

Role and molecular mechanisms of hypoxia-derived exosomal miR-301a on gemcitabine resistance in pancreatic cancer

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Abstract. Pancreatic cancer is a highly aggressive digestive malignancy and gemcitabine (GEM)-based chemotherapy remains a cornerstone of its treatment despite widespread resistance. The present study employed human pancreatic cancer cell lines to construct a hypoxia model, exosomes were isolated and characterized via ultracentrifugation, microRNA (miR)-301a expression was detected using reverse transcription-quantitative PCR, GEM resistance was evaluated via the Cell Counting Kit-8 assay and the molecular mechanisms were validated by western blotting. The present study results demonstrated that under hypoxic stress, pancreatic cancer cells displayed morphological adaptations typical of cellular stress responses, upregulated miR-301a expression and markedly enhanced GEM resistance. Hypoxia increases the number of exosomes, which can transfer miR-301a to normoxic pancreatic cancer cells. Under normoxic conditions, inhibiting miR-301a expression markedly increased the sensitivity of pancreatic cancer cells to GEM. Furthermore, miR-301a promoted the development of drug resistance by regulating acyl-CoA synthetase long-chain family member 4 (ACSL4) expression. In conclusion, the present study revealed a novel mechanism by which hypoxia-derived exosomal miR-301a promotes GEM resistance in pancreatic cancer by regulating ACSL4. These findings provide a novel potential target to reverse GEM resistance, with theoretical and practical implications for future studies.

Introduction

Pancreatic cancer (PC), a highly malignant neoplasm of the digestive system with a 5-year survival rate of <10%, is

recognized as the 'king of cancer types' (1). According to data from the International Agency for Research on Cancer, ~496,000 new PC cases and 466,000 related deaths were reported globally in 2020, which ranks PC as the seventh leading cause of cancer mortality in both sexes (2). The pathogenesis of PC is characterized by complex interactions between multiple genetic and environmental factors. The associated genetic factors include regulatory molecules, such as tRNA-Val-CAC-2, which has been shown to interact with FUBP1 to promote PC metastasis by activating c-MYC transcription (3); and the environmental factors encompass smoking and excessive alcohol consumption. Furthermore, recurrent acute pancreatitis, often linked to the aforementioned environmental triggers, can augment long-term PC risk (4). Current therapeutic modalities remain limited and primarily include surgical intervention, chemotherapy and radiotherapy (5-7). However, due to the aggressive biological behaviour of PC, ~80% of patients present with advanced-stage disease at diagnosis (1), which results in limited opportunities for radical resection and suboptimal outcomes with conventional chemo-radiotherapeutic approaches.

Gemcitabine (GEM) remains one of the most widely used chemotherapeutic agents in PC management (8). Its mechanism of action involves incorporation into DNA during synthesis, thereby inducing strand fragmentation and cell cycle arrest (9). However, in the hypoxic tumour microenvironment (TME), multiple resistance mechanisms have emerged that undermine GEM efficacy. Specifically, these mechanisms include reduced drug uptake (for example, loss of the human equilibrative nucleoside transporter 1), enhanced DNA repair capacity and metabolic reprogramming (10). These resistance mechanisms contribute to the limited clinical efficacy of GEM, which is characterized by an overall response rate of <20% and frequent development of rapid tumour resistance (8,11). Therefore, elucidating the molecular mechanisms underlying chemoresistance and identifying effective therapeutic targets to overcome drug resistance have become key challenges in contemporary PC treatment research.

Although recent advancements have been made in targeted therapies and immunotherapies for PC, its complex TME remains a pivotal factor contributing to therapeutic failure (12). The highly fibrotic stroma and aberrant vascular proliferation

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in PC result in persistent intratumoural hypoxia, which fosters a hypoxic microenvironment. This hypoxic niche, a recognized hallmark of PC, drives tumour progression, metastasis and chemoresistance through hypoxia-inducible factor (HIF)-mediated activation of multiple oncogenic signalling pathways (including the VEGF pathway, the PI3K/Akt/mTOR pathway and the Notch/Wnt/ β -catenin pathways) (13-16). Previous studies have revealed that hypoxia markedly enhances the exosomal secretion capacity of tumour cells (17-19).

Exosomes (Exos), extracellular vesicles measuring 40-160 nm in diameter, serve as molecular carriers within the TME by transporting bioactive cargo (for example, proteins, lipids and nucleic acids) to mediate intercellular communication. Under hypoxic conditions, tumour-derived Exos can modulate drug sensitivity through the horizontal transfer of non-coding RNAs, particularly microRNAs (miRNAs). For example, hypoxia-induced Exos carrying miR-210-3p promote proliferation, migration, invasion, epithelial-mesenchymal transition and apoptosis resistance in triple-negative breast cancer (TNBC) cells through the activation of the nuclear factor I X-Wnt/ β -catenin signalling axis (20). Nevertheless, the mechanistic role of hypoxia-derived exosomal miRNAs in GEM resistance remains to be elucidated, which represents a key frontier in contemporary PC research.

miR-301a, a key member of the miRNA family, is markedly upregulated across multiple malignancies, such as breast cancer and gastric cancer, and participates in oncogenic processes including proliferation, invasion and immune evasion through targeted gene regulation (21,22). Its tumour-promoting effects may be mediated through the suppression of tumour suppressor genes such as PTEN and SMAD4 (23,24). Our preliminary investigations demonstrated that miR-301a acts as a key regulator involved in hypoxia-induced gemcitabine resistance in PC. Furthermore, hypoxia induces the upregulation of miR-301a within tumour-derived Exos (25,26). These Exos can be internalized by neighbouring malignant cells, subsequently modulating their biological behaviours, although the precise molecular mechanisms remain to be fully elucidated.

Acyl-CoA synthetase long chain family member 4 (ACSL4), a key enzyme in fatty acid metabolism, catalyses the conjugation of long-chain fatty acids with coenzyme A to generate acyl-CoA derivatives, thereby regulating membrane phospholipid remodelling, signal transduction and energy metabolism (27). Dysregulated ACSL4 expression has been implicated in the progression of multiple cancer types. For example, in TNBC, aberrant ACSL4 expression mediates cell membrane phospholipid remodelling, inducing lipid raft localization and integrin β 1 activation in a CD47-dependent manner, which promotes tumour metastasis via focal adhesion kinase phosphorylation (28). In hepatocellular carcinoma, ACSL4 enhances tumour cell proliferation and metastasis by regulating *de novo* lipid synthesis (29). Notably, ACSL4 has emerged as a key regulator of ferroptosis, an iron-dependent form of cell death driven by lipid peroxidation (30). MiRNAs have also been revealed to modulate ACSL4-mediated processes: MiR-23a-3p promotes sorafenib resistance in hepatocellular carcinoma by inhibiting ACSL4 expression and blocking ferroptosis (31), whereas miR-20a-5p alleviates ferroptosis in renal ischaemia-reperfusion injury by directly targeting the 3' untranslated region (3'-UTR) of ACSL4

mRNA (32). Notably, emerging evidence suggests that ACSL4 also contribute to GEM resistance in PC, although the underlying mechanisms remain largely undefined (33).

The present study aimed to investigate the functional impact of hypoxic tumour-derived exosomal miR-301a on GEM resistance in PC and elucidate its underlying molecular mechanisms. The present study provides novel insights into the chemoresistance landscape of PC and establishes a theoretical foundation for the potential development of exosomal miRNA-based combinatorial therapeutic strategies in the future.

Materials and methods

Cell culture and transfection

Cell line information. Descriptions of the original cell line derivation are shown in Table I (34).

