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Original Article

microRNA-218 promotes sensitivity of human pancreatic cancer cells to gemcitabine through HMGB1 and the PI3K/Akt pathway

Zhe Liu[1], Jin Long[1], Ruixia Du[2], Chunlin Ge[1], Kejian Guo[1], Yuanhong Xu[1]

[1]Department of Pancreatic Surgery, First Hospital of China Medical University, Shenyang, China; [2]Department of Otorhinolaryngology, Fengtian Hospital, Shenyang Medical University, Shenyang, China

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Abstract: Objective: The purpose of the present study was to investigate the mechanisms and signaling pathways by which microRNA-218 (miR-218) regulates the chemosensitivity of pancreatic cancer cells to gemcitabine (GEM), to explore the mechanisms underlying the resistance of pancreatic cancer cells to GEM and to provide novel approaches and strategies for treatment of pancreatic cancer. Methods: MiR-218 was overexpressed in GEM-resistant PANC-1 cells through transfection of the cells with a miR-218 mimic. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to examine the changes in miR-218 expression in PANC-1 cells. The Cell Counting Kit-8 (CCK-8) was used to examine the effect of miR-218 on the viability of GEM-induced PANC-1 pancreatic cancer cells. An enzyme-linked immunosorbent assay (ELISA) was conducted to investigate effect of miR-218 and GEM on the secretion of high mobility group box I (HMGB1) protein by PANC-1 cells. Western blot analysis was performed to analyze the effect of miR-218 on the expression of HMGB1 and beclin 1 in PANC-1 cells. In addition, beclin 1 expression was knocked down in PANC-1 pancreatic cancer cells using RNA interference (RNAi) technology. After knockdown of beclin 1, the CCK-8 assay was performed to examine the viability of GEM-induced PANC-1 cells. Finally, the effects of miR-218 overexpression on the expression of AKT and phospho-AKT (p-AKT) in GEM-induced PANC-1 cells as well as on cell viability were examined using the phosphatidylinositol-3-kinase (PI3K) pathway inhibitor, wortmannin, with western blot analysis and the CCK-8 assay. Results: The qRT-PCR results showed that compared with the control group, miR-218 expression was significantly increased in PANC-1 cells at 48 h after transfection with the miR-218 mimic ($P<0.01$). The CCK-8 assay results showed that the viability of PANC-1 cells was markedly reduced after transfection with the miR-218 mimic and treatment with 5 μM GEM (miR-218 mimic+GEM group) compared with the mimic ctrl+GEM group and the normal control group ($P<0.01$). The ELISA results showed that GEM induced the secretion of HMGB1 by PANC-1 cells ($P<0.01$), whereas miR-218 and quercetin inhibited the secretion of HMGB1 by GEM-induced PANC-1 cells ($P<0.01$). The western blot analysis showed that miR-218 inhibited the expression of HMGB1 and beclin 1 in PANC-1 cells ($P<0.01$). Transfection of PANC-1 cells with beclin 1 shRNA effectively reduced the expression of beclin 1 in PANC-1 cells ($P<0.01$). Knockdown of beclin 1 expression in PANC-1 cells enhanced the sensitivity of PANC-1 cells to GEM ($P<0.01$), whereas overexpression of HMGB1 effectively reversed the beclin 1 knockdown-induced susceptibility to GEM ($P<0.01$). The results of the western blot analysis and CCK-8 assay showed that compared with the control group, wortmannin significantly inhibited the expression of p-AKT in PANC-1 cells ($P<0.01$). In addition, wortmannin promoted the effect of miR-218 on the sensitivity of GEM-induced PANC-1 cells ($P<0.05$). Conclusion: Overexpression of miR-218 promotes the sensitivity of PANC-1 cells to GEM. The effect of miR-218 is achieved mainly through inhibiting the secretion of HMGB1 by PANC-1 cells and the PI3K/Akt pathway.

Keywords: Pancreatic cancer, miR-218, gemcitabine, HMGB1, beclin 1

Introduction

Pancreatic cancer is a highly malignant tumor. Early pancreatic cancer lacks typical symptoms. By the time pancreatic cancer is discovered, the cancer has often invaded into the surrounding tissues and metastasized to distant sites. Therefore, pancreatic cancer typically has a poor prognosis [1, 2]. The worldwide incidence of pancreatic cancer continues to show an upward trend year after year. Pancreatic cancer has become the fourth leading cause of

cancer-related deaths. Most pancreatic cancers are already in advanced stages when diagnosed, and the 5-year survival rate is less than 5% [3, 4]. Pancreatic cancer is fundamentally a genetic disorder. Pancreatic cancer arises from atypical endothelial hyperplasia, which is developed into carcinoma in situ and further into invasive tumor. The process of pancreatic cancer development and progression involves multiple stages and multiple steps, and it requires continuous accumulation of multiple genetic alterations [5, 6]. During the process, a number of genes that control cell differentiation, proliferation and apoptosis display abnormal changes, including mutation or overexpression of oncogenes and inactivation of tumor suppressor genes. MicroRNAs (miRNAs) are also involved in the pathogenesis of pancreatic cancer [7-9].

MiRNAs are a class of endogenous, non-coding, single-stranded small RNA molecules that are approximately 22 nucleotides (nt) in length. Through complementarily pairing with the 3' untranslated region (3'UTR) of the target gene mRNAs, miRNAs regulate mRNA expression at the posttranscriptional level, thereby participating in physiological processes, such as cell proliferation, apoptosis, differentiation, metabolism and development, as well as pathological processes, such as cardiovascular diseases, neurological disorders and tumors [10-12]. Zhu et al. demonstrated that microRNA-218 (miR-218) is expressed in pancreatic ductal adenocarcinima (PDAC) and is related to the development of pancreatic cancer [13]. The results of our previous study showed that miR-218 expression is downregulated in human pancreatic cancer tissues and cell lines. MiR-218 negatively regulates the expression of high mobility group box I (HMGB1) protein and inhibits the proliferation and invasion of pancreatic cancer cells.

