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ORIGINAL ARTICLE

MiRNA-3662 reverses the gemcitabine resistance in pancreatic cancer through regulating the tumor metabolism

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Abstract

Objectives Gemcitabine (Gem) is one of the most commonly used chemotherapeutic drugs in treating patients with pancreatic ductal adenocarcinoma (PDAC). Acquired drug resistance against Gem presents a major clinical challenge in the chemotherapy of PDAC. It has been shown that miRNA-3662 is lowly expressed and implicated with quantities of biological processes in cancer. However, whether miRNA-3662 regulates chemoresistance in PDAC remains largely unknown.

Materials and methods The level of miRNA-3662 in PDAC tissues was determined by real-time qPCR (RT-qPCR). Functional experiments were used to investigate the biological role of miRNA-3662 on Gem resistance of PDAC in vitro and in vivo. Fluorescence in situ hybridization (FISH), RT-qPCR, western blotting, bioinformatics analysis and luciferase reporter assay were employed to determine the precise regulation mechanisms.

Results In this study, it was investigated that miRNA-3662 was down-regulated in PDAC clinical samples as well as cell lines. Functional assays revealed that miRNA-3662 was sufficient to inhibit Gem resistance in PDAC cells both in vitro and in vivo. Mechanistically, hypoxia-inducible factor 1α (HIF-1α) was one of the transcriptional target of miRNA-3662 and was up-regulated in PDAC samples. Importantly, genetic promoting of HIF-1α largely compromised miR-3662-mediated chemosensitive effects. In addition, miR-3662 could impair the aerobic glycolysis in PDAC cells.

Conclusions This study sheds light on miRNA-3662 inhibits PDAC cell chemoresistance and aerobic glycolysis through a negative feedback loop with HIF-1α. Therefore, the co-delivery of miR-3662 and Gem could be served as a promising therapeutic regimen for PDAC patients.

Keywords Chemoresistance · MiRNA-3662 · HIF-1α · Pancreatic adenocarcinoma

Abbreviations

Gem	Gemcitabine
PDAC	Pancreatic cancer
FISH	Fluorescence in situ hybridization
RT-qPCR	Real-time qPCR
HIF-1α	Hypoxia-inducible factor 1α
miRNAs	MicroRNAs
dCK	Deoxycytidine kinase
IHC	Immunohistochemical
Tunel	Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling

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Introduction

PDAC is a common malignant tumor worldwide representing a serious threat to human's health [1]. The prognosis of PDAC patients is poorest among all solid cancers, with median survival duration less than 6 months [2]. Recently, despite increasing number of therapies and advances in clinical management, most PDAC patients are diagnosed when the tumor has metastasized to other organs [3]. In these cases, Gem-based chemotherapy concurrently with or after radiotherapy is still the main remedy for this disease. Whereas continuous exposure to Gem can cause serious adverse effects, and many patients with PDAC exhibit a poor response to systematic chemotherapy, the long-term clinical prognosis of PDAC patients still remains poor. Among these influencing factors, the production of innate or acquired resistant cancer cells is the major clinical impediment for the curative effect [4, 5].

Energy metabolism alteration is emerged as a common malignant feature of cancers. Cancer cells tend to use glucose-dependent glycolytic pathway rather than mitochondrial oxidative phosphorylation for energy production despite in the presence of adequate oxygen, a phenomenon defined as Warburg effect [6–8]. Although mitochondrial oxidative phosphorylation produces more energy than glycolysis. Promoted Warburg effect in cancer cells is required for cancer cell proliferation by providing adequate carbon sources [9, 10]. Consequently, aerobic glycolysis is highly capable of increasing nonresponsiveness to chemoradiotherapy or reconstituting tumor after successful therapy [11]. Furthermore, it has been well documented that aerobic glycolysis is related to a higher incidence of tumor invasion and metastases [12]. Therefore, development of novel therapeutic modalities on aerobic glycolysis eradication is urgently needed to fight against cancer development and enhance the efficacy of cancer therapies.

As one of non-coding RNA, microRNAs (miRNAs) play important roles in the occurrence of a variety of biological characteristics that are critical for normal physiological development [13, 14]. Some miRNAs have been demonstrated to either inhibit chemoresistance or promote chemoresistance in malignant cells [15]. He et al. reported that miR-186-3p inhibits tamoxifen resistance and aerobic glycolysis in breast cancer [16]. MiRNA-30a-5p is low-expressed in liver cancer cells, and promoting of miRNA-30a-5p can substantially repress aerobic glycolysis and sorafenib resistance [17]. Previous study found that miRNA-29c-3p was remarkably up-regulated in ovarian cancer cells, and inhibited cisplatin resistance through FOXP1/ATG14 pathway [18]. In addition, there is literature indicating that silencing of miRNA-210 can inhibit the Gem resistance of PDAC by mediation of mTOR signaling pathway [5]. Recent study has proved that miRNA-3662 is actively involved in the various biological processes of cancers [19]. However, miR-3665 was rarely studied in drug resistance and glycolysis, and so far there have been only two reports. MiRNA-3662 was found low-expressed in lung cancer cells and tissues, and knockdown of miRNA-3662 can substantially promote aerobic glycolysis and accelerate tumor growth [20]. Another study showed that miRNA-3662 negatively regulates antiviral drug resistance and inhibits the proliferation of T cell leukemia [21]. Nevertheless, the effect of miRNA-3662 in the progression of chemoresistance in PDAC is yet to be elucidated.

The task of our present study was to explore the biological role and regulatory mechanism of miRNA-3662 in PDAC chemoresistance. MiRNA-3662 exhibited a low expression level in PDAC tissues and cell lines. Upregulation of miRNA-3662 repressed the Gem resistance and aerobic glycolysis in PDAC. Moreover, mechanistic analyses indicated that HIF-1α was directly transcribed by miRNA-3662. Collectively, our study exposed that miRNA-3662 exerted antioncogenic function in PDAC through directly targeting HIF-1α, which might provide a novel sight for PDAC treatment.

Materials and methods

Ethics statement and tissue

Prior to this study, all patients were informed and signed informed consent. 56 PDAC clinical specimens were obtained from The First People's Hospital of Yueyang from Jun 2013 to Apr 2015. All the PDAC patients recruited in this study were diagnosed with ductal adenocarcinoma of head of pancreas. This study was approved by the institutional ethics review board of The First People's Hospital of Yueyang. Tissue preparation for laser microdissection was performed as previously described [22].

FISH assay

Ribo™ Fluorescent In Situ Hybridization Kit (Ribo, China) was conducted to detect the localization of miRNA-3622 in PDAC tissues. Specific probes for the miRNA-3662 were designed and synthesized by Gene-seed (Guangzhou, China). DAPI was employed to stain cell nuclei. Confocal fluorescence microscope (Olympus BX53 Biological Microscope) was used to capture the images of cells.

Cell culture

The human PDAC cell lines (Panc-1, MIA PaCa-2, HPAC, BxPC-3, Capan-1, AsPC-1, SW1990 and Capan-2) as well as the normal pancreatic epithelial cell line HPDEC were obtained from ATCC. Panc-1, BxPC-3 and Capan-1 cells were cultured in RPMI 1640 (Life Technologies, Carlsbad, CA), while MIA PaCa-2, HPAC, AsPC-1, SW1990, Capan-2 and HPDEC cells were maintained in DMEM (Life technology). All cell lines were supplemented with 10% fetal bovine serum (PBS, GIBCO, USA) in a humidified incubator at 37 °C with 5% CO_2 condition. The cells in third–eight generation and logarithmic phase were used for experiment.

Construction of Gem-resistant PDAC cell lines

To establish Gem-resistant PDAC cell models, AsPC-1 and SW1990 cells were exposed to gradually increasing doses of Gem in vitro as described previously [5]. The starting concentrations of Gem for AsPC-1 and SW1990 cells were 200 and 10 nmol/L, respectively. The constructed Gem-resistant cell lines were named AsPC-1/Gem and SW1990/Gem. Gem-resistant PDAC cells were maintained in Gem-free medium for 7 days prior to the experiments.