Cell culture. All the cell lines used in the present study [ascites pancreatic cancer 1 (AsPC-1), BxPC-3, cystic fibrosis pancreatic adenocarcinoma (CFPAC-1), malignant inflammatory adenocarcinoma pancreatic carcinoma-2 (MIA PaCa-2) and PANC-1] were purchased from the Shanghai Cell Bank at the Chinese Academy of Sciences. MIA PaCa-2 and PANC-1 cells were cultured in DMEM (cat. no. C11995500BT; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (cat. no. FSP500; Shanghai ExCell Biology, Inc.) and 1% penicillin/streptomycin (cat. no. C0222; Beyotime Institute of Biotechnology). AsPC-1 and BxPC-3 cells were cultured in RPMI-1640 (cat. no. C11875500BT; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1% penicillin/streptomycin. CFPAC-1 cells were cultured in Iscove's Modified Dulbecco's Medium (cat. no. BL312A; Biosharp Life Sciences) supplemented with 10% FBS and 1% penicillin/streptomycin. All the cell lines were cultured in an incubator at 37°C in an atmosphere containing 5% CO₂. During hypoxic treatment, the cells were cultured in a hypoxic incubator (37°C; 5% CO₂; 1% O₂).

Cell transfection. The miR-301a inhibitor, miR-301a mimics and their respective negative control (NC) oligonucleotides were synthesized by Anhui General Bioscience. The sequences are presented in Table II. Transfection was performed using Lipofectamine® 2000 transfection reagent (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.), a widely used cationic lipid transfection reagent, for which the manufacturer's protocol recommends a 4-6 h incubation period in a 37°C incubator for optimal nucleic acid delivery while minimizing cytotoxicity. However, based on our long-term research experience on the effects and molecular mechanisms of non-coding RNAs in the malignant biology of pancreatic tumours, the present study extended the transfection time to 6-8 h to balance transfection efficiency and cell viability. The specific steps were as follows: Cells under good growth conditions, characterized by 50-80% confluence (in the logarithmic growth phase), $\geq 90\%$ cell viability, normal cell morphology and no microbial contamination, were evenly plated in a 6-well plate, 1 day before transfection. Transfection was initiated when the cell confluence reached 60-70%. Preparation of transfection mixtures: For each well, 5 μ l of Lipofectamine® 2000 was added to 250 μ l of Opti-MEM Reduced Serum Medium (cat. no. L530JV; Shanghai Basal

Table I. Cell line characteristics.

Cell line	Derivation	Molecular subtype	Aggressiveness level	Key genetic features
BxPC-3	Primary tumour	Classical	Low-to-moderate	Wild-type KRAS, SMAD4 intact
CFPAC-1	Liver metastasis	Basal-like	Moderate	KRAS G12V, TP53 mutant
AsPC-1	Ascites	Basal-like	High	KRAS G12V, TP53 mutant
MIA PaCa-2	Primary tumour	Classical	High	KRAS G12C, TP53 mutant
PANC-1	Primary tumour	Basal-like	Highly aggressive	KRAS G12D, SMAD4 deleted

TP53, tumour protein 53; CFPAC-1, cystic fibrosis pancreatic adenocarcinoma; AsPC-1, ascites pancreatic cancer 1; MIA PaCa-2, malignant inflammatory adenocarcinoma pancreatic carcinoma-2.

Table II. Inhibitor and mimics sequences.

RNA	Direction	Primer sequences (5'-3')
miR-301a inhibitor	-	GCUUUGACAAUACUAAUUGCACUG
Inhibitor NC	-	UCUACUCUUUCUAGGAGGUUGUGA
miR-301a mimics	S	CAGUGCAAUAGUAAUUGUCAAGC
	AS	GCUUUGACAAUACUAAUUGCACUG
Mimics NC	S	UCACAACCUCUAGAAAGAGUAGA
	AS	UCUACUCUUUCUAGGAGGUUGUGA

miR, microRNA; NC, negative control; S, sense; AS, antisense.

Media Technologies Co., Ltd.) in a sterile centrifuge tube and gently mixed. This mixture was incubated at room temperature for 5 min to allow the Lipofectamine® 2000 to activate. Meanwhile, in another sterile centrifuge tube, 20 nM of the miR-301a inhibitor, mimics or NC oligonucleotide was diluted in 250 µl of Opti-MEM reduced serum medium. The diluted oligonucleotide mixture was then slowly added to the activated Lipofectamine® 2000-Opti-MEM mixture and mixed gently by pipetting up and down several times. The resulting transfection mixture was incubated at room temperature for 20 min to form stable Lipofectamine®-oligonucleotide complexes. The culture medium in each well was carefully aspirated and replaced with 1.5 ml of serum-free medium. The prepared transfection mixture was then added dropwise to each well and the plates were gently swirled to ensure the even distribution of the complexes. After 6-8 h, the medium containing serum and antibiotics was replaced, and the cells were further cultured for 24 or 48 h for subsequent experiments.

Observation of cell morphology. After 24 and 48 h of culture under normoxic or hypoxic conditions, the morphological changes of PC cells (AsPC-1, BxPC-3, CFPAC-1, MIA PaCa-2 and PANC-1) were examined using an inverted light microscope (brightfield mode). Images of cells were captured at x20 magnification.

Western blotting. AsPC-1, BxPC-3, CFPAC-1, MIA PaCa-2 and PANC-1 cells were used to assess HIF-1α protein expression under normoxic and hypoxic conditions. MIA PaCa-2, PANC-1 and BxPC-3 cells were used to evaluate the expression

levels of ACSL4 protein after overexpression of miR-301a. MIA PaCa-2 and PANC-1 cells were utilized to assess the expression levels of ACSL4 protein following the knockdown of miR-301a. Protein extraction and quantification: Collected cells were lysed in RIPA buffer (cat. no. BL1321A; Biosharp Life Sciences) supplemented with 1% PMSF (cat. no. BL1426A; Biosharp Life Sciences) on ice for 15 min. The lysates were centrifuged at 12,000 x g for 15 min at 4°C and the supernatants were collected. The protein concentrations were determined using a BCA assay kit (cat. no. SW201-02; Seven Biotech). For western blot analysis, 20 µg of protein was resolved by 7.5% SDS-PAGE using the Colour PAGE Gel Rapid Preparation Kit (cat. no. PG111; Shanghai Epizyme Biopharmaceutical Technology Co., Ltd.; Ipsen Pharma) and transferred to PVDF membranes (cat. no. IPVH00010; MilliporeSigma). Each membrane was then blocked in 10% skim milk at room temperature for 2 h. Subsequently, anti-HIF-1α (1:500; cat. no. 610958; BD Biosciences), anti-ACSL4 (1:2,000; cat. no. ab155282; Abcam) and anti-β-actin antibodies (1:4,000; cat. no. 60008; Proteintech Group, Inc.) were added and incubated on a shaker at 4°C overnight. The membranes were incubated with horseradish peroxidase-labelled mouse (1:5,000; cat. no. PR30012; Proteintech Group, Inc.)/rabbit (1:5,000; cat. no. PR30011; Proteintech Group, Inc.) secondary antibodies at room temperature for 1 h. Lastly, a high-sensitivity ECL detection reagent (Ultrasensitive ECL Detection Kit; cat. no. PK10003; Proteintech Group, Inc.) was used for visualization of the protein signals and imaged with the Chemiluminescence Imaging System (ChemiDoc MP; Bio-Rad Laboratories, Inc.; Tanon 5200 Multi; Tanon Science and Technology Co., Ltd.).

Table III. RT-qPCR primer sequences.

Gene	Primer use/direction	Primer sequences (5'-3')
miR-301a	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGAC[GCTTTG]
miR-301a	Forward	GCCAGTGAATAGTATTGTCAAAG
miR-301a	Reverse	GTGCAGGGTCCGAGGT
U6	Forward	CGCTTCGGCAGCACATATAC
U6	Reverse	CAGGGGCCATGCTAATCTT

For the miR-301a RT primer, the sequence inside square brackets indicates the region specifically complementary to the 3' end of mature miR-301a. RT-qPCR, reverse transcription-quantitative PCR; F, forward; R, reverse; miR, microRNA.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. FreeZol Reagent (cat. no. R711-01; Vazyme Biotech Co., Ltd.) was used to extract the total RNA from human pancreatic cancer cell lines (AsPC-1, BxPC-3, CFPAC-1, MIA PaCa-2 and PANC-1) according to the instructions, and the RNA concentration was determined and reverse transcribed following the manufacturer's protocol (*Evo M-MLV* RT Kit for qPCR; cat. no. AG11707; Hunan Accurate Biotechnology Co., Ltd.) into complementary DNA (cDNA), which was then amplified using cDNA as a template. Specific RT-qPCR experiments were performed to detect miR-301a expression using a universal high-specificity miRNA quantitative PCR kit (MonAmp™ miRNA Universal Super Specificity qPCR Mix; cat. no. MQ00901; Monad Biotech Co., Ltd.) according to the manufacturer's protocol. The following thermocycling conditions were performed: Initial denaturation at 95°C for 10 min; followed by 40 cycles of denaturation at 95°C for 10 sec and combined annealing/extension at 60°C for 30 sec (two-step PCR). U6 was used as an internal reference and the relative expression was calculated using the $2^{-\Delta\Delta C_q}$ method (35). All RT-qPCR primers were designed against *Homo sapiens* (human) genes and synthesized by Anhui General Bioscience. The primer sequences are listed in Table III.

