# MicroRNA-23b-3p promotes pancreatic cancer cell tumorigenesis and metastasis via the JAK/PI3K and Akt/NF-κB signaling pathways

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**Abstract.** MicroRNA (miR)-23b-3p plays an important role in tumor growth, proliferation, invasion and migration in pancreatic cancer (PC). However, the function and mechanistic role of miR-23b-3p in the development of PC remains largely unknown. In the present study, the miR-23b-3p levels in the serum of patients with PC were found to be elevated, and the phosphorylation levels of Janus kinase (JAK)2, PI3K, Akt and NF-κB were found to be upregulated. In addition, miR-23b-3p was induced in response to interleukin-6 (IL-6), which is known to be involved in the progression of PC. Overexpression of miR-23b-3p, on the other hand, activated the JAK/PI3K and Akt/NF-κB signaling pathways in PC cells, as evidenced by miR-23b-3p-induced upregulation of phosphorylated (p-) JAK2, p-PI3K, p-Akt and p-NF-κB, as well as the downregulation of PTEN; and these effects were found to be reversible by miR-23b-3p inhibition. Furthermore, miR-23b-3p was found to downregulate PTEN by directly targeting the 3'-untranslated region of PTEN mRNA. Notably, in an *in vivo* xenograft mouse model, overexpression of miR-23b-3p accelerated PC cell-derived tumor growth, activated the JAK/Akt/NF-κB signaling pathway and promoted liver metastasis. In contrast, knockdown of miR-23b-3p suppressed tumor growth and metastasis as well as JAK/Akt/NF-κB signaling activity. *In vivo* imaging of the mice further confirmed the metastasis promoting role of miR-23b-3p in PC. These results suggested that miR-23b-3p enhances PC cell tumorigenesis and metastasis, at least, partially via the JAK/PI3K and Akt/NF-κB signaling pathways. Therefore, targeting miR-23b-3p or the JAK/PI3K and Akt/NF-κB signalings may be potential therapeutic strategy against PC.

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## Introduction

Pancreatic cancer (PC) is an incurable solid malignancy that poses a serious threat to human health, worldwide (1). Despite recent advancements in the understanding of its molecular pathogenesis, there is still a lack of effective therapies against this deadly disease (2).

MicroRNAs (miRNAs/miR) are a class of small non-coding RNAs (~22 nucleotides in length) (3). miRNAs can regulate gene expression by directly targeting the 3'-untranslated region (3'UTR) of target gene mRNA (4). Studies have indicated that miRNAs are aberrantly expressed in serum and cancer tissues, and they elicit pro- or antitumorigenic functions (5,6). miRNAs have been shown to serve as potential diagnostic and therapeutic biomarkers for PC (7). For example, a previous study demonstrated that miR-23b-3p accelerates the progression of PC, functioning as an oncogene (8). However, the molecular mechanisms underlying PC development and progression are still not fully understood.

Interleukin (IL)-6/Janus kinase(JAK)/STAT3 signaling axis is a key therapeutic target in PC (9), and contributes to cell invasion in PC (10). The oncogenic PI3K/Akt, which is downstream of the JAK/STAT3 pathway, is also a key pathway in cancer development (11). The IL-6/JAK2/PI3K signaling pathway can regulate the proliferation and metastasis of breast cancer cells (12) and was also found to be a potential therapy strategy for PC (13). PTEN is a negative regulator of the PI3K/Akt signaling pathway. Loss-of-function mutations of PTEN have been found in numerous types of human solid cancer, such as hepatocellular carcinomas, breast cancer and gastric cancer (14,15). Evidence has also indicated that knockdown of PTEN could enhance tumorigenesis and induce tumor development in a variety of different organs, such as prostate, breast and liver (16-19). On the other hand, PI3K/Akt signaling has been frequently upregulated in various types of cancer (20), plays a key role in tumor cell growth and survival, and has been associated with poor prognosis in patients with PC (21). In addition, inactivation of PI3K/Akt signaling has been shown to suppress PC progression (22). In the present study, based on bioinformatics, miR-23b-3p was predicted to target the 3'UTR of PTEN mRNA. However, there has been no experimental evidence demonstrating whether IL-6 induce miR-23b-3p expression and the role of miR-23b-3p in PC development depends on the JAK/PI3K and Akt/NF-κB pathways by targeting PTEN.

In the present study, the miR-23b-3p levels in the serum of patients with PC were examined and the phosphorylation levels of JAK2, PI3K, Akt and NF-κB were measured using immunohistochemical (IHC) staining using PC tissue chip analysis. The target gene of mR-23b-3p was analyzed using both western blot analysis and luciferase reporter assays. Furthermore, *in vitro* and *in vivo* studies were performed to determine whether miR-23b-3p promotes PC cell tumorigenesis and metastasis by activating the JAK/PI3K and Akt/NF-κB signaling pathways. The present study might provide novel insights into the potential application of miR-23b-3p as a predictive marker and therapeutic target for the prevention and treatment of PC.

## Materials and methods

*Statement of ethics*. The current study was approved by the Institutional Ethics Committee of Nanjing Medical University. Written informed consent was provided from all participants included in the study prior to their enrolment.

*Human serum samples and tissue chips*. The diagnosis of PC was based on the National Comprehensive Cancer Network clinical practice guidelines 2016 (23). The inclusion criteria comprised of patients who were diagnosed with PC and had not received chemotherapy or other treatments, while the exclusion criteria included patients with PC who had infections, immune system diseases, chronic diseases or other kinds of cancer at the same time, such as biliary tract or pulmonary infection, rheumatoid arthritis, uremia and leukemia. The healthy controls were selected from the Physical Examination Center of Wuxi People's Hospital, and excluded individuals with any acute or chronic diseases, including cancer.

Serum samples were collected from 10 healthy controls and 10 patients with PC at Wuxi People's Hospital from May 2017 to September 2018. The collected clinical data of the 10 patients with PC is showed in Table I. All the samples were freshly frozen and stored at -80˚C until further use.

The tissue chips, including tumor tissues and matched adjacent tissues of 36 patients with PC were purchased from Shanghai Zuocheng Biological Technology Co., Ltd. The clinical data of the 36 patients with PC was shown in Table II.

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*. Total RNA was extracted from the serum of patients using the RNeasy Mini kit (Qiagen GmbH), according to the manufacturer's instructions. The Bulge-Loop™ miRNA qPCR primer set for hsa-miR-23b-3p (MQPS0000872-1-100) and U6 small nuclear RNA (snRNA, MQPS0000002-1-100) were purchased from Guangzhou RiboBio Co. Ltd. A total of 0.5 $\mu$g total RNA was used to synthesize cDNA in a 20 $\mu$l reaction volume at 42˚C for 45 min using a PrimeScriptsis kit (cat. no. RR047A; Takara Bio, Inc.). RT-qPCR was performed in a 20 $\mu$l reaction volume using iQ™ SYBR Green Supermix (cat. no. 1708882AP; Bio-Rad Laboratories, Inc.) The thermocycling conditions were as follows: 95˚C for 3 min, followed by 40 cycles of 95˚C for 20 sec, 60˚C for 30 sec and 70˚C for 30 sec.

The 5S ribosomal RNA was used as the internal control for the serum miR-23b-3p level, which was quantified using the $2^{-\Delta\Delta Ct}$ method (24). All RT-qPCR experiments were performed in triplicate.