RNA isolation and real-time qPCR (RT-qPCR)

RNAiso Plus (9108Q, Takara, China) or RNAiso for Small RNA (9753Q) were used to isolate total RNA or miRNA, as required. Reverse transcription step was referred to the instructions of PrimeScrip RT reagent Kit (RR037Q) produced by Takara (China). The RT-qPCR detection was constructed in the Mx3000P Real-Time QPCR System (Agilent, USA) with TB Green Premix Ex Taq II (RR820Q, Takara, China), followed by the conditions set as below: pre-denaturation (97 °C, 4 min), denaturation (97 °C, 13 s) and annealing (60 °C, 1 min) for 40 cycles, and finally extended (68 °C, 7 min). U6 was used as control for miRNA and mRNA expression, and the results were quantified in the form of 2-ΔΔCt method [10].

Cell transfection

AgomiRNA-NC, AgomiRNA-3662, AntagomiRNA-NC, and AntagomiRNA-3662 were ordered from Guangzhou RIBOBIO Biotechnology Co. LTD (China). The HIF-1α overexpression (HIF-1α) plasmid was constructed from GenePharma Company (China). When the confluence of AsPC-1 and SW1990 cells in a 24-well plate reached 70–90%, they were ready for transfection. For the transfection method, refer to the direction of Lipofectamine 3000 (L3000015, Invitrogen, USA).

Cell viability assay

Cells were inoculated into a 96-well plate and cultured for 24 h after transfection. 48 h after culture, 10 μL CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added to each well and incubated at 37 °C for 1 h. The optical density (OD) value at 450 nm was determined by a spectrophotometer (Thermo Scientific, Rockford, IL, USA). The IC50 value was calculated as the Gem concentration causing 50% decrease in cell viability.

Flow cytometry analysis for apoptosis

Cells were harvested and fixed with 75% ethanol at − 20 °C overnight. After PBS washing again, cells were stained with FITC-Annexin V and PI (Bio-Rad) for 25 min in dark. Flow cytometry (FACS Calibur and LSR™ II Flow Cytometer; BD Pharmingen) was used to detect the apoptotic rate.

Detection of glucose utilization and lactate release

Cells were cultured in six-well plates at a density of 1×10^6 cells/well without phenol red for 24 h, and the culture media was harvested for detection of glucose consumption and lactate production. The extracellular concentrations of glucose and lactate were quantified with a glucose assay kit (Sigma-Aldrich) and a Lactate Assay Kit (BioVision, Milpitas, CA, USA) according to the direction of manufacturer. The assays were performed in triplicate.

Western blotting

The proteins in this study were lysed using RIPA Lysis Buffer (C05-01001, Bioss, China), followed by quantified with Pierce Rapid Gold BCA Protein Assay Kit (A53225, ThermoFisher, USA). 20 μg of protein was taken to perform electrophoresis with SDS-PAGE, and then transferred to PVDF membrane. After sealing with Blocking Buffer (37,565, Thermo Scientific, USA) for 2 h, membranes were incubated with primary antibodies and secondary antibodies, respectively. Then, protein bands were showed with ECL luminous fluid (WBKlS0100, Millipore, USA) and analyzed with gel imaging system (Tanon 2500, Solarbio, China) and Quantity One image analysis software (Bio-Rad, USA). The antibodies against deoxycytidine kinase (dCK), HIF-1α and β-actin were obtained from Abcam (UK).

Target gene verification

The wild-type or mutant sequences of HIF-1α 3' UTR were inserted into the pmirGLO vector (E1330, Promega, USA) to perform dual-luciferase reporter assay. Different recombinant plasmids together with either wt-HIF-1α or mut-HIF-1α were transfected into BxPC-3 cells, respectively. Luciferase activities were measured in dual-luciferase system (D0010-100 T, Solarbio, China) by GloMax 20/20 detector (Promega, USA).

Tumor induction assay in a nude mouse model

Male BALB/c-nude mice at 4 weeks of age were maintained under specific pathogen-free conditions. AsPC-1/Gem cells were stably transfected with AgomiRNA-NC or AgomiRNA-3662 and harvested. Cells were then washed with phosphate-buffered saline, and re-suspended at a concentration of 5 × 107 cells/ml. The node mice were randomly divided into four groups, 8 mice per group. In two groups of mice, 100 µl of suspended control AsPC-1/Gem cells was inoculated subcutaneously implanted into the armpit of each mouse. In the rest two groups, the mice were subcutaneously implanted with the same number of miR-3662 overexpressing-AsPC-1/Gem cells. After the tumors reached 50 mm^3, one group of mice implanted with the control cells and one group of mice implanted with the miR-3662 overexpressing cells were treated with Gem (50 mg/kg given twice a week by intraperitoneal injection for 28 days). All mice were euthanized 28 days later. This study was approved by the Animal Ethical and Welfare Committee of Hunan Institute of Science and Technology.

Immunohistochemical (IHC) analysis

The removed tumors were fixed in 4% paraformaldehyde for 15 min, embedded in paraffin wax, sectioned into slices, and then processed using hematoxylin and eosin staining. Such samples were then incubated with specific antibody against Ki-67 (1:1000) and HIF-1α (1:300), respectively. Observations were carried out using an optical microscope (Olympus, Japan), and positive staining was brown.

Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (Tunel) assay

The apoptosis of tumor tissues was evaluated using an in situ cell death detection kit from Roche diagnostics as per manufacturer's protocols strictly. Tunel reaction was viewed and photographed using a light microscope (Olympus BX51, Shinjuku, Japan) by two different pathologists unaware of the xenograft tumor groups. Average number of Tunel-positive apoptotic cells of 10 randomly chosen fields from each treatment groups was counted.

Statistical analysis

All statistical analyses were implemented using Graphpad 8.0 software. One-way ANOVA was employed for comparison between multiple groups. Dunnett-*t* test or Tukey test for pairwise comparison between groups. Correlation analysis was calculated using the Spearman's test. $P < 0.05$ was considered statistically significant.

Results

MiRNA-3662 expression was down-regulated in PDAC tissues

To determine the biological role of miRNA-3662 in PDAC, we first performed FISH analysis to measure miRNA-3662 expression in PDAC tissue. As presented in Fig. 1a, miRNA-3662 was positively expressed in cytoplasm of PDAC cells. Further, we explored miRNA-3662 expression in 56 PDAC tissue samples and their matched adjacent normal tissues through RT-qPCR. The results turned out that miRNA-3662 expression was dramatically lower in 78.6% (44/56) of PDAC tissue cases (Fig. 1b). As shown in Fig. 1c, our data also demonstrated that the expression of miRNA-3662 in PDAC tissues was decreased than that of adjacent normal tissues ($P < 0.001$). Taken together, the above results demonstrated that the expression of miRNA-3662 was down-regulated in PDAC tissues, and may acted as a positive prognostic factor in PDAC.

MiRNA-3662 regulates the Gem resistance in PDAC cells in vitro

The effects of miRNA-3662 modification on the Gem resistance of cultured PDAC cells were then explored. We first examined the situation of miRNA-3662 in PDAC cell lines and found that the expression of miRNA-3662 in Panc-1, MIA PaCa-2, HPAC, BxPC-3, Capan-1, AsPC-1, SW1990 and Capan-2 cells was dramatically lower than that of HPDEC cells ($P < 0.001$, Fig. 2a). Among all PDAC cell lines, the expression of miRNA-3662 was highest in SW1990 cells and lowest in AsPC-1 cells, so these two cell lines were selected as the next experimental cells. As depicted in Fig. 2b, AsPC-1 and SW1990 cells were treated with gradient doses of Gem (0–20 µmol/L) for 6 h.

