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Effect of miR-184 on Proliferation and Apoptosis of Pancreatic Ductal Adenocarcinoma and Its Mechanism

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Abstract

Objective: Previous studies have shown that abnormal expression of microRNA-184 leads to a variety of cancers, including pancreatic ductal adenocarcinoma, suggesting microRNA-184 as a new treatment target for pancreatic ductal adenocarcinoma. However, the molecular mechanism of microRNA-184 in pancreatic ductal adenocarcinoma remains unclear. It is important to investigate the effect and role of microRNA-184 in pancreatic ductal adenocarcinoma. **Methods:** The clinical and laboratory inspection data of 120 patients with pancreatic cancer admitted to the First Affiliated Hospital of Anhui Medical University were compared. MicroRNA-184 expression in tumor tissues and cells was evaluated using reverse transcription polymerase chain reaction. Flow cytometry and Annexin V/propidium iodide staining were performed to examine cell cycle and apoptosis. Western blotting analysis was conducted to measure the protein expression of p-PI3K, p-AKT, JNK1, C-Myc, C-Jun, caspase-9, and caspase-3. **Results:** MicroRNA-184 expression was low in patients with pancreatic ductal adenocarcinoma. Survival curve showed that patients with lower expression of microRNA-184 in tumor tissues had a worse prognosis and shorter survival time ($P < .05$), and the multivariate analysis identified that microRNA-184 was an independent prognostic indicator ($P < .05$). *In vitro* studies showed that microRNA-184 overexpression induced apoptosis and suppressed cell cycle transition from G1 to S and G2 phases in pancreatic ductal adenocarcinoma cells. Furthermore, molecular studies revealed that inhibition of microRNA-184 promoted the gene expression of p-PI3K, p-AKT, JNK1, C-Myc, and C-Jun compared with the control group. Overexpression of microRNA-184 led to significantly increased expression of caspase-9 and caspase-3 and significantly decreased expression of Bcl-2. **Conclusion:** This study suggests that microRNA-184 inhibits the proliferation and promotes the apoptosis of pancreatic ductal adenocarcinoma cells by downregulating the expression of C-Myc, C-Jun, and Bcl-2. Our verification of the role of microRNA-184 may provide a novel biomarker for the diagnosis, therapy, and prognosis of pancreatic ductal adenocarcinoma.

Keywords

pancreatic ductal adenocarcinoma, microRNA, proliferation, apoptosis, microRNA-184

Abbreviations

JNK, C-Jun N-terminal kinase; miRNAs, microRNAs; mRNA, messenger RNA; PBS, phosphate-buffered saline; PDAC, pancreatic ductal adenocarcinoma; PI, propidium iodide

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Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death, with a 5-year survival rate of less than 5%, making it one of the most threatening malignancies to humans.[1,2] As most cases of pancreatic adenocarcinoma (PDAC) are difficult to detect in an early stage and show high tolerance to existing radiotherapy and chemotherapy drugs, it is particularly

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important to identify new therapeutic targets.[3] Although several protein biomarkers, such as carbohydrate antigen 19-9 and carcinoembryonic antigen, have been proposed, their sensitivity and/or specificity are low.[4]

MicroRNAs (miRNAs) are endogenous noncoding RNAs, 20 to 25 nucleotides in length, and function as vital regulators of gene expression.[5,6] These molecules act as post-transcriptional regulators by binding to the 3′-untranslated regions of their target messenger RNAs (mRNAs) and are involved in the regulation of proliferation, invasion, apoptosis, and other processes in various tumor cells after transcription by inhibiting the translation or degradation of mRNA.[7,8] An increasing number of studies have demonstrated that miRNA is closely related to the regulation of the biological behaviors of cancer cells, such as apoptosis, response to cytokines, or insulin secretion.[9] MicroRNA-184 is downregulated in glioma cells, and exogenous miRNA-184 significantly decreases cell proliferation and invasion.[10,11] MicroRNAs are also involved in a wide range of biological and pathological processes, highly conserved across different species and highly specific for different tissue and developmental stages, and play crucial roles in regulating important processes, such as cell proliferation, apoptosis, and tumor invasion.[12,13] For example, research has suggested that the miRNA-184/SND1 axis is a useful diagnostic and therapeutic tool for malignant glioma.[14] However, few studies have evaluated the association between miRNA-184 and PDAC in humans. Therefore, further research and verification of the role of abnormal miRNA-184 expression in PDAC may provide novel ideas for the treatment of PDAC.