*IHC staining*. The tumor and adjacent tumor tissues from patients with PC were fixed with 40% formaldehyde at room temperature overnight. Fixed samples were incubated in 70% ethanol at 4˚C overnight, dehydrated in an ascending alcohol series (80, 90 95 and 100%, 30 min for each concentration), cleared in pure xylene and immersed in liquid paraffin at 60˚C for 24 h. Then the samples were embedded in paraffin and the sections (5-$\mu$m) were cut deparaffinised. The sections were blocked (1X PBS containing 10% horse serum and 0.01% Triton X-100) with blocking solution at room temperature for 1 h. The blocking solution was also used for dilution of antibodies. Following which, they were incubated with primary antibodies against p-JAK2 (1:1,000; cat. no. abs130652; Absin Bioscience Inc.) p-Akt (1:100; cat. no. 4060; Cell Signaling Technology, Inc), p-PI3K (1:100; cat. no. 17366; Cell Signaling Technology, Inc.), p-NF-κB (1:100; cat. no. 3033T; Cell Signaling Technology, Inc.) and matrix metalloproteinase-2 (MMP2; 1:100; cat. no. 87809; Cell Signaling Technology, Inc.) overnight at 4˚C, after washing three times with Tris-buffered saline (TBS), the sections were incubated with EnVision-labeled polymer-horseradish peroxidase (HRP)-conjugated rabbit antibody (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 1 h at room temperature and then visualized using diaminobenzidine. The IHC staining was quantified using the 3D Histech Quant Center2.1 (3DHISTECH Ltd.) and all histopathological sections were read, diagnosed and recorded by 2 senior pathologists from Wuxi People's Hospital (Wuxi, China) with >3 years of combined experience at the same time, when there were disagreements of interpretation between them the results were discussed with other pathologists from the aforementioned hospital and a consensus reached. The positive rate was calculated, as the number of positive cells/total cells number.

*Cell culture, IL-6 treatment and transfection/infection*. The human PANC-1, BXPC-3 and 293T cell lines cells were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle medium (DMEM; cat. no. SH30243.01; Hyclone; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (FBS; cat. no. 16140071; Gibco; Thermo Fisher Scientific Inc.), 100 U/ml penicillin, and 100 g/ml streptomycin (cat. no. P1400; Beijing Solarbio Science & Technology Co., Ltd.). PANC-1 cells were seeded on 6-well plates at a density of $5x10^5$/well in growth media in a humidified incubator at 37˚C and 5% $CO_2$. After 24 h of culture, the media was replaced with fresh growth media containing recombinant human IL-6 (cat. no. PHC0064; Invitrogen; Thermo Fisher Scientific Inc.) at the final concentration of 10 ng/ml every day (25). After 3 days, cells were centrifuged at 1,000 x g for 5 min, harvested and total RNA was extracted for RT-qPCR.

Table I. Clinical data of the 10 patients with pancreatic cancer.

| Patient no. | Age, years | Sex | Location, within the pancreas |
|---|---|---|---|
| 1 | 77 | Female | Head |
| 2 | 61 | Female | Body |
| 3 | 63 | Male | Head |
| 4 | 75 | Female | Head |
| 5 | 45 | Male | Body-tail |
| 6 | 84 | Male | Head-body |
| 7 | 69 | Male | Head |
| 8 | 79 | Female | Body |
| 9 | 72 | Female | Tail |
| 10 | 50 | Male | Body-tail |

The miR-23b-3p mimic (23b-3pM sense, 5'-AUCACAUUG CCAGGGAUUACC-3' and antisense, 5'-GGUAAUCCCUGG CAAUGUGAU-3'), non-specific mimic (NSM sense, 5'-UUU GUACUACACAAAAGUACUG-3' and antisense: 5'-CAG UACUUUUGUGUAGUACAAA-3') control, miR-23b-3p inhibitor (23b-3pI, 5'-GGUAAUCCCUGGCAAUGUGAU-3') and non-specific inhibitor (NSI, 5'-CAGUACUUUUGU GUAGUACAAA-3') control were obtained from Guangzhou RiboBio Co., Ltd. PANC-1 cells (5x10$^5$/well) were seeded into the each well of 6-well plates and cultured in growth media in a humidified incubator at 37˚C and 5% $CO_2$. After 24 h, the cells reached 70% confluence and they were transfected with 20 nM 23b-3pM and NSM (control) to upregulate miR-23b-3p, or 50 nM 23b-3pI and NSI (control) to downregulate miR-23b-3p, using Lipofectamine® RNAiMAX reagent (cat. no. 13778075; Invitrogen, Thermo Fisher Scientific, Inc.). After transfection cells were cultured in a humidified incubator at 37˚C and 5% $CO_2$ for 48 h before the initiation of further experiments.

miRNA lentivirus can achieve long-term and stable expression of target gene by integrating foreign gene into host cell genome, has low immunogenicity and can be used for the animal experiments (26). miR-23b-3p lentivirus (Lenti-23b-3p) and negative control lentivirus (NCL) with fluorescein expression were purchased from Shanghai GeneChem Co., Ltd. Infectious fluids were including 3.75 ml DMEM+100 $\mu$l HitransGP (cat. no. REVG005; Shanghai Genechem Co., Ltd.) +7.5 $\mu$l Lenti-23b-3p (2.5x10$^9$ TU/ml) or NCL (1.0x10$^9$ TU/ml) were prepared and the media of the cells was changed to the 2.5 ml infectious fluids. A total of 2x10$^5$ BXPC-3 cells were seeded in a T25 flask and cultured in media, after 24 h when they reached ~30% confluency the cells were infected with Lenti-23b-3p. Infected cells were then selected with puromycin antibiotic (0.5 $\mu$g/ml; cat. no. 631305; Clontech Laboratories, Inc.) treatment for 3 days and the expression of miR-23b-3p was assessed using RT-qPCR (data not shown). The cells stably expressing miR-23b-3p were cultured in a humidified incubator at 37˚C and 5% $CO_2$. The infected cells were centrifuged at 1,000 x g for 5 min and harvested, then the cells were counted with a cell counter and resuspended in 0.9% saline (2x10$^7$ cells/100 $\mu$l) for the following *in vivo* imaging mouse experiment.

Table II. Clinical data of the 36 patients with pancreatic cancer.

| Patient no. | Age, years | Sex | Location, within the pancreas |
|---|---|---|---|
| 1 | 76 | Male | Head |
| 2 | 33 | Female | Body-tail |
| 3 | 81 | Male | Body-tail |
| 4 | 71 | Female | Head |
| 5 | 69 | Female | Head |
| 6 | 48 | Male | Body-tail |
| 7 | 66 | Male | Head |
| 8 | 67 | Female | Head |
| 9 | 62 | Male | Head |
| 10 | 57 | Female | Head |
| 11 | 58 | Male | Head |
| 12 | 74 | Female | Body-tail |
| 13 | 67 | Male | Head |
| 14 | 55 | Female | Head |
| 15 | 79 | Male | Head |
| 16 | 63 | Male | Head |
| 17 | 51 | Male | Body-tail |
| 18 | 58 | Female | Body-tail |
| 19 | 51 | Male | Head |
| 20 | 62 | Male | Head |
| 21 | 75 | Male | Head |
| 22 | 59 | Male | Head |
| 23 | 52 | Male | Body-tail |
| 24 | 73 | Female | Body-tail |
| 25 | 66 | Male | Body-tail |
| 26 | 69 | Female | Body-tail |
| 27 | 61 | Male | Head |
| 28 | 69 | Female | Head |
| 29 | 38 | Male | Head |
| 30 | 60 | Female | Body-tail |
| 31 | 48 | Male | Body-tail |
| 32 | 59 | Male | Body-tail |
| 33 | 46 | Male | Head |
| 34 | 54 | Male | Head |
| 35 | 54 | Male | Head |
| 36 | 57 | Male | Head |