Cytotoxicity assay. AsPC-1, BxPC-3, CFPAC-1, MIA PaCa-2 and PANC-1 cells were used to validate the hypoxic effect on gemcitabine resistance. The MIA PaCa-2, PANC-1 and BxPC-3 and cells were used to validate the effect of miR-301a on gemcitabine resistance. After the cells were transfected with the miR-301a inhibitor for 24 h, they were digested with trypsin and resuspended in a 96-well plate (5×10^3 cells/well). After 6 h of adherence, the GEM drug (Eli Lilly and Company) at various concentrations (0–50 μ M; 0 μ M as the control group) was added. At 48 h after drug addition, the original culture medium was discarded and fresh medium containing 10% Cell Counting Kit-8 (CCK-8; cat. no. GK10001; GLPBIO Technology LLC) was added to each well, followed by incubation at 37°C for 1 h. The absorbance at 450 nm was measured using a microplate reader to calculate the cell viability. Cell viability = $[(As-Ab)/(Ac-Ab)] \times 100\%$ [As, experimental wells (containing cells, medium, CCK-8 solution and drug solution); Ac, control wells (containing cells, medium and CCK-8 solution, but not containing drugs); Ab, blank wells (containing medium and CCK-8 solution, but not containing cells and drugs)].

Isolation and identification of exosomes. The PANC-1 cells were used for the isolation and identification of exosomes.

Extraction and identification of exosomes using ultracentrifugation. Supernatants from cells treated under hypoxic and normoxic conditions were collected, filtered through a 0.22 μ m filter and transferred into 50 ml centrifuge tubes. The tubes were centrifuged at 300 x g for 10 min at 4°C to remove the cells. The resulting supernatants were centrifuged at 4°C and 3,000 x g for 30 min to eliminate cellular debris. The clarified supernatants were transferred into ultracentrifuge tubes and centrifuged at 10,000 x g for 40 min at 4°C. The supernatants from this step were subsequently transferred into new ultracentrifuge tubes and centrifuged at 100,000 x g at 4°C for 2 h. After ultracentrifugation, the supernatants were discarded and the precipitate was resuspended in 100 μ l PBS (cat. no. BL302A; Biosharp Life Sciences) to obtain exosomes. The isolated exosomes were stored at -80°C for subsequent experiments.

Transmission electron microscopy (TEM) was used to observe the morphology and size of the exosomes, with the sample preparation steps as follows: Exosome pellets were first fixed with 2.5% glutaraldehyde (in 0.1 M PBS, Ph 7.4) at 4°C for 1 h, then post-fixed with 1% osmium tetroxide at 4°C for 2 h. After dehydration using gradient concentrations of ethanol (50, 70, 90 and 100%), the samples were embedded in Epon 812 epoxy resin and incubated at room temperature overnight, before being cut into 70–100 nm ultrathin sections. The sections were then stained with 2% uranyl acetate at room temperature for 10 min, followed by 2% lead citrate at room temperature for 10 min. Nanoparticle tracking analysis (NTA) was used to detect the distribution of exosome sizes.

Exosome uptake experiment. The hypoxia-derived exosome pellet was resuspended in PBS and labelled with the membrane phospholipid dye PKH67 (green; cat. no. PKH67GL-1KT; MilliporeSigma) according to the manufacturer's protocol. Exosomes were incubated with 4 μ l of PKH67 at room temperature for 5 min and the reaction was terminated by adding 10% FBS. Unbound dye was removed by ultracentrifugation at 100,000 x g at 4°C for 2 h and labelled exosomes were resuspended in serum-free DMEM. Normoxic PC cells were treated with PKH67-labelled hypoxic exosomes for 12 h at 37°C. Following co-culture, the cells were fixed at room temperature and stained with DAPI (cat. no. C1002; Beyotime Institute of Biotechnology) at room temperature for 5 min to

visualize the cell nuclei. Images of the stained samples were captured under a laser confocal microscope.

Bioinformatics analysis. TargetScan (targetscan.org/vert_80/) and starBase (rnasysu.com/encori/) databases were used to predict the binding sites between miRNAs and the target genes. TargetScan relies on conserved seed matches in the 3'-UTR of genes annotated in RefSeq and University of California, Santa Cruz genome alignments. StarBase integrates 2,725 cross-linking and immunoprecipitation (CLIP)-seq datasets [e.g., photoactivatable ribonucleoside-enhanced-CLIP, a modified CLIP technique that uses photoactivatable nucleosides to improve the accuracy of mapping RNA-protein interaction sites; and high-throughput sequencing-CLIP, an early CLIP derivative that combines cross-linking immunoprecipitation with high-throughput sequencing to identify genome-wide RNA targets of RNA-binding proteins] and 100 degradome-seq datasets to prioritize experimentally supported miRNA-target interactions.

Statistical analysis. GraphPad Prism (version 9.5.0; Dotmatics) was used for statistical analysis and plotting. All the experiments were repeated three times. The quantitative data that conformed to a normal distribution are expressed as the means $\pm$ SE. An unpaired t-test was used for comparisons between two groups. For >2 groups one-way ANOVA was conducted first, followed by two post-hoc tests based on comparison goals: Tukey's honestly significant difference test for pairwise comparisons among all groups (to detect differences between every pair of groups), and Dunnett's test for pairwise comparisons between each experimental group and the control group (to focus on control-centred differences and reduce type I errors). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Hypoxia induces a substantial accumulation of HIF-1 α in PC cells. A physically hypoxic environment was used to establish a hypoxic model. PC cells (AsPC-1, BxPC-3, CFPAC-1, MIA PaCa-2 and PANC-1) were cultured under hypoxic conditions for 24 and 48 h, with normoxic conditions serving as the baseline. Detection of basal HIF-1 α under normoxia serves two key purposes: i) Baseline control to establish the natural expression level of HIF-1 α in oxygen-rich conditions, as even low, non-inducible HIF-1 α levels can occur in certain cell types (such as HCT116, human colorectal cancer cells and CFPAC-1 cells) under normoxia (36); and ii) hypoxia model validation, by comparing normoxic and hypoxic samples, the present study confirmed that observed HIF-1 α upregulation was hypoxia-induced and not an experimental artifact. Western blotting was used to detect the expression levels of HIF-1 α protein in PC cells under normoxic and hypoxic conditions. Compared with the results under normoxia, there was a notable accumulation of HIF-1 α protein in the PC cell lines after 24 and 48 h under hypoxic conditions (Fig. 1). Notably, HIF-1 α accumulation exhibited notable cell line-specific dynamics: BxPC-3, CFPAC-1, MIA PaCa-2 and PANC-1 cells displayed marked HIF-1 α induction at 24 h, with levels plateauing or decreasing marginally by 48 h.

AsPC-1 cells represented an outlier, with HIF-1 α expression increasing from 24 to 48 h.

