

Long non-coding RNA CUDR promotes malignant phenotypes in pancreatic ductal adenocarcinoma via activating AKT and ERK signaling pathways

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Abstract. Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies, with a marked potential for invasion and metastasis. Emerging evidence has suggested that dysregulation of long non-coding RNAs (lncRNAs) is associated with the development of multiple types of cancer. However, the function of lncRNAs in PDAC is poorly known. In the present study, a microarray assay was used to screen for differently expressed lncRNAs in PDAC and it was identified that cancer upregulated drug resistance (CUDR) was upregulated in PDAC. CUDR increased PDAC cell proliferation, migration and invasion, inhibited apoptosis, and promoted drug resistance; it also regulated the PDAC cell epithelial-mesenchymal transition. The CUDR-induced PDAC malignant phenotypes is via the protein kinase B and extracellular-signal-regulated kinase signaling pathways. Downregulation of CUDR may be

a novel therapeutic strategy to prevent PDAC development and drug resistance in the future.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive human digestive malignancies and ranks fourth as the cause of cancer-associated mortality in the USA (1). In total, 90% of patients with PDAC are in an advanced stage at diagnosis, and ~80% are not eligible for radical resection because of tumor invasion of the adjacent organs or extensive metastasis (2). The remaining patients have a poor prognosis regardless of surgical status; their 5-year survival rate is <44%, and their mortality rate is ~100% (3,4). Therefore, an increased understanding of the molecular mechanisms that mediate PDAC proliferation, invasion and metastasis is urgently required.

Increasing evidence has suggested that long non-coding RNAs (lncRNAs) serve multiple functions in tumor initiation and development, and represent novel targets for cancer diagnosis and therapies (5,6). Several lncRNAs have been associated with pancreatic cancer and may be used as biomarkers of PDAC (7-9).

In the present study, a microarray assay was used to screen for differently expressed lncRNAs in PDAC. Furthermore, the expression of the lncRNA cancer upregulated drug resistance (CUDR) was identified in PDAC, and its biological function and underlying molecular mechanism were investigated in PDAC cells.

Materials and methods

Tissue samples. A total of 25 pairs of tumor tissue samples and matched paracarcinoma tissue samples were obtained from patients with PDAC (15 males and 10 females; average age,

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58.3±10.4 years; range, 35-76 years) between February 2012 and August 2013 from Changhai Hospital, Second Military Medical University (Shanghai, China). All patients or their relatives provided written informed consent for their tissues to be used for scientific research, and the present study was approved by the Committee on Ethics of Biomedicine, Second Military Medical University. None of the patients had received radiotherapy, chemotherapy or other antitumor therapy prior to surgery. All diagnoses were confirmed by two certified pathologists on the basis of pathological and/or cytological evidence. All specimens were immediately frozen with RNA-stabilizing agent (RNAlater®; Qiagen GmbH, Hilden, Germany) in liquid nitrogen at the time of surgery, and stored at -80°C until required.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues or cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RNA was reverse-transcribed into cDNA using a PrimeScript RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. The relative expression level of the targets was determined by qPCR using SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd. Thermocycling conditions: Following an initial denaturation for 30 sec at 95°C, 40 cycles of 5 sec at 95°C and 1 min at 60°C were performed, followed by a dissociation stage for 15 sec at 95°C, 1 min at 60°C and 15 sec at 95°C). The expression of CUDR in clinical samples was normalized to that of GAPDH. The relative level of CUDR expression in PDAC tissues (T) was compared with that in adjacent normal tissues (N), and the fold change of the target lncRNAs was calculated using the $2^{-\Delta\Delta C_q}$ method and was \log_2 -transformed [$\log_2(T/N)$] (10). Primers for RT-qPCR are presented in Table I. RT-qPCR for each sample was performed in triplicate.

lncRNA microarrays and analysis. Frozen PDAC tissues and matched adjacent non-tumor samples were sent to KangChen Biotechnology Co. Ltd. (Shanghai, China) for lncRNA extraction, quantity testing, lncRNA labeling and analysis. The Arraystar Human lncRNA Array version 2.0 was designed for profiling lncRNAs and protein-coding RNAs in the human genome. A total of 33,045 lncRNAs were collected from authoritative data sources including RefSeq (www.ncbi.nlm.nih.gov/refseq), University of California Santa Cruz Known Genes (www.genomaize.org), Ensembl (ensemblgenomes.org) and a number of associated references (11-14). A paired t-test was used to compare mean differences when the observations had been obtained in pairs. Hierarchical clustering analysis was performed using Cluster (version 3.0) and Java TreeView software (version 1.1.4r3) (both from Stanford University, Stanford, CA, USA). All the microarray data were published in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) under accession number GSE101094.

Cell culture and transfection. Human pancreatic cancer cell lines (Panc-1, CFPAC-1, Capan-1, Aspc-1, SW1990, BxPC-3, Miapaca-2 and HPAC) were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in RPMI-1640 medium or Dulbecco's modified

Eagle's medium (both from Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare, Logan, UT, USA) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). HPDE cells (provided by Professor Lixing Zhan, University of The Chinese Academy of Sciences, Shanghai, China) were cultured in a keratinocyte serum-free medium supplemented with 5 ng/ml epidermal growth factor (Invitrogen; Thermo Fisher Scientific, Inc.) and 50 mg/ml bovine pituitary extract (Gibco; Thermo Fisher Scientific, Inc.). All cell lines were cultured in a 37°C incubator with 5% CO₂. Plasmids and siRNAs were transfected into cells using FuGENE® HD transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's protocol. In order to inhibit the AKT or ERK activation, cells were treated with a specific AKT-activation inhibitor MK2206 (2 µM) for 24 h, or with a specific ERK-activation inhibitor PD98059 (10 µM) for 24 h before performing MTT, flow cytometric analysis, wound-healing, Transwell or western blot assays.

