GPX3 represses pancreatic cancer cell proliferation, migration and invasion, and improves their chemo-sensitivity by regulating the JNK/c-Jun signaling pathway

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Received April 26, 2023; Accepted November 22, 2023

DOI: 10.3892/etm.2024.12407

Abstract. Pancreatic cancer (PC) is a deadly and aggressive disease, which is characterized by poor prognosis. It has been reported that glutathione peroxidase 3 (GPX3) is involved in the development of several types of cancer. The present study aimed to explore the regulatory role of GPX3 in PC and uncover its underlying mechanism. Bioinformatics analysis was initially carried out to predict the expression profile of GPX3 in PC and its association with prognosis. The expression levels of GPX3 were also detected in PC cells by reverse transcription-quantitative PCR and western blot analysis. Following transfection to induce GPX3 overexpression, the proliferation ability of PC cells was assessed by Cell Counting Kit-8, colony formation and 5-ethynyl-2'-deoxyuridine incorporation assays. In addition, wound healing and Transwell assays were performed to evaluate the migration and invasion abilities of PC cells. Cell apoptosis was assessed by flow cytometric analysis. The expression levels of epithelial-mesenchymal transition (EMT)-, apoptosis-, and JNK signaling-related proteins were detected by western blot analysis. Additionally, for rescue experiments, JNK signaling was activated following cell treatment with anisomycin. The results showed that GPX3 was downregulated in PC and its expression was associated with favorable prognosis. In addition, cell transfection-induced GPX3 overexpression markedly inhibited cell proliferation, migration and invasion, and inhibited EMT. In addition, GPX3 improved the chemo-sensitivity of PC and gemcitabine (GEM)-resistant PC cells to GEM. Furthermore, GPX3 significantly suppressed JNK/c-Jun signaling in PC, while anisomycin treatment reversed the inhibitory effects of GPX3 on the malignant behavior and chemo-resistance of PC cells. The results of the present study indicated that GPX3 could serve as a tumor suppressor in PC via inhibiting JNK/c-Jun signaling, thus providing novel insights into the treatment of PC.

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Key words: pancreatic cancer, gemcitabine, glutathione peroxidase 3, JNK signaling

Introduction

Pancreatic cancer (PC) is a deadly and aggressive disease, accounting for 1.8% of all types of cancer worldwide, and is characterized by increasing morbidity and mortality rates. Pancreatic ductal adenocarcinoma (PDAC), arising from non-invasive precancerous lesions, accounts for ~90% of all PC cases (1). PC is characterized by poor prognosis, with a 5-year survival rate of <5% and an average survival time without prompt treatment of no more than 6 months (2,3).

Currently, surgical resection combined with neoadjuvant therapy is considered as the mainstay therapy approach for PC. However, due to the lack of early symptoms and screening strategies, the majority of patients with PC are initially diagnosed at an advanced stage of the disease, presented with unresectable or metastatic PC (4). Chemotherapy is the most common strategy for treating metastatic PC. However, prevailing resistance to chemotherapy greatly restricts its utilization (5). Therefore, exploring the intrinsic mechanism of PC to overcome chemotherapy resistance and prevent cancer metastasis is of great importance.

Glutathione peroxidase 3 (GPX3), located on chromosome 5q33.1, is a glycosylated tetramer composed of four subunits of 226 amino acids. It is the only exocrine member of the GPX family, which catalyzes the detoxification of hydro- and soluble lipid hydroperoxides by reducing glutathione and protects cells from oxidative stress-related damage (6,7). In recent years, the effect of GPX3 in cancer has attracted increasing attention (8,9). Emerging evidence has suggested that GPX3 has a diverse role in different types of human cancers, serving as a pro-survival protein in myeloid leukemia and clear cell renal

cell carcinoma, and as a tumor suppressor in lung, ovarian and gastric cancer (10-14). Consistently, a previous study demonstrated that GPX3 was downregulated in human cancers, but it was positively associated with poor outcomes (15), thus supporting the controversial role of GPX3 in cancer. It has been also reported that GPX3 is highly involved in cancer metastasis and chemotherapy resistance (15). Nevertheless, the particular role of GPX3 in PC has not been extensively investigated.

Therefore, the present study aimed to explore the substantial effect of GPX3 on the tumorigenesis and metastasis of PC and to elucidate its underlying molecular mechanism of action, thus providing novel insights into the development of effective therapeutic strategies against PC.

Materials and methods

Application of bioinformatics databases. The expression pattern of GPX3 in pancreatic adenocarcinoma (PAAD; n=178) and the normal tumor-adjacent tissues (n=4) was downloaded from UALCAN database (https://ualcan.path.uab.edu/index.html) (16). The association between GPX3 expression (cut-off value, 50%; to determine high and low GPX3 expression levels) and prognosis [overall survival (OS), disease-free survival (DFS) and relapse-free survival (RFS)] in PAAD was obtained from the GEPIA (http://gepia.cancer-pku.cn/) (17) and Kaplan-Meier Plotter (http://kmplot.com/analysis/) databases (18).

Cell lines. The human pancreatic ductal epithelial cell line HPDE6c7 (cat. no. BNCC359453) was obtained from BeNa Culture Collection. The PC cell lines BxPC-3 (cat. no. CL-0042), SW1990 (cat. no. CL-0448B) and PANC-1 (cat. no. CL-0184) were purchased from Wuhan Procell Life Science & Technology Co., Ltd. HPDE6c7 and BxPC-3 cells were cultured in RPMI-1640 medium, while SW1990 and PANC-1 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; MilliporeSigma) and 1% penicillin/streptomycin solution (Life Technologies; Thermo Fisher Scientific, Inc.). All cells were cultured at 37˚C in a humidified incubator with 5% CO_2.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells utilizing a TRIzol reagent (MilliporeSigma), according to the manufacturer's instructions. After purity verification and concentration measurement, 1 μg total RNA was reverse transcribed into complementary DNA using the riboScript Reverse Transcription Kit (Guangzhou RiboBio Co., Ltd.) according to the manufacturer's instructions. Subsequently, qPCR was carried out using the SYBR Premix Ex Taq II kit (Takara Bio Inc.) on a real-time PCR instrument (Bio-Rad Laboratories, Inc.). The reaction protocol was as follows: Initial denaturation at 95˚C for 30 sec; 40 cycles at 95˚C for 5 sec and 60˚C for 20 sec, and a final extension step at 72˚C for 10 min. The primer sequences used in this study were as follows: GPX3 forward, 5'-AGCAGTATGCTGGCAAATATGTCC-3' and reverse, 5'-CAGACCGAATGGTGCAAGCTCTTC-3'; β-actin forward, 5'-AGCGAGCATCCCCCAAAGTT-3' and reverse, 5'-GGGCACGAAGGCTCATCATT-3'. The expression levels of GPX3 were calculated using the $2^{-\Delta\Delta Cq}$ method (19) and β-actin served as an endogenous control.

