishes. Following 24 h, when cell confluence reached 60-70%, cells were washed with prechilled PBS and proteins were extracted using sample buffer [62.5 mmol/l Tris-HCl (pH 6.8), 2% SDS, 10% glycerol and

293FT cells (cat. no. CRL-1573) (both from ATCC) using the standard calcium phosphate transfection method, as previously described (25). A total of 36 h following co-transfection, supernatants were collected and incubated with cells to be infected for 24 h in the presence of polybrene (2.5 µg/ml). Following infection, puromycin (1.5 µg/ml) was used to select stably transduced cells over a 10-day period. The 3'-UTR regions of BCL2, BIRC3 and XIAP were amplified from genomic DNA using qPCR with 2x Phanta HS Master mix (cat. no. P512-01; Vazyme Biotech Co., Ltd, Nanjing, China) and using the thermocycling conditions described in the preceding paragraph. The primers used in the

5% 2-β-mercaptoethanol]. The concentration of protein was determined using the BCA method. Equal amounts (30 μg) of protein from the supernatant were loaded per lane and resolved using 9% SDS-PAGE. Proteins were transferred onto a PVDF membrane (EMD Millipore, Billerica, MA, USA), blocked with 5% nonfat milk for 1 h at room temperature and probed with primary antibodies against BCL2 (cat. no. ab194583; Abcam, Cambridge, UK), BIRC3 (cat. no. 3130), XIAP (cat. no. 14334), cytochrome *c* (cat. no. 11940, all dilution 1:1,000) (all from Cell Signaling Technology, Inc., Danvers, MA, USA) and mouse anti-α-tubulin antibody (cat. no. ab7291, dilution, 1:5,000; Abcam) overnight at 4°C. Membranes were washed three times (10 min/wash) in TBS-T buffer and incubated for 40 min at room temperature with horseradish peroxidase-conjugated anti-mouse (cat. no. 7076) or anti-rabbit (cat. no. 7074; Cell Signaling Technology, Inc.) secondary antibodies (both, dilution 1:5,000). Blots were then washed three times (10 min/wash) in TBS-T and developed using an enhanced chemiluminescence system (BeyoECL Plus, cat. no. P0018; Beyotime Institute of Biotechnology, Haimen, China). Protein loading was normalized by reprobing the blots with the mouse anti-α-tubulin antibody.

Analysis of cell apoptosis. Flow cytometric analysis of apoptosis was performed using the fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection kit I (BD Biosciences, San Jose, CA, USA) and performed as previously described (27). Briefly, cells were dissociated with trypsin and resuspended at 1x10^6 cells/ml in binding buffer with 50 μl/ml FITC Annexin V and 50 μl/ml propidium iodide (PI). Cells were subsequently incubated for 15 min at room temperature and analyzed using a Gallios flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). The cell's inner mitochondrial membrane potential ($\Delta\psi$m) was detected by flow cytometry using a MitoScreen JC-1 staining kit (BD Biosciences) following a previously described protocol (27). Briefly, cells were dissociated with trypsin, resuspended at 1x10^6 cells/ml in assay buffer and then incubated at 37°C for 15 min with 10 μl/ml JC-1. Cells were washed twice with the assay buffer prior to analysis with the flow cytometer. Flow cytometric data were analyzed using FlowJo 7.6 software (FlowJo LLC, Ashland, OR, USA).

Quantification of pancreatic cell viability following treatment with gemcitabine. All pancreatic cancer cells, including BxPC-3, PANC-1, AsPC-1, SW1990, Capan-1, Capan-2, CFPAC-1 and MIA PaCa-2, were cultured in 96-well plates and treated with dimethyl sulfoxide (vehicle) or 10 μM gemcitabine (cat. no. 1288463; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 36 h under a humidified atmosphere of 5% CO_2 at 37°C. The number of live cells was quantified using the CellTiter-Glo® Luminescent Cell Viability Assay (cat. no. G7570; Promega Corporation). Surviving cells were calculated as the fraction of vehicle controls and the results were averaged between six wells per dose in three independent experiments.

Caspase-9 and -3 activity assays. Caspase-9 and -3 activity was analyzed by spectrophotometry using the caspase-9 (cat. no. KGA403) and caspase-3 (cat. no. KGA202) colorimetric assay kits, respectively (Nanjing Keygen Biotech, Co., Ltd.,

Nanjing, China), following the manufacturer's protocol. Briefly, 5x10^6 cells or 100 mg fresh tumor tissues were washed with cold PBS, resuspended in lysis buffer and incubated on ice for 30 min. A total of 50 μl cell suspension, 50 μl reaction buffer and 5 μl caspase-3/-9 substrate was mixed and then incubated at 37°C for 4 h. Absorbance was then measured at 405 nm and the results of BCA protein quantitative analysis were used as the reference to normalize expression in each experimental group.

High throughput data processing and visualization. Datasets regarding miRNA expression and the clinical profile of pancreatic cancer were downloaded from The Cancer Genome Atlas (TCGA: https://cancergenome.nih.gov/) and ArrayExpress (http://www.ebi.ac.uk/arrayexpress/). The $\log_2$ values of miRNAs in each sample were analyzed using Excel 2010 (Microsoft Corporation, Redmond, WA, USA) and GraphPad 5 (GraphPad, Inc., La Jolla, CA, USA). Differences in the miRNA expression of all pancreatic cancer tissues between the two groups were assessed using paired or unpaired t-tests. Datasets were downloaded from GSE24279 and GSE29352 (https://www.ncbi.nlm.nih.gov/geo/) to assess the expression of miRNAs in pancreatic cancer. The expression of miRNAs in each sample were analyzed as previously described (28). The expression of miR-374b-5p in pancreatic cancer datasets taken from TCGA and GSE was analyzed. Targetscan (http://www.targetscan.org/vert_71/) and miRanda (http://34.236.212.39/microrna/home.do) were used to predict the potential targets of miR-374b-5p and assessed using a previously described protocol (29,30).

Tumor xenografts. A total of 20, 6-week-old female BALB/c-nu mice weighing 10-12 g were randomly divided into 4 groups (each, n=5). Mice were kept at a temperature of 18-29°C, humidity of 40-70% and under a 12-h light-dark cycle. Mice also had *ad libitum* access to food and water mice. Cells (2x10^6) were inoculated subcutaneously into the inguinal folds of the nude mice. In 2 groups of mice, 2x10^6 control AsPC-1 cells were inoculated subcutaneously in the left dorsal flank; in the other 2 groups, the mice were subcutaneously injected with the same number of miR-374b-5p overexpressing-AsPC-1 cells. At 7 days after the cell inoculation, one group of mice injected with the control cells and one group of mice injected with the anti-miR-149-5p cells received 50 μg/g gemcitabine (cat. no. 1288463, Sigma-Aldrich; Merck KGaA) via intraperitoneal injection twice a week for 4 weeks. Tumor volume was determined using an external caliper and calculated using the following equation: Length x (Weight)^2/2. Tumor volume was assessed using the IVIS Spectrum In Vivo Imaging System (Perkin Elmer, Inc., Waltham, MA, USA) at 5-day intervals. On day 40, animals were euthanized via cervical dislocation. Tumors were excised, weighed and stored in liquid nitrogen tanks. Ethical approval for the animal study was obtained from the Institutional Research Ethics Committee from the First Hospital of Jilin University (ethics no. 2005-001).