Morphological changes in PC cells under hypoxic conditions. PC cells (AsPC-1, BxPC-3, CFPAC-1, MIA PaCa-2 and PANC-1) were cultured under normoxic conditions or hypoxic conditions for 24 and 48 h and their morphologies were examined under an inverted microscope. Compared with the normoxic state observations, the BxPC-3 and CFPAC-1 cells under hypoxic conditions displayed notable morphological changes characterized by a shift from an epithelial morphology (oval shape with indistinct cell boundaries) to a mesenchymal morphology (a spindle-shaped morphology with well-defined cell margins). By contrast, AsPC-1, MIA PaCa-2 and PANC-1 cells presented minimal morphological alterations under hypoxia. While AsPC-1 and MIA PaCa-2 cells occasionally demonstrated subtle cellular rounding or cytoplasmic vacuolization at 48 h, potentially reflecting hypoxic stress or early apoptotic changes, these changes did not represent a fully mesenchymal phenotype and lacked the elongated, spindle-shaped morphology observed in BxPC-3 and CFPAC-1 cells (Fig. 2).

Hypoxia upregulates the expression levels of miR-301a and enhances GEM resistance in PC cells. The expression level of miR-301 was detected through RT-qPCR experiments, which revealed that miR-301a in PC cells under hypoxic conditions was significantly upregulated compared with that in the normoxic group (Fig. 3A-E). Notably, MIA PaCa-2 and BxPC-3 cells presented a unique temporal pattern of miR-301a expression. Under hypoxia, the miR-301a levels significantly increased at 24 h [1.4-fold in MIA PaCa-2 cells ($P < 0.01$) and 1.8-fold in BxPC-3 cells ($P < 0.001$; Fig. 3A and D). However, by 48 h, the expression had declined to baseline levels.

CCK-8 cytotoxicity assays were employed to measure the cell viability in both the hypoxic and normoxic treatment groups following their exposure to various concentrations of GEM. The results (Fig. 3F-J) revealed that the cell viability of the hypoxic treatment group was significantly greater compared with that of the normoxic group at different concentrations ($P < 0.05$).

Hypoxia increases the number of exosomes and facilitates their transfer to normoxic cells. After the hypoxia model was established, PANC-1 cells were selected for exosome experiments due to their robust miR-301a upregulation under hypoxia. As shown in Fig. 3B, PANC-1 cells exhibited a 2.18-fold increase in miR-301a expression at 48 h compared with normoxia ($P < 0.0001$), which makes PANC-1 cells ideal for modelling exosome-mediated miR-301a transfer. Equal numbers of PANC-1 cells were cultured under normoxic and hypoxic conditions for 48 h. Exosomes from the supernatants of normoxic and hypoxic PANC-1 cells were collected and identified. TEM was used to observe the morphology of the isolated exosomes, which revealed that both normoxic and hypoxic PANC-1-derived exosomes exhibited a characteristic cup-shaped double-layered membrane structure (Fig. 4A). NTA was used to analyse the size distribution of the exosomes and the findings revealed that the concentration of exosome particles in the 'Hypoxia-Exos' group was significantly greater

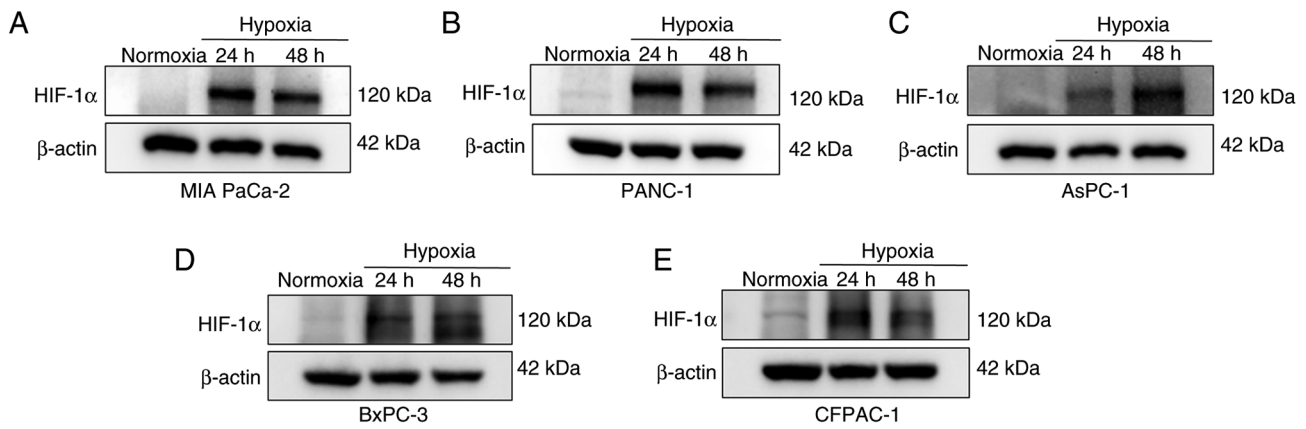


Figure 1. Hypoxia induces a substantial accumulation of HIF-1 α in PC cells. Western blotting was used to detect the expression levels of HIF-1 α protein in PC cell lines: (A) MIA PaCa-2, (B) PANC-1, (C) AsPC-1, (D) BxPC-3 and (E) CFPAC-1 after culture under normoxia conditions and hypoxia conditions for 24 and 48 h. HIF-1 α , hypoxia inducible factor-1 α ; PC, pancreatic cancer; CFPAC-1, cystic fibrosis pancreatic adenocarcinoma; AsPC-1, ascites pancreatic cancer 1; MIA PaCa-2, malignant inflammatory adenocarcinoma pancreatic carcinoma-2.

compared with that in the 'Normoxia-Exos' group ($P < 0.0001$) and that this secretion could be inhibited ($P < 0.0001$) by the exosome inhibitor GW4869 (Fig. 4B and C).

To investigate intercellular exosome transfer, hypoxic PANC-1-derived exosomes were labelled with the membrane dye PKH67 and co-cultured with normoxic PANC-1 recipient cells for 12 h. Laser confocal microscopy demonstrated that hypoxic-derived exosomes could be endocytosed by normoxic PANC-1 cells (Fig. 4D). After the collected normoxic and hypoxic-derived exosomes were co-cultured with normoxic PANC-1 cells for 24 h, RT-qPCR was performed to detect the relative expression level of the miR-301a gene. The results revealed that the expression levels of miR-301a in the hypoxic Exos-treated group was significantly greater compared with that in the Normoxic Exos-treated group ($P < 0.001$; Fig. 4E). These results indicated that hypoxia induces an increase in the number of exosomes and facilitates the transfer of a large amount of miR-301a to normoxic cells.

Effect of miR-301a on GEM resistance in PC cells under normoxia. To explore the effect of hypoxia-derived exosomal miR-301a on normoxic PC cells, a miR-301a inhibitor was transfected into PC cells. Due to their high basal expression of miR-301a, MIA PaCa-2, PANC-1 and BxPC-3 cells were selected for these experiments and RT-qPCR was used to verify the knockdown efficiency of miR-301a. The results demonstrated that the knockdown efficiency of miR-301a in the three cell lines was $>70\%$ ($P < 0.05$; Fig. 5A). Subsequently, CCK-8 cytotoxicity assays were performed to detect the resistance of PC cells to GEM in both the inhibitor NC group and the miR-301a inhibitor group. Notably, the GEM concentration gradients in Fig. 5B-D were tailored to the drug sensitivity of each cell line, as determined by preliminary dose-response experiments. The results indicated that, when the cells were treated with different drug concentrations, the viability of the cells in the miR-301a inhibitor group was lower compared with that of the cells in the inhibitor NC group ($P < 0.05$; Fig. 5B-D).

Regulatory mechanism of hypoxia-derived exosomal miR-301a on GEM resistance in normoxic PC cells. It has been

reported that ACSL4 is associated with GEM resistance (33). Further bioinformatics analysis revealed a potential binding site between miR-301a and ACSL4 (Fig. 6A). To determine whether GEM resistance in PC cells is enhanced by miR-301a through the regulation of ACSL4 expression, miR-301a mimics were first transfected into PC cells and the effect of miR-301a overexpression was verified using RT-qPCR. The results revealed that the expression levels of miR-301a were significantly increased in all three studied PC cell lines ($P < 0.05$; Fig. 6B). Furthermore, western blotting results indicated that when the expression level of miR-301a was upregulated, the protein expression level of ACSL4 also increased significantly (Fig. 6C-E). Conversely, when miR-301a expression was inhibited, the ACSL4 protein level decreased markedly (Fig. 6F and G). These results suggested that hypoxia-derived exosomal miR-301a further influences the GEM resistance of PC cells by regulating the expression level of ACSL4 under normoxic conditions.