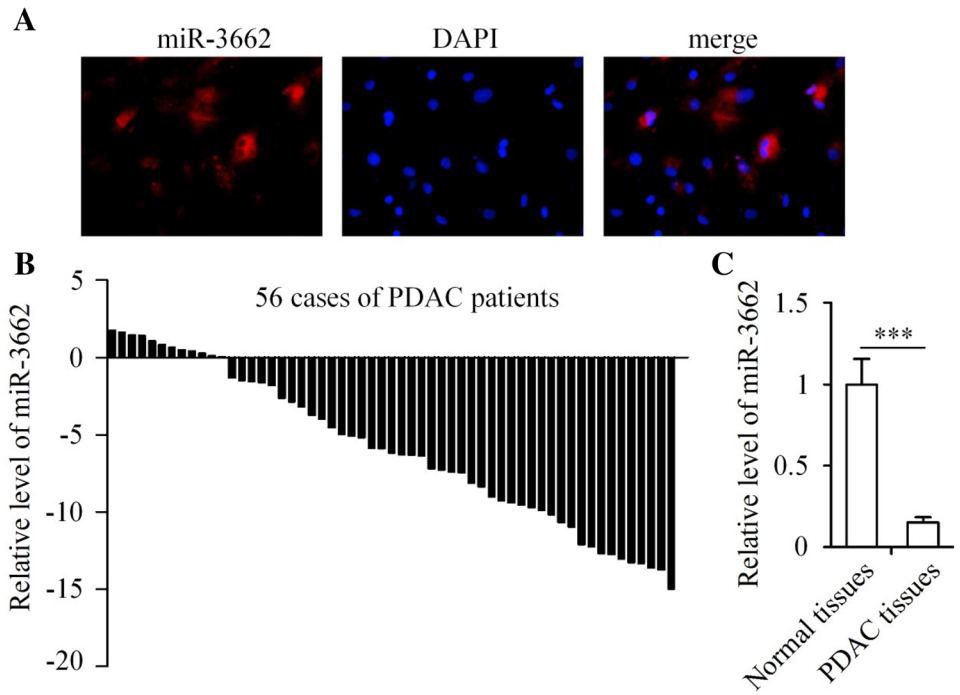


Fig. 1 Relative expression of miRNA-3662 in PDAC tissues. **a** FISH showing the localisation of miRNA-3662 in PDAC tissues. **b** The relative level of miRNA-3662 was markedly decreased in 78.6% (44/56) of PDAC specimen cases. **c** Analysis of miRNA-3662 expression in PDAC tissues and adjacent non-cancerous samples by RT-qPCR

Treatment with Gem dose-dependently reduced the level of miRNA-3662 in both PDAC cell lines. The relationship between miRNA-3662 and the chemotherapeutic response of PDAC cells was ascertained by calculating the IC50 value of Gem. As depicted in Fig. 2c, PDAC cell line with low expression of miRNA-3662 displayed relatively high resistance to Gem. Moreover, the abundance of miRNA-3662 was inversely correlated with the IC50 value of Gem in PDAC cell lines (Fig. 2d).

In this study, by exposing AsPC-1 and SW1990 cells to increasing doses of Gem for 3 months, Gem-resistant PDAC cell lines (AsPC-1/Gem and SW1990/Gem) were established successfully. As shown in Fig. 3a, the IC50 values of Gem in AsPC-1/Gem and SW1990/Gem cells are nearly three times higher than their parental cell lines. The expression of dCK was decreased in both Gem-resistant PDAC cell lines (Fig. 3b). We next explored whether acquired resistance to Gem associated with the dysregulation of miRNA-3662 in PDAC cells. As shown in Fig. 3c, RT-qPCR assay disclosed that the level of endogenous miRNA-3662 was markedly repressed in Gem-resistant cell lines in comparison with their matched parental cell lines. In addition, AgomiRNA-3662 or AntagomiRNA-3662 was transfected into PDAC cells. As expected, transfection of AgomiRNA-3662 or AntagomiRNA-3662 were indeed significantly increased or decreased the level of miRNA-3662 in PDAC cells (Fig. 3d). Moreover, CCK-8 assay showed that the IC50 value of AsPC-1/Gem cells was repressed remarkably by miRNA-3662 overexpression. In contrast, miRNA-3662 knockdown significantly promoted the IC50 value of SW1990/Gem (Fig. 3e). The subsequent western blot results exhibited that the expression of dCK was elevated in the cells transfected with AgomiRNA-3662 and reduced in the cells transfected with AntagomiRNA-3662 (Fig. 3f). Subsequently, the apoptotic ratio in each group was analyzed by flow cytometry. As presented in Fig. 3g, the results disclosed that the numbers of apoptotic cells induced by Gem were significantly promoted in AsPC-1/Gem cells with overexpression of miRNA-3662, whereas the depletion of miRNA-3662 remarkably attenuated the apoptosis induction effect of Gem on SW1990/Gem cells. Collectively, above results strongly support the assumption that miRNA-3662 is effective in inhibiting Gem resistance of PDAC in vitro.

MiRNA-3662 regulates the Warburg effect in PDAC cells

Since increasing evidence showed that Warburg metabolism was characterized by promoted chemoresistance efficiency, and implicated in cancer initiation and metastasis. To better understand the mechanisms of miRNA-3662 on the chemoresistance of PDAC cells, we next attempted to determine the effect of miRNA-3662 on the aerobic glycolysis in