Dual-luciferase reporter assay. Cells (4x10^4) were seeded in triplicate in 24-well plates and cultured for 24 h. Subsequently, a dual-luciferase reporter assay was performed following a previously described protocol (31). Briefly, cells were plated

Table III. Primer sequences used in the reactions for clone polymerase chain reaction.

Gene	Sequence (5'-3')
miR-374b-5p-clone-F	CGGATTAGGCACTGTGAATACAAAG
miR-374b-5p-clone-R	TCTGCCAGGTAGAGTGGGAAAC
BCL2-3'-UTR-F	GGGGGTTATCTGTACATCCTGGG
BCL2-3'-UTR-R	AACGATCACCTTTGCTCACAAATAG
BIRC3-3'-UTR-F	GTGGAAGAACAATTGCGGAGAC
BIRC3-3'-UTR-R	CCTGGCTTCATGTTCCCGGTATTAG
XIAP-3'-UTR-F	AGAACGTCCAGGGTTTACATTACAAG
XIAP-3'-UTR-R	GTGGCACCTGCCTGTAATTCC

F, forward; R, reverse; UTR, untranslated region; miR, microRNA; BCL2, B-cell lymphoma 2; BIRC3, Baculoviral IAP Repeat Containing 3; XIAP, X-linked inhibitor of apoptosis. The primer sequences for U6 were synthesized and purified by Guangzhou RiboBio (patent no. MQP-0202; http://www.ribobio.com/sitecn/product_info.aspx?id=51, Guangzhou, China).

in 60-mm cell culture dishes and cultured for 24 h until they reached 60-80%. Subsequently, 100 ng BCL2-3'-UTR, BIRC3-3'-UTR or XIAP-3'-UTR luciferase plasmids, plus 5 ng pRL-TK *Renilla* plasmid (Promega Corporation) were transfected into cells using Lipofectamine 3000. Following 12-h incubation, the transfection medium was replaced; cells were harvested and washed with PBS and lysed using passive lysis buffer (Promega Corporation). Cell lysates were analyzed immediately using a Synergy™ 2 microplate system (BioTek Instruments, Inc., Winooski, VT, USA). Luciferase and *Renilla* luciferase were measured using a dual-Luciferase Reporter assay system (Promega Corporation) following the manufacturer's protocol. The luciferase activity of each lysate was normalized to *Renilla* luciferase activity. The relative transcriptional activity was converted into fold induction above the vehicle control value.

miRNA immunoprecipitation (IP). Cells were co-transfected with pIRESneo-FLAG/human influenza hemagglutinin (HA)-Ago2 plasmid (cat. no. 10822; Addgene, Inc., Cambridge, MA, USA), followed by HA-Ago2 IP using HA-antibody (dilution, 1:1,000; cat. no. H3663, Sigma-Aldrich; Merck KGaA), following a previously described protocol (32). Briefly, cells ($4x10^4$) were plated in 60-mm cell culture dishes and cultured for 24 h until they reached 60-80% confluence. pIRESneo-FLAG/HA-Ago2 plasmids were transfected into cells using Lipofectamine 3000. Following 48-h transfection, cells were washed and lysed in radioimmunoprecipitation buffer (Sigma-Aldrich; Merck KGaA) containing 10% proteinase inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride. A fraction of the whole cell lysate was used for RNA isolation and the remaining lysate was subjected to IP using antibodies against HA (dilution, 1:1,000; cat. no. H3663; Sigma-Aldrich; Merck KGaA) or immunoglobulin G (dilution, 1:2,000; cat. no. ab109489; Abcam). RNA from whole cell lysates and RNA IP (RIP) fractions were extracted using TRIzol (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Relative levels of mRNA were determined using RT-qPCR, following the aforementioned protocol. The relative mRNA

enrichment in the RIP fractions was computed based on the ratio of relative mRNA levels in the RIP fractions and the relative mRNA levels in the whole cell lysates.

Statistical analysis. All values are presented as the mean ± standard deviation. Significant differences were determined using GraphPad 5.0 software (GraphPad, Inc.). One-way analysis of variance was used to determine statistical differences between ≥2 groups, followed by Tukey's post hoc test. Unpaired or paired t-tests were used to determine statistical differences between 2 groups. The χ^2 test was used to analyze the association between miR-374b-5p expression and patient clinicopathological characteristics. Survival curves were plotted using the Kaplan Meier method and compared using the log-rank test using X-tile software version 3.6.1 (Yale University, New Haven, CT, USA). Pearson's correlation coefficient was used to analyze the correlation between miR-374b-5p expression levels and the apoptotic ratio following treatment with gemcitabine. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were repeated three times.

Results

miR-374b-5p expression is reduced in pancreatic cancer tissues. By analyzing several miRNA sequencing datasets of pancreatic cancer from TCGA and GSE using ArrayExpress, it was demonstrated that miR-374b-5p expression was significantly decreased in pancreatic cancer tissues compared with adjacent normal tissues (Fig. 1A-C). Subsequently, miR-374b-5p expression levels in the pancreatic cancer tissues taken from 8 patients recruited in the current study were measured and compared with miR-374-5p expression in the adjacent normal tissues. It was demonstrated that that miR-374b-5p expression was significantly decreased in 7 of the pancreatic cancer tissues compared with the respective adjacent normal tissue (Fig. 1D). Furthermore, miR-374-5p expression in the 87 pancreatic cancer tissues was significantly decreased compared with the normal tissues (Fig. 1E). The association between miR-374b-5p expression and the clinicopathological characteristics of patients with pancreatic cancer was then assessed and it was demonstrated that miR-374b-5p expression was inversely associated with node classification in patients with pancreatic cancer (Table IV). Kaplan-Meier survival analysis indicated that patients with pancreatic cancer that exhibited low expression of miR-374b-5p had significantly poorer overall and progression-free survival than those exhibiting high expression of miR-374b-5p (Fig. 1F and G). This was also the case in patients from the TCGA dataset (Fig. 1H and I). These results indicate that miR-374b-5p expression is sigificantly reduced in pancreatic cancer tissues and that this is assiciated with increased mortality in patients with pancreatic cancer.