Discussion

PC is a highly malignant tumour of the digestive system, with persistently high incidence and mortality rates. As a first-line chemotherapy drug for PC, GEM has been widely used in clinical practice, yet the emergence of drug resistance has notably limited its efficacy (8,37). In recent years, the role of the TME, especially the hypoxic microenvironment, in tumour chemotherapy resistance has attracted increasing attention (38). Previous studies have reported that the hypoxic microenvironment increases glucose metabolism and pyrimidine synthesis in tumour cells by inducing the upregulation of HIF-1 α , thereby leading to the resistance of PC cells to GEM (39). Yoo *et al* (40) reported that hypoxia can also regulate metabolic reprogramming through HIF-2 α -mediated generation of SLC1A5 variants, further enhancing the resistance of PC cells to GEM. In the present study, a hypoxic model for PC cells was established using physical hypoxia and the effectiveness of the model was validated by detecting the level of HIF-1 α protein. Compared with that under normoxia, the protein level of HIF-1 α was markedly elevated after

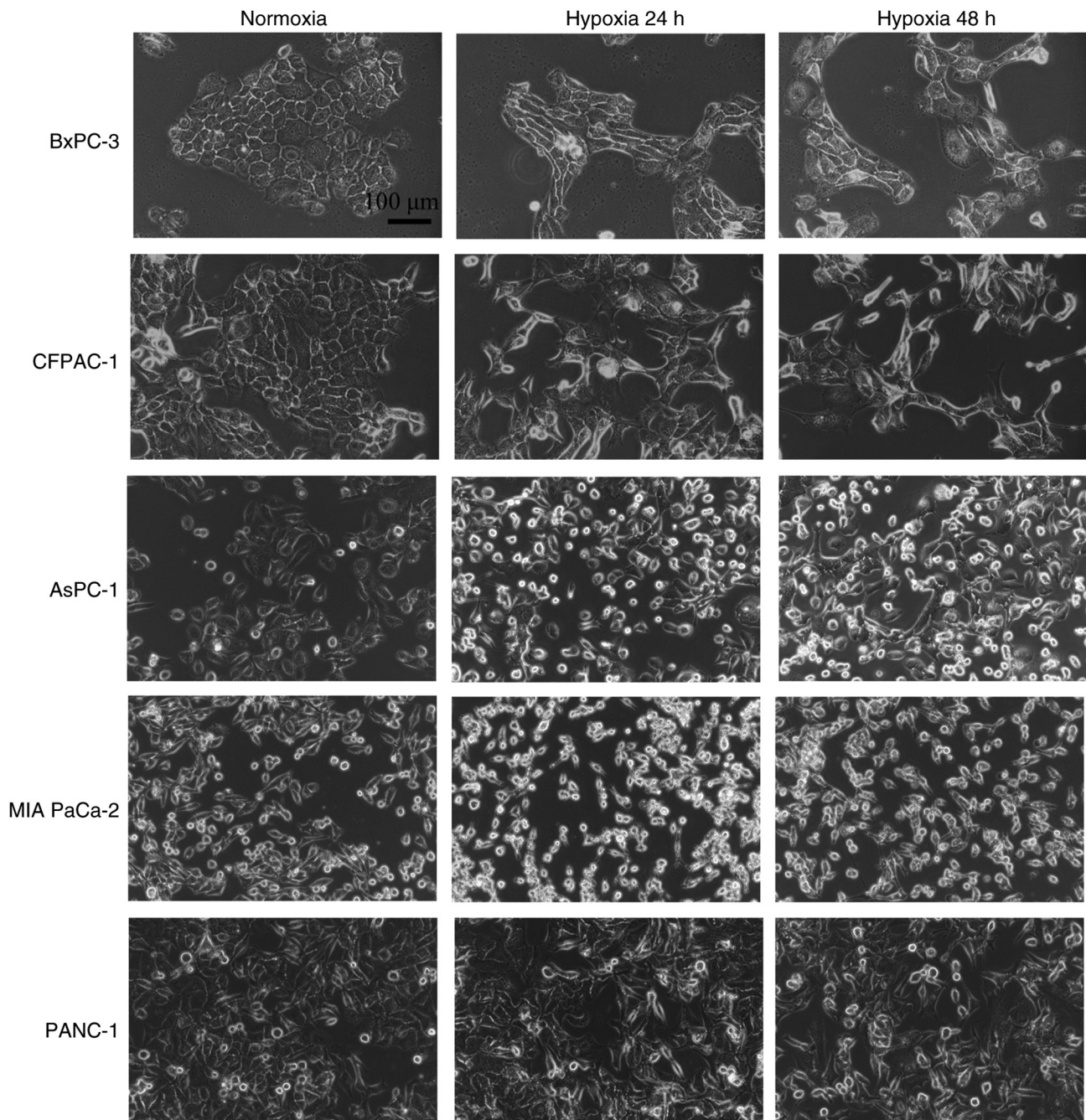


Figure 2. Morphological changes of PC cells under hypoxic conditions. The morphologies of BxPC-3, CFPAC-1, AsPC-1, MIA PaCa-2 and PANC-1 cells were observed under an inverted microscope (magnification, x20; scale bar, 100 μ m) following culture under normoxic and hypoxic conditions for 24 and 48 h. PC, pancreatic cancer; CFPAC-1, cystic fibrosis pancreatic adenocarcinoma; AsPC-1, ascites pancreatic cancer 1; MIA PaCa-2, malignant inflammatory adenocarcinoma pancreatic carcinoma-2.

hypoxia treatment for 24 and 48 h. Additionally, morphological changes in PC cells were observed under hypoxic conditions. Further evaluation of cytotoxicity through CCK-8 assays revealed that hypoxia treatment significantly reduced the sensitivity of PC cells to GEM, which was consistent with previously reported findings that hypoxia promoted chemotherapy resistance in tumours (41).

Exosomes can carry various bioactive molecules, such as proteins, lipids and nucleic acids (for example, mRNAs, miRNAs), and participate in intercellular communication and material exchange. In various disease models, hypoxic stimulation not only leads to an increase in the secretion of

exosomes but also causes alterations in their contents, which further affects the biological effects on recipient cells. For example, in pancreatic neuroendocrine tumours (17) and chondrosarcomas (18,42), the hypoxic microenvironment induces the production of several exosomes (such as carcinoembryonic antigen-related cell adhesion molecule 5, long non-coding RNA RAMP2 antisense RNA 1) by cells, resulting in M2 polarization of tumour-associated macrophages, which in turn promotes tumour invasion and metastasis. Furthermore, Liu *et al* (19) reported that the number of exosomes derived from hypoxic bone marrow mesenchymal stem cells increased and could enhance fracture healing.