Table 1. Characteristics of Patients With Pancreatic Ductal Adenocarcinoma.

Characteristic	Number
Gender	
Male	72
Female	48
Age (years)	
Median	66
Range	40-75
Histological grade	
III-IV	56
I-II	48
Tumor size (cm)	
≥2	72
<2	48
Lymph nodes metastasis	
Absence	96
Presence	24
MiR-184	
Low	51
High	69

Methods and Materials

Tissue Samples

In total, 120 pairs of PDAC tissues and matched normal adjacent tissues more than 2 cm from the tumor edge were obtained from the patients (age range, 40-75 years; 72 males and 48 females) who had been diagnosed with PDAC and had undergone surgery at the First Affiliated Hospital of Anhui Medical University between March 2015 and March 2017 (Table 1). Pancreatic ductal adenocarcinoma tissues and matched normal adjacent tissues were immediately frozen in liquid nitrogen for 10 minutes and stored at −80 °C until use. The patients were followed up for 3 years by telephone, WeChat, or clinic medical records once every 3 months to record their survival.

Cell Culture

The human PDAC cell line BXPC-3 and normal pancreatic duct epithelial cell NPDC were purchased from the Shanghai Institute of Biochemistry and Cell Biology. All cells were cultivated in Dulbecco's modified Eagle medium containing 10% fetal bovine serum at a constant temperature of 37 °C and at 5% CO_2 and then digested with 0.25% trypsin for 2 to 3 days to allow the cells to grow at a steady rate. Cells in the logarithmic phase of growth were used in our experiments.

MicroRNA-184 Inhibitor Transfection

The miRNA-184 inhibitor LY294002 was purchased from Guangzhou RiboBio Co, Ltd. The miRNA-184 mimic sequence was 5′-GGC AUU CUG UAU ACA UCG GAG-3′. Cell transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol to determine the effect of LY294002 on inhibiting miRNA-184.

RNA Extraction, Reverse Transcription, and Quantitative PCR

RNA was isolated from BXPC-3 cells at 48 hours after transfection using the Potent SYBR Green Cells-to-CT kit. The quality and integrity of RNA was quantified, after which the miRNA were reverse-transcribed into complementary DNA. MicroRNA-184 analyses were conducted using an miRNA real-time quantitative reverse transcriptase polymerase chain reaction SYBR green PCR kit (Vazyme) and the 7500 Real-Time PCR system (Applied Biosystems). Complementary DNA was synthesized by GenePharma. Primer sequences for miRNA-184 were as follows: forward, 5′-TTTCCAGCC-CAGCT-3′ and reverse, 5′-CCTTATCAGTTCTCC-3′. The primer sequences for the internal reference gene U6 were as follows: forward, 5′-GCTTCGGCAGCACATATAC-TAAAAT-3′ and reverse, 5′-CGCTTCACGAATTTGCGTGT-CAT-3′. The thermal cycling conditions were as follows: predenaturation at 95 °C for 5 minutes, followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 40 seconds, and extension at 72 °C for 35 seconds. The relative

expression of miRNA-184 was calculated using $2^{-\Delta\Delta Ct}$ method. After 48 hours of transfection, the inhibition effect of LY294002 on miRNA-184 expression in BXPC-3 cells was determined.

Cell Apoptosis Analyses

BXPC-3 cells transfected with miRNA-184 were centrifuged at 1000*g* for 5 minutes and resuspended in phosphate-buffered saline (PBS), followed by double staining with an Annexin V-fluorescein isothiocyanate/propidium iodide (PI) Apoptosis Detection Kit UCA1 (Beyotime) according to the manufacturer's instructions. Cell apoptotic rates were assessed by flow cytometry (FC500, Beckman Coulter).