Stable cell line construction. The full-length cDNA of CUDR was synthesized, digested with *Bam*HI and *Eco*RI restriction enzymes, cloned into pBABE-puro plasmid (provided by Professor Lixing Zhan, University of The Chinese Academy of Sciences, Shanghai, China), and were validated by sequencing (Biosune Biotechnology Co. Ltd., Shanghai, China). RNA interference (RNAi) sequences were designed using the short hairpin (sh)RNA library of RNAi Codex (codex.cshl.edu). They were synthesized and cloned into the plko.1-puro retroviral expression vector (Addgene, Inc., Cambridge, MA, USA) using flanking *Eco*RI and *Age*I restriction sites. The sequences are presented in Table II.

The stable CUDR-overexpressing Panc-1 cell line (Panc-1-CUDR) and the control Panc-1 cell line (Panc-1-Control) were generated using retroviral vectors. Retrovirus was produced by transiently transfecting 293-GPG packaging cells seeded in 10-cm dishes with 10 µg pBABE-puro-CUDR vector or empty pBABE-puro vector. At 2 days after transfection, viral supernatant was collected, filtered and added to the Panc-1 cells. After 2 days, cells were selected with 2 mg/ml puromycin for 2 weeks. Stable CUDR-knockdown CFPAC-1 cell lines (CFPAC-1-shCUDR-1 and CFPAC-1-shCUDR-2) and the control cell line (CFPAC-1-shControl) were generated using lentiviral vectors. Lentivirus was produced by transiently transfecting 293T packaging cells seeded in 6-cm dishes with 2.7 µg packaging construct, 0.9 µg construct expressing vesicular stomatitis virus glycoprotein and 2.7 µg plko.1-puro-shCUDR-1, plko.1-puro-shCUDR-2 or empty plko.1-puro vectors, respectively. The media from the transfected cells were collected as the cells were re-fed each day for 3 or 4 days. The virus-containing media were pooled, filtered and added to the CFPAC-1 cells. After 2 days, cells were selected with 2 mg/ml puromycin for 2 weeks.

MTT proliferation assay. Cells were seeded in 96-well plates at a density of 2,000 cells/well in triplicate and incubated overnight. MTT solution (20 µl at 5 mg/ml; Sigma; Thermo Fisher Scientific, Inc.) was added to each well and plates were incubated for 4 h at 37°C. The supernatant was aspirated and the formazan crystals that had formed were dissolved by the

Table I. Primer sequences used.

Gene	Forward primer	Reverse primer
CUDR	5'-CCCCAACTGATCAATCAACC-3'	5'-TGGAGAGATGATGGGACTCA-3'
E-cadherin	5'-CTGCTGCAGGTCTCCTCTTG-3'	5'-TGTCGACCGGTGCAATCTTC-3'
N-cadherin	5'-GACAATGCCCCTCAAGTGTT-3'	5'-CCATTAAGCCGAGTGATGGT-3'
Vimentin	5'-AAGGCGAGGAGAGCAGGATT-3'	5'-GGTCATCGTGATGCTGAGAAG-3'
Slug	5'-TGATGAAGAGGAAAGACTACAG-3'	5'-GCTCACATATTCCTTGTCACAG-3'
ZEB1	5'-TGCACTGAGTGTGGAAGAGC-3'	5'-TGGTGATGCTGAAAGAGACG-3'
GAPDH	5'-TGTTCGACAGTCAGCCGCATCTTC-3'	5'-CATGTAGTTGAGGTCAATGAAGG-3'

CUDR, cancer upregulated drug resistance; E-cadherin, epithelial cadherin; N-cadherin, neuronal cadherin; ZEB1, zinc finger E-box-binding homeobox 1.

Table II. RNA interference sequences of CUDR.

shRNA symbol	Forward	Reverse
shCUDR-1	5'-CCGGGGTAATGTATCATCGGCTTAGCTCGA GCTAAGCCGATGATACATTACCTTTTTTG-3'	5'-AATTCAAAAAGGTAATGTATCATCGGCTT AGCTCGAGCTAAGCCGATGATACATTACC-3'
shCUDR-2	5'-CCGGGGAATACTATTCGTATGATGGCTC GAGCCATCATACGAATAGTATTCCTTTTTTG-3'	5'-AATTCAAAAAGGAATACTATTCGTATGAT GGCTCGAGCCATCATACGAATAGTATTC-3'

CUDR, cancer upregulated drug resistance; shRNA/sh, short hairpin RNA.

addition of 150 μ l dimethylsulfoxide. The absorbance was measured at 490 nm in a Biotek Elx800 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The mean values of 6 wells were calculated to determine cell proliferation every 24 h.

Wound-healing assay. PDAC cells were cultured until reaching 80% confluence. Scratches were made by a 96 wounding replicator (V&P Scientific, Inc., San Diego, CA, USA), and the medium was replaced with fresh RPMI-1640 medium containing 0.5% FBS. The cells were washed twice with PBS to remove the floating cells and were incubated with fresh RPMI-1640 medium containing 0.5% FBS. Images of the scratches were captured at 0, 8 and 24 h under a fluorescence microscope (IX71; Olympus Corporation, Tokyo, Japan) and wound-healing rates were determined.

Transwell migration and invasion assays. For the Transwell migration assay, PDAC cells were collected with serum-free medium and seeded into the upper chamber of 24-well Transwell dishes (Corning Incorporated, Corning, NY, USA) at an initial concentration of 4×10^4 cells/well, and the lower chamber was filled with culture medium containing 10% FBS as the chemoattractant. Following incubation at 37°C for 24 h, non-migrating cells were removed with a cotton swab, and the remaining cells were fixed, stained with crystal violet at 37°C for 10 min and images were captured under a fluorescence microscope (IX71; Olympus Corporation). The invasion assay was similar to the migration assay except that the Transwell insert was coated with Matrigel. The number of migratory and

invasive cells were determined in five randomly selected fields at x200 magnification.