Western blot analysis. Protein samples were prepared following cell lysis with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology) containing protease and phosphatase inhibitors (Roche Diagnostics). Following protein concentration assessment using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.), equal amounts of protein extracts (30 μg/lane) were separated by 15% SDS-PAGE and were then transferred onto polyvinylidene fluoride membranes (MilliporeSigma). Non-specific binding was blocked following membrane incubation with 5% skimmed milk for 2 h at room temperature. Subsequently, membranes were incubated with primary antibodies, including anti-GPX3 (1:1,000; cat. no. ab275965, Abcam), anti-E-cadherin (1:1,000; cat. no. ab40772, Abcam), anti-N-cadherin (1:5,000; cat. no. ab76011, Abcam), anti-Snail (1:1,000; cat. no. ab216347, Abcam), anti-Bax (1:1,000; cat. no. ab32503, Abcam), anti-Bcl-2 (1:1,000; cat. no. ab32124, Abcam), anti-p-JNK (1:1,000; cat. no. 9251, Cell Signaling Technology, Inc.), anti-JNK (1:1,000; cat. no. 9252, Cell Signaling Technology, Inc.), anti-p-c-Jun (1:1,000; cat. no. ab32385, Abcam), anti-c-Jun (1:1,000; cat. no. ab40766, Abcam), anti-p21 (1:1,000; cat. no. ab109520, Abcam), anti-c-Myc (1:1,000; cat. no. ab32072, Abcam), and anti-GAPDH (1:2,500; cat. no. ab9485, Abcam) at 4˚C overnight followed by incubation with HRP-conjugated secondary antibody (1:2,000; cat. no. ab6721, Abcam) for 2 h at room temperature. Finally, the protein bands were visualized utilizing an Enhanced Chemiluminescence System Reagent (Nanjing KeyGen Biotech, Co., Ltd.) and quantified using ImageJ software version 1.48 (National Institutes of Health).

Cell transfection and anisomycin treatment. The coding sequences of *GPX3* (accession no. NM_002084.5) were cloned into pcDNA3.1 (Shanghai Genechem Co., Ltd.) to construct GPX3-overexpressing vector (Oe-GPX3), and the empty pcDNA3.1 vector served as the negative control (Oe-NC). Upon achieving 60-70% confluency, PANC-1 cells were transfected with 5 μg/ml Oe-NC or Oe-GPX3 using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 48 h, according to the manufacturer's protocol. At 48 h following transfection, the cell transfection efficiency was assessed by RT-qPCR and western blot analysis. For the rescue experiment, the GPX3-overexpressing PANC-1 cells were treated with 0.01 μM anisomycin (Shanghai Yuan Ye Bio-Technology Co., Ltd.), an activator of JNK, for another 24 h at 37˚C.

Cell viability assay. Cell viability was assessed using a Cell Counting Kit-8 (CCK-8) assay. Briefly, PANC-1 cells were seeded into 96-well plates at a density of $2x10^3$ cells/well and cultured at 37˚C in an incubator with 5% CO_2. Following incubation for 24, 48 and 72 h, each well was supplemented with 10 μl CCK-8 reagent (Dojindo Laboratories, Inc.) and cells were incubated for an additional 3 h. The absorbance at a wavelength of 450 nm was measured in each well using a microplate reader (BioTek Instruments, Inc.).

Colony formation assay. A total of $2x10^3$ PANC-1 cells were inoculated into each well of 6-well plates and cultured at 37˚C in an incubator with 5% CO_2 for 2 weeks. The culture medium was changed with fresh one every 3 days. The formed colonies were fixed with 4% paraformaldehyde for 10 min at room temperature and then stained with 1% crystal violet for 30 min at room temperature. The colonies (>50 cells) were observed under a microscope and counted using ImageJ software version 1.48.

5-Ethynyl-2'-deoxyuridine (EdU) incorporation assay. An EdU assay was carried out using the Cell-Light EdU DNA Cell Proliferation Kit (Guangzhou RiboBio Co., Ltd.). Briefly, PANC-1 cells were labeled with EdU for 2 h at 37˚C. Subsequently, cells were stained with DAPI solution (MilliporeSigma) for 5 min. The fluorescence signal was visualized under an inverted fluorescence microscope (Olympus Corporation), and then quantified using ImageJ software version 1.48.

Wound healing assay. A wound healing assay was conducted to assess the migration ability of PC cells. When PANC-1 cells reached 100% confluency in 6-well plates, a wound was created on the cell monolayer using a 200-μl sterile pipette tip. Following washing with PBS for three times to remove detached cells, cells were incubated in serum-free medium for 24 h. Images of the wound at 0 and 24 h were captured under a light microscope (Olympus Corporation). The cell migration rate was calculated depending on the shortened wound distance of each group: (wound distance at 0 h-wound distance at 24 h)/wound distance at 0 h x100.

Transwell assay. To evaluate the invasion ability of PC cells, a Transwell assay was performed in a 24-well plate using a Transwell chamber (Corning; Corning, Inc.) pre-coated with Matrigel (BD Biosciences) at 37˚C for 30 min. Briefly, PANC-1 cells ($5x10^5$ cells/ml) were resuspended in serum-free medium and were then added onto the upper chamber of the Transwell insert. The lower chamber was supplemented with complete medium containing 10% FBS. Following incubation at 37˚C for 48 h, the remaining cells on the upper surface of the membrane were removed using cotton swabs. The invasive cells were fixed in 4% paraformaldehyde and were then stained with 1% crystal violet for 30 min at room temperature. Finally, the invasive cells were observed and counted under a light microscope (Olympus Corporation). The cell invasion rate was calculated depending on the number of invasive cells of each group.

Gemcitabine (GEM) chemosensitivity assay. PANC-1 cells were treated with increasing concentrations of GEM (0, 1, 2, 5, 10 and 20 μM; APeXBIO Technology LLC) for 48 h. Meanwhile, GEM-resistant PANC-1 cells (PANC-1/GEM; cat. no. IMD-015; Xiamen Immocell Biotechnology Co., Ltd.) were treated with 0, 5, 10, 20, 40, 60, 80 and 100 μM GEM for 48 h. Subsequently, the cell viability of each group was assessed as aforementioned. The half maximal inhibitory concentration (IC_{50}) values were calculated using the concentration-response data in GraphPad Prism 8.0 software (GraphPad Software, Inc.).