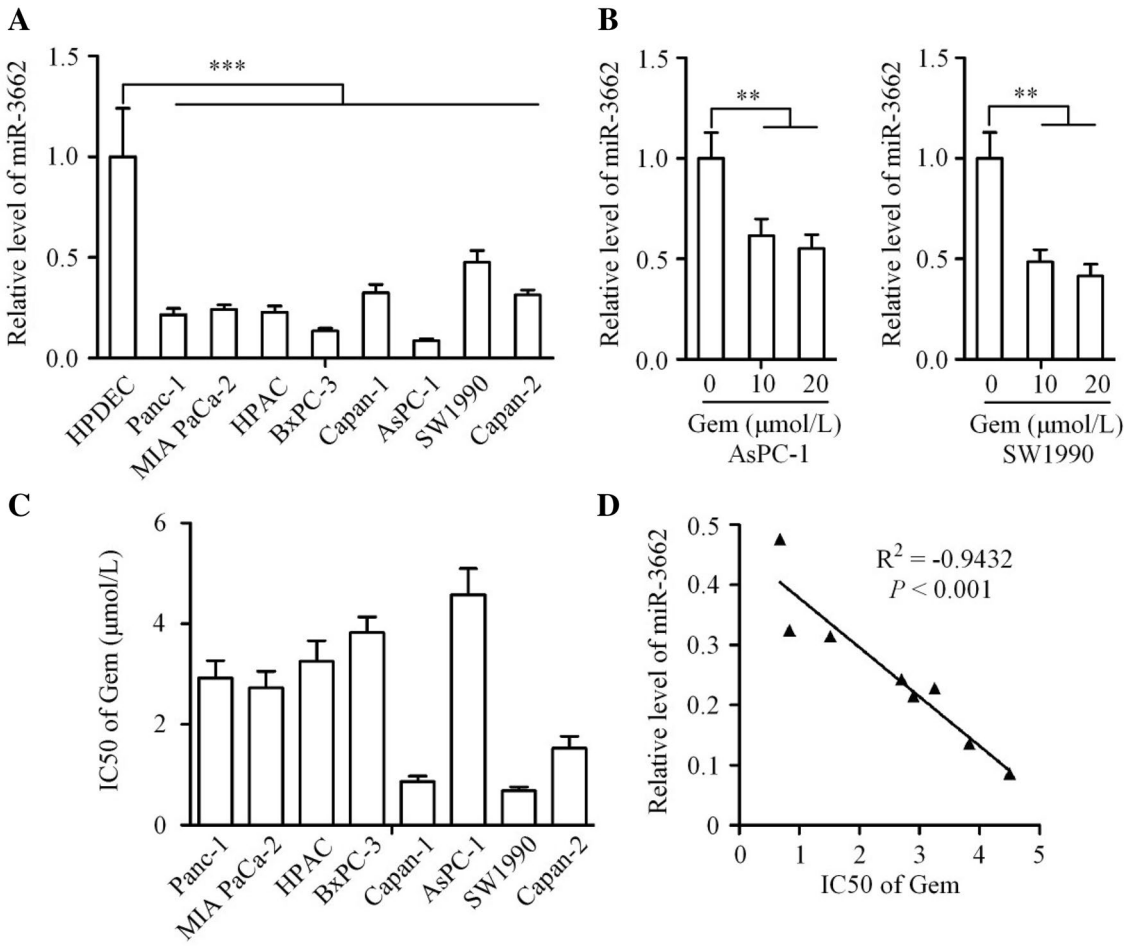


Fig. 2 The relationship of miRNA-3662 between the sensitivity of Gem. **a** The expression of miRNA-3662 was detected in eight human PDAC cell lines and one non-malignant pancreatic epithelial cell line HPDEC by RT-qPCR. **b** Analysis of miRNA-3662 expression in PDAC cells after treated with indicated concentrations of Gem for 6 h. **c** The IC50 values of Gem in eight PDAC cell lines. **d** The correlation of miRNA-3662 and IC50 values of Gem in PDAC cell lines. $^{**}P < 0.01$, $^{***}P < 0.001$. Results are reported as the mean ± SD of four independent experiments

AsPC-1 and SW1990 cells. First, glucose utilization and lactate release as two key glycolytic parameters were detected in the Gem-resistant PDAC cells and their parental cells. As expected, the consumption of glucose and the production of lactate were both increased in Gem-resistant PDAC cell lines than their parental cell lines (Fig. 4a). Importantly, the results showed that miRNA-3662 overexpression resulted in significant decrease in glucose utilization and lactate release, while silencing of miRNA-3662 dramatically promoted glucose consumption and lactate production (Fig. 4b). To further validate this observation, RT-qPCR analysis showed that miRNA-3662 overexpression inhibited the RNA levels of glucose transporter SLC2A1 as well as glycolytic enzymes (PFKP, PKM, and LDHA), whereas miRNA-3662 knockdown reversed these effects (Fig. 4c). Collectively, these findings above suggest that miRNA-3662 antagonizes the glycolytic phenotype of PDAC cells.

HIF-1α is a target of miRNA-3662

To determine the specific mechanism of miRNA-3662 functioning, we predicted possible target genes bound by miRNA-3662 through the TargetScan website. Our data showed that miRNA-3662 had a binding site with 3' UTR of HIF-1α mRNA (Fig. 5a). Next, we verified that miRNA-3662 could bind to HIF-1α by dual-luciferase reporter assay ($P < 0.001$, Fig. 5b). Furthermore, genetic silencing of miRNA-3662 led to significant up-regulation

of HIF-1α protein level in AsPC-1 cells, while overexpression of miRNA-3662 down-regulated HIF-1α protein level in SW1990 cells (Fig. 5c). Since miRNA-3662 expression was down-regulated in PDAC tissues, we further detected the expression of HIF-1α in these samples. Consistent with previous reports, we found that the level of HIF-1α was considerably increased in PDAC tissues in comparison with normal tissues (Fig. 5d). More importantly, when the relative level of HIF-1α was plotted against that of miRNA-3662 in PDAC tissues, linear correlation analysis demonstrated a significant inversely correlation between HIF-1α and miRNA-3662 (Fig. 5e). Furthermore, the HIF-1α protein was considerably promoted in eight PDAC cell lines than that in HPDEC (Fig. 5f). Above data demonstrated that miRNA-3662 could directly bind with 3' UTR of HIF-1α and negatively regulate the level of HIF-1α.

MiRNA-3662 attenuates the chemoresistance of PDAC in vivo

Above date showed that miRNA-3662 attenuate the Gem resistance of PDAC cells in vitro. Then, the PDAC subcutaneous xenograft tumor model was established to evaluate the tumor therapeutic potential of miRNA-3662 on the Gem resistance of PDAC cells in vivo. Our results show that mice subjected to intraperitoneal injection of Gem does not greatly alter weight tumor formed by AgomiRNA-NC-AsPC-1/Gem cells, demonstrating that the xenograft model designed in this study displayed great tolerance to Gem treatment. As expected, tumor weight indicated that the up-regulation of miRNA-3662 combined with Gem treatment significantly influenced in decreasing the mean weigh of tumors compared to Gem group (Fig. 6a). Furthermore, RT-qPCR result suggested that overexpression of miRNA-3662 enhanced the expression of miRNA-3662 in tumors (Figs. 6b).

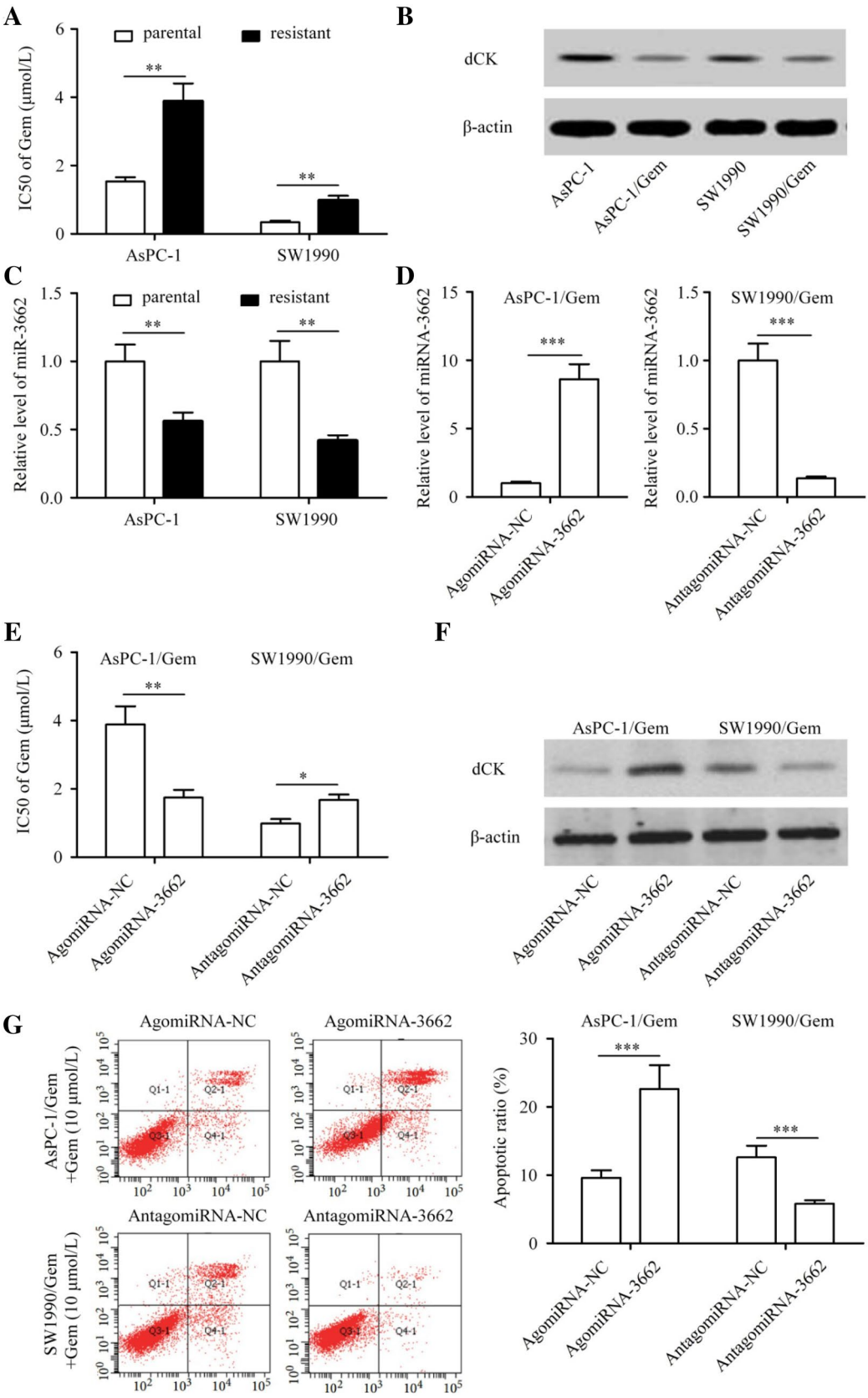
In addition, we performed immunohistochemistry to identify the expression of Ki-67 in tumors from four groups. As shown in Fig. 6c, tumors in AgomiRNA-3662 group exhibited lower percentage of Ki-67 positive stained cells relative to the Gem group. Immunohistochemistry results also demonstrated that the percentage of HIF-1α-positive cells was down-regulated in miRNA-3662 overexpressing xenografts (Fig. 6d). Furthermore, the expression of dCK was promoted by overexpression of miRNA-3662, even in the existence of Gem (Fig. 6e). Subsequently, Tunel results demonstrated that the percentage of Tunel-positive cells was down-regulated in tumor samples from mice treated miRNA-3662-overexpressing and Gem injection (Fig. 6f). Overall, these in vivo results further confirms our hypothesis that miRNA-3662 is a negative regulator of PDAC chemoresistance in vivo.