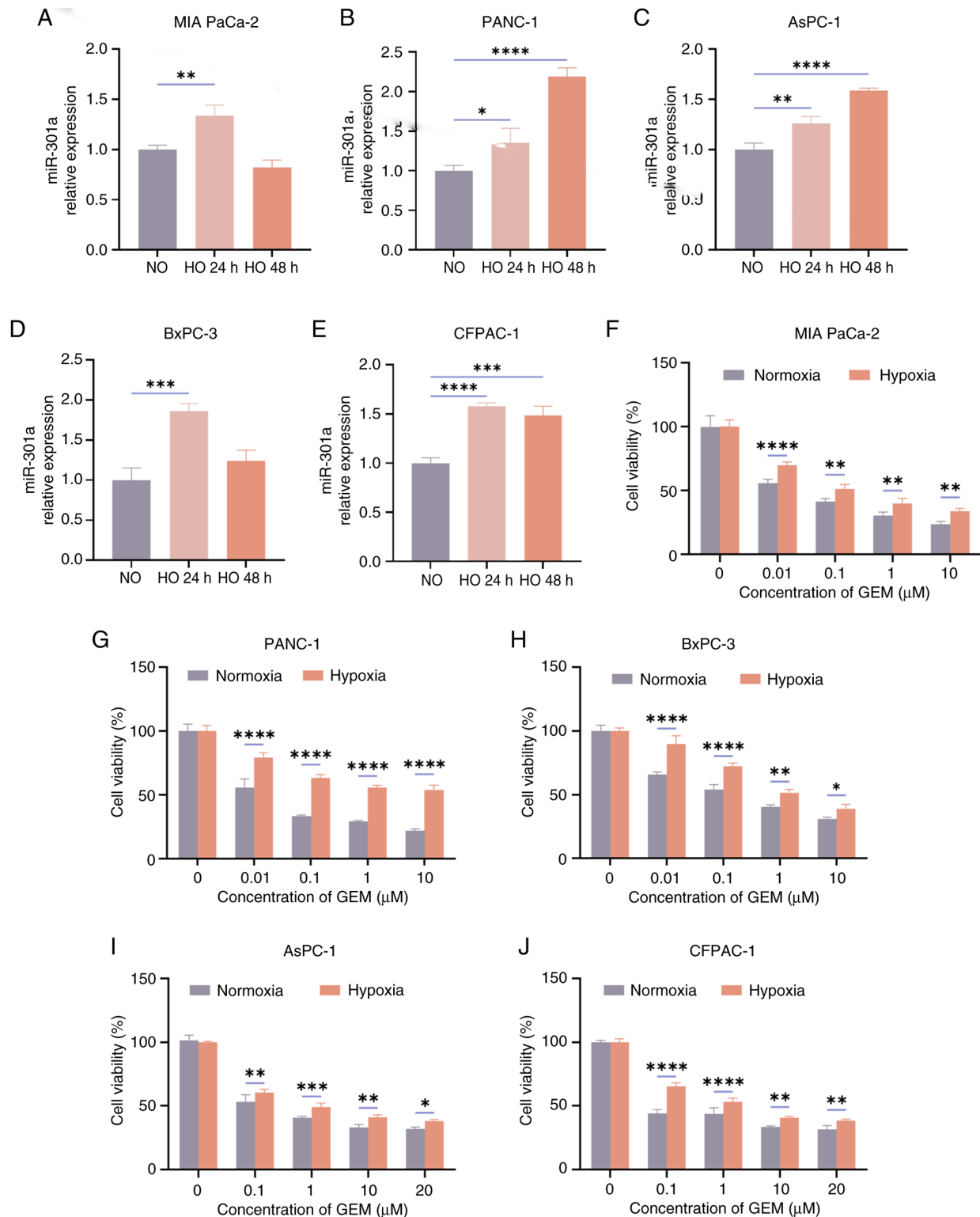


Figure 3. Hypoxia upregulates the expression levels of miR-301a and enhances GEM resistance in PC cells. (A-E) Relative expression levels of miR-301a in PC cell lines [(A) MIA PaCa-2, (B) PANC-1, (C) AsPC-1, (D) BxPC-3 and (E) CFPAC-1] were detected using RT-qPCR following culture under normoxia conditions and hypoxic conditions for 24 and 48 h. (F-J) Cell viability of PC cells [(F) MIA PaCa-2, (G) PANC-1, (H) BxPC-3, (I) AsPC-1 and (J) CFPAC-1] in both hypoxic and normoxic treatment groups was measured using CCK-8 cytotoxicity assays upon exposure to various concentrations of GEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. NO, normoxic; HO, hypoxic; PC, pancreatic cancer; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; CCK-8, Cell Counting Kit-8; GEM, gemcitabine; CFPAC-1, cystic fibrosis pancreatic adenocarcinoma; AsPC-1, ascites pancreatic cancer I; MIA PaCa-2, malignant inflammatory adenocarcinoma pancreatic carcinoma-2.

Exosomes released by tumour cells serve key roles in tumour progression and drug resistance, particularly through their small RNA molecules, such as miRNAs, which can

regulate the expression levels of target genes and influence the biological characteristics of cells. Victor Ambros and Gary Ruvkun were awarded the Nobel Prize in 2024 for their

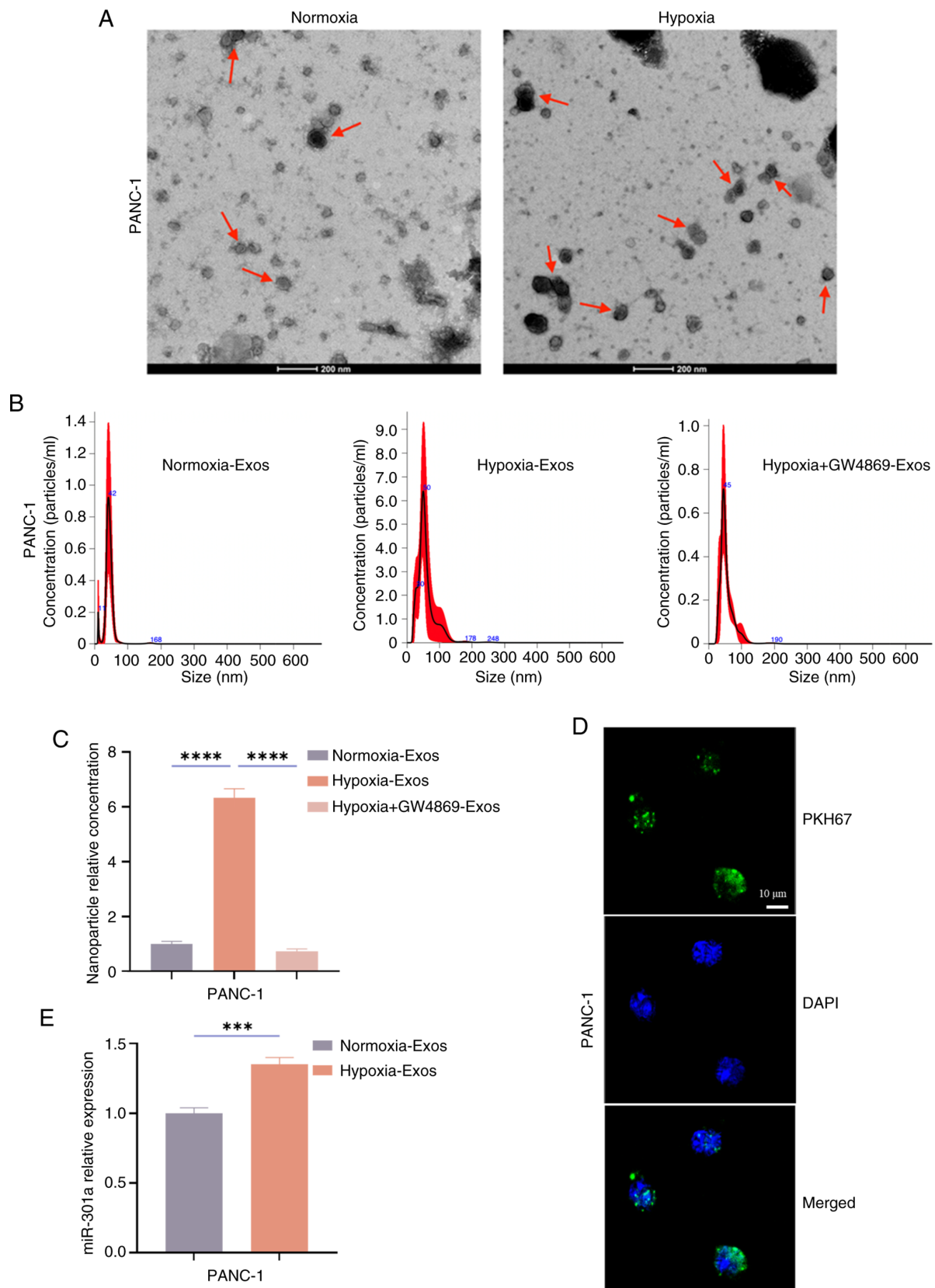


Figure 4. Hypoxia induces an increase in exosomes and facilitates their transfer to normoxic cells. (A) The exosomes from PANC-1 cells cultured under normoxic and hypoxia condition for 48 h were collected and their morphology was observed by TEM; red arrows indicate the exosomes (scale bar, 200 nm). (B and C) PANC-1 cells were cultured under normoxic and hypoxia condition for 48 h and exosome inhibitor GW4869 was added to hypoxia culture, exosomes were collected and NTA was used to analyse the relative particle concentration in the Normoxia-Exos, Hypoxia-Exos and Hypoxia + GW4869-Exos group. (D) Hypoxic PANC-1-derived exosomes were collected and labelled with the membrane phospholipid dye PKH67. After co-culture with normoxic PANC-1 cells for 12 h, laser confocal microscopy was employed to observe the endocytosis of labelled exosomes by normoxic PANC-1 cells (scale bar, 10 μ m). (E) Following co-culturing the collected normoxic and hypoxic-derived exosomes with normoxic PANC-1 cells for 24 h, RT-qPCR was performed to detect the relative expression level of the miR-301a. *** P <0.001 and **** P <0.0001. TEM, transmission electron microscopy; NTA, nanoparticle tracking analysis; RT-qPCR, reverse transcription-quantitative PCR; miR, microRNA.