Flow cytometric analysis. Cell apoptosis was assessed using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (BD Biosciences). PANC-1/GEM cells were seeded into 6-well plates ($5x10^5$ cells/per well). Following incubation with GEM (80 μM) or PBS at 37˚C for 48 h, PANC-1/GEM cells were washed with ice-cold PBS and resuspended in binding buffer, followed by the addition of Annexin V-FITC and PI, according to the manufacturer's protocol. Following incubation at 37˚C for 15 min in the dark, the apoptotic cells, including early apoptotic cells and late apoptotic cells, were analyzed with the FACScan flow cytometer (BD Biosciences) and CellQuest Pro software version 3.3 (BD Biosciences).

Statistical analysis. The experimental data were statistically analyzed using GraphPad Prism 8.0 software (GraphPad Software, Inc.; Dotmatics). All experiments were repeated at least three times. The experimental data were normally distributed and were expressed as the mean ± standard deviation (SD). The differences among multiple groups were compared using one-way ANOVA, followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

GPX3 is downregulated in PC. Bioinformatics analysis was performed to assess the effect of GPX3 on PC. As shown in Fig. 1A, the expression levels of GPX3 in tumor tissues were lower compared with those in normal ones, thus suggesting that GPX3 was downregulated in PC. In addition, the increased expression levels of GPX3 were positively associated with enhanced OS, DFS and RFS (Fig. 1B and C). Additionally, the expression levels of GPX3 were also detected in PC cell lines and the results consistently disclosed that both the mRNA and protein expression levels of GPX3 were markedly decreased in the PC cell lines BxPC-3, SW1990 and PANC-1 compared with HPDE6c7 cells (Fig. 1D). Since the lowest expression levels of GPX3 were observed in PANC-1 cells, this cell line was selected for the subsequent *in vitro* experiments.

GPX3 inhibits the proliferation, migration and invasion of PANC-1 cells. To explore the regulatory role of GPX3 in PC, gain-of-function experiments were carried out. PANC-1 cells were first transfected with Oe-GPX3. The markedly increased mRNA and protein expression levels of GPX3 in the Oe-GPX3 group confirmed that the cells were successfully transfected (Fig. 2A). As shown in Fig. 2B and C, GPX3 overexpression significantly inhibited cell viability and reduced the colony formation ability of PC cells, thus supporting the anti-proliferative capacity of GPX3 in PANC-1 cells. The above finding was further verified by the reduced number of EdU-positive cells in the Oe-GPX3 group (Fig. 2D). Furthermore, wound healing and Transwell assays revealed that GPX3 overexpression not only inhibited the healing velocity of PANC-1 cells within 48 h, but also reduced their invasion ability (Fig. 2E), thus suggesting that GPX3 could inhibit the migration and invasion of PANC-1 cells. The results also demonstrated that GPX3 notably increased the protein expression levels of E-cadherin and decreased those of N-cadherin and Snail (Fig. 2F), thus