MiR-3662 regulates Gem resistance of PDAC cells via HIF-1α

HIF-1α is frequently dysregulated in Warburg effect and plays crucial roles in the regulation of tumorigenesis and chemoresistance [23]. To assess the role of HIF-1α in miRNA-3662 regulated PDAC Gem resistance, we performed "rescue" experiments. In line with former results, Fig. 7a shows that the expression of HIF-1α was promoted in both Gem-resistant PDAC cell lines in comparison with their parental cell lines. Interesting, transfection with AgomiRNA-3662 into AsPC-1/Gem cells decreased expression of HIF-1α, while co-transfection with overexpression plasmid of HIF-1α reversed the inhibitory effect of AgomiRNA-3662 on the expression of HIF-1α (Fig. 7b). Then, the effects of HIF-1α restoration on Gem resistance were determined. As depicted in Fig. 7c, HIF-1α up-regulation offset the inhibition of Gem resistance induced by AgomiRNA-3662. Similarly, ectogenic HIF-1α obviously abrogated the apoptosis induction activity of AgomiRNA-3662 in vitro (Fig. 7d). Further experiments were employed to explore the function of HIF-1α on miRNA-3662 regulated aerobic glycolysis in PDAC cells. Our results also confirmed that the reintroduction of HIF-1α effectively reversed the suppression of glucose consumption (Fig. 7e) and lactate production (Fig. 7f) caused by AgomiRNA-3662. These results suggested that miRNA-3662 regulated chemoresistance process and Warburg effect of PDAC cells via HIF-1α.

Transcriptional regulation of miRNA-3662 by HIF-1α in PDAC cells

Hypoxia is a distinguishing feature of PDAC microenvironment and HIF-1α is a crucial regulator in the reprogramming of hypoxic stress [21]. Given that the inverse correlation between miRNA-3662 and HIF-1α, we hypothesized that miRNA-3662 might be regulated by hypoxia in PDAC cells. To clarify this hypothesis, we cultured AsPC-1 and SW1990 cells under normoxic or hypoxic condition for 24 h. As depicted in Fig. 8a, miRNA-3662 expression was drastically inhibited by hypoxia in both tested cell lines. As a chemical inducer of HIF-1α, $CoCl_2$ also attenuated the level of miRNA-3662 in both PDAC cell lines (Fig. 8b). Above data demonstrated that miRNA-3662/HIF-1α loop played a crucial role in regulating PDAC aerobic glycolysis.


A
parental
resistant
IC50 of Gem (μmol/L)
AsPC-1
SW1990
B
dCK
β-actin
AsPC-1
AsPC-1/Gem
SW1990
SW1990/Gem
C
Relative level of miR-3662
D
AsPC-1/Gem
SW1990/Gem
Relative level of miRNA-3662
AgomiRNA-NC
AgomiRNA-3662
AntagomiRNA-NC
AntagomiRNA-3662
E
F
G
AgomiRNA-NC
AgomiRNA-3662
AntagomiRNA-NC
AntagomiRNA-3662
AsPC-1/Gem +Gem (10 μmol/L)
SW1990/Gem +Gem (10 μmol/L)
Apoptotic ratio (%)

◂**Fig. 3** MiRNA-3662 inhibits the Gem resistance of PDAC cells. **a** The IC50 values of Gem on Gem-resistant PDAC cell lines were significantly higher than their parental cell lines. **b** The expression of dCK was decreased in both Gem-resistant PDAC cell lines. **c** The expression of miRNA-3662 was markedly lower than their parental cell lines. **d** The transfection efficiency of miRNA-3662 in AsPC-1 and SW1990 cells was analyzed by RT-qPCR. **e** CCK-8 assay was utilized to detect the IC50 values of Gem in PDAC cells after dysregulation of miRNA-3662. **f** The level of dCK in PDAC cells after transfection with AgomiRNA-3662 or AntagomiRNA-3662 was examined by western blot. **g** Cell apoptosis was explored by flow cytometry in PDAC cells. $*P<0.05$, $**P<0.01$, $***P<0.001$. Similar results were obtained in three independent experiments

Discussion

At present, the incidence of PDAC in China has been growing year by year, which is the tenth leading cause of cancer-related death in China [1]. The most effective assistant therapeutic method of PDAC is chemotherapy, Gem is widely used in the clinical treatment of PDAC. However, the chemoresistance of cancer cells to Gem causes poor prognosis, recurrence and metastasis [3, 4]. Thus, it is imminently needed to discover the key players implicated in chemoresistance and develop new therapeutic strategies with less toxicity and high anti-cancer efficacy. In the present study, we discovered that miRNA-3662, a miRNA with unknown function in PDAC chemoresistance so far, was down-regulated in PDAC clinical samples and cells, and the abnormal expression of miRNA-3662 was significantly associated with lymphatic invasion and tumor stage in patients with PDAC. Accordingly, we hypothesized that miRNA-3662 dysregulation is implicated with malignant biological behaviors of PDAC. Our results showed that miRNA-3662 is act as a tumor inhibitor in PDAC by negatively targeting HIF-1α, and ulteriorly suppressing chemotherapeutic resistant PDAC in vitro and in vivo. All these findings demonstrate that miRNA-3662/HIF-1α loop plays a momentous role in Gem resistance and maintenance of PDAC.

The aberrant expression of miRNA-3662 is common in many different types of cancers. The downregulation of miRNA-3662 promotes the downstream oncogenic activity, which in turn directly linking to enhanced cell proliferation and promoted cell motility and tumor invasion [19–21]. This is the first report describing the therapeutic benefit and mechanism of miRNA-3662 against resistant PDAC cells. Our study indicated that the expression level of miRNA-3662 was inversely correlated with the IC50 value of Gem in PDAC cell lines. Therefore, we propose that miRNA-3662 plays a vital role in Gem resistance of PDAC, and this hypothesis was supported by our observations. In in vitro experiment, we found that the overexpression of miRNA-3662 could partly reverse the tolerance of cultured resistant PDAC cells to Gem, and vice versa. Further experiments were used to testify the hypothesis that the chemosensitive activity of miRNA-3662 in PDAC is sustained in vivo. The in vivo results disclosed that miRNA-3662 markedly enhance the cytotoxic effect of Gem on chemoresistant PDAC. dCK is a central protein that contribute to gemcitabine cytotoxicity because it is the rate-limiting enzyme in the phosphorylation process to generate the active metabolites of Gem. Previous reports have showed that the level of dCK implicates with Gem efficacy and prognosis of PDAC patients [24, 25]. The pharmacokinetics data achieved in this study demonstrated that the expression of dCK was regulated by the dysregulation of miRNA-3662 in vitro and in vivo. Our in vivo findings are in accordance with the changes observed in the cultured cells. The data could strongly support the idea that miRNA-3662 is more directly and importantly affect the chemoresistance of PDAC cells both in vitro and in vivo, and miRNA-3662 might be employed as a "supplement" in Gem-based chemotherapy to improve the overall response rate to Gem.