The low expression of miR-374b-5p is positively correlated with chemoresistance in pancreatic cancer. miRNA sequencing of the TCGA dataset containing patients with pancreatic cancer indicated that miR-374b-5p expression was significantly decreased in tissues from patients with drug-resistant pancreatic cancer compared with those from patients
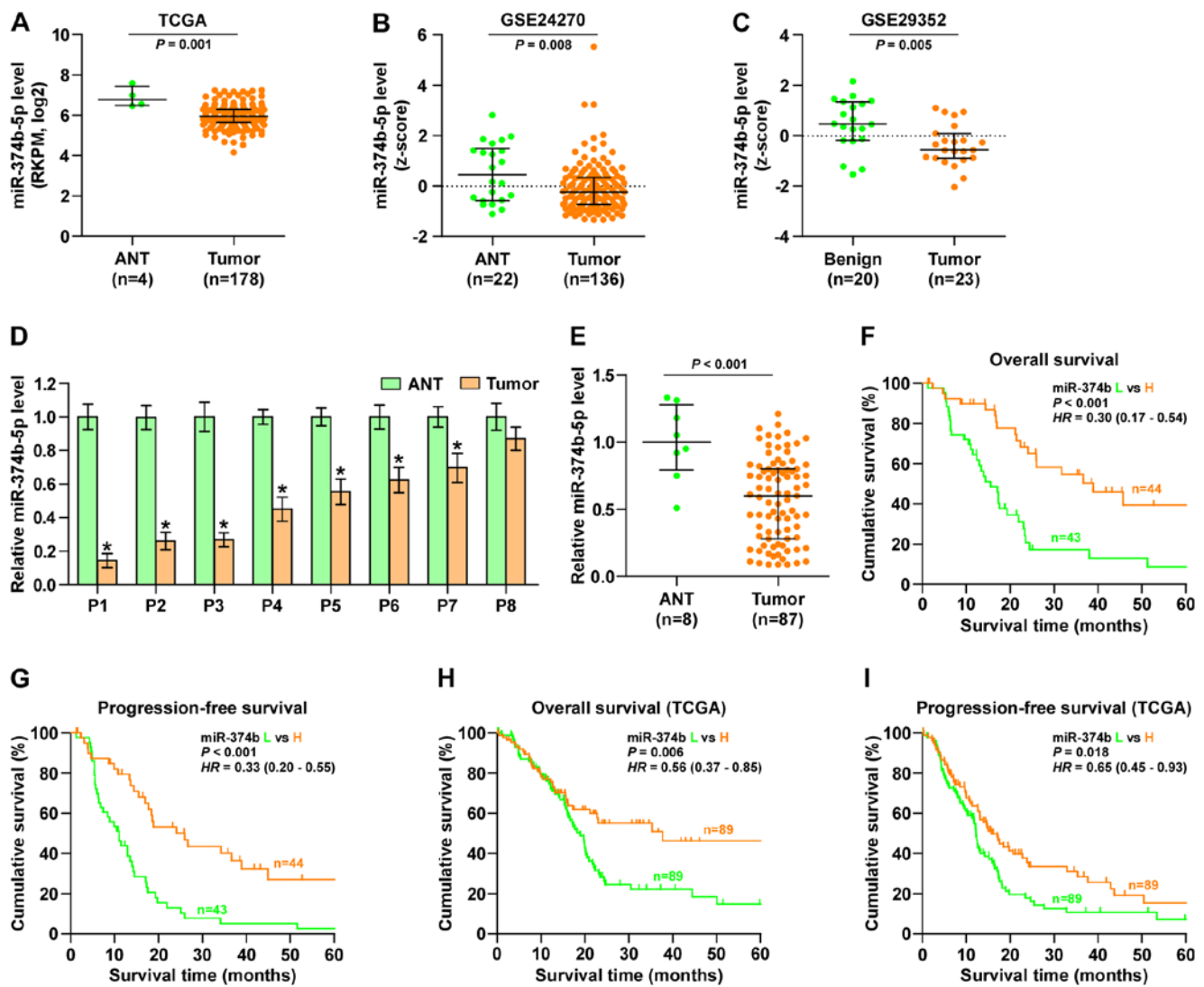
Figure 1. miR-374b-5p expression is decreased in pancreatic cancer tissues. (A) Analyzing the TCGA pancreatic cancer miRNA sequencing dataset indicated that miR-374b-5p expression was significantly decreased in pancreatic cancer tissues compared with ANTs. (B) Analysis of the GSE24270 pancreatic cancer miRNA sequencing dataset indicated that miR-374b-5p expression was decreased in pancreatic cancer tissues compared with ANTs. (C) Analysis of the GSE29352 pancreatic cancer miRNA sequencing dataset indicated that miR-374b-5p expression was decreased in pancreatic cancer tissues compared with ANTs. (D) miR-374b-5p expression was decreased in pancreatic cancer tissues compared with the 8 paired matched normal pancreatic tissues taken from patients in the current study. *P<0.05 vs. corresponding ANT (E) Analysis of patients included in the current study indicated that miR-374b-5p expression was decreased in pancreatic cancer tissues compared with ANTs. Kaplan-Meier analysis of (F) overall and (G) progression-free survival curves of patients with pancreatic cancer with low miR-374b-5p expression compared with those with high miR-374b-5p expression included in the current study. Kaplan-Meier analysis of (H) overall and (I) progression-free survival curves of patients with low miR-374b-5p expression compared with those with high miR-374b-5p expression in the TCGA pancreatic cancer dataset. The optimal cutoff point was selected using the log-rank test using X-tile software. The results are presented as the mean ± standard deviation of three independent experiments. ANT, adjacent normal tissue; miR, microRNA; TCGA, The Cancer Genome Atlas; L, low; H, high.

with drug-sensitive pancreatic cancer (Fig. 2A). This was also the case in patients recruited in the current study (Fig. 2B). The association between miR-374b-5p expression and the chemotherapeutic response of pancreatic cancer cells was evaluated by calculating the apoptotic ratio of pancreatic cancer cells treated with gemcitabine. As presented in Fig. 2C and D, pancreatic cancer cells with low expression of miR-374b-5p exhibited significantly increased resistance to gemcitabine compared with those that had high expression of miR-374b-5p. Furthermore, there was a positive correlation between miR-374b-5p expression and the apoptotic ratio of pancreatic cancer cells (Fig. 2E). Collectively, these results indicate that the low expression of miR-374b-5p is strongly associated with chemoresistance in pancreatic cancer.