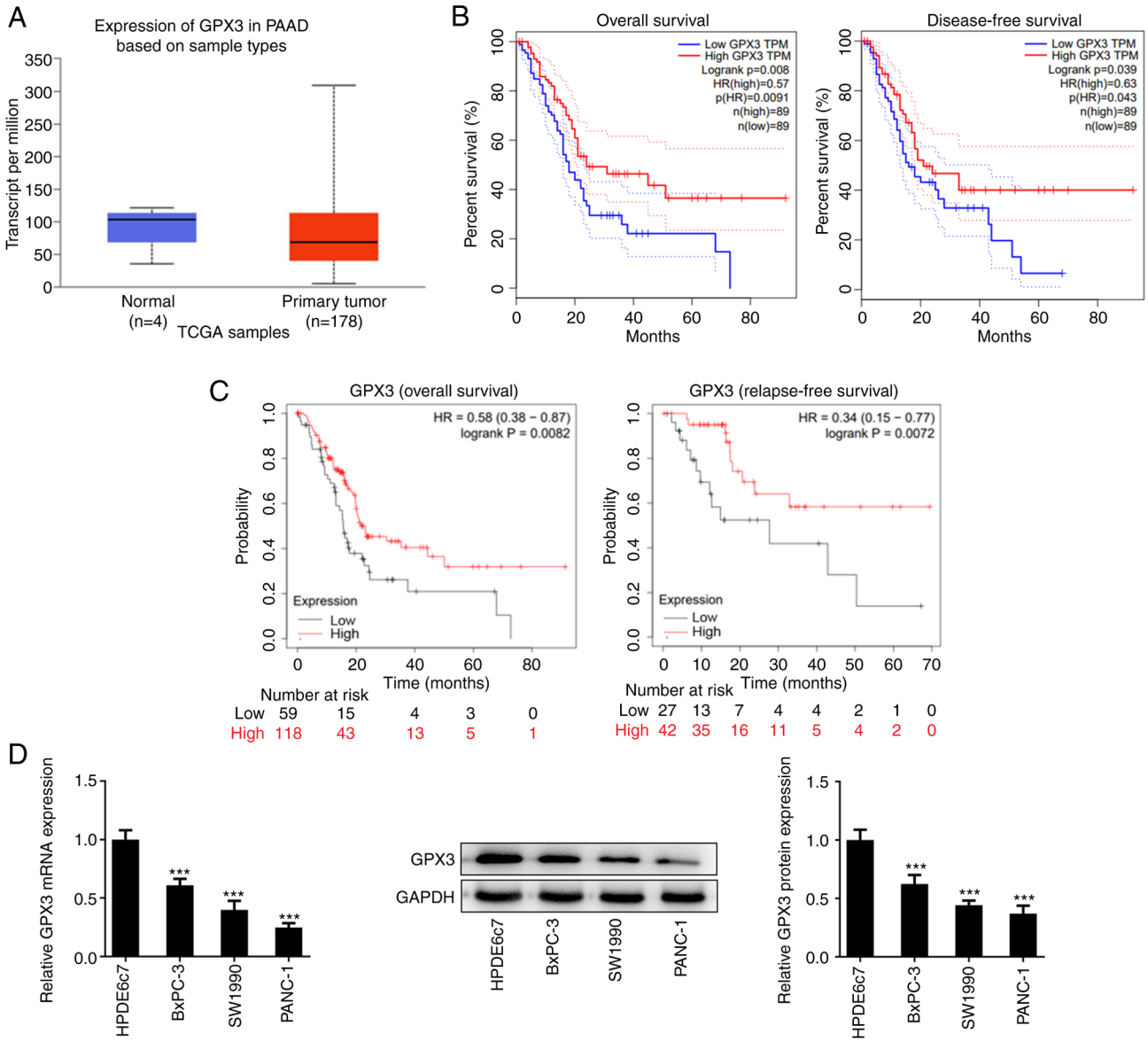

Figure 1. GPX3 is downregulated in PC. (A) The expression of GPX3 in PAAD and normal-adjacent tissues was presented by UALCAN database (https://ualcan.path.uab.edu/index.html). (B) The association between GPX3 expression and OS and disease-free survival in PAAD was obtained from GEPIA database (http://gepia.cancer-pku.cn/). (C) The association between GPX3 expression and OS and relapse-free survival in PAAD was obtained from Kaplan-Meier Plotter database (http://kmplot.com/analysis/). (D) The mRNA and protein expression levels of GPX3 in the PC cell lines, BxPC-3, SW1990 and PANC-1, and human pancreatic ductal epithelial cell line, HPDE6c7 cells, were determined using reverse transcription-quantitative PCR and western blot analysis, respectively. ***P<0.001 vs. HPDE6c7 cells. GPX3, glutathione peroxidase 3; PC, pancreatic cancer; PAAD, pancreatic adenocarcinoma; OS, overall survival; TPM, transcript per million.

indicating that GPX3 attenuated the epithelial-mesenchymal transition (EMT) of PANC-1 cells.

GPX3 sensitizes PANC-1 and PANC-1/GEM cells to GEM. In addition to the enhanced proliferation and invasion abilities of cancer cells, chemo-resistance is also significantly associated with poor prognosis in PC (20). Therefore, the present study also aimed to investigate the effect of GPX3 on the chemo-resistance of PANC-1 cells to GEM. The results revealed that PANC-1/GEM cells possessed a higher GEM IC_{50} value compared with PANC-1 cells, while GPX3 overexpression markedly reduced the IC_{50} value of GEM in PANC-1 and PANC-1/GEM cells (Fig. 3A). The flow cytometric analysis showed that cell treatment with GEM promoted the apoptosis of PANC-1/GEM cells, which was further enhanced by GPX3 overexpression (Fig. 3B). Consistently, the western blot analysis results showed that Bax was upregulated and Bcl-2 was downregulated in the Oe-GPX3 group, thus further verifying the anti-apoptotic activity of GPX3 in PANC-1/GEM cells (Fig. 3C). The aforementioned findings indicated that GPX3 overexpression could robustly improve the chemo-sensitivity of PC and GEM-resistant PC cells to GEM.

GPX3 inhibits the activity of JNK signaling in PANC-1 cells. Subsequently, the present study aimed to uncover the detailed mechanism underlying the effect of GPX3 on antagonizing the malignant behavior of PC cells. Therefore, western blot analysis showed that compared with the Oe-NC group, the