*Plasmid construction and luciferase reporter assay.* The luciferase plasmid was constructed based on TargetScan (http://www.targetscan.org/). The pmiR-RB-REPORT™ vector (Guangzhou RiboBio Co., Ltd.) was used to clone the wild-type (NM_000314.6: 896-1686) and mutated 3'-untranslated region (3'-UTR) of PTEN cDNA. In brief, the 3'-UTR of human PTEN cDNA (791 bp in length) containing the putative target site of miR-23b-3p (1608-1615) was amplified from genomic DNA of 293T cell lines using PCR and cloned into the pmiR-RB-REPORT™ vector downstream of the Renilla luciferase reporter gene. The binding site-containing region was amplified using linker primers (h-PTEN-3'UTR forward [896 (SgfI)], ACTGCGATCGCCAATTGTAACGACTTC

TCCATC and h-PTEN-3'UTR-reverse [1686 (XhoI)], GCG CTCGAGTCAAATGTCAAGGTGAAGGT; h-PTEN-3'UTR mutant forward, GTAAAGCTTTACACTAGATATTATTAA AAAGGT and h-PTEN 3'UTR mutant reverse, TAATAT CTAGTGTAAAGCTTTACAATAGTAGTT) containing the *Xho*I and *Sgf*I restriction sites. The thermocycling conditions were as follows: 95˚C for 2 min followed by 33 cycles of 95˚C for 30 sec, 57˚C for 30 sec and 72˚C for 30 sec. The mutants were generated with 5 ng template by replacing seven nucleotides (1608-1615-bp: AATGTGA>TTACACT) in the miR-23b-3p-binding site using a QuikChange XL Site-Directed Mutagenesis kit (Agilent Technologies, Inc.). The thermocycling conditions were as follows: 95˚C for 2 min followed by 33 cycles of 95˚C for 30 sec, 50˚C for 30 sec and 72˚C for 30 sec. These constructed plasmids were amplified and the DNA sequence was confirmed by first generation sequence analysis (Guangzhou RiboBio Co., Ltd.).

For the luciferase reporter assay, 293T cells were seeded into 96-well plates ($1.5x10^4$/well) with 90 $\mu$l DMEM in each well and cultured in a humidified incubator at 37˚C and 5% $CO_2$ for 24 h. Prior to transfection, the following mixtures were prepared: i) miR-23b-3p mimic (20 nM), or NSM (20 nM) mixed with 5 $\mu$l OPTI-MEM (cat. no. 3138013; Gibco; Thermo Fisher Scientific Inc.) respectively; ii) pmiR-RBREPORT™ luciferase reporter plasmids (50 ng/well) containing either the wild-type or the mutated PTEN 3'UTR mixed with 0.1 $\mu$l P3000 (Lipofectamine™ 3000; cat. no. L3000015; Invitrogen; Thermo Fisher Scientific Inc.); and iii) 0.225 $\mu$l Lipofectamine™ 3000 mixed with 5 $\mu$l OPTI-MEM. Mixtures i), ii) and iii) were mixed together (total volume was about 10 $\mu$l) and after 15 min the mixture was added to the aforementioned 96-well plates. Next, 48 h after cotransfection, the luciferase reporter assay was performed using a Dual-Luciferase Reporter Assay System (cat. no. P1041; Promega Corporation). The luminescence intensity was determined using a Veritas microplate luminometer (Turner BioSystems; Thermo Fisher Scientific, Inc.). The luciferase activity was measured with a TD-20/20 luminometer (Turner Designs, Inc.), using the Dual-Glo Luciferase Assay System (Promega Corporation). Firefly luciferase activity was used for normalization of the *Renilla* luciferase activity. All experiments were performed in triplicate and repeated at least three times.

*Western blot analysis*. Cells were centrifuged at 1,000 x g for 5 min at 4˚C, harvested and then lysed with radioimmunoprecipitation assay buffer (Pierce; Thermo Fisher Scientific, Inc.). Cell lysates were cleared by centrifugation with the speed of 12,000 x g for 15 min at 4˚C, and the protein concentration was determined using the bicinchoninic acid assay. Proteins were separated using 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skimmed in TBS-Tween-20 (TBST), followed by overnight incubation at 4˚C with rabbit anti-human JAK2 (1:1,000; cat. no. abs131625; Absin Bioscience Inc.) and p-JAK2 (1:1,000; cat. no. abs130652; Absin Bioscience Inc.), Akt (1:1,000; cat. no. 9272; Cell Signaling Technology, Inc.), p-Akt (1:2,000; cat. no. 4060; Cell Signaling Technology, Inc.), PI3K (1:1,000; cat. no. 4255; Cell Signaling Technology, Inc.), p-PI3K (1:1,000; cat. no. 17366; Cell Signaling Technology, Inc.), MMP2 (1:1,000; cat. no. 87809; Cell Signaling Technology, Inc.), NF-κB (1:1,000; cat. no. 8242T; Cell Signaling Technology, Inc.), p-NF-κB (1:1,000; cat. no. 3033T; Cell Signaling Technology, Inc.), PTEN (1:1,000; cat. no. 9559; Cell Signaling Technology, Inc.), p-PTEN (1:1,000; cat. no. 9551T; Cell Signaling Technology, Inc.) and β-actin (1:1,000; cat. no. 4967; Cell Signaling Technology, Inc.) primary antibodies. The membranes were then washed with TBST three times, followed by incubation with HRP-conjugated goat anti-rabbit IgG (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 1 h at room temperature. The protein bands on the membrane were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce; Thermo Fisher Scientific, Inc.) and quantified using Image-Pro Plus software version 6.0.0.260 (Media Cybernetics, Inc.).

*Subcutaneous tumor xenograft model*. Female nude mice (6-8-weeks-old) were purchased from Changzhou Kaiwen Laboratory Animal Co., Ltd. The mice were maintained under specific pathogen-free conditions and housed in ventilated cages with free access to food and water. The mice were allowed to acclimatize for one week prior to the start of the experiment. The animal procedures were performed in accordance with the guidelines of the Nanjing Medical University Laboratory Animal Center.

PANC-1 cells were cultured in 10% FBS-containing DMEM and then harvested by centrifugation with the speed of 1,000 x g for 5 min at room temperature. A total of ~$4x10^7$ cells were resuspended in 100 ml saline and inoculated subcutaneously on the right flank of mice. At 40 days following inoculation, the negative control agomir (NCA), miR-23b-3p agomir (23b-3pA), or miR-23b-3p antagomir (23b-3pAnt) were injected every 3 days into the tumor, and the tumor width and length were measured every 5 days, the volume was calculated using the formula: Volume = width$^2$ x length x 0.5 (27). The mice were anesthetized with 2% isoflurane prior to euthanasia using cervical dislocation to confirm the death, and the tumors were harvested for measurement of their diameter and for histopathological investigation, 10 days later. Mice injected with 0.9% saline solution only into the right flank were used as the normal group. A total of 3 mice were used in each experimental group and the experiment was repeated three times.