Silencing miR-374b-5p promotes chemoresistance in pancreatic cancer cells. The role of miR-374b-5p in the chemoresistance of pancreatic cancer was further investigated. The BxPC-3 and SW 1990 chemosensitive pancreatic cancer cells exhibited the highest expression of miR-374-5p (Fig. 2C); therefore, miR-374b-5p expression was knocked down by transfecting anti-miR-374b-5p into BxPC-3 and SW 1990 cells (Fig. 3A). An Annexin V apoptosis assay was performed and the results demonstrated that silencing miR-374b-5p expression significantly decreased the apoptotic ratio in BxPC-3 and SW 1990 cells following treatment with gemcitabine (Fig. 3B). A mitochondrial membrane potential assay was then performed and the results indicated that silencing miR-374b-5p significantly enhanced the mitochondrial potential of BxPC-3 and SW
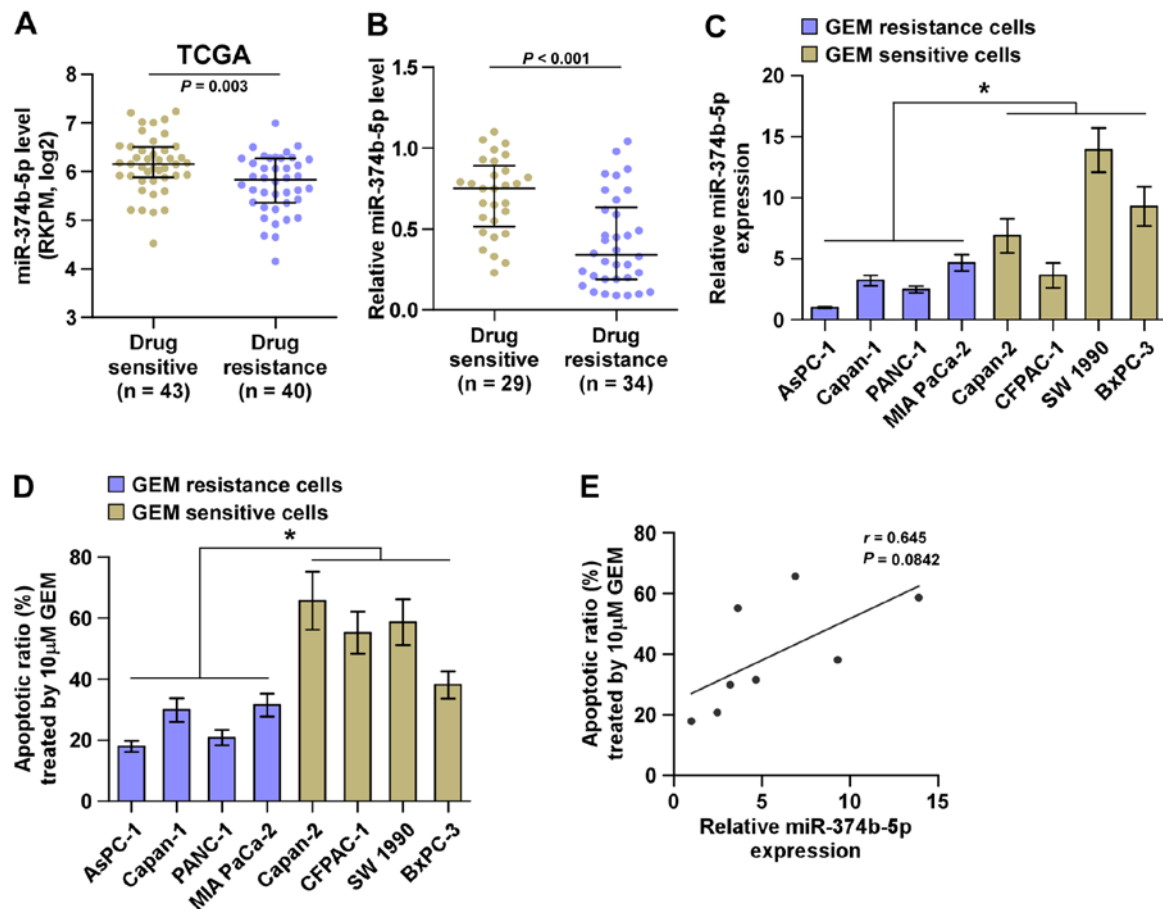
Figure 2. The decreased expression of miR-374b-5p is associated with chemoresistance in pancreatic cancer. (A) In the TCGA dataset, miR-374b-5p expression was reduced in chemoresistant pancreatic cancer tissues compared with chemosensitive pancreatic cancer tissues. (B) In tissues taken from patients included in the present study, miR-374b-5p expression was reduced in chemoresistant pancreatic cancer tissues compared with chemosensitive pancreatic cancer tissues. (C) Reverse transcription-quantitative polymerase chain reaction analysis of miR-374b-5p expression in pancreatic cancer cells. Transcript levels were normalized to U6 expression. (D) The apoptotic ratio of different pancreatic cancer cell lines following treatment with GEM. (E) The correlation between miR-374b-5p expression and the apoptotic ratio in pancreatic cancer cells following treatment with GEM. The results are presented as the mean ± standard deviation of three independent experiments. *P<0.05. TCGA, The Cancer Genome Atlas; miR-374-5p, microRNA-374-5p; GEM, gemcitabine.

1990 cells following treatment with gemcitabine (Fig. 3C). Furthermore, silencing miR-374b-5p decreased the expression of cytochrome *c* (Fig. 3D) and significantly decreased caspase-3 and -9 activity in BxPC-3 and SW 1990 cells following treatment with GEM (Fig. 3E). These results indicate that silencing miR-374b-5p induces the chemoresistance of pancreatic cancer cells to gemcitabine.

Ectopic expression of miR-374b-5p attenuates chemoresistance in pancreatic cancer cells. Subsequently, miR-374b-5p was overexpressed in AsPC-1 and PANC-1 cells via viral transduction (Fig. 4A), as these two cell lines expressed the lowest levels of miR-374b-5p (Fig. 2C). The upregulation of miR-374b-5p significantly increased the apoptotic ratio, the expression of cytochrome *c* and caspase-3 and -9 activity, but significantly reduced the mitochondrial potential in AsPC-1 and PANC-3 cells following treatment with gemcitabine (Fig. 4B-E). These results demonstrate that the upregulation of miR-374b-5p abrogates the resistance of pancreatic cancer cells to gemcitabine.