Pancreatic cancer typically has a poor prognosis. The 5-year survival rate of pancreatic cancer patients is less than 50%. Chemotherapy-based adjuvant therapy plays an important role in treatment of pancreatic cancer [14]. Chemotherapy not only improves the symptoms of advanced pancreatic cancer but also prolongs the survival time [15]. Chemotherapy remains one of the important means of prolonging the survival time of patients with pancreatic cancer. However, lack of chemosensitivity in tumors has been an important reason underlying the poor efficacy of chemotherapy [16, 17]. Compared with 5-fluorouracil, gemcitabine (GEM) significantly improves the symptoms of patients and prolongs the median survival time of patients. Therefore, GEM has become the preferred chemotherapy drug for advanced pancreatic cancer [18-21]. In long-term clinical practice, however, the efficacy of GEM in treatment of pancreatic cancer is far from ideal. The overall efficacy of GEM is less than 20%. The main underlying reason is the acquired or intrinsic drug resistance of pancreatic cancer cells, which leaves many patients who receive GEM-based chemotherapy to suffer the side effects of chemotherapy without obtaining the desired therapeutic effect [22-24]. A previous study has demonstrated that miR-218 is a tumor suppressor miRNA. In addition, Li et al. reported that miR-218 inhibits the growth of cervical cancer and enhances the sensitivity of cervical cancer to cisplatin-based chemotherapy [25]. The results of our previous study showed that miR-218 expression is downregulated in a GEM-resistant cell line. MiR-218 promotes the sensitivity of PANC-1 cells to GEM, and the effect of miRNA is exerted mainly through regulation of HMGB1 expression in PANC-1 cells. The mechanisms and molecular signaling pathways by which miR-218 enhances the sensitivity of PANC-1 cells to GEM remain to be identified.

HMGB1 is widely distributed and present in the nuclei and cytoplasm of nearly all metazoan and plant cells. HMGB1 was initially discovered as a chromatin-associated protein containing a large number of acidic and basic amino acids [26-28]. HMGB1 undergoes extensive post-translational modifications, including glycosylation, acetylation, methylation, oxidation and phosphorylation. However, only acetylated HMGB1 effectively forms complexes with homologous DNA polymerase α and promotes the activity of DNA polymerase α [29]. Conventional anti-tumor therapies, such as radiotherapy and chemotherapy, induce tumor stress or death [30, 31]. The presence of extracellular HMGB1 suggests that some cells are under stress or have already died. In addition, extracellular HMGB1 serves as an alert signal to other cells and activates anti-damage mechanisms [32]. Necrotic tumor cells release HMGBI, thereby

regulating tumor immunity and interfering with the efficacy of chemotherapy and radiotherapy. HMGB1 and other factors released during chemotherapy or radiotherapy are important components of the disordered tumor microenvironment [33]. A recent study has shown that HMGB1 is capable of interacting with beclin 1 and regulating the occurrence of autophagy [34]. Therefore, in the present study, we examined the relationship between miR-218 and the chemosensitivity of pancreatic cancer cells to GEM. We also investigated the effect of miR-218 on HMGB1 release and beclin 1 expression in GEM-induced pancreatic cancer cells, and we explored the mechanisms and molecular signaling pathways by which miR-218 promotes the sensitivity of PANC-1 cells to GEM. This study provided novel ideas and experimental evidence for the identification of effective treatment for pancreatic cancer.

Materials and methods

Main reagents

The GEM-resistant pancreatic cancer cell line, PANC-1, was purchased from American Type Culture Collection (ATCC, MD, USA). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, L-glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Lipofectamine 2000 and TRIzol reagent were purchased from Life Technologies (USA). Cell culture plates and dishes were purchased from Corning Inc. The reverse transcription (RT) kit was purchased from Qiagen. The HMGB1 ELISA kit was purchased from Shino-Test Corporation (Kanagawa, Japan). The pGEM-T and pcDNA3.1 vectors were purchased from Life Technologies (USA).

The TaqMan miRNA Isolation Kit, TaqMan microRNA Assay Kit, TaqMan microRNA Assay and TaqMan Universal PCR Master Mix were purchased from Life Technologies, Inc. (Waltham, MA, USA). The miR-218 RT primer, miR-218 mimic and non-specific control (mimic ctrl) were synthesized by Shanghai Gene Pharma Co., Ltd. The beclin 1 shRNA and negative control (control shRNA) were purchased from Sigma-Aldrich (San Diego, CA, USA).

The following primers used for construction of the recombinant HMGB1 expression vector were synthesized by Shanghai Invitrogen Biotechnology Co., Ltd.: upstream primer, 5'-CGGAA TTC ATG GGC AAA GGA GAT CCT AA-3' (containing the *EcoR* I restriction site); and downstream primer, 5'-CG GGA TCC TTC ATC ATC ATC ATC TTC TT-3' (containing the *Bam*H I restriction site). The *EcoR* I and *Bam*H I restriction endonucleases were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. The Taq DNA polymerase was purchased from Fermentas, Inc. (Glen Burnie, MD, USA).

GEM was purchased from Eli Lilly and Company (USA and Canada). Quercetin was purchased from Sigma-Aldrich (San Diego, CA, USA). Wortmannin, an inhibitor of the phosphoinositide 3-kinase (PI3K) pathway, was purchased from Axxora Life Sciences Inc. (San Diego, CA). Protein Extraction and Quantitation Kits were purchased from Bio-Rad Laboratories, Inc. The Cell Counting Kit-8 (CCK-8) was purchased from Beyotime Institute of Biotechnology (Shanghai). Trypsin and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (USA). The rabbit anti-human HMGB1 monoclonal antibody, mouse anti-human β-actin monoclonal antibody and rabbit anti-human beclin 1 polyclonal antibody were purchased from Abcam (UK). Primary antibodies against AKT and phospho-AKT (p-AKT; Ser473) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The horseradish peroxidase (HRP)-conjugated affinity-purified goat anti-mouse IgG and HRP-conjugated affinity-purified goat anti-rabbit IgG secondary antibodies were purchased from Sigma-Aldrich (San Diego, CA).