Dysregulation of Warburg effect in cancer cells implicated with tumorigenicity and chemosensitivity, suggesting that targeting the Warburg effect is an potential therapeutic target in a variety of cancers [26]. It has been shown that miRNA-3662 attenuates cellular proliferation, metastasis and glucose metabolism in liver cancer by targeting HK2 [19]. Therefore, we hypothesized that miRNA-3662 could inhibit cell growth and Warburg effect of PDAC, and our observations supported this hypothesis. The dysregulation of miRNA-3662 is required to maintain aerobic glycolysis of PDAC cells in vitro. These findings could strongly support the idea that miRNA-3662 plays a critical role in Warburg effect of PDAC cells. In summary, we for the first time uncovered a new role of miRNA-3662 in regulating cancer cell aerobic glycolysis. This finding further extends our insight into the antioncogenic activities of miRNA-3662 on PDAC. However, the signaling of how miRNA-3662 regulates chemoresistance and aerobic glycolysis in PDAC is not yet understood.

Evidence suggests that HIF-1α is increased with advanced stages and related to Warburg effect in many invasive and metastatic cancers [27]. Cancer cells adapt to hypoxic microenvironment is of great significance for their malignant proliferation and distant metastasis [28]. One of the irreversible mechanisms participating the hypoxic response is HIF-1α, which dominates alteration of energy metabolism by directly targeting metabolism-related genes [29]. The cooperation of miRNAs and HIF-1α has been investigated in previous studies. MiR-210 is up-regulated in lung cancer and mediates

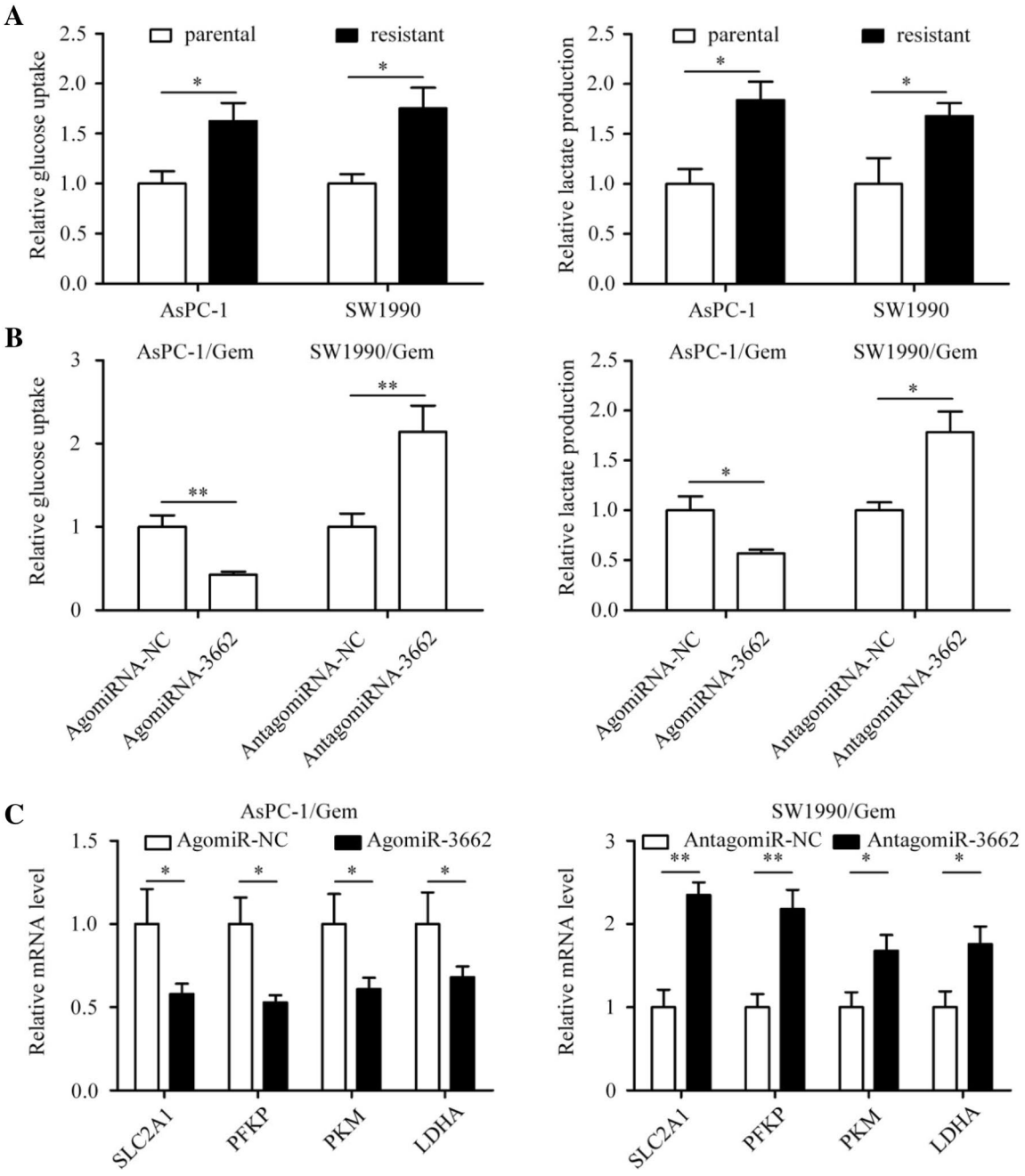


Fig. 4 miRNA-3662 suppresses PDAC cells metabolic rewiring. **a** The glucose uptake and lactate production were analyzed in AsPC-1/Gem and SW1990/Gem cells and their parental cell lines. **b** The dysregulation of miRNA-3662 on the glucose utilization, lactate production, and expression levels of glycolytic components **c** in PDAC cells. $^{*}P<0.05$, $^{**}P<0.01$. Data were derived from 3 experiments with 6 replicates

mitochondrial alterations implicated with modulation of HIF-1α activity [30]. Additionally, miRNA-124 involved in cell proliferation, survival, migration, and tumorigenesis by regulating STAT3/HIF-1α signaling pathway [31]. Emerging evidence has suggested that HIF-1-regulated glucose metabolism played a key role in apoptosis resistance of human