Upregulation of miR-374b-5p ameliorates the resistance of pancreatic cancer cells to gemcitabine in vivo. To investigate

the effect of miR-374b-5p on the chemoresistance of pancreatic cancer cells *in vivo*, mice were randomly divided into 4 groups (n=5/group). In the first two groups of mice, 2x10⁶ control AsPC-1 cells were inoculated subcutaneously into the left dorsal flank; in the other two groups, mice were subcutaneously injected with the same number of miR-374b-5p-overexpressing-AsPC-1 cells. Following 1 week, one control and one miR-374-5p-overexpressing group received an intraperitoneal injection of 50 µg/g gemcitabine twice per week over a 4-week period. Mice that received miR-374b-5p-overexpressing cells and gemcitabine treatment exhibited significantly smaller tumor volumes and weights (Fig. 5A-C) and significantly longer survival times compared with other three groups (Fig. 5D). Furthermore, caspase-3 and -9 activity were significantly increased in the mice injected with miR-374b-5p-overexpressing cells and receiving gemcitabine treatment (Fig. 5E). These results indicate that the upregulation of miR-374b-5p restores the sensitivity of pancreatic cancer cells to gemcitabine *in vivo*.

miR-374b-5p targets multiple anti-apoptotic proteins in pancreatic cancer cells. By analyzing several public databases, including TargetScan and miRanda, it was determined
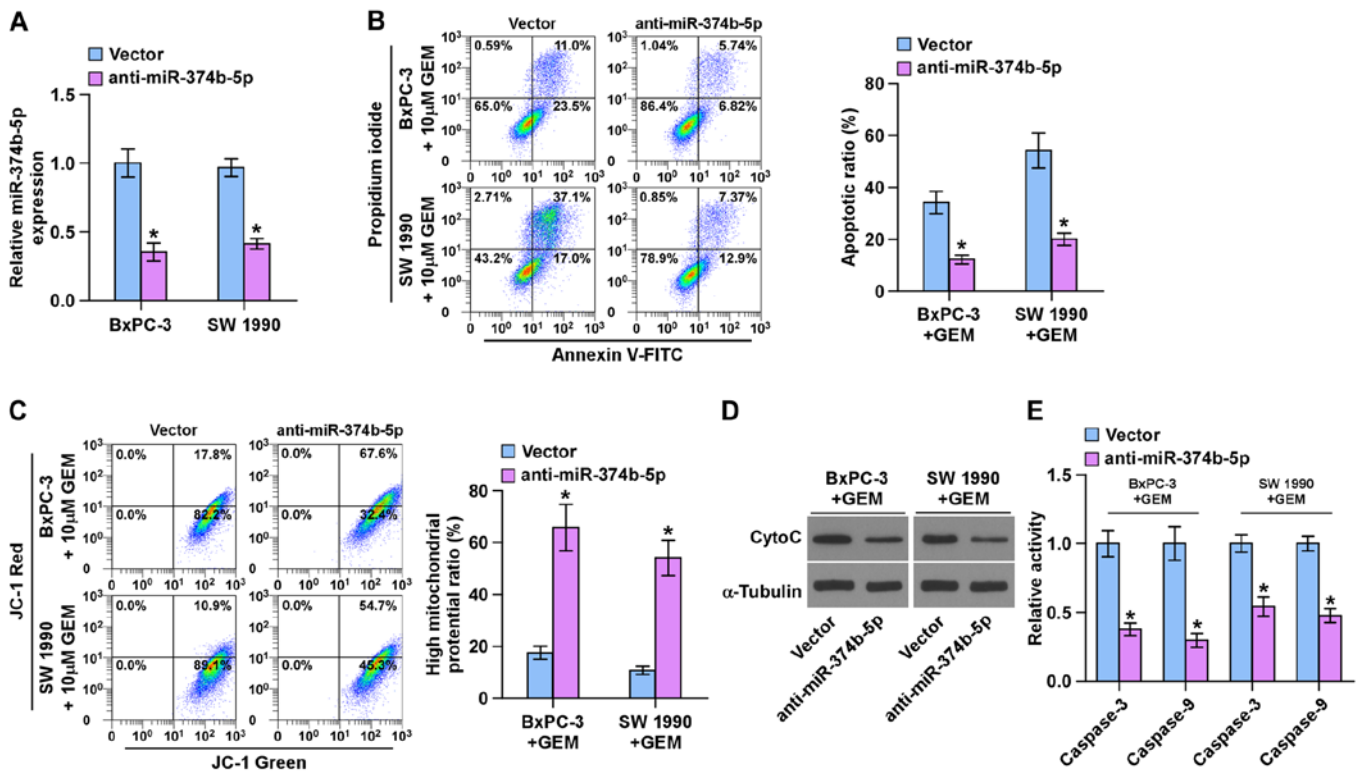
Figure 3. Downregulation of miR-374b-5p induces chemoresistance in BxPC-3 and SW1990 cells. (A) Reverse transcription-quantitative polymerase chain reaction analysis of miR-374b-5p expression. Transcript levels were normalized to U6 expression. (B) Annexin V-FITC/PI staining of cells treated with gemcitabine (10 μM) for 36 h. (C) JC-1 staining of the cells treated with GEM. (D) Western blotting analysis measuring CytoC expression. α-tubulin served as the loading control. (E) Analysis of caspase-3 and -9 activity. All results are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. vector. miR-374b-5p, microRNA-374b-5p; GEM, gemcitabine; CytoC, cytochrome *c*; FITC, fluorescin isothiocyanate; PI, propidium iodide.

that several anti-apoptotic proteins, including BCL2, BIRC3 and XIAP were potential targets of miR-374b-5p (Fig. 6A). The results of western blotting demonstrated that silencing miR-374b-5p increased the expression of BCL2, BIRC3 and XIAP in GEM-sensitive pancreatic cancer cell lines (Fig. 6B). Furthermore, the upregulation of miR-374b-5p significantly repressed the luciferase reporter activity of the 3'-UTRs of BCL2, BIRC3 and XIAP in pancreatic cancer cells (Fig. 6C), whereas silencing miR-374b-5p significantly elevated it (Fig. 6D). The results of the RNA IP assay identified a direct association between miR-374b-5p and BCL2, BIRC3 and XIAP transcripts (Fig. 6E and F). Collectively, these results demonstrate that BCL2, BIRC3 and XIAP are direct targets of miR-374b-5p in pancreatic cancer cells.

BCL2, BIRC3 and XIAP are crucial for the pro-chemoresistance effects of anti-miR-374b-5p on pancreatic cancer cells. It was then investigated whether BCL2, BIRC3 and XIAP mediate the functional effects of anti-miR-374b-5p on the chemoresistance of ovarian cancer cells. BCL2, BIRC3 and XIAP expression were silenced following transfection of siRNAs against BCL2, BIRC3 and XIAP into pancreatic cancer cells. The results of RT-qPCR demonstrated that silencing BCL2, BIRC3 and XIAP significantly reduced BCL2, BIRC3 and XIAP expression compared with the respective scramble controls (Fig. 6G). The silencing of BCL2, BIRC3 and XIAP expression significantly attenuated the stimulatory effects of anti-miR-374b-5p on the mitochondrial potential and caspase-3 and -9 activity, and reversed the inhibition of apoptosis induced

by anti-miR-374b-5p in pancreatic cancer cells (Fig. 6H-K). Taken together, these results indicate that BCL2, BIRC3 and XIAP are crucial for the effects of anti-miR-374b-5p on the chemoresistance of pancreatic cancer cells.