Construction of recombinant HMGB1 expression vector

The human *HMGB1* mRNA sequence was obtained from GenBank (NM_002128.4 in GenBank), and the primers were designed to flank the *HMGB1* open reading frame (ORF). Both the primer design and restriction enzyme analysis were performed by Primer Premier 5 software.

Total RNA was extracted from PANC-1 cells using the TRIzol reagent according to the instruction manual followed by quantification. cDNA was synthesized from the mRNA template from PANC-1 cells, and PCR was conducted using the *HMGB1* primers. The PCR prod-

ucts were cloned into the pGEM-T vector. After cleavage and identification with restriction endonucleases, the correct recombinant plasmid was sequenced. The pcDNA3.1 vector and the pGEM-*HMGB1* recombinant plasmid were simultaneously cleaved with *Bam*H I and *Eco*R I restriction endonucleases, and the target fragments were purified and joined by T4 DNA ligase. The resulting pcDNA3.1-*HMGB1* recombinant plasmid was transformed into DH5α competent cells.

Processing of cells

PANC-1 cells were cultured in DMEM containing 10% FBS, 1.5 g/L $NaHCO_3$ and 4 mM L-glutamine under standard conditions (37°C, 5% CO_2 and saturated humidity). The growth state of the cells was monitored using an inverted microscope. Once grown to 70% to 80% confluence, the cells were digested with 0.25% trypsin and passaged. The culture medium was changed every other day, and the cells were passaged every 3-4 days. Cells in logarithmic growth phase were harvested for the assays.

Normally cultured PANC-1 cells were seeded uniformly into 6-well culture plates at a density of 3×10^5 cells/ml in a volume of 1 ml. After the cells adhered to the culture surface, the miR-218 mimic, non-specific control (mimic ctrl), recombinant *HMGB1* expression vector (pcDNA3.1-*HMGB1*), empty pcDNA3.1 vector (vector ctrl), beclin 1 shRNA and shRNA ctrl were transfected into the cells using the Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. The normal control group (normal ctrl) was established at the same time. The miR-218 mimic, mimic ctrl, pcDNA3.1-*HMGB1*, vector ctrl, beclin 1 shRNA and shRNA ctrl were diluted in serum-free Minimum Essential Media (MEM). Subsequently, Lipofectamine 2000 was diluted by gently mixing the liposome with MEM and incubating at room temperature (RT) for 5 min. The MEM-diluted Lipofectamine 2000 was then evenly mixed with diluted miR-218 mimic, mimic ctrl, pcDNA3.1-*HMGB1* and vector ctrl. The mixtures were incubated at RT for 20 min to allow the formation of complexes. The complexes were added to the cell culture plates containing PANC-1 cells. After addition of the complexes, the cells were placed into a humidified incubator (37°C and 5% CO_2). After 5 h of incubation, the complex-containing medium was replaced with either fresh MEM supplemented with 10% FBS or MEM containing 10% FBS and 5 μM GEM (final concentration). The cells were then cultured for an additional 48 h.

Measuring the effect of miR-218 on the viability of GEM-induced pancreatic cancer cells using the CCK-8 assay

MiR-218 was overexpressed in PANC-1 cells by transfecting PANC-1 cells with miR-218 mimic. At 48 h after transfection of PANC-1 cells with either miR-218 mimic or mimic ctrl, the normal ctrl group was established. RNAs were extracted from all groups of PANC-1 cells using the TaqMan miRNA Isolation Kit. Changes in miR-218 expression in various groups of PANC-1 cells were examined using quantitative real-time polymerase chain reaction (qRT-PCR).

Cell viability was determined using the CCK-8 Assay Kit in accordance with the manufacturer's instructions. PANC-1 cells were transfected with either miR-218 mimic or mimic ctrl. Subsequently, GEM was added to each well of cell culture medium at a final concentration of 5 μM. After treating the cells with GEM for 48 h, CCK-8 solution was added to the cells, and the cells were incubated (at 37°C at 5% CO_2) for 2 h. The normal ctrl group was set up at the same time. The optical density (OD) of the cells was measured at 450 nm (reference wavelength of 630 nm) using a microplate reader (Bio-Tek, USA). Ten replica wells were set up for each experimental group, and each experiment was repeated three times.

Examination of the effect of miR-218 on HMGB1 secretion by GEM-resistant pancreatic cancer cells using an enzyme-linked immunosorbent assay (ELISA)

During chemotherapy, HMGB1 released by tumor cells affects the chemosensitivity of the tumor cells [35]. MiR-218 promotes the sensitivity of pancreatic cancer cells to GEM-based chemotherapy. Therefore, we tested if miR-218 affects the sensitivity of pancreatic cancer cells to GEM through regulating HMGB1 secretion. In the present study, an ELISA was conducted to analyze the effect of miR-218 on HMGB1 secretion by GEM-induced pancreatic cancer cells.

PANC-1 cells were transfected with the miR-218 mimic or mimic ctrl. Following transfection, GEM was added to the cells at a final concentration of 5 μM. The normal ctrl group was set up at the same time. After 48 h of GEM treatment, the cell culture supernatant was collected. According to the instruction manual of the ELISA kit, the OD value of each well was measured at a wavelength of 450 nm. Based on the concentrations of the standard and the corresponding OD values, the linear regression equation of the standard curve was determined. Subsequently, the HMGB1 concentration in each group of samples was calculated from the corresponding OD value using the regression equation. Quercetin inhibits the release of HMGB1 by cells [36]. In the present study, the CCK-8 assay was performed to determine if quercetin affects the viability of pancreatic cancer cells after GEM induction.