Flow Cytometry Analysis

Cells in the logarithmic growth phase were digested with trypsin without EDTA. After 48 hours of transfection, the cells were seeded into a 6-well culture plate (1×10^6). After the cells had attached, they were cultured for 12 hours, and the primary culture was discarded. The cells were obtained, washed twice with PBS, and centrifuged, and then the supernatant was resuspended in precooled 75% ethanol, fixed at −20 °C overnight, centrifuged with PBS to remove the supernatant, and washed twice. Each sample was suspended in 450 μL PBS, and then PI (0.5 mg/mL) was added and mixed. The samples were incubated in a water bath at 37 °C for 30 minutes. The supernatant was removed by centrifugation, the cells were resuspended in PBS, and red fluorescence was recorded by flow cytometry (model: FACSCalibur; analysis of cell cycle distribution). Cell fractions with a DNA content lower than the G0/G1peak, known as the sub-G0/G1 fraction, were quantified and considered as a marker of the percentage of dead/apoptotic cells.

Western Blot Assay

After 48 hours of transfection with the miRNA-184 mimic or inhibitor, the cells were lysed in lysis solution and incubated for 20 minutes in an ice bath. The cells were centrifuged, and the supernatant was removed. The protein concentration was measured by the bicinchoninic acid method using a protein assay kit (Beyotime). Proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel and blotted onto polyvinylidene fluoride membranes (Merck Millipore). Nonspecific protein binding to the membranes was then blocked with 5% non-fat dried milk for 1 hour followed by incubation of the membranes with the following primary antibodies at 40 °C overnight: glyceraldehyde-3-phosphate dehydrogenase, p-PI3, p-AKT, C-Jun N-terminal kinase (JNK1), C-Jun, and C-Myc (1:300 dilution), Bcl-2, caspase-9, and caspase-3 (1:200 dilution; Santa Cruz Biotechnology). The membranes were washed 3 times for 15 minutes, each time with PBS containing 0.05% Tween 20 to remove unbound primary antibodies, before incubation with secondary antibodies at room temperature for 1 hour. The protein bands were imaged with an electrochemiluminescence system (Wanlei Biotechnology). Glyceraldehyde-3-phosphate dehydrogenase was evaluated as an internal reference. The relative expression of the proteins was analyzed with ImageJ software (NIH).

Statistical Analysis

All quantitative data obtained in this study were expressed as the mean ± SD (X ± S) and then analyzed with SPSS version 19.0 statistical software (SPSS, Inc). Overall survival from the date of surgery was estimated using the Kaplan-Meier method. Multivariate Cox regression analysis was performed to determine survival trends adjusted for clinical and demographic factors. Student *t* test was used to compare the differences between 2 or more groups. *P* value of <.05 indicated a significant difference.

Results

Expression of miRNA-184 in Histological Level of Patients With Pancreatic Cancer

Table 1 summarizes the patient characteristics. The ΔCt values of miRNA-184 in the cancer tissue of patients with 120 pancreatic cancer was 5.492 ± 2.048 and in the normal tissue control group was 7.264 ± 2.375 ($P < .05$). The value of ΔCt higher than or equal to 7.264-2.375 was defined as the high expression group and the lower one as the low expression group. The results showed that the expression level of miRNA-184 was decreased in tumor tissues. Statistical analysis revealed that the expression level of miRNA-184 in cancer tissues was significantly lower than that in normal tissues ($P < .05$; Figure 1B). Table 2 lists the multivariate analysis results, where low miRNA-184 expression was an independent prognostic factor ($P < .05$). Moreover, survival curve showed that patients with lower expression of miRNA-184 in tumor tissues had a worse prognosis and shorter survival time ($P < .05$; Figure 1C).

Inhibition Effect of LY294002 on MiRNA-184 Expression

The miRNA-184 inhibitor LY294002 was added to BXPC-3 cells as experimental group, whereas an equal volume of ultrapure water was added to the control group. Reverse transcriptase polymerase chain reaction was performed to detect the inhibitory effect of LY294002 on miRNA-184 in BXPC-3 cells. As a result, the miRNA-184 content in BXPC-3 cells of the experimental group was significantly lower than that of the control group (Figure 1D).