*Imaging of metastatic role of miR-23b-3p upregulation in vivo*. A total of 100 $\mu$l of $2x10^7$ BXPC-3 cells (overexpressing the miR-23b-3p lentivirus) were injected in the caudal vein of each nude mice, the cells infected with negative control lentivirus were used as the control (NCL). After 52 days, D-Luciferin (15 mg/ml) was subsequently injected intraperitoneally at a dose of 10 ml/g. After 15 min, animals were anesthetized with 40 mg/kg sodium pentobarbital by intraperitoneal injection. After several minutes, the animals were placed in an *in vivo* imaging system (PerkinElmer, Inc.) for scanning. The data were collected and analyzed using the Living Image version 4.5 software (Xenogen Corporation) according to the manufacturer's instructions. Quantification of luminescence signals was achieved using the Region of Interest (ROI) function within the same Living Image software, for each mouse, an ROI area was drawn with the red circle and total flux within the ROI was considered as the signal intensity.

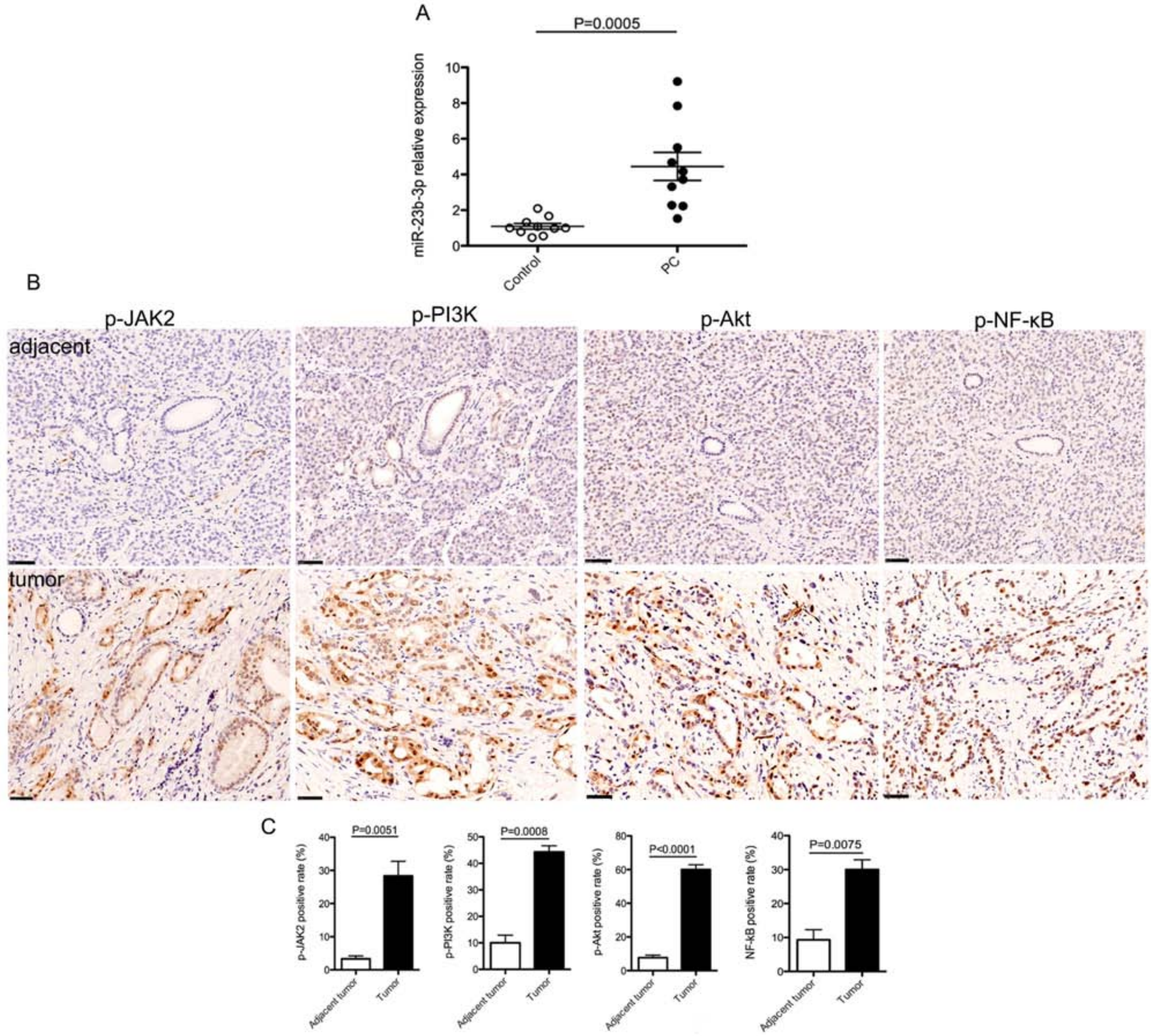


Figure 1. Serum miR-23b-3p levels and tissue expression of p-JAK2, p-PI3K p-Akt and p-NF-κB in patients with PC. (A) Reverse transcription-quantitative PCR was performed to determine the miR-23b-3p levels in the serum of 10 patients with PC and 10 healthy controls. (B) IHC staining was performed to investigate the protein expression level of p-JAK2, p-PI3K p-Akt and p-NF-κB in tumor and adjacent normal tissues using tissue chips. Scale bar, 50 $\mu$m. (C) Quantification of IHC staining, indicating the rate of positive staining between the tumor and the control samples (n=40). IHC, immunohistochemical; JAK2, Janus kinase 2; miR, microRNA; PC, pancreatic cancer; p, phosphorylated.

*Statistical analysis*. All experiments were repeated in triplicate and all data were presented as the mean ± standard error of the mean. GraphPad Prism software (version 5; GraphPad Software, Inc.) was used to perform statistical analyses. Differences between groups were analyzed using one-way analysis of variance followed by Bonferroni's post hoc test. The paired datasets (adjacent normal and tumor tissues) were analyzed using Wilcoxon signed-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Serum miR-23b-3p levels are upregulated and JAK2/Akt/NF-κB signaling is activated in patients with PC*. To investigate the possible role of miR-23b-3p in PC development, the levels of miR-23b-3p in the serum of patients with PC were examined using RT-qPCR. Results showed that the serum miR-23b-3p levels were significantly upregulated in patients with PC, compared with that in healthy controls (Fig. 1A). As JAK/PI3K and Akt/NF-κB signaling pathways have well-known roles in the progression of PC (28,29), the expression patterns of p-JAK2, p-PI3K, p-Akt and p-NF-κB were analyzed in PC tissues. The results showed that the expression of p-proteins examined were markedly enhanced in PC tissues compared with that in tumor-adjacent tissues (Fig. 1B and C). These data suggested a positive association between the levels of serum miR-23b-3p and JAK/PI3K and Akt/NF-κB signaling activity in PC and that miR-23b-3p might promote PC development and progression through the activation of JAK/PI3K and Akt/NF-κB signaling pathways.