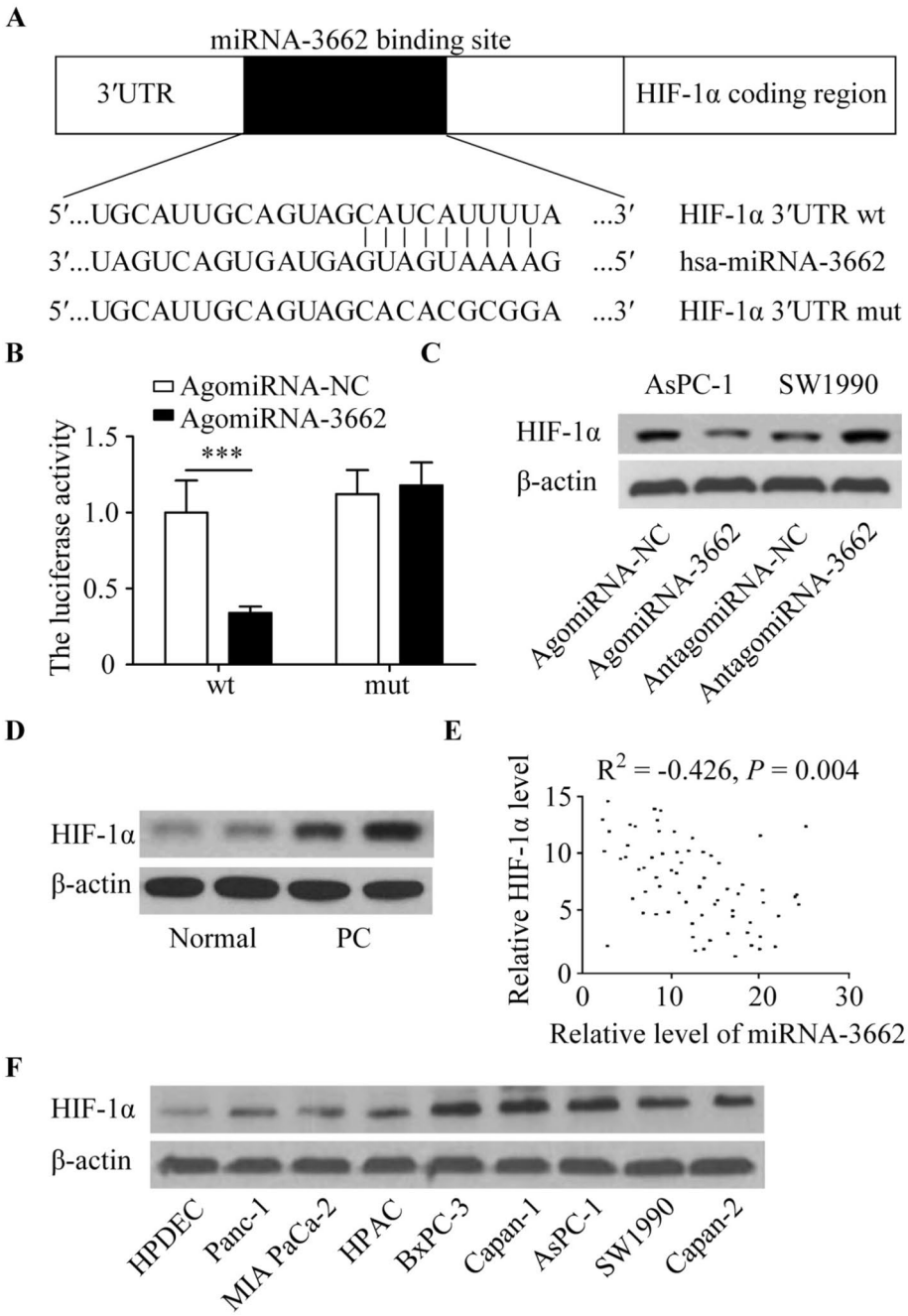


Fig. 5 The relationship between miRNA-3662 and HIF-1α in PDAC cells. **a** miRNA-3662 and its putative binding region in the HIF-1α 3' UTR using the TargetScan website. **b** Dual-luciferase report analysis verified the targeting relationship between miRNA-3662 and HIF-1α. **c** Western blot results of HIF-1α expression in PDAC cells. **d** The expression of HIF-1α was markedly increased in PDAC specimens compared with normal tissues. **e** The negative correlation between HIF-1α and miRNA-3662 was analyzed by Spearman's correlation. **f** The expression of HIF-1α was surprisingly enhanced in PDAC cell lines than that in HPDEC. All experiments have been performed in independent triplicate. ***$P < 0.001$. Similar results were obtained in three independent experiments

cancers [32]. Meanwhile, HIF-1α was activated by a series of upstream genes, such as ERK and JNK, which play an indispensable role in the tumorigenesis and the promotion of cancer deterioration [33]. However, the interaction between miRNA-3662 and biological functions of HIF-1α has not been investigated so far. Here we revealed that miRNA-3662 can attenuate chemoresistance and aerobic glycolysis by targeting HIF-1α, which is constantly overexpressed in

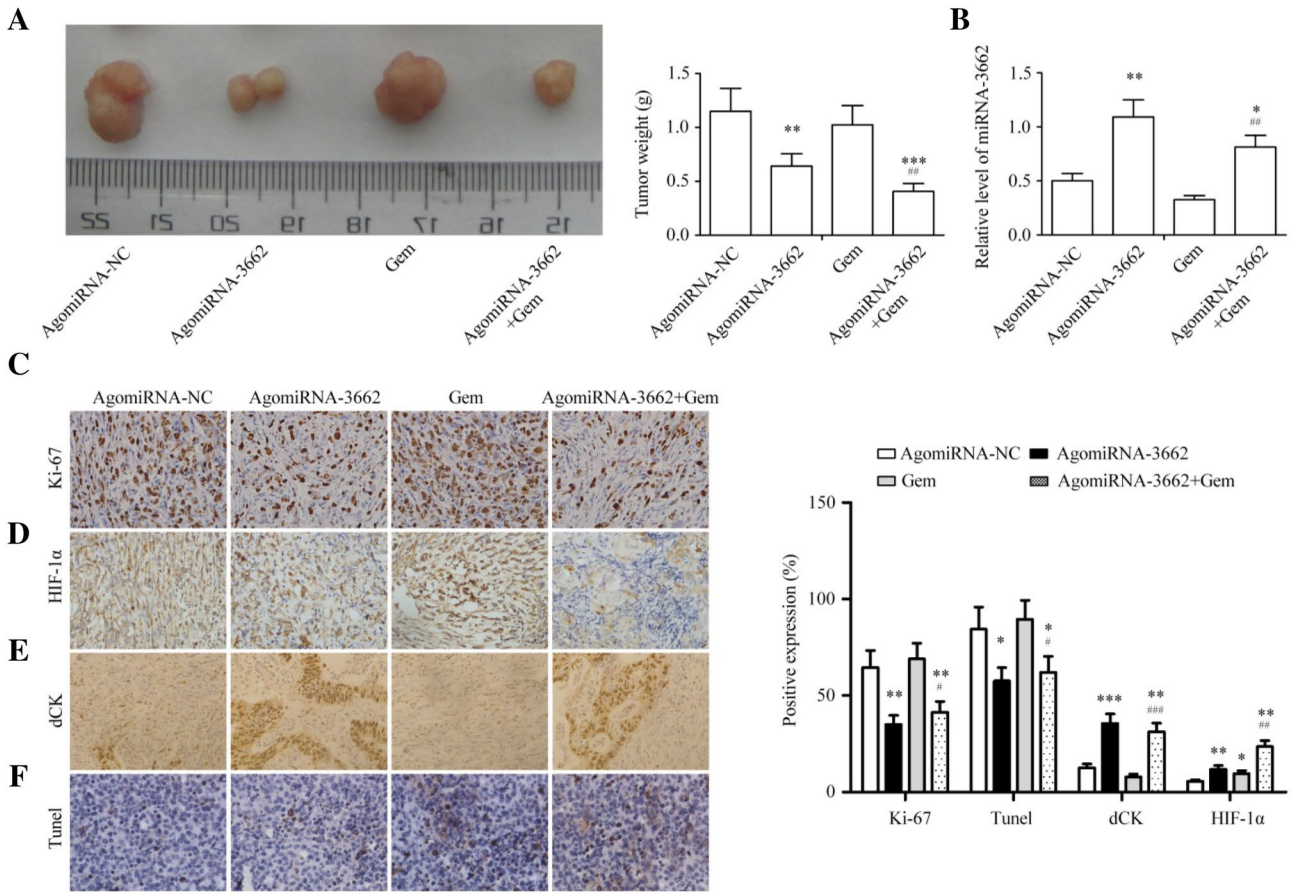


Fig. 6 The ectopic expression of miRNA-3662 represses PDAC chemoresistance in vivo. **a** Representative images of xenograft tumor showing the therapeutic benefit of miRNA-3662. **b** The expression of miRNA-3662 in tumor neoplasm from tumor-bearing mice was explored by RT-qPCR. Immunochemical analysis of Ki-67 (**c**), dCK (**d**) and HIF-1α (**e**) in tumor xenograft. **f** The apoptosis of tumor xenograft was analyzed by Tunel assay. Similar results were obtained in three independent experiments. $^{*}P<0.05$ versus the AgomiRNA-NC, $^{**}P<0.01$ versus the AgomiRNA-NC, $^{***}P<0.001$ versus the AgomiRNA-NC, $^{\#}P<0.05$ versus the Gem, $^{\#\#}P<0.01$ versus the Gem, $^{\#\#\#}P<0.001$ versus the Gem. Similar results were obtained in three independent experiments

PDAC. Increased HIF-1α expression is closely correlated with Gem resistance [34]. Interestingly, this study identified that miRNA-3662 inversely regulates HIF-1α expression in PDAC cells both in vitro and in vivo. Importantly, restoration of HIF-1α was able to attenuate the suppression of chemoresistance and aerobic glycolysis caused by miRNA-3662, suggesting the importance of miRNA-3662-HIF-1α loop in PDAC development.