Effect of miRNA-184 on Cell Apoptosis

The apoptosis of BXPC-3 cells was detected by Annexin V and PI staining. After transfection of the cells with miRNA-184 inhibitors and compared with BXPC-3 cells transfected with

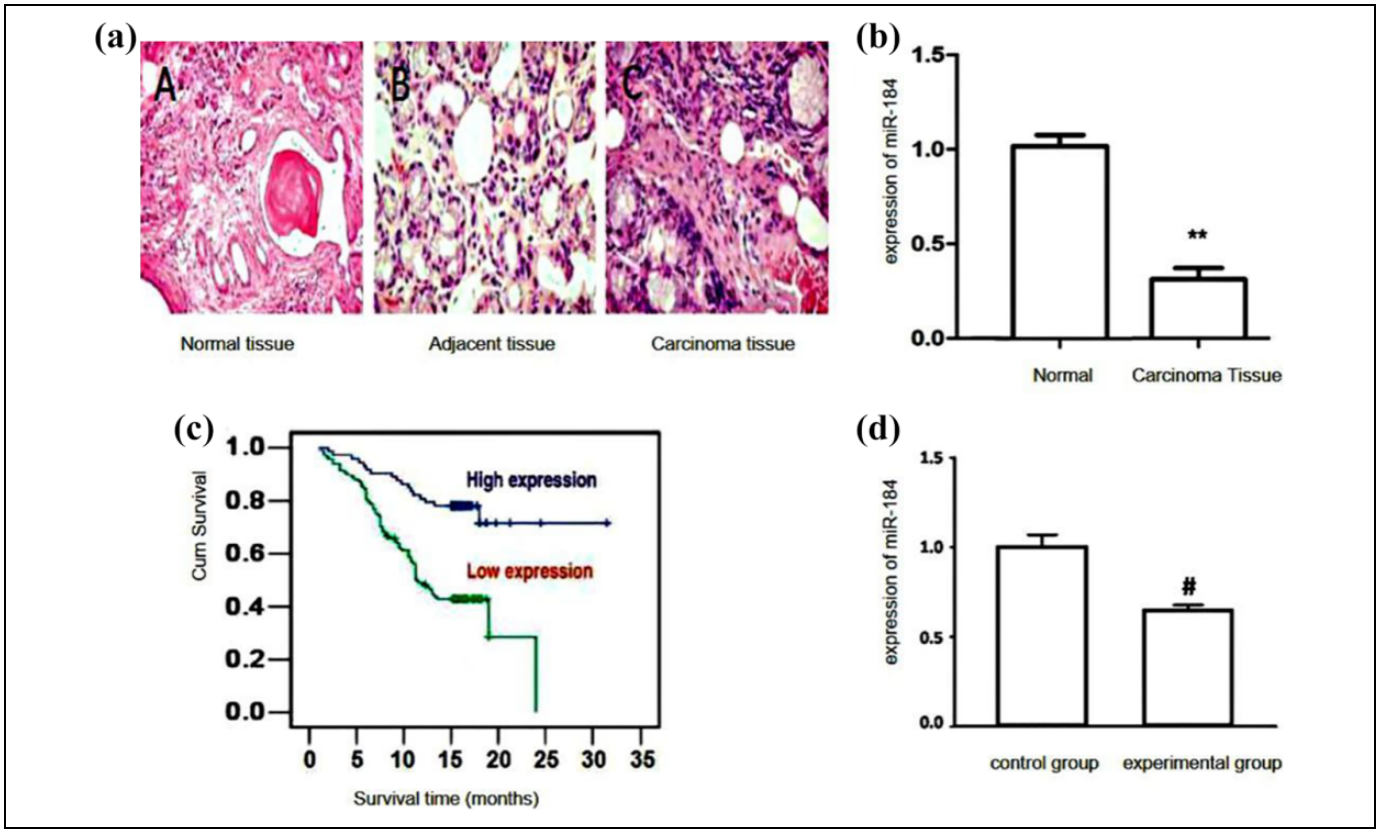

Figure 1. a, Representative histological images of tissues from patients with pancreatic cancer (HE staining, ×100): (A) Normal tissue; (B) adjacent tissue; (C) carcinoma 455 tissue. b, MiR-184 expression level of patients' normal tissue, adjacent tissue, and 456 carcinoma tissue. $^{**}P < .01$. c, Kaplan-Meier postoperative survival curve showing 457 between the miR-184 High expression and low expression groups, and the median 458 overall survival (OS) in the high expression group was longer than that in the low 459 expression group (17 vs 9 months, respectively; $^{*}P < .001$). D, Reverse transcriptase polymerase chain reaction (RT-PCR) to detect the 460 effect of LY294002, an miR-184 inhibitor, $^{\#}P < .05$.

Table 2. Multivariate Cox Regression analysis of Pancreatic Cancer Risk Factors.