Examination of the effect of miR-218 on beclin 1 expression in pancreatic cancer cells by western blot analysis

A recent study has demonstrated that HMGB1 can interact with beclin 1 and regulate the occurrence of autophagy [34]. In the present study, western blot analysis was performed to examine the effect of miR-218 on the expression of the autophagy-related protein, beclin 1, in pancreatic cancer cells.

Normally cultured PANC-1 pancreatic cancer cells were seeded uniformly into 6-well culture plates at a density of 3×10^5 cells/ml. PANC-1 cells were transfected with the miR-218 mimic or mimic ctrl, and the normal ctrl group was set up at the same time. At 48 h after transfection, 1 ml of cell lysis buffer was added to each well of PANC-1 cells. The resulting cell lysates were transferred to 1.5 ml centrifuge tubes and centrifuged at 160,00 g for 30 min. The supernatants were collected, and the protein concentrations in the supernatants were measured using the bicinchoninic acid (BCA) assay. Subsequently, a 5% stacking gel and 15% separating gel were poured. Total protein (50 μg) was loaded in each lane, separated by electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane using a wet transfer system (Bio-Rad Laboratories, Inc., USA). The membrane was blocked at RT in Tris-Buffered Saline-Tween 20 (TBST) solution (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; and 0.1% Tween-20) containing 5% nonfat dry milk for 1 h and then incubated with the rabbit anti-human HMGB1 monoclonal antibody (1:1000 dilution), rabbit anti-human beclin 1 polyclonal antibody (1:500 dilution) or mouse anti-human β-actin monoclonal antibody (1:1000 dilution) at 4°C overnight. The membrane was washed three times with TBST (5 min each) and incubated with HRP-labeled goat anti-rabbit IgG or HRP-labeled goat anti-mouse IgG secondary antibody at 37°C for 1 h. The membrane was washed three times with TBST (5 min each), incubated with enhanced chemiluminescence (ECL) reagent and autoradiographed. The relative contents of HMGB1 and beclin 1 were expressed as the grayscale ratios of HMGB1/β-actin and beclin 1/β-actin, respectively. Changes in the relative expression of HMGB1 and beclin 1 were analyzed using PDQuest software (Bio-Rad Laboratories, Inc., Richmond, CA).

Investigation of the effect of miR-218 on the sensitivity of pancreatic cancer cells to GEM-based chemotherapy through regulation of HMGB1 and beclin 1 using RNA interference (RNAi)

Normally cultured PANC-1 pancreatic cancer cells were seeded uniformly into 6-well culture plates at a density of 3×10^5 cells/ml. PANC-1 cells were transfected with beclin 1 shRNA or control shRNA, and the normal ctrl group was set up at the same time. At 48 h after transfection, western blot analysis was conducted to determine the interference efficiency of beclin 1 shRNA.

After knockdown of beclin 1 or simultaneous knockdown of beclin 1 and overexpression of HMGB1 in PANC-1 pancreatic cancer cells, the CCK-8 assay was conducted to examine the viability of PANC-1 cells treated with GEM. PANC-1 cells were divided into the following five experimental groups: normal ctrl group, control shRNA+GEM group, beclin 1 shRNA+GEM group, pcDNA3.1-HMGB1+control shRNA+GEM group and pcDNA3.1-HMGB1+beclin 1 shRNA+GEM group. After 48 h of treatment, CCK-8 solution was added to the cells, and the cells were incubated (at 37°C and 5% CO_2) for 2 h. The normal ctrl group was set up at the same time. The OD of the cells was measured at 450 nm (reference wavelength of 630 nm) using a microplate reader (Bio-Tek, USA). Ten replica wells were set up for each experimental group,

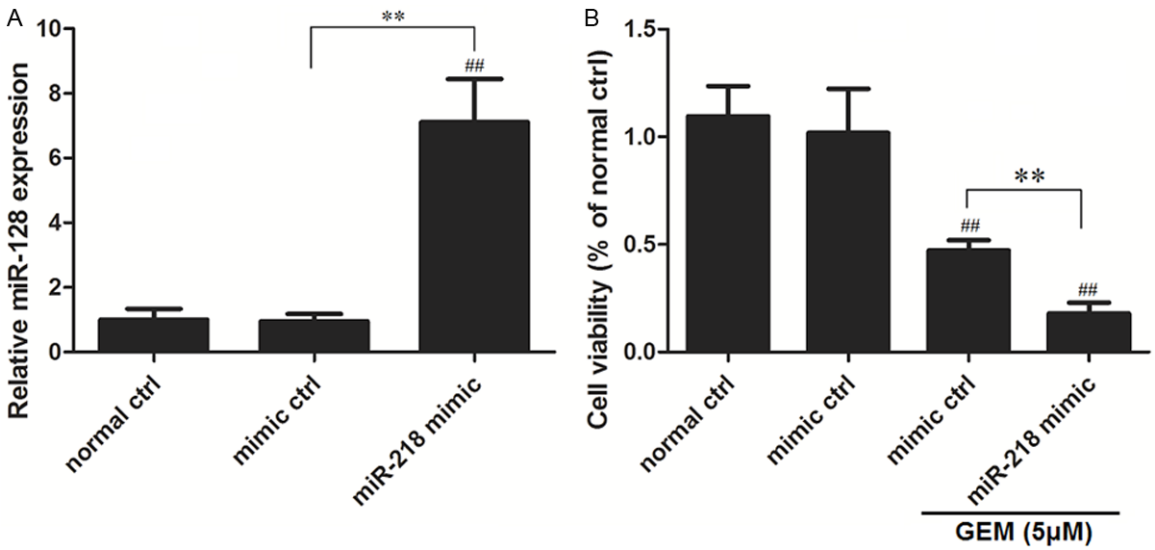

Figure 1. Examination of the effect of miR-218 on the viability of GEM-induced pancreatic cancer cells. A. The effect of transfection of PANC-1 pancreatic cancer cells with miR-218 mimic or mimic ctrl on miR-218 expression. B. Analysis of the effect of miR-218 overexpression on the viability of GEM-induced pancreatic cancer cells using the CCK-8 assay. ##P<0.01 versus normal control group (normal ctrl), **P<0.01.

and each experiment was repeated three times.