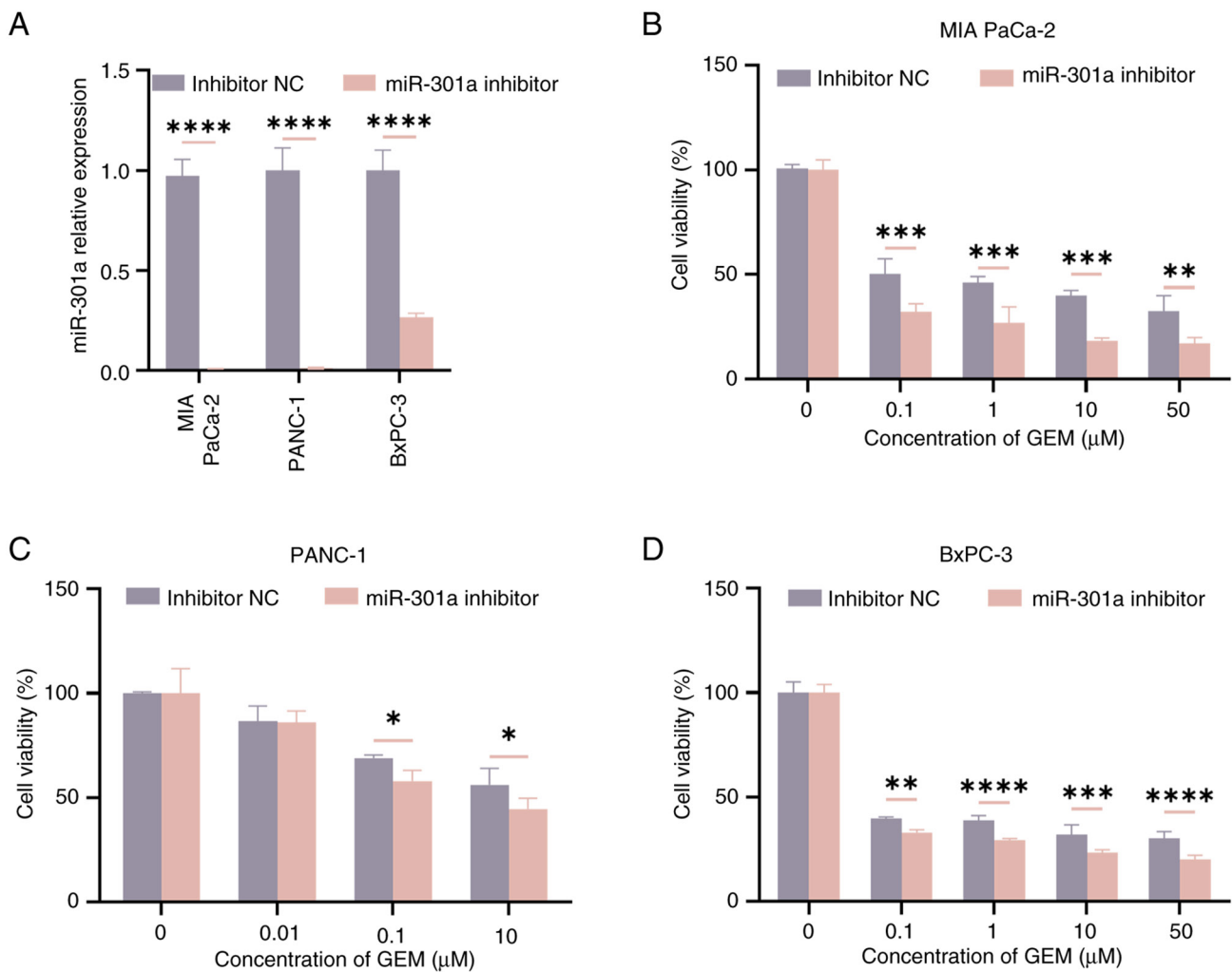


Figure 5. Regulatory mechanism of hypoxia-derived exosome miR-301a on GEM resistance in normoxic PC cells. (A) PC cells were transfected with inhibitor NC and miR-301a inhibitor for 24 h and then the relative expression level of miR-301a was detected by RT-qPCR. (B) MIA PaCa-2, (C) PANC-1, and (D) BxPC-3 cells were treated with a gradient concentration of GEM for 48 h. CCK-8 cytotoxicity assays were conducted to detect cell viability in both the inhibitor NC group and the miR-301a inhibitor group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. GEM, gemcitabine; miR, microRNA; PC, pancreatic cancer; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; CCK-8, Cell Counting Kit-8.

discovery of miRNAs and their role in post-transcriptional gene regulation (43), which underscores the importance of miRNAs in intercellular communication and gene expression regulation and provides a theoretical foundation for the present study experiments. Zhao *et al* (44) demonstrated that exosomal miR-934 can induce M2 polarization of macrophages, thereby promoting liver metastasis of colorectal cancer. Furthermore, exosomal miR-522 secreted by cancer-associated fibroblasts in the tumour microenvironment is involved in chemotherapy resistance in gastric cancer (45). Through a literature review, it was found that miR-301a is differentially expressed in a variety of malignant tumours and affects tumorigenesis and development through different regulatory mechanisms. A previous study has demonstrated that miR-301a is abnormally upregulated in breast cancer and further promotes breast cancer cell proliferation, metastasis, and cell cycle progression through the cytoplasmic polyadenylation element binding protein 1/sirtuin 1/Sox2 axis, accelerating the malignant progression of breast cancer (21). Furthermore, miR-301a can promote the proliferation of prostate cancer cells by inhibiting the expression of p21 and SMAD4 (46). Qi *et al* (47) demonstrated that miR-301a

is highly expressed in PC. Rather than verifying or further exploring the differential expression and regulatory mechanisms of miR-301a itself, the present study focused on the impact and underlying mechanisms of exosomal miR-301a on GEM drug resistance in PC under hypoxic conditions, which aimed to provide a novel strategy for PC treatment. These results indicated that the hypoxic microenvironment markedly enhanced the resistance of PC cells to GEM, accompanied by a marked increase in exosome secretion and notable upregulation of miR-301a expression. Notably, MIA PaCa-2 and BxPC-3 cells presented a unique temporal pattern of miR-301a expression. Under hypoxia, miR-301a levels increased significantly at 24 h. However, by 48 h, expression had declined to baseline levels. A literature review revealed that HIF-2 α can regulate the expression levels of miR-301a in PC cells under hypoxic conditions (48). Furthermore, compared with that in the 24 h hypoxic treatment, the protein level of HIF-2 α decreases at 48 h of hypoxic treatment. Therefore, it can be hypothesized that the upregulation of miR-301a expression in MIA PaCa-2 and BxPC-3 cell lines at 24 h and its downregulation at 48 h are due to the regulation of HIF-2 α . Our research