Discussion

The present study indicates that miR-374b-5p serves a tumor suppressive role in chemotherapeutic resistant pancreatic cancer. miR-374b-5p expression was decreased in pancreatic cancer tissues, particularly in chemoresistant pancreatic cancer tissues, and the low expression of miR-374b-5p was strongly associated with chemotherapeutic resistance, as well as poor overall and progression-free survival in patients with pancreatic cancer. The upregulation of miR-374b-5p decreased, whereas miR-374b-5p silencing increased, the chemoresistance of pancreatic cancer cells to gemcitabine *in vitro* and *in vivo*. Furthermore, the results of the current study revealed that the upregulation of miR-374b-5p restored the chemotherapeutic sensitivity of pancreatic cancer cells to gemcitabine via the direct targeting of several anti-apoptotic proteins, including BCL2, BIRC3 and XIAP. Therefore, the results of the current study indicate that miR-374b-5p serves a tumor suppressive role in the development of chemotherapeutic resistance in pancreatic cancer.

BCL2 belongs to the Bcl-2 family and is an inhibitor of apoptosis. Previous studies have demonstrated that the upregulation of BCL2 contributes to intrinsic and acquired drug resistance in different types of human cancer (33-35). In
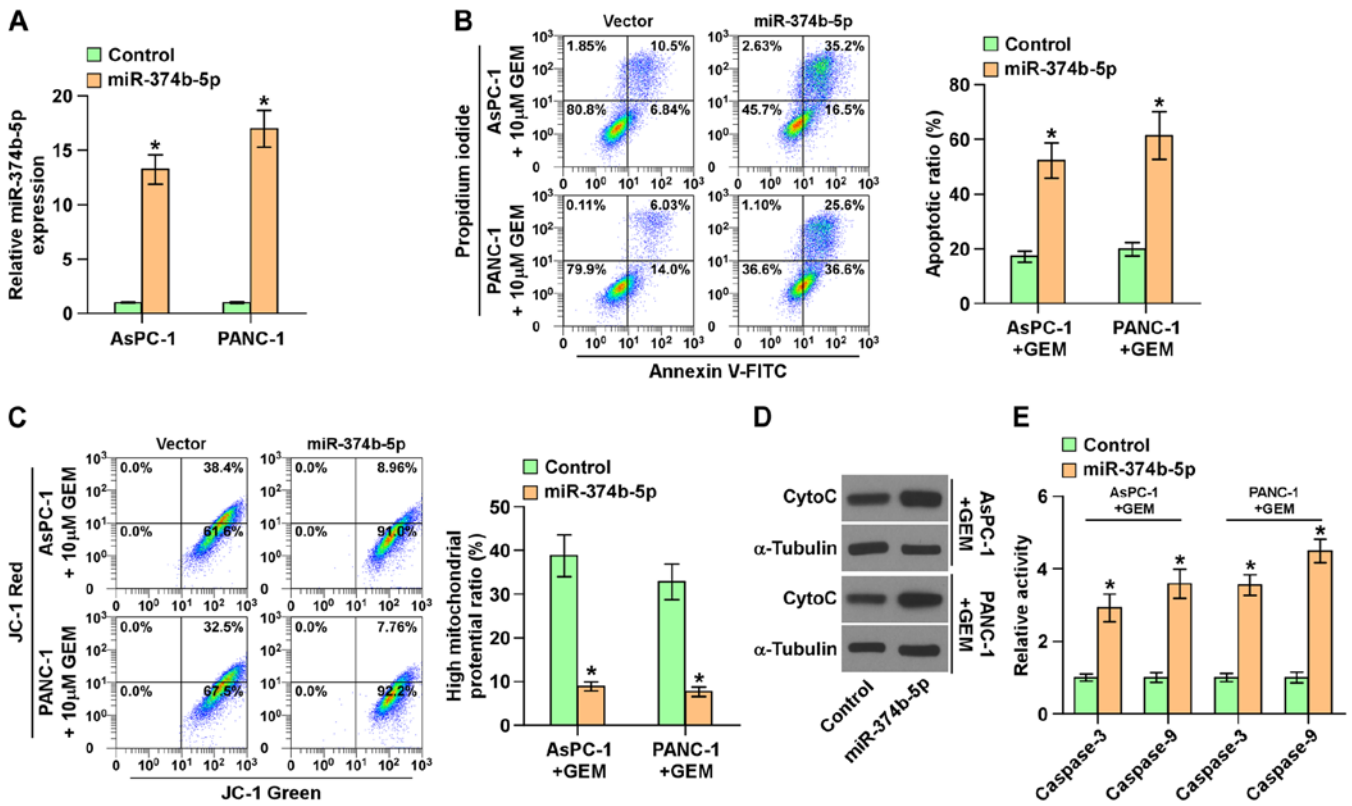
Figure 4. Upregulation of miR-374b-5p decreases the chemoresistance of AsPC-1 and PANC-1 cells. (A) Reverse transcription-quantitative polymerase chain reaction analysis of miR-374b-5p expression in the indicated pancreatic cancer cells. Transcript levels were normalized to U6 expression (B) Annexin V-FITC/PI staining of the cells treated with gemcitabine (10 μM) for 36 h. (C) JC-1 staining of cells treated with GEM. (D) Western blotting measuring CytoC expression. α-tubulin served as the loading control. (E) Analysis of caspase-3 and -9 activity. Results are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. respective control. miR-374b-5p, microRNA-374b-5p; FITC, fluorescin isothiocyanate; PI, propidium iodide; CytoC, cytochrome *c*; GEM, gemcitabine.
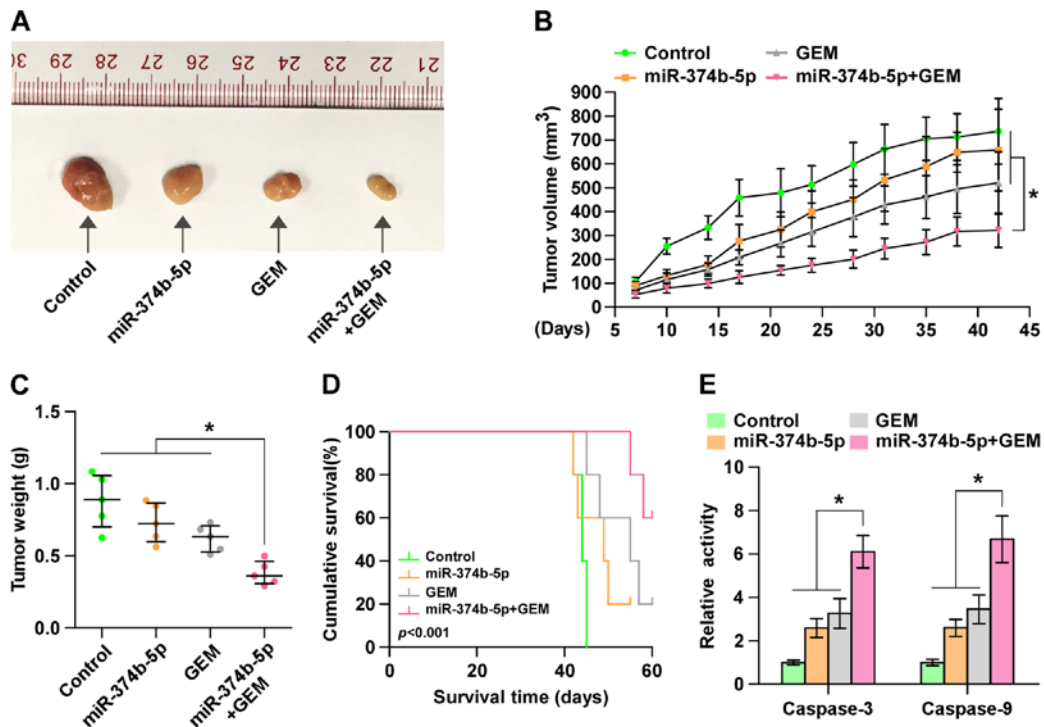