*Overexpression of miR-23b-3p activates JAK/Akt/NF-κB signaling in PC cells*. The possible role of miR-23b-3p in the activation of the JAK/PI3K and Akt/NF-κB signaling

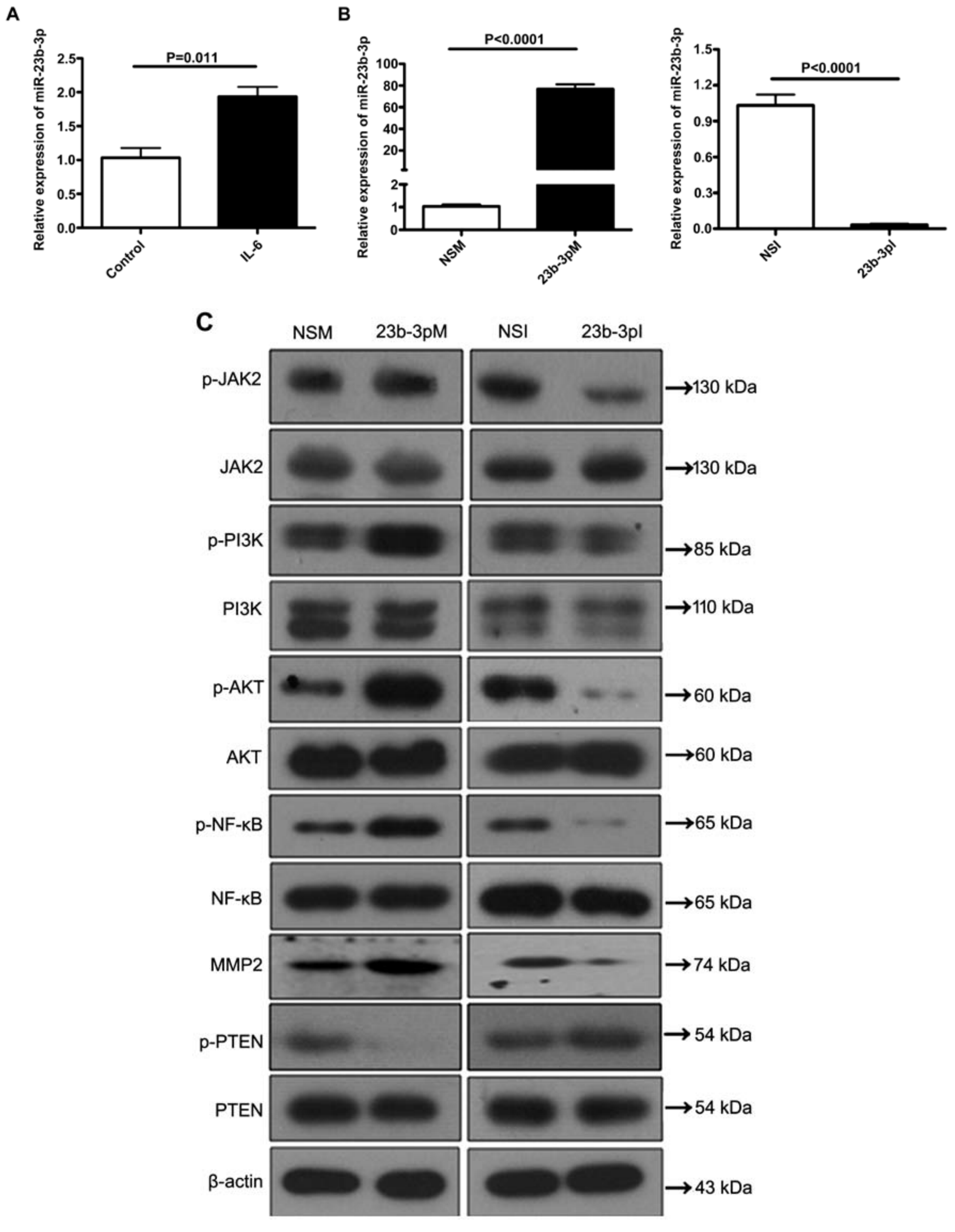


Figure 2. miR-23b-3p regulates the JAK/Akt/NF-κB signaling pathway in pancreatic cancer cells. (A) RT-qPCR was performed to detect the mRNA levels of miR-23b-3p in PANC-1 cells in response to IL-6 stimulation. (B) PANC-1 cells were transfected with 23b-3pM, 23b-3pI and the respective controls, NSM and NSI for 72 h, and the relative expression of miR-23b-3p was measured using RT-qPCR. (C) The effect of miR-23b-3p overexpression and inhibition on the expression levels of proteins in the JAK/Akt/NF-κB signaling pathway. Western blot assays were performed to detect the expression of JAK2, p-JAK2, PI3K, p-PI3K Akt, p-Akt, NF-κB, p-NF-κB, MMP2, PTEN and p-PTEN. β-actin was used as the internal control for total protein, while total protein was used as the internal control for the phosphorylated protein..

pathways in PC was further supported by the finding that miR-23b-3p could be induced by IL-6, a critical inflammatory cytokine involved in the progression of PC (30) (Fig. 2A). Notably, overexpression of miR-23b-3p in PANC-1 cells, with the transfection of 23b-3pM (Fig. 2B), significantly upregulated the protein expression level of p-JAK2, p-PI3K, p-Akt and p-NF-κB, as well as MMP2, which is a downstream target of the PI3K/Akt signaling pathway (Fig. 2C and D). In addition, p-JAK2, p-PI3K, p-Akt, p-NF-κB and MMP2 were significantly downregulated by miR-23b-3p inhibition with the transfection of 23b-3pI in PANC-1 cells (Fig. 2B-D). Meanwhile, activation of PTEN, which inactivates PI3K/Akt