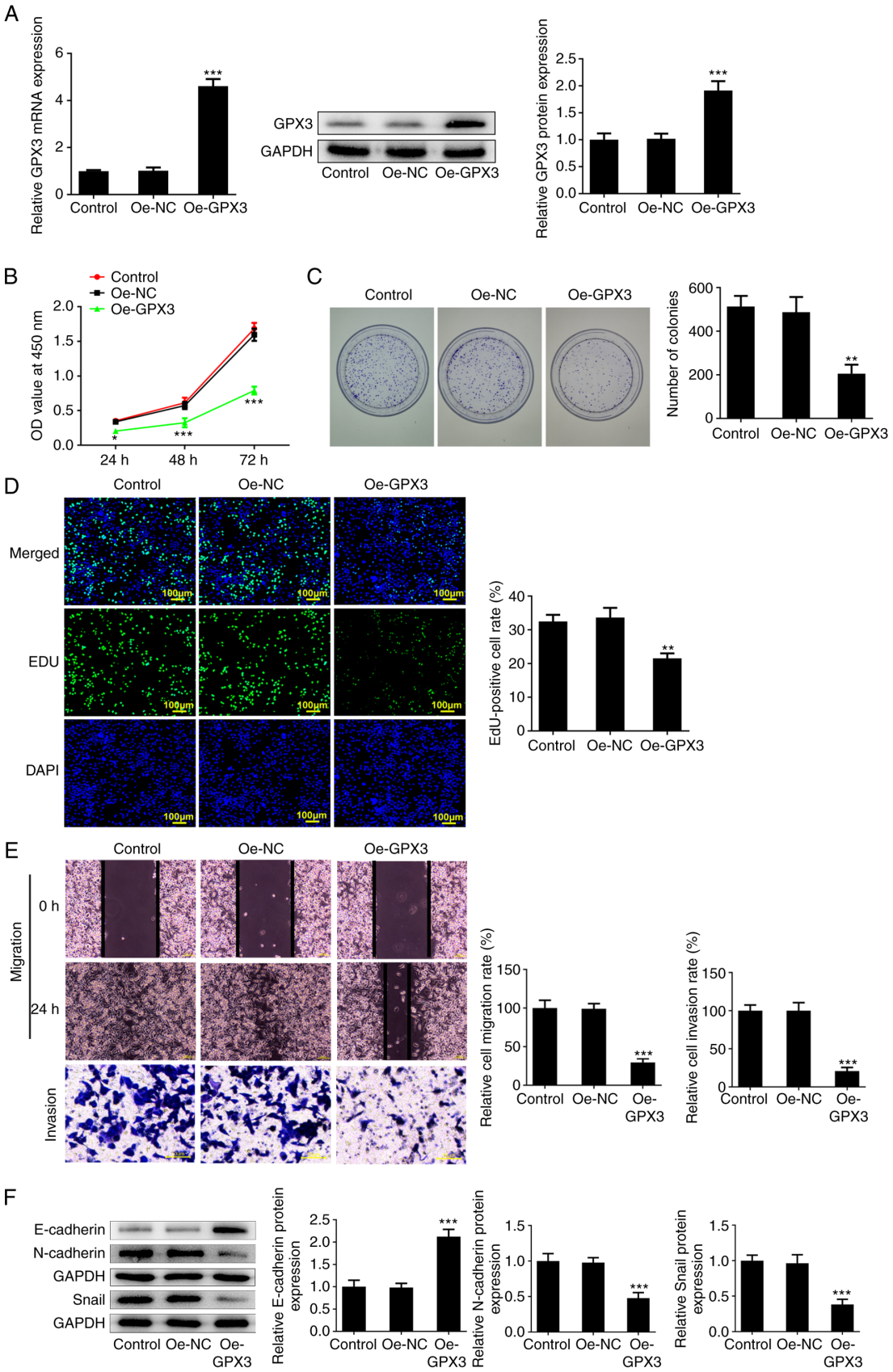

Figure 2. GPX3 restricts the proliferation, migration and invasion of PANC-1 cells. (A) PANC-1 cells were transfected with Oe-NC or Oe-GPX3 and the mRNA and protein expression levels of GPX3 were detected using reverse transcription-quantitative PCR and western blot analysis, respectively. (B) Cell viability at 24, 48 and 72 h was assessed using Cell Counting Kit-8 assay. (C) Colony formation assays were then performed and the colonies were counted under a microscope. (D) 5-Ethynyl-2'-deoxyuridine incorporation assay was performed to assess cell proliferation. (E) Wound healing and Transwell assays were carried out to evaluate the cell migration and invasion abilities, respectively. (F) The protein expression levels of E-cadherin, N-cadherin and Snail were detected using western blot analysis. *P<0.05, **P<0.01 and ***P<0.001 vs. the Oe-NC group. GPX3, glutathione peroxidase 3; Oe-NC, negative control overexpression vector; Oe-GPX3, GPX3-overexpressing vector.

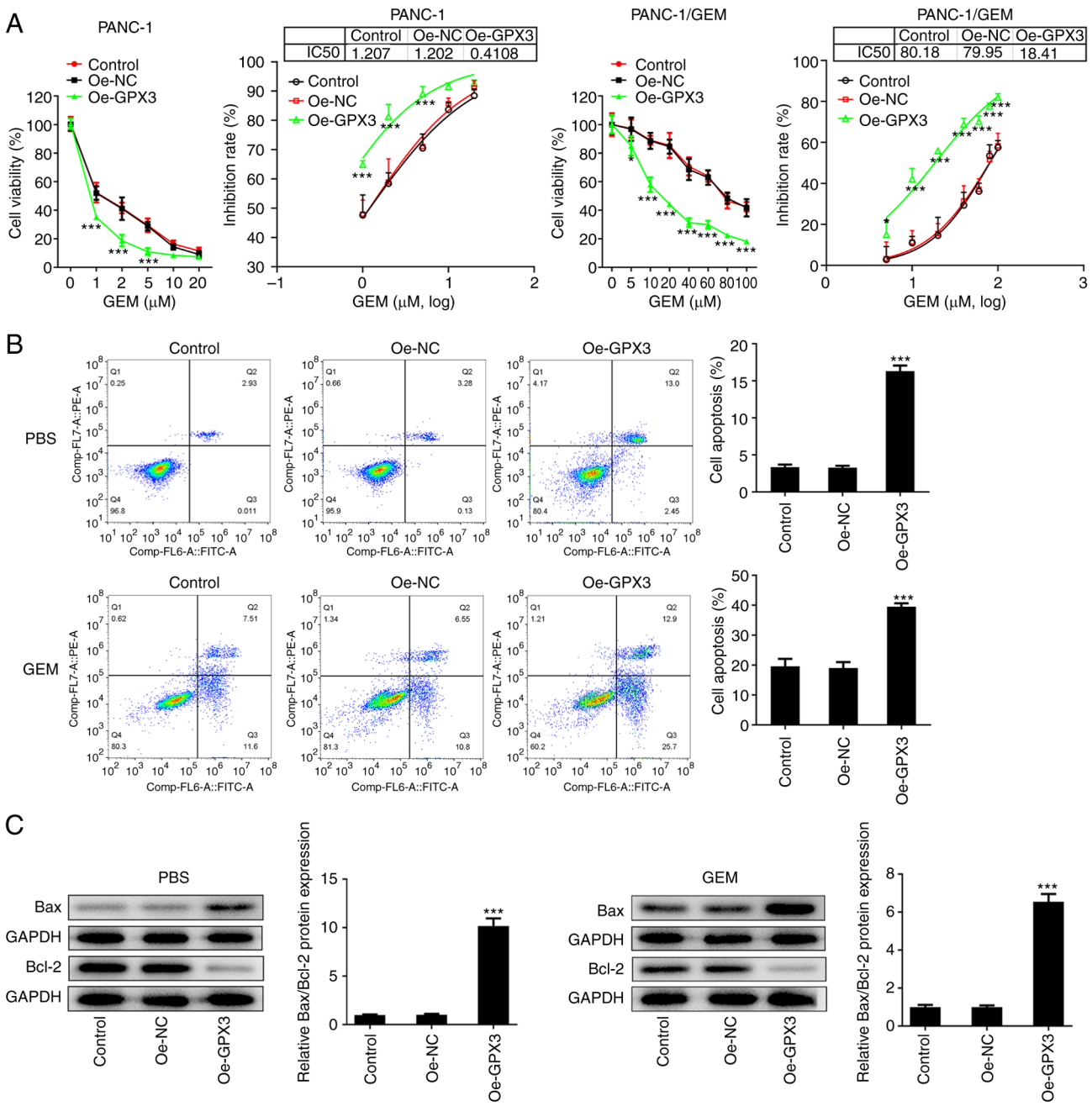

Figure 3. GPX3 sensitizes PANC-1 and PANC-1/GEM cells to GEM. (A) PANC-1 and PANC-1/GEM cells were treated with increasing concentrations of GEM for 48 h and cell viability was assessed using Cell Counting Kit-8 assay. The half maximal inhibitory concentration value of GEM was then calculated. (B) Flow cytometric analysis was used to evaluate the apoptosis rate of PANC-1/GEM cells treated with or without GEM. (C) The protein expression levels of Bax and Bcl-2 were detected using western blot analysis and the Bax/Bcl-2 ratio was then calculated. *P<0.05 and ***P<0.001 vs. the Oe-NC group. GPX3, glutathione peroxidase 3; PANC-1/GEM, gemcitabine-resistant PANC-1; Oe-NC, negative control overexpression vector; Oe-GPX3, GPX3-overexpressing vector.

protein expression levels of phosphorylated (p)-JNK, p-c-Jun, c-Jun and c-Myc were markedly decreased, while those of p21 were robustly elevated in the Oe-GPX3 group (Fig. 4). The aforementioned finding indicated that GPX3 significantly inhibited JNK/c-Jun signaling in PANC-1 cells.