Figure 5. Upregulation of miR-374b-5p restores the sensitivity of pancreatic cancer cells to gemcitabine *in vivo*. (A) Representative images of the tumors in the xenograft model of nude mice are presented. (B) Following 10 days of inoculating the indicated AsPC-1 cells, mice were intraperitoneally injected with 50 μg/g GEM twice a week for 4 weeks. Tumor volumes were measured from the fifth day at intervals of 5 days. (C) Tumor weights of each group. (D) The overall survival of mice in the indicated group. (E) Caspase-3 and -9 activity in the indicated tumors. Data are presented are the mean ± standard deviation. *P<0.05. miR-374b-5p, microRNA-374b-5p; GEM, gemcitabine.

pancreatic cancer, the upregulation of BCL2 by the transcription factor cut like homeobox 1 significantly increased drug resistance (36). IAP proteins are another well-known family that act as endogenous inhibitors of apoptosis by directly binding to and inhibiting caspase proteins (37). The most well characterized IAP proteins include XIAP and BIRC3, which have been implicated in the chemoresistance of different types of cancer (38-40). Targeting Bcl-2 or IAP proteins markedly enhance the therapeutic effects of chemotherapeutic agents in various types of cancer (41,42); however, it remains unknown how these anti-apoptotic proteins are simultaneously repressed in cancer. The results of the current study demonstrated that miR-374b-5p simultaneously targets BCL2, BIRC3 and XIAP, thus contributing to the chemoreistance of pancreatic cancer cells to gemcitabine. Therefore, the results of the current study identified a novel mechanism by which miR-374b-5p improves the chemotherapeutic response of pancreatic cancer to gemcitabine.

The dysregulation of miR-374b-5p has been implicated several disorders, including obesity, calcific aortic stenosis, ischemic stroke, IgA nephropathy and infertility (43-47). In cancer, the overexpression of miR-374b-5p has been identified in breast, head and neck, gastric and prostate cancer, as well as in melanoma (48-53). However, the downregulation of miR-374b-5p has also been identified in T-cell lymphoblastic lymphoma and colorectal cancer (54,55). These results indicate that miR-374b-5p serves different roles in different types of tumor. Notably, a study by Schreiber *et al* (56) indicated that, following step-wise treatment with increasing concentrations of cisplatin over >20 passages, the downregulation of miR-374b-5p was directly involved in the development of the cisplatin-resistant phenotype. This suggests that the decreased expression of miR-374b-5p is associated with chemoresistance in pancreatic cancer. However, the clinical significance and underlying mechanism mediating the functional role of miR-374b-5p in pancreatic cancer are yet to be elucidated. The current study demonstrated that miR-374b-5p was downregulated in pancreatic cancer tissues and was associated with poor responses to chemotherapy, as well as poor overall and progression-free survival in patients with pancreatic cancer. Furthermore, the results of the current study indicate that the upregulation of miR-374b-5p reduces the chemotherapeutic resistance of pancreatic cancer cells to gemcitabine by inhibiting the expression of several anti-apoptotic proteins, including BCL2, BIRC3 and XIAP. This clarifies that miR-374b-5p serves a tumor suppressive role during the development of chemotherapeutic resistance in pancreatic cancer.

It has been suggested that miR-374b-5p may be used as a diagnostic marker in different types of cancer. Hanniford *et al* (50) reported that miR-374-b-5p has a diagnostic signature in patients with melanoma and brain metastasis compared with those without brain metastasis. Analysis of the TCGA dataset indicated that miR-374b-5p may be a prognostic marker of breast cancer (49). The results of the current study indicated that miR-374b-5p expression was dramatically reduced in pancreatic cancer tissues and the low expression of miR-374b-5p predicted the poor overall and progression-free survival of patients with pancreatic cancer. This indicates that miR-374b-5p may be used as a diagnostic marker in patients with pancreatic cancer. Importantly,

Table IV. The association between miR-374b-5p expression and the clinicopathological characteristics of the 87 patients with pancreatic cancer.

Parameters	No. of cases	miR-374b-5p expression Low	High	P-values
Sex				
Male	51	29	22	0.099
Female	36	14	22	
Age (years)				
≤60	28	16	12	0.321
>60	59	27	32	
Histopathology				
Ductal adenocarcinoma	70	35	35	0.828
Other	17	8	9	
Location				
Head of pancreas	71	38	33	0.108
Other	16	5	11	
Grade				
G1-G2	63	31	32	0.947
G3	24	12	12	
T classification				
T1-2	20	7	13	0.142
T3-4	67	36	31	
N classification				
N0	25	6	19	0.003[a]
N1-2	62	37	25	
M classification				
M0	83	39	44	0.055
M1	4	4	0	
Stage				
I-II	77	36	41	0.167
III-IV	10	7	3	
Chemotherapeutic response				
Sensitivity	29	9	20	<0.001[a]
Resistance	34	25	9	

[a]P<0.05. Other histopathology includes: adenocarcinoma not otherwise specified, mucinous carcinoma, and neuroendocrine carcinoma. miR-374b-5p, microRNA-374b-5p.

Summerer *et al* (51) reported that high levels of miR-374b-5p in the plasma predicted the poor prognosis of patients with head and neck cancer, suggesting that miR-374b-5p may be used as a minimally invasive diagnostic marker in patients with cancer. However, further studies are required to determine whether miR-374b-5p levels in the serum or plasma may be used as a minimally invasive diagnostic or prognostic marker for patients with pancreatic cancer.