Cell cycle and apoptosis analyses. For the cell cycle assay, cells were seeded in 6-well plates at a density of 2×10^5 cells/dish. Following incubation at 37°C for 48 h, cells were collected, mixed with 70% ice-cold ethanol and stored overnight at 4°C. DNA of the cells was then stained with propidium iodide (PI)/RNase staining buffer (0.5 mg/ml PI and 1 mg/ml RNase A; BD Biosciences, Franklin Lakes, NJ, USA). Following incubation at 37°C for 30 min in the dark, the cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). To investigate the effect on apoptosis, cells were seeded in 6-well plates at a density of 2×10^5 cells/dish. Following incubation at 37°C for 24 h, cells were treated with gemcitabine (GEM; 2 μ g/ml) or 5-fluorouracil (5-FU; 50 μ g/ml) for 48 h. PBS served as a negative control. The cells were harvested and stained with Annexin V-fluorescein isothiocyanate/PI (Trevigen, Inc., Gaithersburg, MD, USA) for 15 min in the dark at room temperature. Finally, the cell populations were analyzed using a FACSCalibur flow cytometer. Cells were treated in triplicate and assayed separately.

Immunofluorescence analyses. Cells were grown on coverslips and fixed with 4% paraformaldehyde according to the manufacturer's protocol. Following overnight incubation at 4°C with mouse anti-epithelial (E-)cadherin antibody (cat. no. ab76055; 1:200 dilution; Abcam, Cambridge, MA, USA), proteins were visualized by incubation with Alexa Fluor 647-conjugated donkey anti-mouse secondary antibody (cat. no. A31571;

1:50 dilution; Invitrogen; Thermo Fisher Scientific, Inc.). The coverslips were mounted with mounting medium containing DAPI (Prolong Gold Antifade Reagent; Invitrogen; Thermo Fisher Scientific, Inc.). Fluorescence was analyzed using confocal laser microscopy (Olympus Corporation).

Western blotting. Cells were harvested and lysed in radioimmunoprecipitation buffer (Cell Signaling Technology, Inc., Danvers, MA, USA), supplemented with a protease inhibitor cocktail and phenylmethylsulfonyl fluoride (both from Roche Molecular Diagnostics, Pleasanton, CA, USA). The protein concentration was assessed using the Quick Start™ Bradford protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's protocol. Equivalent amounts of proteins (40 µg) from each sample were separated by SDS-PAGE (10% gel) and then transferred onto pre-activated polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The blotted membranes were blocked in 5% fat-free milk in Tris-buffered saline (20 mM Tris/HCl, pH 7.6, 127 mM NaCl and 0.1% Tween-20) for 1 h at room temperature, and incubated with polyclonal primary antibodies against E-cadherin (cat. no. ab40772), neuronal (N)-cadherin (cat. no. ab76011), vimentin (cat. no. ab92547), Slug (cat. no. ab183760), zinc finger E-box-binding homeobox 1 (ZEB1; cat. no. ab203829) and GAPDH (cat. no. ab9485) (all from Abcam) (all at 1:1,000 dilution) at 4°C overnight, then with the goat anti-rabbit IgG secondary antibody (cat. no. 7074; 1:2,000 dilution; Cell Signaling Technology, Inc.) at room temperature for 1.5 h. The specific bands were visualized using an enhanced chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.) and were subjected to densitometric analysis using AlphaEaseFC software (version 3.1.2; Protein Simple, San Jose, CA, USA). GAPDH was used as a control.

Subcutaneous xenograft transplantation. Female nude mice (BALB/c, nu/nu, n=5 animals per group, four-week-old, 16–18 g) were purchased from the Shanghai Experimental Animal Center (Shanghai, China) and maintained under pathogen-free conditions (temperature, 26–28°C; air pressure, 0.65 cmH₂O; relative humidity, 40–60%; ventilation, 10–15 counts/h; 10-h light/14-h dark cycle; free feed and water intake). All animals were used in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute of Nutritional Sciences. Mice were injected subcutaneously into the right posterior flanks with 2×10⁶ Panc-1 cells (15). When the tumor reached a volume of ~100 mm³, mice were randomized into the indicated groups and subjected to treatment with 5-FU (5 mg/kg) intraperitoneally, MK2206 (20 mg/kg) by gastrogavage or PD98059 (10 mg/kg) by gastrogavage three times weekly. Tumor growth was determined every 5 days for a total of 35 days. At day 35, mice were sacrificed and images of tumors were captured. Tumor volume was determined using Vernier calipers [tumor volume = largest diameter × (smallest diameter)² × 0.5].

Statistical analysis. Statistical analysis was calculated using SPSS (version 19.0 software; IBM Corp., Armonk, NY, USA) and GraphPad Prism (version 6; GraphPad Software, Inc., La Jolla, CA, USA). Results are presented as the mean ± standard deviation. Student's t-test and analysis of variance were

used to compare quantitative variables. χ^2 test and Fisher's exact test were used to compare categorical variables. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CUDR is upregulated in PDAC tissues and cell lines. To explore the aberrantly expressed lncRNAs in PDAC, five pairs of human clinical samples were collected, and microarrays were used to analyze differentially expressed lncRNAs and mRNAs between PDAC tissues and matched adjacent non-tumor tissues. A total of 883 lncRNAs and 949 mRNAs were identified that were differentially expressed ($P < 0.05$; data not shown). Hierarchical clustering analysis of these aberrantly expressed lncRNAs and mRNAs exhibited systematic variations between pancreatic cancer tissues and adjacent non-tumor tissues (Fig. 1A and B). As a result, the lncRNAs and mRNAs identified were likely to be representative, and these genes that have differential expression possibly participate in the development of pancreatic cancer. Among the differentially expressed lncRNAs, it was observed that lncRNA CUDR was significantly overexpressed in PDAC (fold change 10.6; $P < 0.0001$).