Determination of the signaling pathways through which miR-218 affects the chemosensitivity of GEM-induced pancreatic cancer cells

Normally cultured PANC-1 cells were seeded uniformly into 6-well culture plates at a density of 3×10^5 cells/ml. PANC-1 cells were transfected with the miR-218 mimic or mimic ctrl. After transfection, GEM was added to the PANC-1 cells at a final concentration of 5 μM. Subsequently, the PI3K pathway inhibitor, wortmannin, was added to the transfected PANC-1 cells (final concentration of 5 μM), and the normal ctrl group was set up at the same time. After 48 h, the cells were washed one to two times with PBS. Protein was extracted from the various groups of cells, and protein concentrations in the supernatants were measured using the BCA method. The proteins were separated by electrophoresis and transferred to PVDF membrane using a wet transfer system (Bio-Rad Laboratories, Inc., USA). The membrane was blocked at RT in TBST solution containing 5% nonfat dry milk for 1 h and then incubated with the anti-AKT, anti-p-AKT (Ser473) (1:500 dilution) or mouse anti-human β-actin monoclonal antibody (1:1000 dilution) at 4°C overnight. Subsequently, the membrane was incubated with the corresponding HRP-labeled secondary antibodies (diluted 1:2000 in PBS) at 4°C overnight. The membrane was washed with TBST, incubated with ECL reagent and autoradiographed. The grayscale ratio of p-AKT/AKT was analyzed using Quantity One software. The effects of the PI3K pathway inhibitor, wortmannin, on the ectopic expression of miR-218 and the viability of GEM-induced PANC-1 cells were analyzed using the CCK-8 assay.

Statistical analysis

Experimental data were statistically analyzed using SPSS17.0 statistical analysis software. Comparison between two groups of data was conducted using a t test, while comparisons between multiple groups of data were performed using analysis of variance (ANOVA). *P* values less than 0.05 were considered statistically significant.

Results

Effect of miR-218 on the viability of GEM-induced pancreatic cancer cells using the CCK-8 assay

MiR-218 was overexpressed in PANC-1 cells by transfection with the miR-218 mimic. Differences in miR-218 expression among the groups of PANC-1 cells were examined using qRT-PCR. The qRT-PCR results showed that miR-218

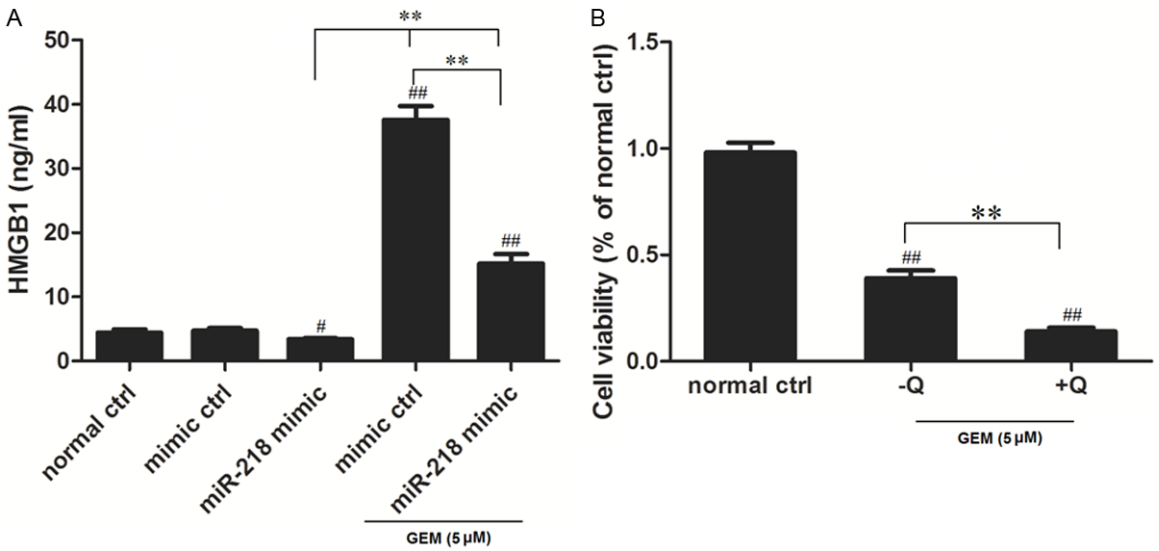

Figure 2. Examination of the effect of miR-218 on HMGB1 secretion by GEM-induced pancreatic cancer cells. A. Examination of the effect of miR-218 on HMGB1 secretion by GEM-induced pancreatic cancer cells using ELISA. B. Analysis of the effect of quercetin (Q) on the viability of GEM-induced pancreatic cancer cells using the CCK-8 assay. #P<0.05 and ##P<0.01 versus normal control group (normal ctrl), **P<0.01.

expression was significantly elevated in PANC-1 cells transfected with the miR-218 mimic compared to the normal ctrl and negative control (mimic ctrl) groups (P<0.01). The results are shown in **Figure 1A**.

The effect of miR-218 on the viability of GEM-induced pancreatic cancer cells was analyzed using the CCK-8 assay. The viability of GEM-treated PANC-1 cells was significantly decreased in comparison with the untreated normal ctrl and negative control (mimic ctrl) groups (P<0.01). In addition, cell viability was markedly reduced in the GEM-treated miR-218-transfected group compared to the negative control (mimic ctrl) group (P<0.01). The results are shown in **Figure 1B**. The results indicated that miR-218 enhances the sensitivity of pancreatic cancer cells to GEM-based chemotherapy.