Anisomycin reverses the inhibitory effects of GPX3 on the proliferation, invasion, EMT and chemo-resistance of PC cells. Finally, to clarify the significance of JNK/c-Jun signaling in the antitumor activity of GPX3 in PC, GPX3-overexpressing PANC-1 cells were treated with 0.01 μM anisomycin, an activator of JNK, and then a series of *in vitro* experiments were performed. As shown in Fig. 5A-C, anisomycin significantly weakened the anti-proliferative effect of GPX3 on PANC-1 cells, as evidenced by the enhanced cell viability and cell colony formation ability, and the increased number of EdU-positive cells in the anisomycin + Oe-GPX3 group compared with the Oe-GPX3 group. In addition, compared with the Oe-GPX3 group, cell treatment with anisomycin enhanced the migration and invasion rates of PC cells (Fig. 5D). Furthermore, E-cadherin downregulation and N-cadherin and Snail upregulation following cell treatment

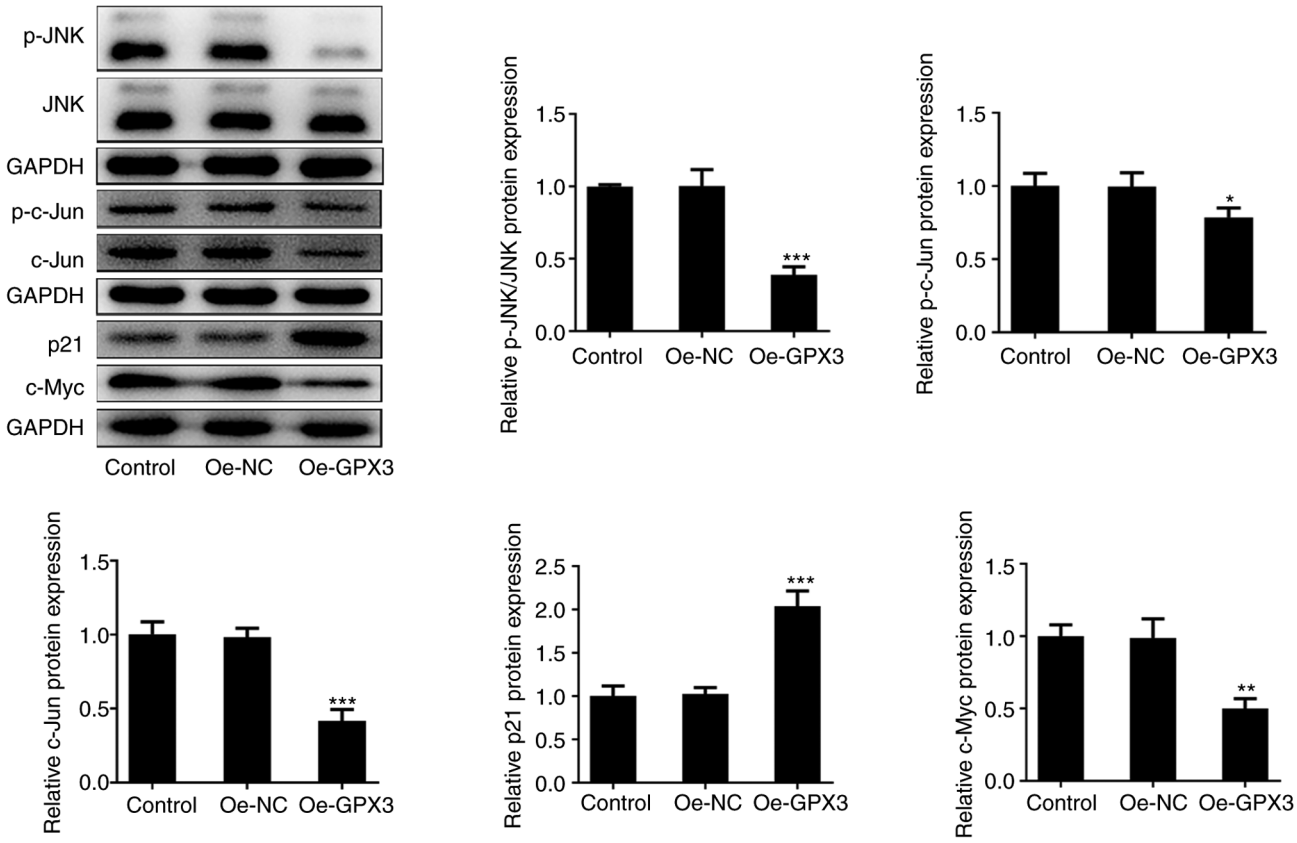

Figure 4. GPX3 inhibits the activity of JNK signaling in PANC-1 cells. The protein expression levels of p-JNK, JNK, p-c-Jun, c-Jun, p21 and c-Myc in PANC-1 cells transfected with Oe-NC or Oe-GPX3 were determined using western blot analysis. *P<0.05, **P<0.01 and ***P<0.001 vs. the Oe-NC group. GPX3, glutathione peroxidase 3; p-, phosphorylated; Oe-NC, negative control overexpression vector; Oe-GPX3, GPX3-overexpressing vector.

with anisomycin indicated that the inhibitory effect of GPX3 overexpression on EMT was partially restored by anisomycin treatment (Fig. 5E). In addition, compared with the Oe-GPX3 group, the reduced cell apoptosis rate and Bax/Bcl-2 ratio in the anisomycin + Oe-GPX3 group suggested that cell treatment with anisomycin abrogated the beneficial effect of GPX3 on the chemo-sensitivity of PC cells to GEM (Fig. 6A and B). These fidings indicated that GPX3 inhibited cell proliferation, migration, invasion and chemo-resistance in PC cells via inhibiting the JNK/c-Jun signaling pathway.

Discussion

In the present study, GPX3 was discovered to be downregulated in PC and positively associated with the prognosis of patients with PC. More particularly, *in vitro* experiments revealed that GPX3 overexpression could not only markedly suppress the proliferation, migration and invasion of PANC-1 cells, but it could also enhance the chemo-sensitivity of PANC-1/GEM cells to GEM, suggesting that GPX3 exhibited tumor suppressive activity during the malignant metastasis and chemo-resistance of PC. In addition, the results demonstrated that the antitumor activity of GPX3 in PC was partially mediated by JNK/c-Jun signaling inhibition.