The CUDR expression level was analyzed using RT-qPCR in a larger clinical sample size of 20 pairs to validate the conclusions of microarray analysis. The result suggested an increased level of CUDR in 16/20 PDAC tissues compared with their matched adjacent normal tissues (median fold change, 2.17±1.61; $P < 0.05$; Fig. 1C). CUDR expression was investigated in 8 PDAC cell lines and the normal pancreatic ductal epithelial cell line HPDE. Consistent with the results for clinical tissues, the level of CUDR was significantly higher in PDAC cell lines compared with in HPDE cells (Fig. 1D). Thus, these results confirmed that CUDR is upregulated in PDAC tissues and cell lines, suggesting a potential function for CUDR in the development of pancreatic cancer.

CUDR overexpression promotes proliferation by regulating cell cycle progression in PDAC cells. The potential function of CUDR in PDAC cell survival was investigated. According to the different CUDR expression levels in the 8 PDAC cell lines, CUDR was the highest in CFPAC-1 and the lowest in Panc-1 cells. Stable cell lines expressing CUDR was constructed for the relatively lower CUDR-expressing Panc-1 cells (Fig. 2A). An MTT assay indicated that aberrant CUDR expression significantly promoted the proliferation of Panc-1 cells (Fig. 2B). To confirm the effect of CUDR in Panc-1 cells, the expression of endogenous CUDR was stably knocked down using virus-mediated RNAi in the relatively highly expressed CFPAC-1 cells (shCUDR-1 and shCUDR-2; Fig. 2C). The MTT assay also indicated that CUDR depletion significantly decreased the survival of CFPAC-1 cells, whereas shCUDR-2 was more efficient compared with shCUDR-1 (Fig. 2D).

To determine whether CUDR affects cell cycle progression, cell cycle analysis was performed using fluorescence-activated cell sorting. The results indicated that the proportion of cells in G₂/M phase was increased in CUDR-overexpressing Panc-1 cells ($P < 0.001$), and the corresponding proportion of cells in G₀/G₁ phase was decreased ($P < 0.001$) (Fig. 2E). Compared with the control group, knocking down CUDR in

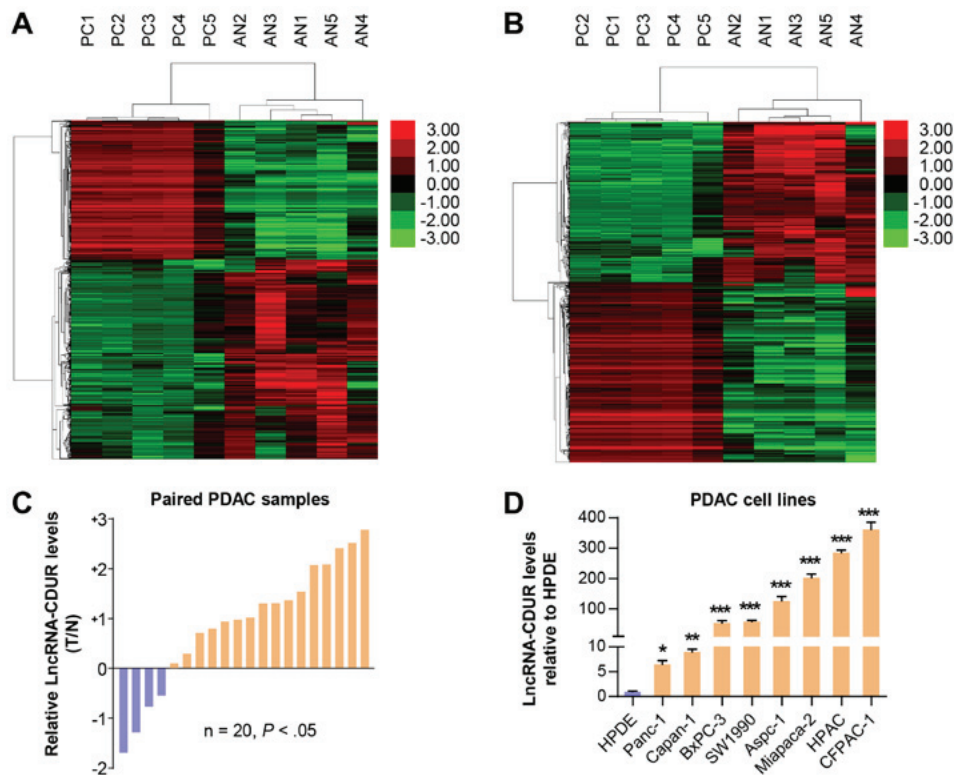


Figure 1. Reverse transcription-quantitative polymerase chain reaction analysis of CUDR in PDAC tissues and cell lines. (A) Hierarchical clustering analysis of aberrantly expressed mRNAs between pancreatic cancer tissues and adjacent non-tumor tissues based on the five pairs of microarrays. (B) Hierarchical clustering analysis of aberrantly expressed long non-coding RNAs between pancreatic cancer tissues and adjacent non-tumor tissues based on the five pairs of microarrays. (C) Relative expression of CUDR in 20 paired human pancreatic cancer tissues and matched adjacent non-tumor tissues. CUDR expression from all tissues was normalized to GAPDH expression (ΔC_q) and then compared with a non-tumorous tissue and converted into the fold change ($2^{-\Delta\Delta C_q}$). The median fold change was 2.17 ± 1.61 ($P < 0.05$). Relative CUDR expression levels were determined as $\log_2(T/N)$. The symbols + and - indicate that the expression is higher or lower, respectively, in the tumor sample compared with in their respective adjacent control tissues. (D) Relative expression of CUDR in 8 PDAC cell lines (Canpan-1, Aspc-1, Panc-1, SW1990, CFPAC-1, BxPC-3, Miapaca-2 and HPAC) and the normal pancreatic ductal epithelial cell line HPDE. The results are presented as the mean \pm standard deviation ($n=3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. CUDR, cancer upregulated drug resistance; PDAC, pancreatic ductal adenocarcinoma; T, tumor tissue; N, match adjacent non-tumor tissue.