Risk factors	HR	95% CI	*P* value
Gender (male or female)	-	-	-
Age (years) (≥65 or <65)	1.891	0.994-2.613	.102
Histological grade (III-IV or I-II)	2.469	1.408-3.516	.000
Tumor size (cm; ≥2 or <2)	-	-	-
Lymph nodes metastasis (absence or presence)	-	-	-
MiR-184 (low or high)	1.475	0.753-1.003	.041

Abbreviation: HR, hazard ratio.

a negative control, the apoptosis rate of miRNA-184-overexpressing cells was increased significantly ($P < .05$). After inhibiting the expression of miRNA-184, the apoptotic rate was decreased (Figure 2A). These results indicate that miRNA-184 promotes apoptosis.

Effect of miRNA-184 on Cell Cycle

The cell cycle was analyzed by flow cytometry. The results showed that the ratio of cells in the G1 phase was significantly decreased in the inhibitor group compared with the control group. Additionally, the proportion of cells in S phase was significantly increased, and the cell proliferation index was significantly increased (Figure 2B).

Regulation of PI3K-AKT and JNK Signaling Pathway by Inhibiting miRNA-184

After inhibiting miRNA-184, proteins in the PI3K, AKT, and JNK signaling pathway were detected by Western blotting and statistically analyzed. The results showed that the expression of p-PI3k (Figure 3A), p-AKT (Figure 3B), and JNK1 (Figure 3C) was significantly increased after the inhibition of miRNA-184.

Regulation of C-Myc and C-Jun Genes by Inhibiting MiRNA-184

Western blotting showed that the expression of C-Jun (Figure 3D) and C-Myc (Figure 3E) was significantly increased after inhibition of miRNA-184 with LY294002. This suggests that inhibition of miRNA-184 can promote activation of the