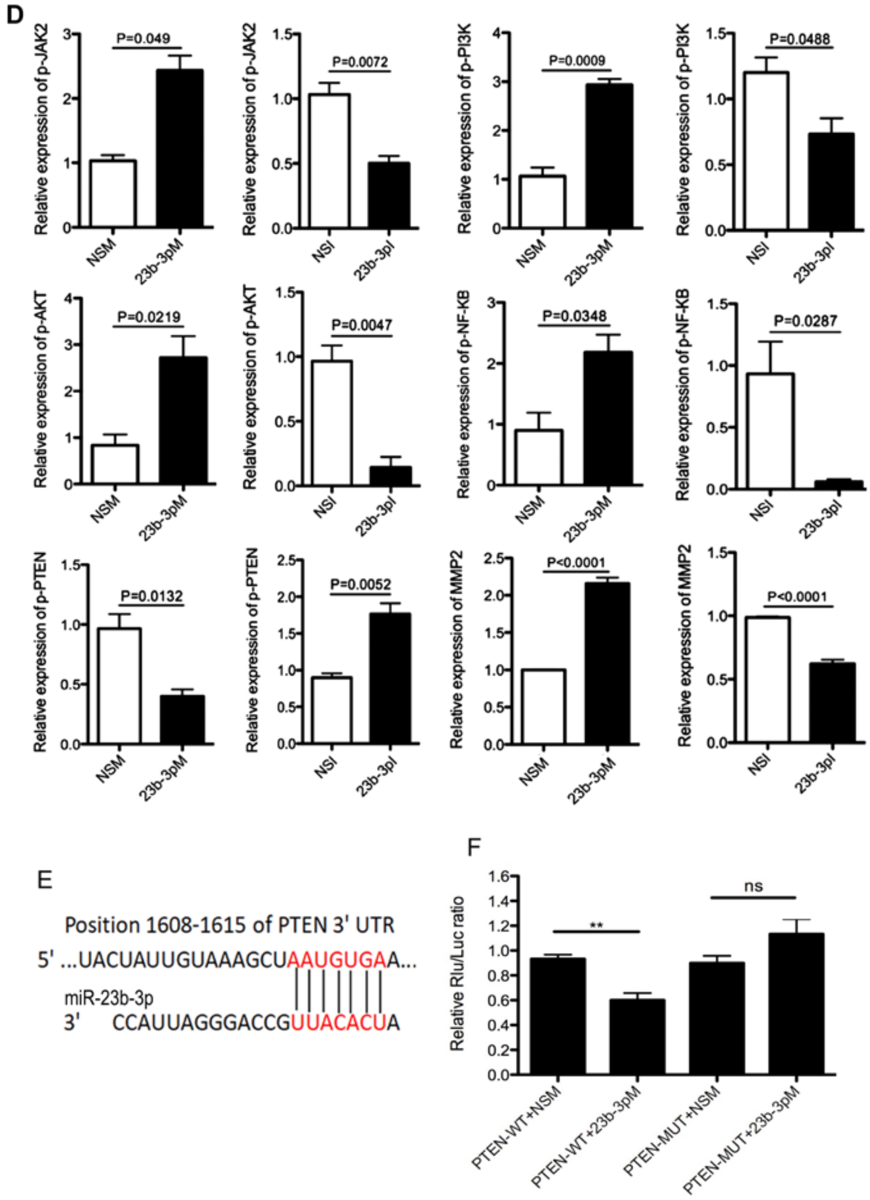


Figure 2. Continued. (D) The bar graphs show the quantification of western blot analysis of the phosphorylated proteins and MMP2. (E) The bioinformatics analysis using TargetScan shows that miR-23b-3p could bind at positions 1,608 to 1,615 of PTEN 3'UTR (the binding sites were indicated as red color). (F) The effect of miR-23b-3p overexpression on the transcriptional activity of the PTEN gene. PANC-1 cells were transfected with 23b-3pM or NSM for 24 h. The luciferase reporter assay was performed to determine the transcriptional activity of the WT PTEN gene and the 3'-UTR-MUT PTEN gene. $^{**}$P<0.01. 23b-3pI, miR-23b-3p inhibitor; 23b-3pM, miR-23b-3p mimic; IL, interleukin; JAK2, Janus kinase 2; miR, microRNA; MMP, matrix metalloproteinase; MUT, mutated; NS, not significant; NSI, non-specific inhibitor; NSM, non-specific mimic; p-, phosphorylated; RT-qPCR, reverse transcription-quantitative PCR; UTR, untranslated region; WT, wild-type; Rlu, *Renilla* luciferase.

signaling, was significantly downregulated by 23b-3pM and significantly upregulated with 23b-3pI in PANC-1 cells (Fig. 2C and D). The expression of the total proteins JAK2, PI3K, Akt, NF-κB and PTEN had no difference in cells treated with 23b-3pM or 23b-3pI when compared to their respective controls (data not shown). TargetScan software was used to identify the putative binding site between miR-23b-3p and PTEN, which was located between 1,608 and 1,615 bp in the 3'-UTR of PTEN (Fig. 2E). Therefore, to further confirm the binding of miR-23b-3p to the 3'-UTR of PTEN, the transcriptional regulation of PTEN by miR-23b-3p in PANC-1 cells was examined. As shown in Fig. 2F, overexpression of miR-23b-3p significantly repressed the transcriptional activity of the wild-type PTEN gene; however, the levels seemed to increase compared to the transcriptional activity of the 3'-UTR-mutated PTEN gene, however, this change was not significant. This result indicated that miR-23b-3p downregulated PTEN by directly targeting the 3'-UTR of PTEN. Taken together, these data show that miR-23b-3p is a positive regulator of the JAK2/Akt/NF-κB signaling pathway in PC cells.

*Overexpression of miR-23b-3p promotes PC cell-derived tumor growth in vivo and JAK/PI3K and Akt/NF-κB signaling activation*. To further investigate the *in vivo* role of miR-23b-3p in PC cell tumorigenesis, the miR-23b-3p agomir was injected into a PANC-1 cell-derived subcutaneous tumor, in a xenograft mouse model. The intratumoral injection of the miR-23b-3p agomir lead to a significant increase in the

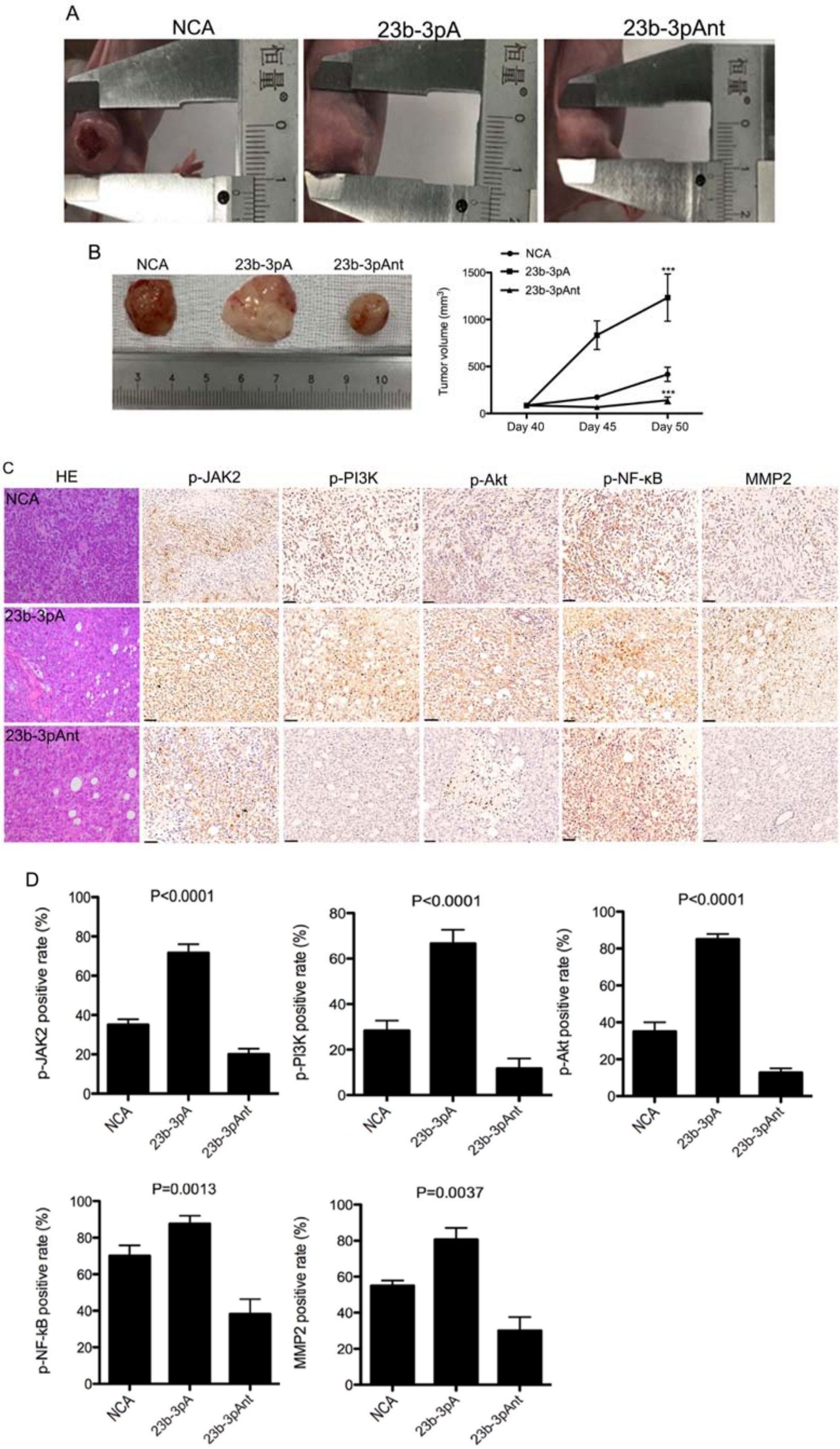


Figure 3. Effect of miR-23b-3p on cell tumorigenesis and JAK/Akt/NF-κB signaling pathway activation in pancreatic cancer. (A) At day 40, 23b-3pA, 23b-3pAnt or NCA was injected into PANC-1 cell-derived subcutaneous tumors in a xenograft mouse model every 3 days. The width and length of tumor were measured and the volume was calculated *in vivo* every 5 days. (B) At 50 days, subcutaneous tumors were harvested. The tumor volume curve was created. ***P<0.001 compared with control. (C) Hematoxylin and eosin staining of PANC-1 cell-derived tumors and immunohistochemical staining of p-JAK2, p-PI3K p-Akt, p-NF-κB and MMP2 in PANC-1 cell-derived tumor sections. Scale bar, 50 $\mu$m. (D) Quantification of the positive staining rate for p-JAK2, p-PI3K p-Akt, p-NF-κB and MMP2 expression. 23b-3pAnt, miR-23b-3p antagomir; 23b-3pA, miR-23b-3p agomir; NCA, negative control agomir; JAK2, Janus kinase 2; miR, microRNA; MMP, matrix metalloproteinase.