CFPAC-1 cells led to a significantly decreased proportion of cells in G_2/M phase ($P < 0.001$) and an increased proportion of cells in G_0/G_1 phase ($P < 0.01$) (Fig. 2F). Taken together, these results suggested that CUDR is sufficient to induce a more marked proliferative activity via accelerating the transition of G_0/G_1 to $S-G_2/M$, thus promoting cell cycle progression in PDAC cells.

CUDR overexpression promotes pancreatic cancer cell migration and invasion in vitro. An increased ability of cell migration and invasion leads to cancer progression and poor prognosis. Therefore, wound-healing and Transwell chamber assays to investigate the effects of CUDR in regulating PDAC cell migration and invasion. The wound-healing assay revealed that ectopic expression of CUDR in Panc-1 cells significantly increased cell motility compared with cells transfected with empty vectors (Fig. 3A), whereas CUDR knockdown in CFPAC-1 cells significantly decreased cell motility compared with the controls (Fig. 3B). Consistently, the Transwell assay also revealed that the ectopic expression of CUDR significantly increased Panc-1 cell migration and invasion (Fig. 3C), and CUDR knockdown significantly decreased CFPAC-1 cell migration and invasion (Fig. 3D). These results demonstrated the critical function of CUDR in promoting PDAC cell migration and invasion.

CUDR overexpression inhibits cell apoptosis and promotes drug resistance in PDAC. In addition to proliferation, increased survival could also be due to changes in apoptosis. To determine whether CUDR influences PDAC cell apoptosis, flow cytometric analysis was performed and it was identified that CUDR overexpression had a marked effect on inhibiting apoptosis in Panc-1 cells (Fig. 4A), whereas CUDR knockdown promoted apoptosis in CFPAC-1 cells (Fig. 4B). To verify whether CUDR had any effect on PDAC cell sensitivity to traditional chemotherapy drugs, PDAC cells were treated with $2 \mu\text{g/ml}$ GEM or $50 \mu\text{g/ml}$ 5-FU for 48 h, and the percentage of apoptotic cells was determined. GEM and 5-FU are chemotherapy agents traditionally used to treat PDAC. As presented in Fig. 4A, the proportion of apoptotic cells was decreased in CUDR-overexpressing Panc-1 cells when treated with GEM or 5-FU. Conversely, when CUDR was knocked down by shCUDR-1 or shCUDR-2, the proportion of apoptotic cells was increased in CFPAC-1 cells treated with GEM or 5-FU (Fig. 4B). Furthermore, the CUDR-overexpressing Panc-1 cells exhibited less sensitivity to the chemotherapy agents in the MTT assay, whereas the CUDR-knockdown CFPAC-1 cells exhibited more sensitivity (Fig. 4C) compared with the controls. When treated with 5-FU, the CUDR-overexpressing Panc-1 cells also exhibited less sensitivity in restricting cell migration, compared with the controls (Fig. 4D and E).