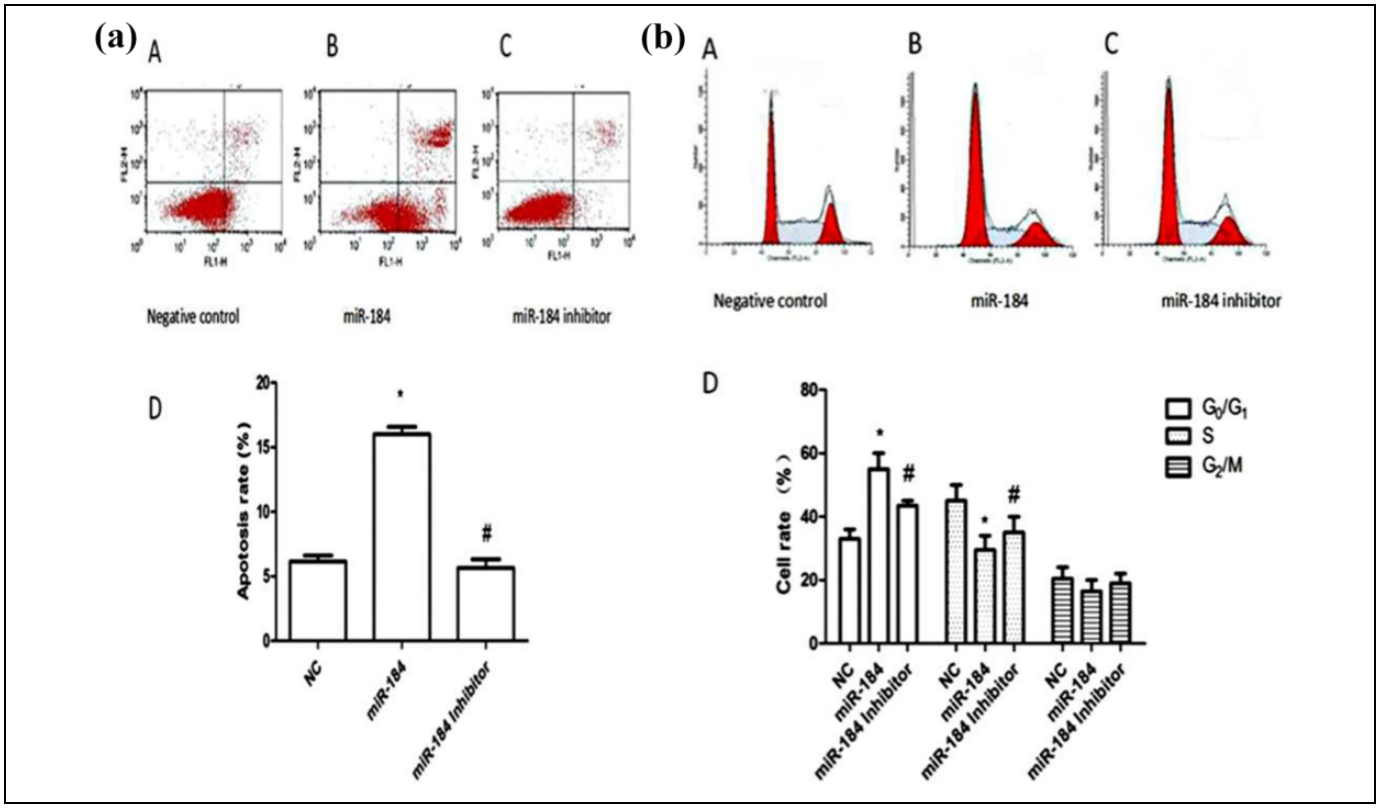

Figure 2. a, Flow cytometry to detect CFPAC-1 cell apoptosis: (A) negative control; (B) 464 miR-184 overexpression; (C) miR-184 inhibitor; (D) summary of flow cytometry 465 data. $^{*}P < .05$, $^{\#}P < .05$. b, Flow cytometry to detect the cell cycle of CFPAC-1 cells: (A) negative control; (B) miR-184 overexpress; (C) miR-184 inhibitor; (D) summary of flow cytometry data.$^{*}P < .05$, $^{\#}P < .05$.

C-Jun and C-Myc genes, thereby promoting tumor cell proliferation and inhibiting cell apoptosis.

Regulation of Bcl-2, Caspase-9, and Caspase-3 by Overexpressing miRNA-184

The expression of apoptosis-related proteins in BXPC-3 cells and BXPC-3 cells overexpressing miRNA-184 was detected by Western blotting. After overexpression of miRNA-184, the expression of Bcl-2 (Figure 3F) was significantly decreased, whereas that of caspase-9 (Figure 3G) and caspase-3 (Figure 3H) was significantly increased.

Discussion

In the present study, miRNA-184 was found to be associated with the cell cycle and cell apoptosis in PDAC. The only clinically available Food and Drug Administration–approved PDAC serum biomarker is CA19-9. However, CA19-9 is primarily used to monitor tumor recurrence after surgery rather than as a primary diagnostic and prognostic biomarker because of its low sensitivity and specificity.[15] Studies have shown that miRNA-1 inhibits gastric cancer,[16] and MiR-29c plays a tumor suppressor role in hepatocellular carcinoma.[17] Increasing evidence suggests that miRNA is an effective marker for cancer diagnosis, prognosis, and prediction.[18] Therefore, miRNA-184 is a promising therapeutic target for the therapy of PDAC.

As a member of the miRNA family, miRNA-184 has gradually become a research hotspot in tumor cells. Many studies have confirmed its anticancer effect in various tumor cells, such as its inhibition of the proliferation and invasion of colorectal cancer.[19] MicroRNA-184 targets Notch2 as a tumor suppressor miRNA, inhibiting the invasion, migration, and metastasis of nasopharyngeal carcinoma.[20] This miRNA has also been shown to be carcinogenic in certain tumors. For example, overexpression of miRNA-184 in hepatocellular carcinoma promotes tumor cell proliferation and cell cycle progression by modulating SOX7.[21] Our results showed that miRNA-184 was downregulated in PDAC tissues and cells, negatively regulating the progression of PDAC.

Previous studies showed that the PI3K/Akt and JNK signaling pathways, which play a central role in apoptosis, proliferation, and protein synthesis by phosphorylating various downstream molecules such as Akt, are classical signaling pathways in tumor cells[22,23] and are frequently activated in many human cancer types, such as breast cancer, non-small cell lung cancer, and so on.[24] Numerous studies have shown that JNK activation is frequently accompanied by activation of Akt and PI3K in tumor cells, indicating that the JNK pathway exhibits crosstalk with the PI3K/Akt pathway and may share the same upstream signaling components.[25,26] In fact, PI3K