Effect of miR-218 on the secretion of HMGB1 by GEM-induced pancreatic cancer cells using an ELISA

PANC-1 cells were transfected with the miR-218 mimic or mimic ctrl. After transfection, GEM was added to the cells at a final concentration of 5 μM. The normal ctrl group was set up at the same time. After treatment for 48 h, cell culture supernatants were collected, and the concentrations of HMGB1 in the supernatants were determined by ELISA. The HMGB1 concentration was lower in the supernatant of the miR-218 mimic-transfected group compared to the normal ctrl and negative control (mimic ctrl) groups (P<0.05). In addition, the HMGB1 concentration was significantly higher in the supernatant of GEM-treated PANC-1 cells compared with the untreated group (P<0.01). The results are shown in **Figure 2A**. The results indicated that GEM promoted the release of HMGB1 by PANC-1 cells, whereas miR-218 inhibited the release of HMGB1 by GEM-induced PANC-1 cells.

Quercetin inhibits the release of HMGB1 by cells [36]. In the present study, the CCK-8 assay was conducted to determine if quercetin affects the viability of GEM-induced pancreatic cancer cells. The results showed that cell viability was significantly decreased in the quercetin-treated group compared with the untreated group (P<0.01). The results are shown in **Figure 2B**. The results indicated that quercetin inhibits the release of HMGB1 by PANC-1 cells and promotes the sensitivity of pancreatic cancer cells to GEM.

Effect of miR-218 on the expression of beclin 1 in pancreatic cancer cells by western blot analysis

A recent study has shown that HMGB1 can interact with beclin 1 and regulate the occur-

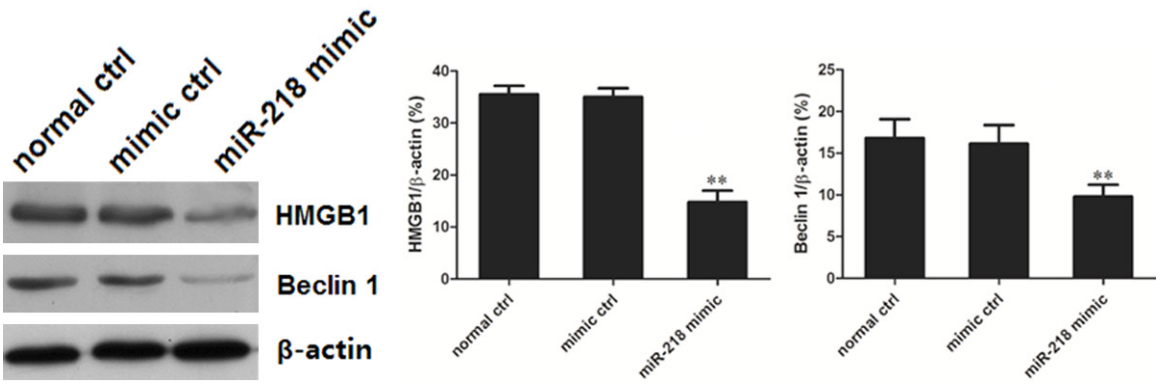

Figure 3. Examination of the effect of miR-218 on the expression of HMGB1 and beclin 1 in pancreatic cancer cells by western blot analysis. **P<0.01 versus normal control group (normal ctrl).

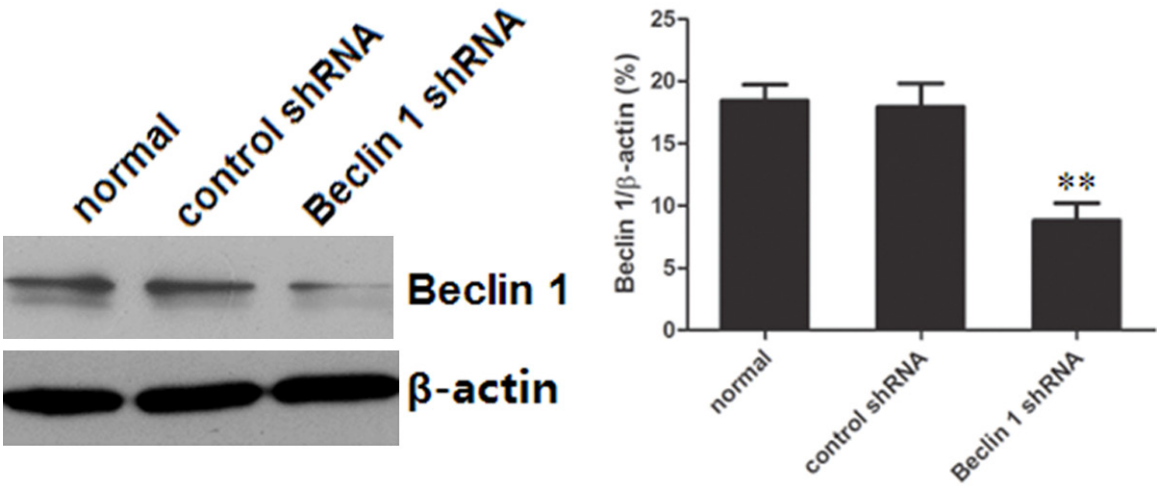

Figure 4. Analysis of beclin 1 expression in pancreatic cancer cells by western blot analysis. **P<0.01 versus normal control group (normal).

rence of autophagy [34]. In the present study, western blot analysis was performed to examine the effect of miR-218 on the expression of the autophagy-related protein, beclin 1, in pancreatic cancer cells. The expression levels of HMGB1 and beclin 1 were significantly lower in the miR-218 mimic-transfected group compared to the normal ctrl and negative control (mimic ctrl) groups (P<0.01). The results are shown in **Figure 3**. The results indicated that miR-218 inhibits the expression of HMGB1 and beclin 1 in PANC-1 cells.

Effect of miR-218 on cell sensitivity to GEM-based chemotherapy through regulation of HMGB1 and beclin 1 using RNAi

Normally cultured PANC-1 pancreatic cancer cells were seeded uniformly into 6-well culture plates at a density of 3×10^5 cells/ml. PANC-1 cells were then transfected with beclin 1 shRNA or control shRNA, and the normal ctrl group was set up at the same time. At 48 h after transfection, western blot analysis was performed to determine the interference efficiency of beclin 1 shRNA. The western blot analysis showed that the expression level of beclin 1 was significantly decreased in the beclin shRNA-transfected group compared to the normal ctrl and negative control (control shRNA) groups (P<0.01). The results are shown in **Figure 4**.