In conclusion, the results of the current study clarify that the upregulation of miR-374b-5p restores the chemotherapeutic resistance of pancreatic cancer cells to gemcitabine by directly repressing BCL2, BIRC3 and XIAP expression. Comprehensive understanding of the underlying mechanism of miR-374b-5p in the chemoresistance of pancreatic cancer
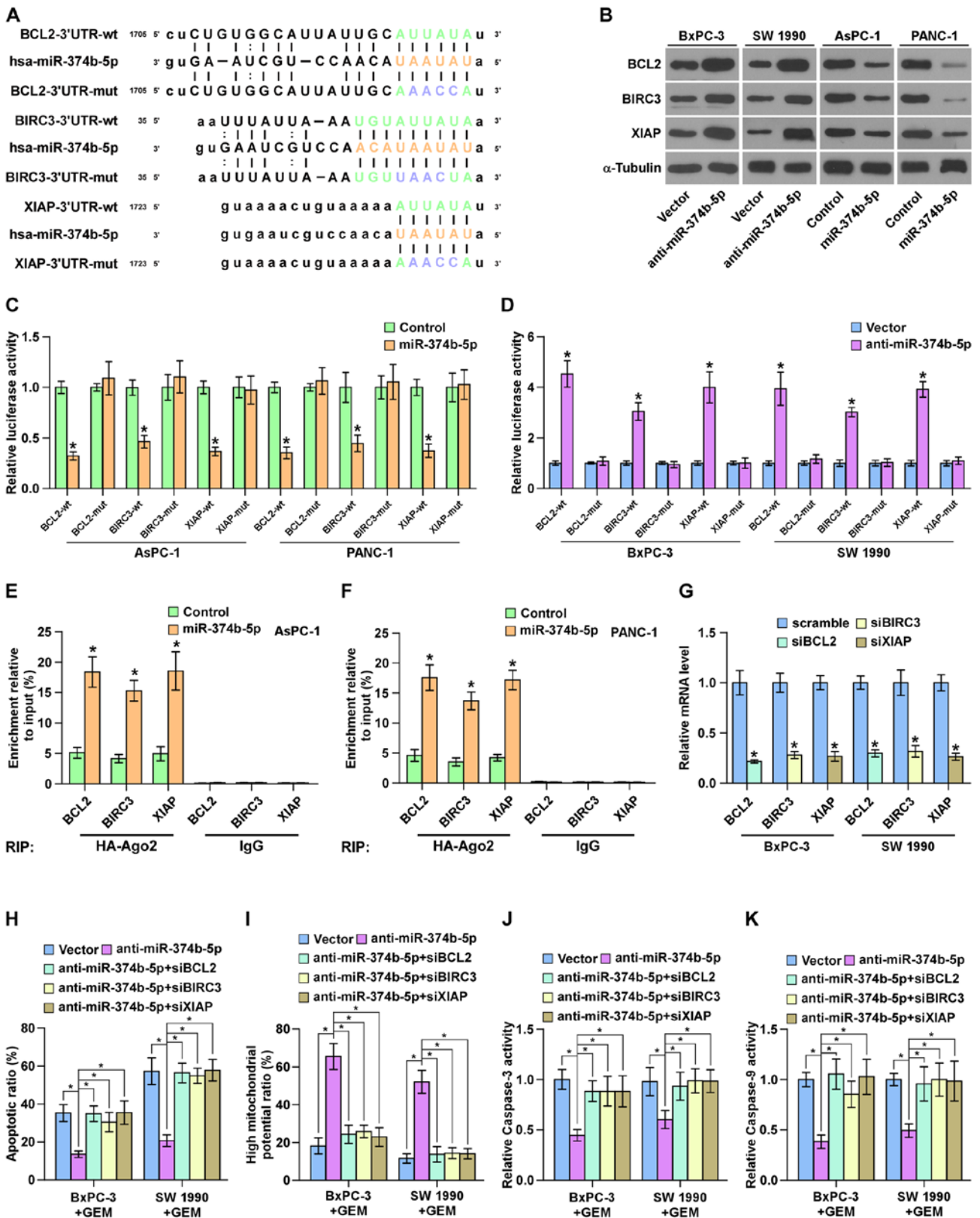
Figure 6. miR-374b-5p targets BCL2, BIRC3 and XIAP in pancreatic cancer cells. (A) Predicted miR-374b-5p target sequence in the 3'-UTRs of BCL2, BIRC3 and XIAP. (B) Western blotting measuring BCL2, BIRC3 and XIAP expression. α-tubulin served as the loading control. (C and D) Luciferase assay of the cells transfected with the indicated reporter following transfection with (C) miR-374b-5p mimic or (D) anti-miR-374b-5p. *P<0.05 vs. control or vector, respectively. (E and F) RNA immunoprecipitation assay indicating the association between miR-374b-5p and BCL2, BIRC3 and XIAP transcripts in the indicated cells. *P<0.05 vs. vector (G) Reverse transcription-quantitative polymerase chain reaction analysis of BCL2, BIRC3 and XIAP expression in the indicated pancreatic cancer cells. Transcript levels were normalized to U6 expression. *P<0.05 vs. scramble. (H) The protective effect of anti-miR-374b-5p on the apoptotic rate of pancreatic cancer cells was attenuated by the individual silencing of BCL2, BIRC3 and XIAP. (I) The stimulatory effect of anti-miR-374b-5p on the mitochondrial potential of pancreatic cancer cells was attenuated by the individual silencing of BCL2, BIRC3 and XIAP. (J and K) The stimulatory effects of anti-miR-374b-5p on caspase-3 and -9 activity of pancreatic cancer cells was attenuated by the silencing of BCL2, BIRC3 and XIAP. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05. miR-374b-5p, microRNA-374b-5p; BCL2, B-cell lymphoma 2; BRIC3, Baculoviral IAP Repeat Containing 3; XIAP, X-linked inhibitor of apoptosis; UTR, untranslated region; mut, mutant; wt, wild-type; si, small interfering; GEM, gemcitabine.

may provide insights into the development of chemoresistance in pancreatic cancer, which may facilitate the development of novel therapeutic methods to treat patients with pancreatic cancer.

Availability of data and materials

The datasets generated and analyzed in the current study are available at TCGA (https://cancergenome.nih.gov/) and ArrayExpress (http://www.ebi.ac.uk/arrayexpress/)

Authors' Contributions

PZ conceived the experiments and drafted the manuscript. DS and XW conducted the experiments and helped to analyze the data. GS performed data analysis. SC and MY performed data analysis and revised the manuscript. All authors contributed to revise the manuscript and approved the final version for publication.

Ethics approval and consent to participate

Written informed consent from each patient enrolled in the study was obtained and ethical approval for the use of human tissues and for the animal study was obtained from the Institutional Research Ethics Committee at The First Hospital of Jilin University (ethics no. 005-001).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflicts of interest.

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