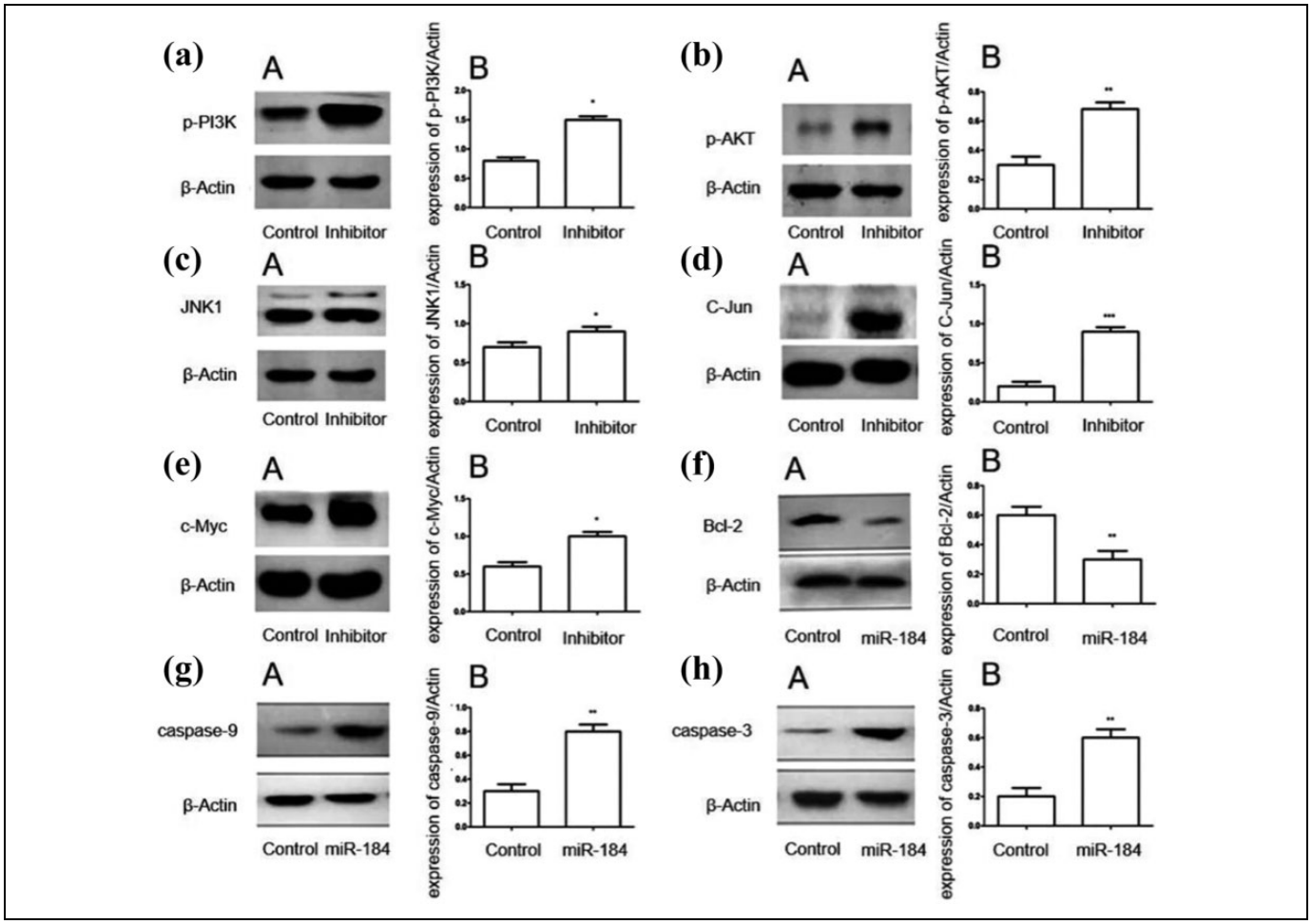

Figure 3. a-e, Inhibition of miR-184 promoted the expression of p-PI3K, p-AKT, JNK1, 470 C-Jun, and C-Myc. A, Representative Western blotting images. B, Summary of 471 Western blotting data. *$P < .05$. f-h, Overexpression of miR-184 promoted the 472 expression of Bcl-2, caspase-9, and caspase-3.