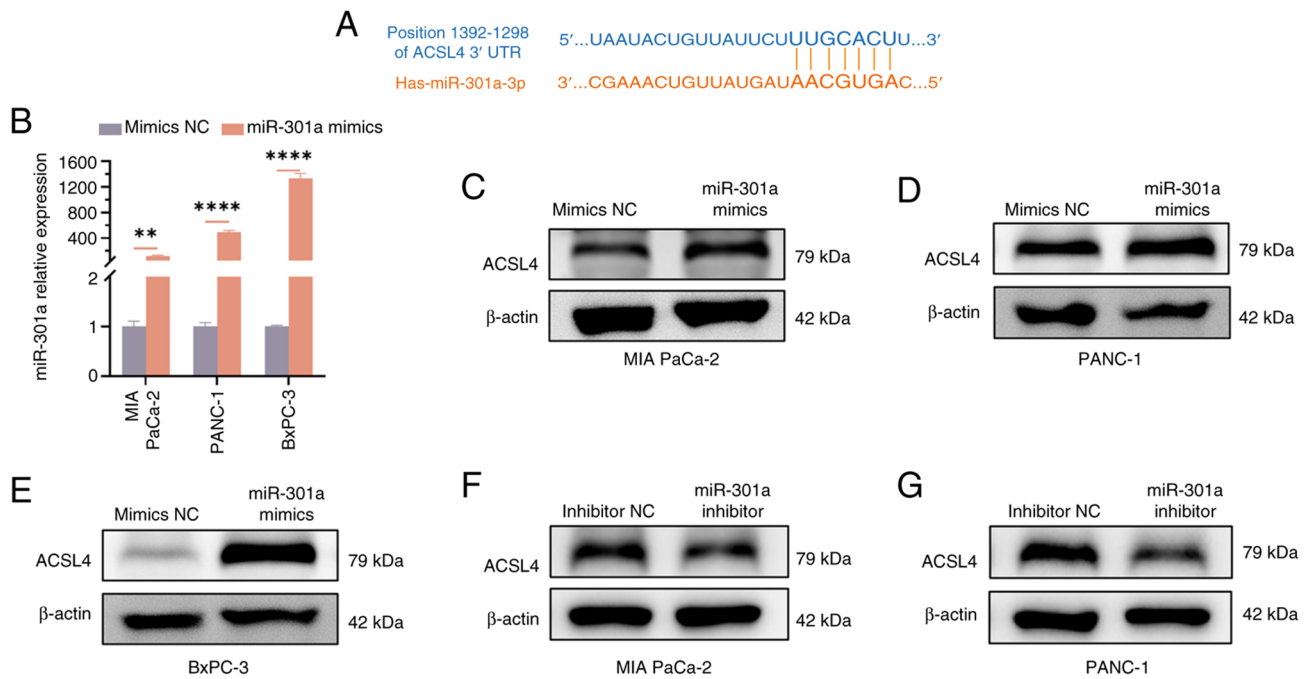


Figure 6. Regulatory mechanism of hypoxia-derived exosome miR-301a on GEM resistance in normoxic PC cells. (A) Target binding sites between miR-301a and ACSL4 were predicted using bioinformatics databases (TargetScan, and starBase). (B) PC cells were transfected with mimics NC and miR-301a mimics for 24 h and then the relative expression level of miR-301a was detected by RT-qPCR. ACSL4 protein expression was detected by western blotting after miR-301a overexpression in (C) MIA PaCa-2 cells, (D) PANC-1 cells and (E) BxPC-3 cells. Following miR-301a knockdown in (F) MIA PaCa-2 and (G) PANC-1 cells, the expression level of the ACSL4 protein was detected by western blotting. **P<0.01 and ****P<0.0001. GEM, gemcitabine; miR, microRNA; PC, pancreatic cancer; NC, negative control; ACSL4, acyl-CoA synthetase long-chain family member 4.

group previously reported that miR-301a expression was markedly increased in hypoxic exosomes (25). Using exosome co-culture experiments, it was verified that the number of hypoxia-induced exosomes increased and that these exosomes could be delivered to normoxic PC cells. To explore the role of exosomal miR-301a, miR-301a knockdown experiments were conducted and the key role of miR-301a in the resistance of PC to GEM was confirmed. Compared with the NC group, miR-301a knockdown markedly reduced the resistance of normoxic PC cells to GEM. These findings suggested that the upregulation of miR-301a in hypoxic-derived exosomes is one of the key mechanisms underlying GEM resistance in normoxic PC. According to previous studies, miRNAs primarily act by binding to the 3'-UTR of mRNAs, inducing mRNA degradation or inhibiting their translation, thereby downregulating the expression of target genes. In certain cases, they bind to the 5'-UTRs of mRNAs to positively regulate target genes.

Previous studies have reported that miRNAs can participate in disease progression by targeting and regulating ACSL4 expression. For example, miR-23a-3p inhibits ferroptosis in hepatocellular carcinoma by targeting ACSL4, thereby participating in the resistance of hepatocellular carcinoma cells to sorafenib (31). Shi *et al* (32) reported that miR-20a-5p alleviates renal ischaemia/reperfusion injury by inhibiting ACSL4 expression. Other studies have demonstrated that ACSL4 is also involved in PC resistance to GEM, but the specific mechanism has not been fully elucidated (33,49). In the present study, using bioinformatics analysis, potential binding sites between miR-301a and ACSL4 were identified, which led to the hypothesis that miR-301a acts by binding to the 3'-UTR of ACSL4 mRNA. However, validation experiments revealed

that miR-301a overexpression increased ACSL4 protein levels, whereas miR-301a knockdown decreased ACSL4 expression, findings that contradict the traditional negative regulatory pattern of miRNAs. It was speculated that miR-301a might bind to the 5'-UTR of ACSL4 mRNA, but after consulting the bioinformatics databases, it was revealed that there was no binding site for miR-301a on the 5'-UTR of ACSL4 mRNA. Based on these results, the present study proposes that miR-301a may not directly target ACSL4 but instead it regulates ACSL4 through an intermediate molecule, which provides a novel direction for future research. Subsequent studies could focus on identifying this potential intermediate molecule, with the aim of uncovering novel therapeutic strategies and targets for PC and other tumours.

In summary, a key mechanism was revealed in the present study, whereby hypoxia-induced upregulation of miR-301a is transferred to normoxic PC cells via exosomes and contributes to GEM resistance by regulating ACSL4. This finding not only provides novel insights into the molecular mechanisms underlying GEM resistance in PC but also offers potential targets for the development of novel therapeutic strategies against PC in the future. The specific interaction mechanism between miR-301a and ACSL4, as well as potential treatment approaches targeting this axis, remains to be explored in future research.

Notwithstanding the aforementioned findings, the present study has certain limitations to note. First, the intermediate molecule mediating miR-301a-induced regulation of ACSL4 was not identified, leaving the exact regulatory cascade unresolved. Second, *in vivo* validation using animal models (such as orthotopic PC xenografts) is lacking, which limits the translational

relevance of the *in vitro* results. Finally, no clinical specimens were analysed to determine the association of miR-301a/ACSL4 expression with GEM response or patient outcomes, thus hindering evaluation of their clinical potential as biomarkers or targets. Addressing these limitations in future work will help strengthen the clinical applicability of the current findings.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

WQ performed all cell culture, exosome isolation and molecular biology experiments, including RT-qPCR, western blot and CCK-8 assays. WQ conducted data analysis, prepared the initial data summaries and drafted the manuscript. WaT conducted bioinformatics prediction and analysis on the potential target genes of miR-301a, and performed subsequent experimental validation using western blotting, while MY provided key reagents, including PC cell lines and exosome inhibitors and offered expertise in hypoxia modelling. WeT and MY participated in data analysis and figure preparation. WeT made substantial contributions to the analysis and interpretation of experimental data (specifically verifying the reliability of exosome isolation efficiency and miR-301a detection data), revised the key intellectual content of the manuscript, and reviewed the consistency of experimental descriptions and data presentation. LZ designed and conducted supplementary experiments (including CCK analysis for BxPC-3, AsPC-1 and CFPAC-1 cells treated with GEM under normoxia/hypoxia) to address reviewer feedback, obtained funding and helped respond to reviewers, including clarifying methodologies and data interpretation. GL conceived and designed the present study, supervised all experimental work and interpreted the results, oversaw the overall validation of data integrity and the accuracy of research outcomes. GL revised the manuscript for intellectual content and ensured the integrity of the data and the accuracy of the findings. WQ and GL confirm the authenticity of all the raw data. All authors 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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