In this scenario, our current findings highlight that miRNA-3662 is involved in the Gem resistance of PDAC. Mechanistically, we further disclosed a reciprocal regulation between miRNA-3662 and HIF-1α, and this loop plays a pivotal role in the Warburg effect and tumor growth. Interference of miRNA-3662-HIF-1α loop may hold promise for preventing PDAC development.

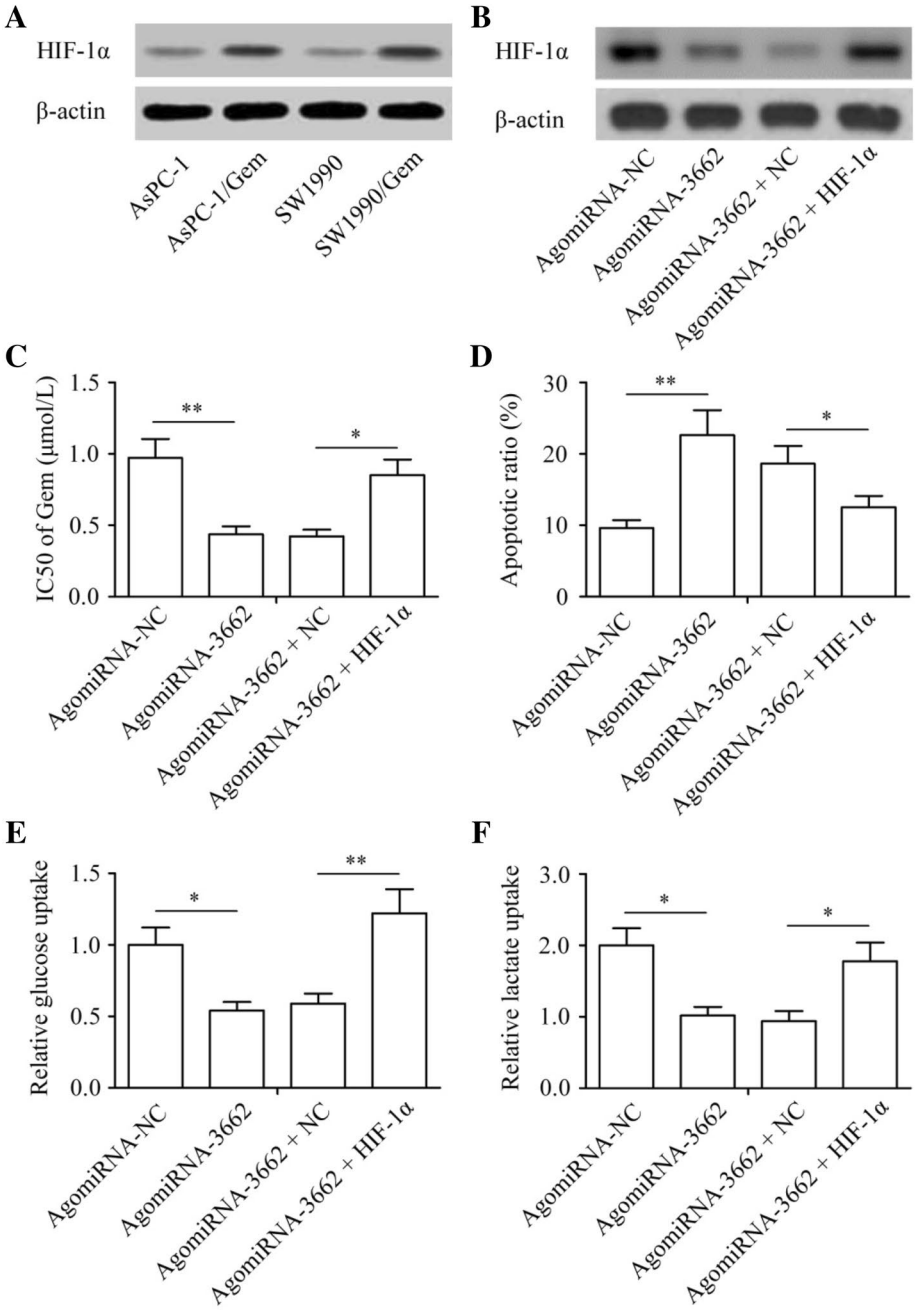


Fig. 7 miRNA-3662 regulated chemoresistance and aerobic glycolysis of PDAC cells via HIF-1α. **a** Western blot analysis of HIF-1α expression in Gem-resistant PDAC cell lines and their parental cell lines. **b** Expression of HIF-1α in AsPC-1/Gem cells after co-transfected with AgomiRNA-3662 and HIF-1α overexpression plasmid by western blot. **c** Promotion of HIF-1α abrogated the inhibitory effect of miRNA-3662 on the Gem resistance of AsPC-1/Gem cells. **d** Overexpression of HIF-1α abolished the promotional effect of miRNA-3662 up-regulation on the apoptosis of AsPC-1/Gem cells. Cellular glucose utilization **e** and lactate production **f** in PDAC cells after co-transfected with AgomiRNA-3662 and HIF-1α overexpression plasmid. $^{**}P < 0.001$, $^{*}P < 0.05$. Similar results were obtained in three independent experiments

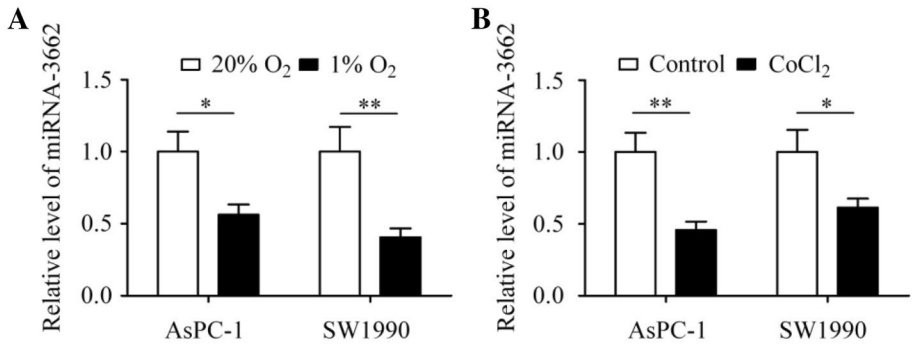


Fig. 8 Transcriptional regulation of miRNA-3662 by HIF-1α in PDAC cells. **a** RT-qPCR analysis of miRNA-3662 expression in AsPC-1 and SW1990 cells under hypoxia (1% O2) and normoxia (20% O2) condition. **b** Analyze of miRNA-3662 expression in PDAC cells after 100 μmol/L CoCl2 treatment for 24 h. $^{**}P<0.01$, $^{*}P<0.05$. Similar results were obtained in three independent experiments

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Declarations

Conflict of interest The author declares that he has no competing interests.

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