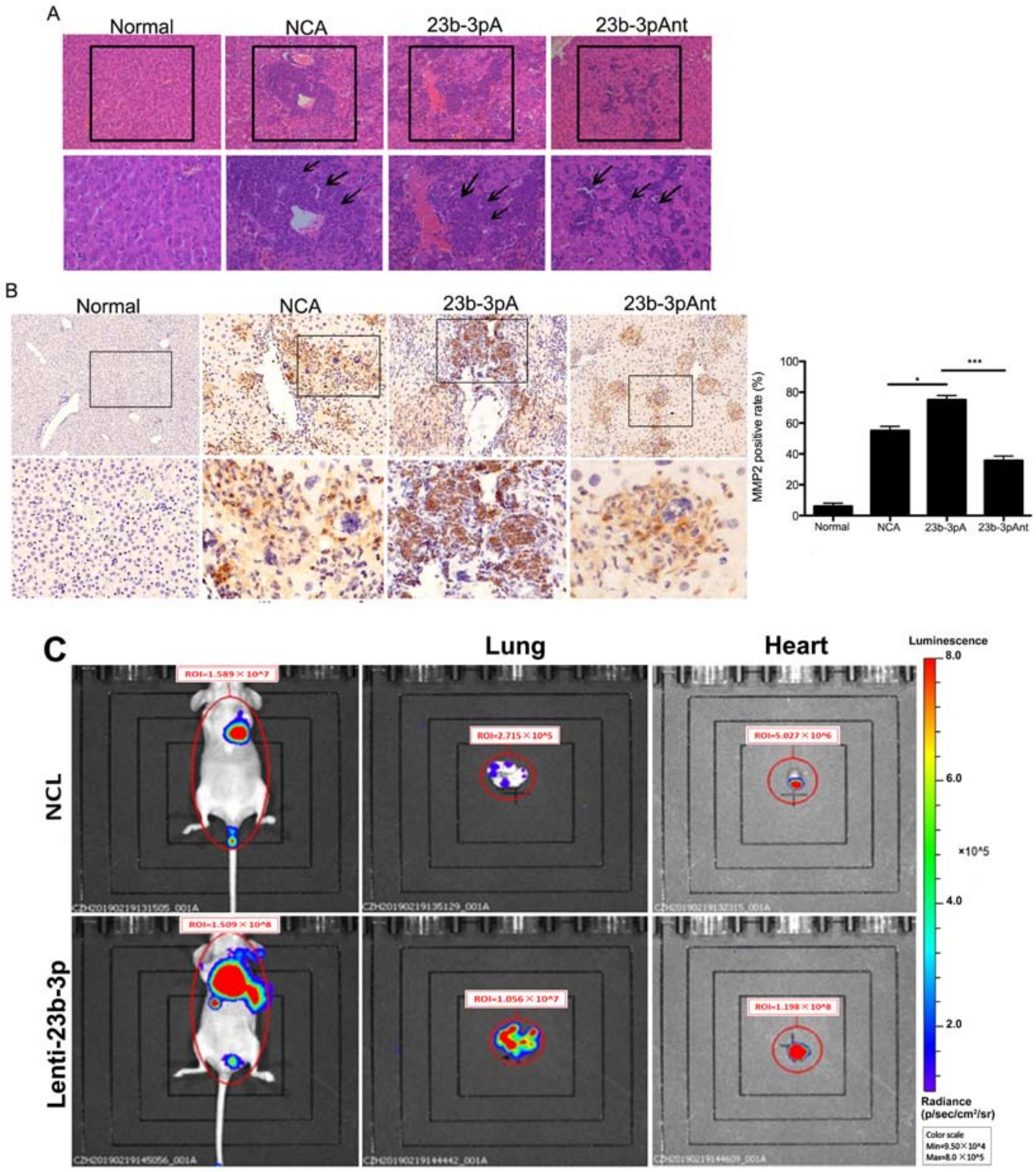


Figure 4. Effect of miR-23b-3p on pancreatic cancer metastasis. (A) At 50 days following creation of the xenograft mouse model, the liver tissues of mice were harvested, and histological analysis was performed to examine the effect of miR-23b-3p on liver metastasis. The images in the upper panel were x100 magnified, and images in the lower panel are a magnification of the indicated boxes, at x200 magnification. The metastatic lesions were indicated with black boxes and the inflammatory cells were indicated with black arrows. (B) IHC staining of MMP2 in liver tissues. The images in the upper panel were x100 magnified, and images in the lower panel are a magnification of the indicated boxes, at x200 magnification. The bar graph shows the quantification of IHC staining. $^{*}$P<0.05; $^{***}$P<0.001. (C) The BXPC-3 cells stably expressing miR-23b-3p were injected into the caudal vein of nude mice, after 52 days, the mice were injected with D-luciferin for 15 min, then the whole-body imaging was acquired using Living Image software. The red circle represents the ROI, and the total luminescence within the ROI was considered as the signal intensity. The mice injected with saline solution only were used as Normal group. 23b-3pAnt, miR-23b-3p antagomir; 23b-3pA, miR-23b-3p agomir; control, negative control agomir; NCL, negative control lentivirus; IHC, immunohistochemical; lenti-23b-3p, miR-23b-3p lentivirus; miR, microRNA; MMP, matrix metalloproteinase; ROI, Region of Interest.

tumor size in a time-dependent manner, whereas injection of the miR-23b-3p antagomir reduced the tumor size, compared with that in the negative control agomir group (Fig. 3A and B), indicating that miR-23b-3p plays a positive role in PC cell tumorigenesis *in vivo*. In addition, miR-23b-3p overexpression could activate JAK/PI3K and Akt/NF-κB signaling pathways, as evidenced by miR-23b-3p-induced upregulation of p-JAK2, p-PI3K, p-Akt, p-NF-κB and MMP2 in mouse subcutaneous tumor tissues (Fig. 3C and D), suggesting that activation of the JAK/PI3K and Akt/NF-κB signaling pathways might be mechanism by which miR-23b-3p promotes PC cell tumorigenesis *in vivo*.