After knockdown of beclin 1 or simultaneous knockdown of beclin 1 and overexpression of HMGB1 in PANC-1 pancreatic cancer cells, the CCK-8 assay was conducted to examine the viability of GEM-treated PANC-1 cells. Treatment of the beclin 1 shRNA-transfected group with

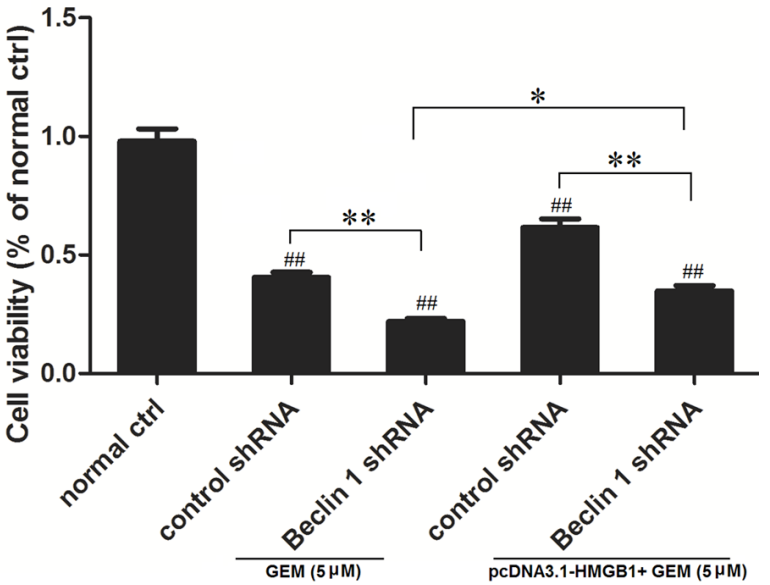

Figure 5. Examination of the viability of GEM-treated PANC-1 pancreatic cancer cells after knockdown of beclin 1 or simultaneous knockdown of beclin 1 and overexpression of HMGB1 in PANC-1 cells using the CCK-8 assay. ##P<0.01 versus normal control group (normal ctrl), *P<0.05 and **P<0.01.

GEM resulted in significantly decreased cell viability compared to the negative control (control shRNA) group (P<0.01). Cell viability was significantly higher in the pcDNA3.1-HMGB1+ Beclin 1 shRNA+GEM group compared with the beclin 1 shRNA+GEM group (P<0.05). The results are shown in **Figure 5**. The results indicated that knockdown of beclin 1 expression enhances the sensitivity of pancreatic cancer cells to GEM, whereas overexpression of HMGB1 reverses the effect of suppressed beclin 1 expression.

Signaling pathways through which miR-218 affects the chemosensitivity of GEM-induced pancreatic cancer cells

The PI3K pathway inhibitor, wortmannin, was utilized to explore the signaling pathways mediated by miR-218. The western blot results showed that treatment of the miR-218 mimic-transfected PANC-1 cells with 5 μM GEM for 48 h resulted in a markedly reduced p-AKT/AKT ratio in comparison to the normal ctrl group (P<0.01). However, no significant difference was detected when compared with the wortmannin treated group. The results are shown in **Figure 6**.

The CCK-8 assay results showed that treatment of the miR-218 mimic-transfected PANC-1 cells with 5 μM GEM and 5 μM wortmannin resulted in significantly reduced cell viability compared with the normal ctrl (P<0.01) and miR-218 mimic+GEM (P<0.05) groups. The results are shown in **Figure 7**. The results indicated that overexpression of miR-218 enhances the sensitivity of PANC-1 cells to GEM and that miR-218 exerts its effect through the PI3K/Akt signaling pathway.

Discussion

GEM (2',2'-difluorodeoxycytidine, dFdC) is a structural analogue of a DNA nucleotide. In cells, GEM is converted to active gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP) by the action of nucleotide kinases [37, 38]. GEM pairs with multiple nucleotides, but GEM exhibits a different terminal structure, which is unrecognizable to DNA exonucleases. As the result, DNA exonuclease-mediated DNA repair is inhibited, which induces apoptosis during the S phase of cell cycle (DNA synthesis phase) via the caspase cascade amplification system [39, 40]. Chemotherapy is an important adjunctive therapy for pancreatic cancer, and it plays a role in improving the quality of life of patients and prolonging the survival time of patients [41, 42]. Currently, GEM is the first-line drug utilized in chemotherapies for pancreatic cancer. However, the overall efficacy of GEM is less than 20% [43, 44]. The main problem causing the low efficacy is that the majority of pancreatic cancer patients will develop resistance to GEM [45]. Therefore, identification of the GEM resistance-related genes in pancreatic cancer and enhancement of the efficacy of GEM are the issues that urgently need to be solved in clinical practice.

Studies on the relationship between miRNAs and tumor chemosensitivity have received increasing attention from scholars in the field of cancer research and have become a research hotspot in recent years. Previous studies have demonstrated that miR-218 is a tumor-suppressing miRNA. In addition, Li et al. demon-

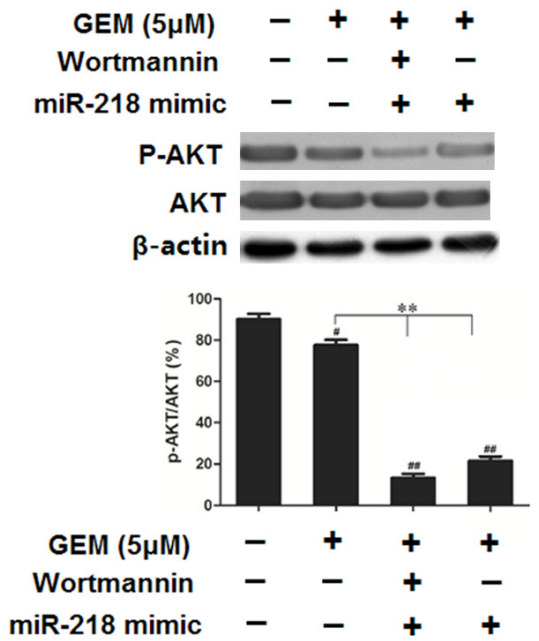

Figure 6. Western blot analysis of the protein expression of p-AKT, AKT and β-actin in various groups of PANC-1 cells. #P<0.05 and ##P<0.01 versus normal control group (normal ctrl), **P<0.01.