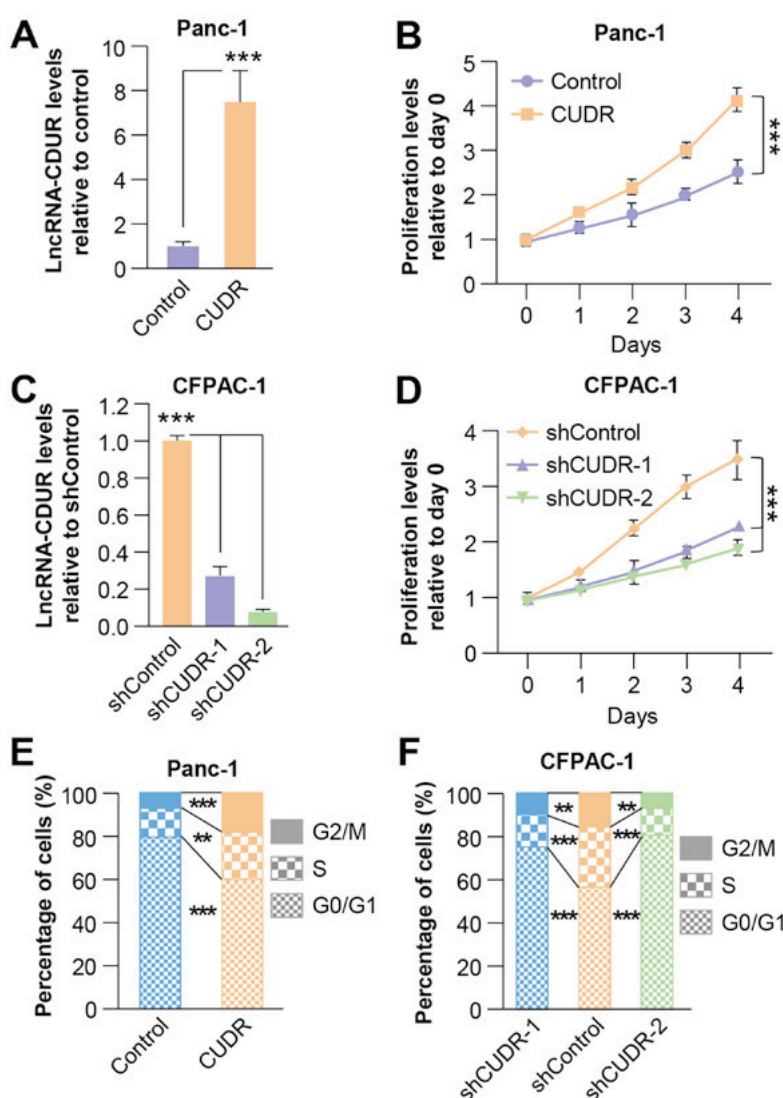


Figure 2. CUDR overexpression promotes proliferation by regulating cell cycle progression. (A) Overexpression of CUDR in the Panc-1 cell line. (B) MTT proliferation assay of Panc-1 cells. (C) Knockdown of CUDR in the CFPAC-1 cell line. (D) MTT proliferation assay of CFPAC-1 cells. Cell cycle analysis of (E) Panc-1 and (F) CFPAC-1 cells. The proportion of cells at various phases (G_0/G_1 , S and G_2/M) was compared between CUDR-overexpressing and CUDR-knocked down cell lines. Results are presented as the mean \pm standard deviation ($n=3$). ** $P<0.01$, *** $P<0.001$. CUDR, cancer upregulated drug resistance; sh, short hairpin RNA; lncRNA, long non-coding RNA.

Immunodeficient mice xenografted with CUDR-overexpressing Panc-1 cells or controls were investigated. At 10 days after injection of Panc-1 cells, palpable tumors were present in all mice, and cohorts of mice were treated with 5-FU or PBS. As presented in Fig. 4F and G, treatment of mice with 5-FU resulted in limited growth inhibition on the cohorts of CUDR-overexpressing Panc-1 tumors. In contrast, it elicited significant growth inhibition of tumors of the control group. Taken together, these results indicated that CUDR inhibits apoptosis, and induces the chemotherapy resistance of GEM and 5-FU in PDAC cells *in vitro* and *in vivo*. In addition, the growth of CUDR-overexpressing Panc-1 tumors was also significantly increased compared with controls treated with PBS. This result indicated a potent growth promotion on CUDR-overexpressing Panc-1 tumors, which is in agreement with results from *in vitro* assays.

CUDR overexpression promotes PDAC cell epithelial-mesenchymal transition (EMT) in vitro. As EMT is one of the

crucial mechanisms in cancer metastasis and chemotherapy resistance, it was further investigated whether CUDR induces EMT in PDAC cells. The expression of the epithelial marker E-cadherin was investigated in the constructed PDAC cell lines via immunofluorescence staining. The results revealed that CUDR overexpression decreased E-cadherin expression from the Panc-1 cell membrane (Fig. 5A), whereas CUDR depletion in CFPAC-1 cells led to the opposite effect (Fig. 5B). To support this result, total mRNAs and proteins were extracted from constructed stable Panc-1 and CFPAC-1 cell lines, and the expression of EMT-associated proteins was analyzed using qPCR and western blotting. As presented in Fig. 5C and D, Panc-1 cells with overexpressed CUDR exhibited increased levels of N-cadherin and vimentin, and a decreased level of E-cadherin. CUDR also upregulated the expression level of the EMT-inducing transcription factors Slug and ZEB1. In contrast, CFPAC-1 cells with depleted CUDR levels exhibited a decreased level of N-cadherin, vimentin, Slug and ZEB1, whereas E-cadherin expression was markedly