Chemotherapy is the most common strategy for treating PC metastasis. GEM, as a standard first-line chemotherapeutic agent, is widely utilized for the palliative treatment of patients with PC. However, the effect of GEM on prolonging the prognosis of patients with PC is limited due to drug resistance (21). Emerging evidence has suggested that multiple genes and proteins are involved in regulating the chemo-resistance of PC cells to GEM. For instance, a previous study showed that HEAT repeat containing 1 (HEATR1) was closely associated with the prognosis of patients with PC, while HEATR1 depletion could greatly enhance the proliferation of PC cells and their resistance to GEM, thus indicating that HEATR1 may be a promising therapeutic target for PC (22). In addition, cancerous inhibitor of protein phosphatase 2A (CIP2A) was found to be highly expressed in PC tissues and CIP2A depletion could significantly repress the proliferation and increase the chemo-sensitivity of PC cells to GEM, thus attenuating the progression of PC (23). In the present study, the results showed that GPX3 was downregulated in PC cell lines. Furthermore, the gain-of-function experiments revealed that GPX3 overexpression could not only notably attenuate the malignant behavior of PC cells, including cell proliferation, migration and invasion, but it could also markedly sensitize PC and GEM-resistant PC cells to GEM. The aforementioned findings suggested that GPX3 could function as a tumor suppressor in PC and may possibly serve as a biomarker to guide the GEM chemotherapy of PC.

It is widely recognized that the MAPK signaling pathway is one of the most attractive targets for cancer therapy. JNK, one of the two major MAPK pathways, can regulate the expression of target genes involved in modulating cell survival and apoptosis via activating c-Jun (24,25). Emerging evidence has also

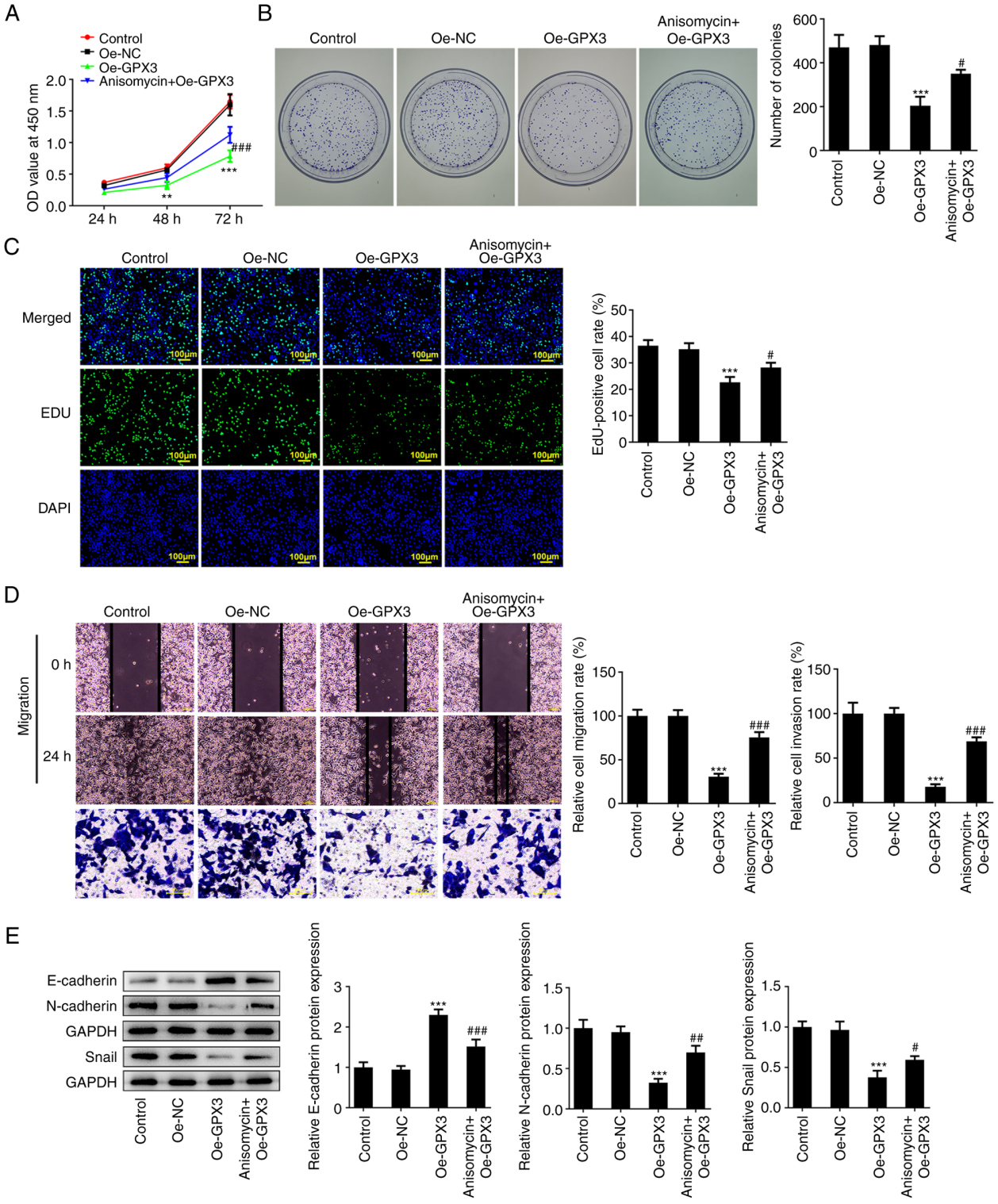

Figure 5. Anisomycin reverses the inhibitory effects of GPX3 on the proliferation, invasion and epithelial-mesenchymal transition of pancreatic cancer cells. (A) PANC-1 cells were transfected with Oe-NC or Oe-GPX3 and GPX3-overexpressing PANC-1 cells were then treated with 0.01 μM anisomycin, a JNK activator. Cell viability was measured at 24, 48 and 72 h using Cell Counting Kit-8 assay. (B) A colony formation assay was performed and the formed colonies were counted under a microscope. (C) 5-Ethynyl-2'-deoxyuridine incorporation assay was performed to assess cell proliferation. (D) Wound healing and Transwell assays were carried out to evaluate the cell migration and invasion abilities, respectively. (E) The protein expression levels of E-cadherin, N-cadherin and Snail were detected using western blot analysis. **P<0.01 and ***P<0.001 vs. the Oe-NC group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the Oe-GPX3 group. GPX3, glutathione peroxidase; Oe-NC, negative control overexpression vector; Oe-GPX3, GPX3-overexpressing vector; Oe-GPX3, GPX3-overexpressing vector.

suggested that JNK/c-Jun signaling is involved in the development of PC and the chemosensitivity of PC cells (25,26). Previous studies revealed that the phosphorylation of JNK induced the migration and invasion of PC cells. c-Jun, the main downstream molecule of JNK, was expansively expressed in pancreatic tumor lesions and it was closely associated with PC