*Overexpression of miR-23b-3p enhances the metastasis of PC in vivo*. PC metastasis commonly occurs in the liver (31). To investigate whether miR-23b-3p plays a role in PC liver metastasis, liver tissues were harvested from miR-23b-3p agomir-administered nude mice for morphological and histological examination. Treatment with the miR-23b-3p agomir was found to increase the number of metastatic lesions and the amount of inflammatory cell infiltration (black arrows) in liver tissues, compared with that in the normal and control groups (Fig. 4A). Whereas the treatment with the miR-23b-3p antagomir had the opposite effect on liver metastasis (Fig. 4A). These results indicated that overexpression of miR-23b-3p

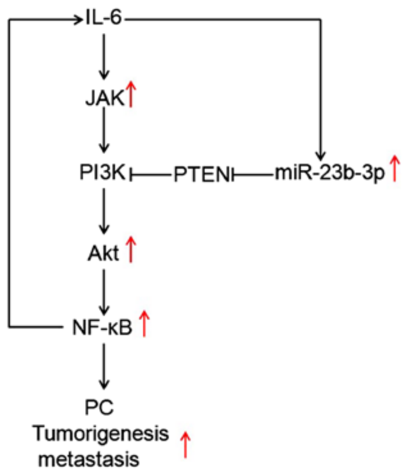


Figure 5. Signaling pathway diagram for PC tumorigenesis and metastasis regulated by miR-23b-3p. The activation role is indicated by the black arrow, while the inhibition role is indicated by the suppression symbol, and the red arrow indicates upregulation. IL-6, interleukin-6; JAK, Janus kinase; miR, microRNA; PC, pancreatic cancer; miR, microRNA.

enhanced liver metastasis from subcutaneous tumor derived in PANC-1 cells. In addition, the miR-23b-3p agomir significantly upregulated the expression of the MMP2 in liver metastasis, while the miR-23b-3p antagomir downregulated MMP2 expression (Fig. 4B). MMP2 is one of the key regulators for the metastasis of PC (32), hence in this experiment, only MMP2 protein expression in the liver was investigated. To further confirm the role of miR-23b-3p in PC metastasis, the BXPC-3 cells infected with miR-23b-3p lentivirus (lenti-23b-3p) were injected into the caudal vein of nude mice, then the whole-body mice imaging was performed using an animal CT scan. The results demonstrated that miR-23b-3p overexpression enhanced the total fluorescence signals from the ROI in mice, and the fluorescence signals were stronger in the lung and heart when compared with that in the NCL group (Fig. 4C). These findings were different from the subcutaneous tumor model as no fluorescence signals were found in liver, this could be due to different cell lines or different animal PC models causing different metastasis modes.

**Discussion**

Metastasis is a key factor influencing the prognosis of PC, and it is well-known that miRNAs can regulate the development, progression and metastasis of breast cancer (33,34). In PC, miRNAs have been found to affect tumor metastasis and function as biomarkers, and prognostic and therapeutic modulators of PC (35-38). For example, miR-141 was associated with the growth and metastasis of PC, whereas miR203a-3p inhibited PC cell growth, proliferation, epithelia-mesenchymal transition and apoptosis (39). However, the exact mechanism by which miRNAs promote metastasis in PC has not been fully understood. miR-23b-3p has been shown to promote the development of a variety of different cancers, such as lung cancer, gastric cancer and ovarian cancer, and it was also associated with poor prognosis as well (40-43). A previous study revealed that miR-23b-3p promotes the proliferation, invasion and migration of PANC-1 cells (8). However, the *in vivo* effects and the mechanistic role of miR-23b-3p in PC development remain to be elucidated.

The activation of the PI3K/Akt signaling pathway was found to promote proliferation and inhibit apoptosis of PC cells (44,45). In colorectal cancer, miRNAs were found to regulate numerous genes, such as BRCA1, PIK3CG and IL-6R involved in this PI3K/Akt signaling pathway (46). In the present study, miR-23b-3p enhanced PC cell tumorigenesis and metastasis *in vivo*. In addition, overexpression of miR-23b-3p significantly increased the phosphorylation of JAK2, PI3K, Akt, NF-κB and the total expression levels of MMP2 in PC cells, as well as in the subcutaneous tumors of the mouse model, all of which are major components of the JAK/PI3K/Akt signaling pathway. Notably, increased phosphorylation of JAK2, PI3K, Akt, NF-κB and the total expression levels of MMP2 were also observed in the tumor tissues from patients with PC, suggesting that the activation of the JAK/PI3K/Akt/NF-κB signaling pathway mediates the observed effect of miR-23b-3p on the development, progression and metastasis of PC. The findings of the present study were consistent with a previous study where the miR-1224/ELF3 axis was found to regulate the metastasis of PC via the PI3K/Akt signaling pathway (47).

IL-6 is an important inflammatory cytokine that plays a critical role in tumor development, progression, invasiveness and metastasis by activating the JAK2/STAT3, PI3K/Akt, and MAPK signaling pathways or by mediating all miRNA expression (48). In addition, it has been found that IL-6 induces miR-224 expression but inhibits miR-370 expression in cholangiocarcinoma. In addition, miR-224 promoted, but miR-370 suppressed tumor growth of cholangiocarcinoma (25,49). The transcriptional expression of IL-6 can be promoted by NF-κB (50). In the present study, IL-6 was shown to upregulate miR-23b-3p in PACN-1 cells, suggesting that IL-6 might regulate PC development by inducing the miR-23b-3p/PI3K/Akt/NF-κB cascade, however, further investigation is required.

In addition, it was also revealed that miR-23b-3p decreased the protein expression level of PTEN, which is a negative regulator of PI3K/Akt signaling, by directly targeting PTEN mRNA (51). Similarly, in renal cancer, PTEN has been found to also be inhibited by miR-23b-3p (52). Furthermore, in PC, miR-107 inhibits PC metastasis through the inhibition of the PI3K/Akt signaling pathway via PTEN (53).

MMP2 is one of the key regulators for the invasion and metastasis of PC (32), and the MMP2 protein expression levels have been associated with lymph node metastasis in PC (54). The present findings suggested that miR-23b-3p could promote liver metastasis of PC cell-derived tumors, possibly via the induction of MMP2. This effect was further confirmed by the administration of a miR-23b-3p antagomir, which significantly suppressed liver metastasis and was accompanied by the downregulation of MMP2 protein expression levels. Notably, MMP2 has been reported to be a downstream target of PI3K/Akt signaling in tumor cells (55). As a result, regulation of the PTEN/PI3K/Akt/NF-κB/MMP2 cascade might be the primary mechanism underlying the *in vivo* role of miR-23b-3p in PC cell tumorigenesis and metastasis.

However, the results of current study require further investigation, as the miR-23b-3p serum levels were only investigated in

10 PC samples, while the mRNA expression of miR-23b-3p in the tumor and adjacent tumor tissues of PC have not been investigated. The role of miR-23b-3p in tumor metastasis should also be studied in different PC models generated using different PC cell lines in future studies. In future research, the number of samples should be increased and the levels of miR-23b-3p should also be measured in the tumor tissues, as well as in the serum of patients with PC at different stages of the disease and the expression of other proteins apart from MMP2 should be investigated in an *in vivo* model of PC. In addition, the exact mechanism of how IL-6 can induce the expression of miR-23b-3p and the role of miR-23b-3p in the JAK/PI3K and Akt/NF-κB signaling pathways will be investigated further.

In conclusion, the present study found that IL-6 could regulate miR-23b-3p, which contributed to the activation of JAK/PI3K and Akt//NF-κB by targeting PTEN, which in turn promoted PC cell tumorigenesis and metastasis *in vivo* (Fig. 5). Together, these findings might provide potential therapeutic strategies in the future for the treatment of PC.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

FA and QZ designed the experiments. YZ and DC conducted the experiments. GZ, XW and LZ performed the animal experiments and prepared the figures. YL and JD analyzed the data. FA and QZ wrote and edited the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The current study was approved by the Institutional Ethics Committee of Nanjing Medical University. Written informed consent was obtained from all patients included in this study prior to their enrolment.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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