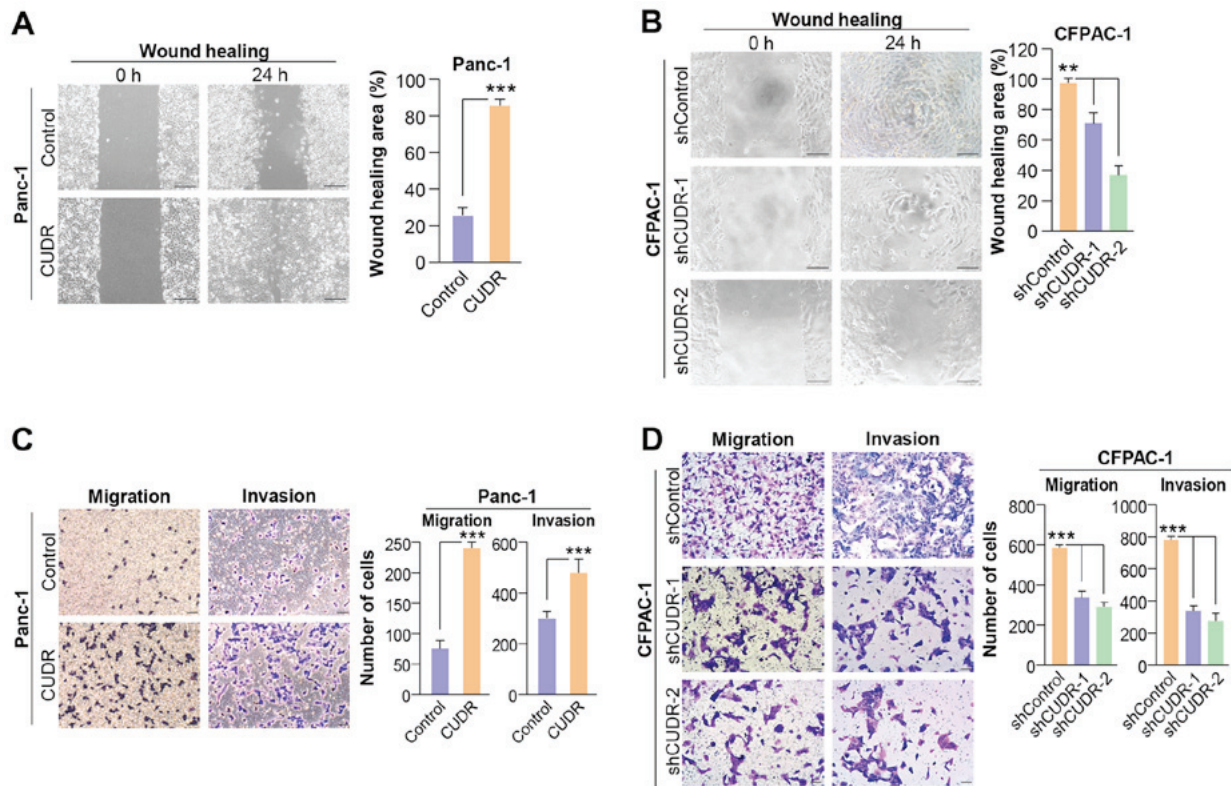


Figure 3. CUDR overexpression promotes pancreatic cancer cell migration and invasion *in vitro*. Effects of CUDR on the migration of (A) Panc-1 and (B) CFPAC-1 cells was estimated using a wound-healing assay. Effects of CUDR on the migration and invasion of (C) Panc-1 and (D) CFPAC-1 cells was estimated using a Transwell assay. Results are presented as the mean \pm standard deviation (n=3). **P<0.01, ***P<0.001. Scale bar, 100 μ m. CUDR, cancer upregulated drug resistance; sh, short hairpin RNA.

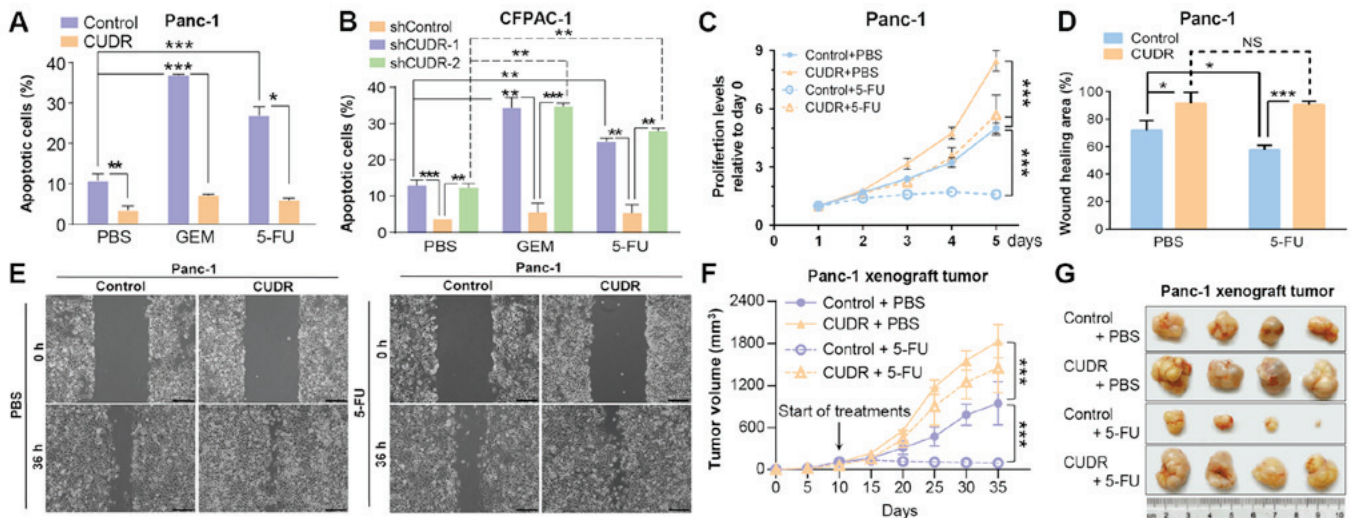


Figure 4. CUDR overexpression inhibits cell apoptosis and promotes drug resistance. (A) Apoptosis analysis of CUDR-overexpressing and control Panc-1 cells and following treatment with 2 μ g/ml GEM or 50 μ g/ml 5-FU for 48 h. (B) Apoptosis analysis of CUDR-knocked down and control CFPAC-1 cells following treatment with 2 μ g/ml GEM or 50 μ g/ml 5-FU for 48 h. (C) MTT proliferation assay of CUDR-overexpressing and control Panc-1 cells following treatment with 50 μ g/ml 5-FU for 24 h (D) Quantification and (E) images of wound-healing assay of CUDR-overexpressing and control Panc-1 cells following treatment with 50 μ g/ml 5-FU for 24 h. The results are presented as the mean \pm standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001. (F) Quantification and (G) images of *in vivo* xenograft tumor formation assays were performed using Panc-1 cells (2x10⁶) stably transfected with CUDR cDNA or an empty vector, subcutaneously injected into the right posterior flanks of 4-week-old female BALB/c nude mice, followed by treatment with 5-FU (5 mg/kg) or PBS three times weekly when the tumor reached a volume of ~100 mm³ in size. Tumor growth was determined every 5 days. At day 35, mice were sacrificed and images of tumors were captured. Results are presented as the mean \pm standard deviation (n=5). *P<0.05, **P<0.01, ***P<0.001. Scale bar, 100 μ m. CUDR, cancer upregulated drug resistance; GEM, gemcitabine; 5-FU, 5-fluorouracil; sh, short hairpin.

increased (Fig. 5E and F). These results suggested that CUDR overexpression promotes EMT in PDAC cells.

CUDR regulates PDAC development via activating protein kinase B (AKT) and extracellular-signal-regulated kinase (ERK)