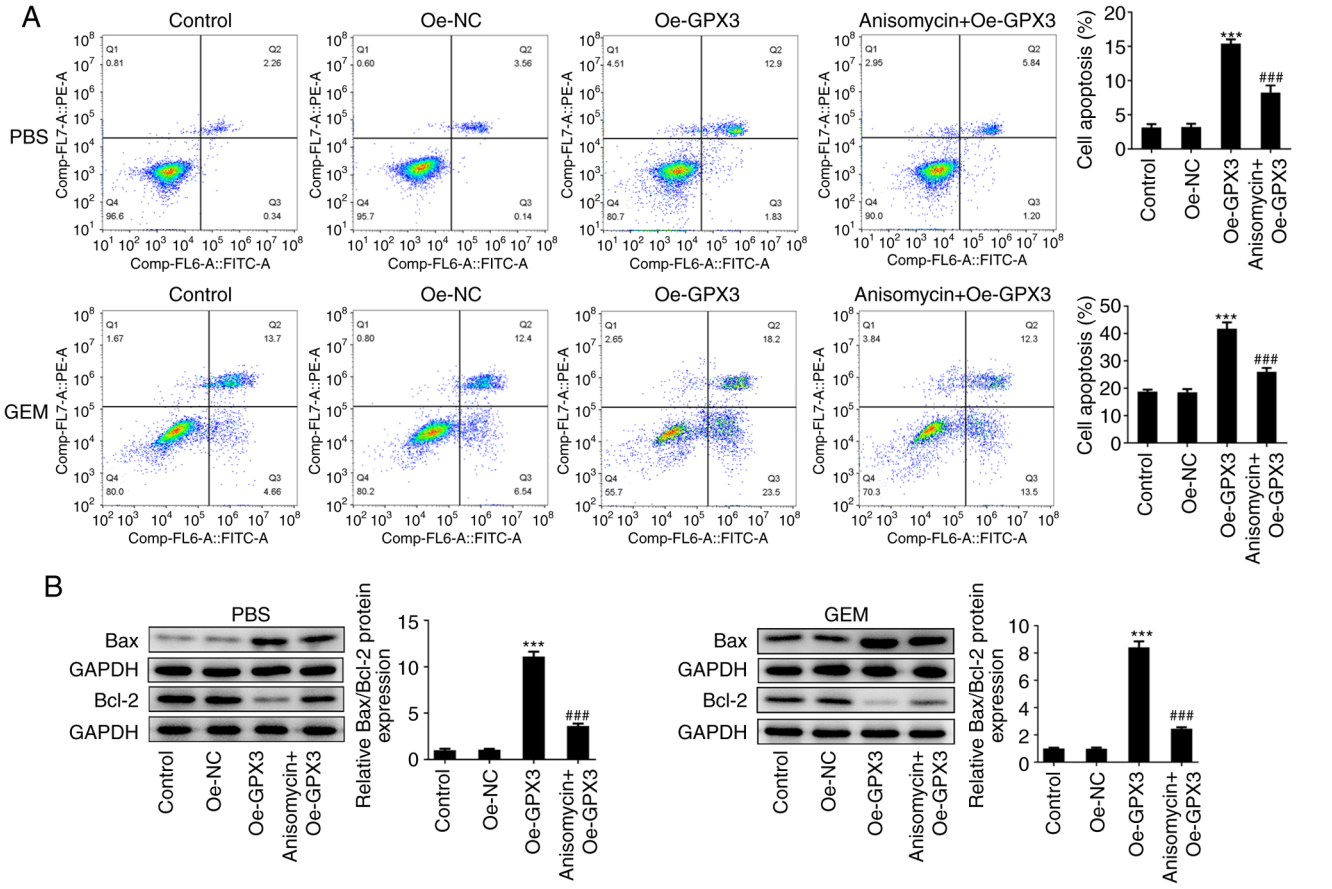

Figure 6. Anisomycin attenuates the inhibitory effect of GPX3 on the chemo-resistance of pancreatic cancer cells to GEM. (A) Flow cytometric analysis was used to determine the apoptosis rate of GEM-resistant PANC-1 cells treated with or without GEM. (B) The protein expression levels of Bax and Bcl-2 were detected using western blot analysis and the Bax/Bcl-2 ratio was then calculated. ***P<0.001 vs. the Oe-NC group; ###P<0.001 vs. the Oe-GPX3 group. GPX3, glutathione peroxidase; GEM, gemcitabine; Oe-NC, negative control overexpression vector; Oe-GPX3, GPX3-overexpressing vector.

progression (27-30). Liu *et al* (31) reported that the Zrt-Irt-like protein 4/zinc finger E-box-binding homeobox 1 axis mediated the resistance of pancreatic tumors to GEM via regulating JNK/c-Jun signaling. Shi *et al* (26) also demonstrated that IX, a JNK inhibitor, restrained PC via regulating p53 and p21. Therefore, inhibiting JNK/c-Jun signaling may be a practicable approach for alleviating the metastasis and chemoresistance of PC (26,32). In the present study, GPX3 overexpression significantly inhibited the activity of JNK/c-Jun signaling. To verify the aforementioned regulatory mechanism, rescue experiments were carried out using anisomycin, a JNK activator, and the results revealed that the inhibitory effects of GPX3 on PC cell proliferation, invasion and chemo-resistance were partially restored by anisomycin, thus confirming that GPX3 exerted its antitumor effect in PC partly via inhibiting JNK/c-Jun signaling.

However, there are some limitations in the present study. It would be beneficial to perform animal experiments to verify the *in vitro* findings. Furthermore, all data were obtained from PANC-1 cells, and the experiments in other PC cell lines may be beneficial for further validation. In addition, the present study preliminarily revealed the regulatory role of GPX3 and the potential mechanism focusing on JNK/c-Jun signaling during the progression of PC; however, other specific players involved and the molecular mechanisms in PC require further investigation. These limitations need to be addressed and may be future directions of subsequent research.

To the best of the authors' knowledge, the present study, for the first time, clarified the precise role of GPX3 in PC, and elucidated the regulatory mechanism. The findings of the present study revealed that GPX3 may serve as a tumor suppressor in PC via inhibiting the malignant behavior of PC cells and improving their chemosensitivity to GEM, which could be partially triggered via inhibiting JNK/c-Jun signaling. The present study suggested that GPX3 may be considered as a potentially valuable target for improving PC treatment, thus providing novel insights into the development of more effective therapeutic strategies for treating PC.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

DZ designed the experiments. YM, LZ and XG obtained, analyzed and interpreted the data. YM and LZ drafted the manuscript and DZ revised the manuscript. YM and DZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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