activity is critical for maintaining the activity of Bax in the cytoplasm, Akt inhibits the translocation of Bax to the mitochondria, and the PI3K/Akt-mediated interaction between Bad and Bcl-XL maintains mitochondrial integrity and blocks cytochrome c efflux and inhibits apoptosis.[27] The JNK pathway is involved in tumorigenesis and tumor suppression, which not only exerts tumor-suppressive effects through tumor monitoring by the immune system and apoptosis promotion[28] but also plays a proapoptotic role by directly increasing the ratio of Bax/Bcl-2.[29]

Cell proliferation is associated with the phase distribution of the cell cycle. Changes in cell proliferation are regulated by the cell cycle, including the G1, G2, and S phases.[30] The mitogen-activated protein kinase signaling pathway plays an important role in transforming extracellular stimuli into various cellular responses, consisting of 3 parts: extracellular signal-regulated kinase, p38 kinase, and JNK family.[31] Stress, inflammation, and other factors can phosphorylate JNKs and activate many nuclear and non-nuclear proteins, such as transcription factor activator protein-1, which is formed by the dimerization of Jun protein (C-Jun, JunB, JunD) with Fos proteins (c-Fos, FosB, Fra-1, Fra-2) to control cell proliferation, differentiation, and cell death,[32] causing MYC to be constantly expressed.

Once the C-Myc gene encoding transcription factors is deregulated, variable version of MYC occurs in various cancers, which not only plays a crucial role in cell proliferation but also participates in various functions, such as cell differentiation, apoptosis, cell cycle progression, and cell transformation, leading to tumorigenesis, and its knockdown or inhibition seriously affects cell proliferation.[33,34] C-Myc is a core component of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway and is carefully managed at different levels.[35] Our results indicate that inhibition of miRNA-184 can promote tumor cell proliferation and inhibit apoptosis by partially activating the JNK/C-Jun pathway and C-Myc gene.

Impaired apoptosis is critical for cancer progression, including endogenous and exogenous pathways.[36] Several studies have demonstrated that the expression of Bcl-2 family proteins is tightly regulated by JNK signaling pathways, which can stimulate apoptosis via transcriptional regulation Bcl-2 family proteins. Bcl-2 and Bax are important regulatory factors in the mitochondria-dependent apoptotic pathway. During apoptosis,

the apoptosis-promoting protein Bax is elevated, and Bax apoptosis-promoting homodimers are formed via the action of death signals.[37] Bcl-2 family proteins regulate the intrinsic pathway: stimulation of proapoptotic molecules induces mitochondria to release cytochrome c into the cytoplasm and bind to apoptotic peptidase activator 1. This complex binds to caspase-9, activates caspase-3, and induces cell death.[38] The B-cell CLL/lymphoma 2 (Bcl-2) family includes pro-life proteins (such as Bcl-2, Bcl-XL) and pro-death proteins (such as Bax, Bak), which crucially regulate mitochondrial membrane integrity and the release of cytochrome C and apoptosis factors that influence cell survival and cell death.[39] Caspase activation plays a vital role in apoptosis. Caspase-9 mediates the activation of caspase-3, marking an important event in apoptosis induction.[40] The level of miRNA-184 in pancreatic cancer tissues from the same patient was significantly lower than that in normal tissues of the pancreas. Tracking of the prognosis of patients revealed that lower expression of miRNA-184 in cancer tissues was associated with a worse prognosis and shorter survival time, suggesting that miRNA-184 inhibits PDAC. The flow cytometry results showed that after transfecting the BXPC-3 cells with miRNA-184, the G1 phase was prolonged, S phase was shortened, and cell growth was delayed. These results demonstrate that miRNA-184 significantly inhibited the proliferation and promoted the apoptosis of PDAC cells. Moreover, we explored the molecular mechanisms underlying miRNA-184-mediated PDAC cell proliferation and apoptosis. For patients with pancreatic cancer, our findings may be useful for designing new targeted drugs for miRNA-184.

In conclusion, our results reveal that miRNA-184 is an effective marker for the prognosis of pancreatic cancer, which may affect the biological function of pancreatic cancer cells through the PI3K/AKT and JNK signaling pathways.

Authors' Note

The protocols were approved by the Ethical Committee of the First Affiliated Hospital of Anhui Medical University (Anhui, China; Approval number 20180037). Additionally, written informed consent was obtained from the patients or guardians. In the present study, all experimental protocols were performed according to approved guidelines.

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Declaration of Conflicting Interests

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