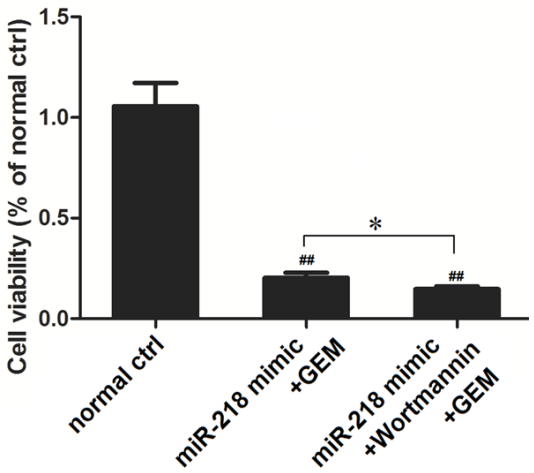

Figure 7. Examination of the viability of various groups of PANC-1 cells using the CCK-8 assay. ##P<0.01 versus normal control group (normal ctrl), *P<0.05.

strated that miR-218 inhibits the growth of cervical cancer and enhances the sensitivity of cervical cancer to cisplatin-based chemotherapy [25]. The results of our previous study showed that miR-218 expression is downregulated in human pancreatic cancer tissues and cell lines. MiR-218 negatively regulates the expression of HMGB1 protein and inhibits the proliferation and invasion of pancreatic cancer cells. In the present study, we first transfected PANC-1 pancreatic cancer cells with the miR-218 mimic and performed the CCK-8 assay to examine the relationship between miR-218 and the chemosensitivity of pancreatic cancer cells to GEM. The results showed that miR-218 enhances the sensitivity of pancreatic cancer cells to GEM-based chemotherapy.

HMGBl is a member of the high mobility group protein (HMG) family. HMGB1 is abundantly expressed in the nuclei of eukaryotic cells [46, 47]. Research on HMGB1 and tumors has become one of the most active fields in life science. Tremendous progress has been made enabling the public to gain a more profound understanding of the pathogenesis of tumors. HMGB1 is often highly expressed in immature and malignant cells. Upregulated HMGB1 expression has been observed in leukemia, colorectal cancer, breast cancer, pancreatic cancer, melanoma and chest tumors. In addition, the ligand of HMGB1, receptor for advanced glycation end products (RAGE), is expressed in various types of tumor cells. Blockage of the interaction between HMGB1 and its receptor inhibits the metastasis of solid tumors [26-28]. Necrotic tumor cells release HMGB1, thereby regulating tumor immunity and interfering with the efficacy of chemotherapy and radiotherapy [33]. The results of our previous study showed that miR-218 inhibits the proliferation and invasion of pancreatic cancer cells and promotes the sensitivity of pancreatic cancer cells to GEM through negatively regulating the expression of HMGB1 protein. Thus, we speculated that miR-218 affects the sensitivity of pancreatic cancer cells to GEM through regulating the release of HMGB1. Therefore, an ELISA was performed in the present study to examine the effects of miR-218 on the secretion of HMGB1 by pancreatic cancer cells. The results showed that GEM promotes the release of HMGB1 by PANC-1 cells, while miR-218 inhibits the release of HMGB1 by GEM-induced PANC-1 cells.

Binding of HMGB1 to RAGE promotes autophagy, inhibits apoptosis and promotes the survival of colon cancer cells [48]. A recent study has shown that HMGB1 interacts with beclin 1 to regulate autophagy [34]. In the present study, the effect of miR-218 on the expression of beclin 1 in PANC-1 pancreatic cancer cells was

analyzed using western blot analysis. In addition, the hypothesis that miR-218 affects the sensitivity of pancreatic cancer cells to GEM-based chemotherapy through regulating HMGB1 and beclin 1 was examined using RNAi technology and the CCK-8 assay. The results showed that miR-218 inhibits beclin 1 expression in PANC-1 cells. Knockdown of beclin 1 expression promotes the sensitivity of pancreatic cancer cells to GEM, whereas overexpression of HMGB1 reverses the effect of suppressed beclin 1 expression.

PI3K is a type of cytosolic phosphatidylinositol kinase. The PI3K protein family is involved in the regulation of various cell activities, including cell proliferation, differentiation, apoptosis and glucose transport. Activity of the PI3K pathway is enhanced in a variety of tumors where their pathogenesis is related to receptor tyrosine kinases (RTKs). Moreover, activation of the PI3K pathway is strictly regulated by RTKs [49]. The PI3K pathway is closely related to autophagy [50]. Using the PI3K pathway inhibitor, wortmannin, the present study demonstrated that overexpression of miR-218 enhances the sensitivity of PANC-1 cells to GEM and that the effect of miR-218 is likely exerted through the PI3K/Akt signaling pathway.

In conclusion, overexpression of miR-218 enhances the sensitivity of PANC-1 cells to GEM. The effect of miR-218 is caused by inhibiting HMGB1 release by pancreatic cancer cells and by reducing the expression of the autophagy-related protein, beclin 1. The PI3K/Akt pathway is the molecular pathway underlying the effect of miR-218.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yuanhong Xu, Department of Pancreatic Surgery, First Hospital of China Medical University, Shenyang, China. Tel: +86-24-83283350; E-mail: xuyhsy@yeah.net

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