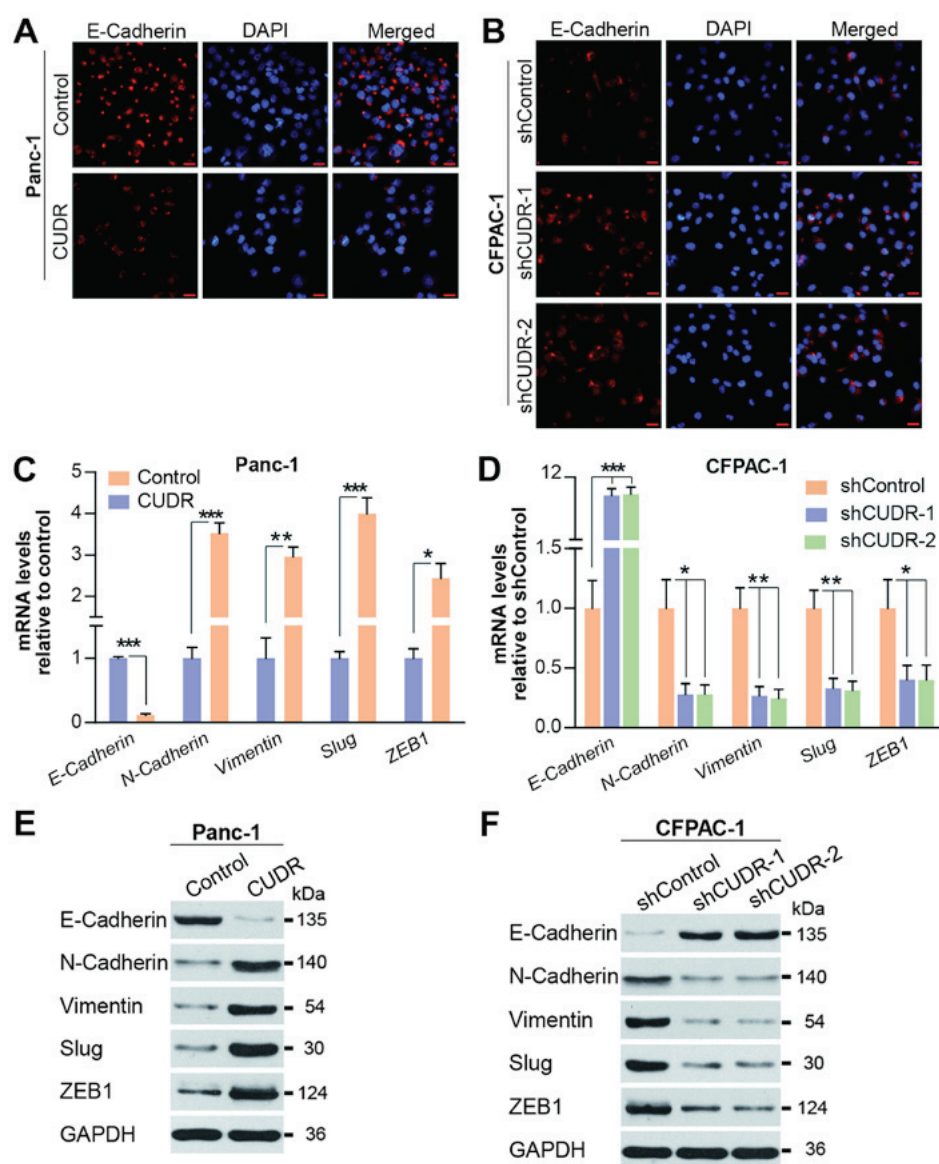


Figure 5. Effect of CUDR in regulating EMT progression. (A) Immunofluorescence images with staining for the epithelial marker E-cadherin for CUDR-overexpressing and control Panc-1 cells. (B) Immunofluorescence images with staining for the epithelial marker E-cadherin of CUDR-knockdown and control CFPAC-1 cells. (C) RT-qPCR analysis of E-cadherin, N-cadherin, vimentin, Slug and ZEB1 expression in CUDR-overexpressing Panc-1 cells compared with the cells transfected with empty vectors. (D) RT-qPCR analysis of E-cadherin, N-cadherin, vimentin, Slug and ZEB1 expression in CFPAC-1 cells transfected with CUDR shRNAs (shCUDR-1 and shCUDR-2) compared with controls. (E) Western blot analysis of E-cadherin, N-cadherin, vimentin, Slug and ZEB1 expression in CUDR-overexpressing Panc-1 cells compared with the cells transfected with empty vectors. (F) Western blot analysis of E-cadherin, N-cadherin, vimentin, Slug and ZEB1 expression in CFPAC-1 cells transfected with CUDR shRNAs (shCUDR-1 and shCUDR-2) compared with controls. The expression of E-cadherin, N-cadherin, vimentin, Slug and ZEB1 was normalized to that of GAPDH. Results are presented as the mean \pm standard deviation ($n=3$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Scale bar, 50 μ m. CUDR, cancer upregulated drug resistance; E-cadherin, epithelial cadherin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; N-cadherin, neuronal cadherin; ZEB1, zinc finger E-box-binding homeobox 1; shRNA/sh, short hairpin RNA.

pathways. The underlying molecular mechanisms of the effect of CUDR on PDAC were investigated. Using western blot analysis, it was identified that CUDR overexpression in Panc-1 cells significantly increased phosphorylated (p-) focal adhesion kinase (FAK) and p-AKT levels, whereas the total FAK and AKT were not altered compared with in Panc-1 cells transfected with an empty vector. Reciprocally, CUDR knockdown significantly decreased p-FAK and p-AKT expression, whereas total AKT and FAK levels were not altered in CFPAC-1 cells. p-FAK and p-AKT levels were decreased in the CFPAC-1 cells stably transfected with CUDR-siRNA plasmids with unchanged levels of total FAK and AKT compared with the controls. It was also observed that ERK phosphorylation was markedly increased

with unchanged levels of total ERK in CUDR-overexpressed Panc-1 cells and was markedly decreased when CUDR expression was knocked down in CFPAC-1 cells (Fig. 6A). These data suggested that ectopic CUDR expression activates the AKT and ERK signaling pathways by activating their phosphorylation, possibly leading to the promotion of PDAC development.

As the AKT and ERK signaling pathways serve important functions in regulating proliferation, migration and invasion, and promoting EMT progression, it was investigated whether CUDR regulated PDAC cell development via the AKT and ERK signaling pathways. Thus, CUDR-overexpressing Panc-1 cells were treated with MK2206 (2 μ M), a specific AKT-activation inhibitor, for 24 h and the MTT assay, flow

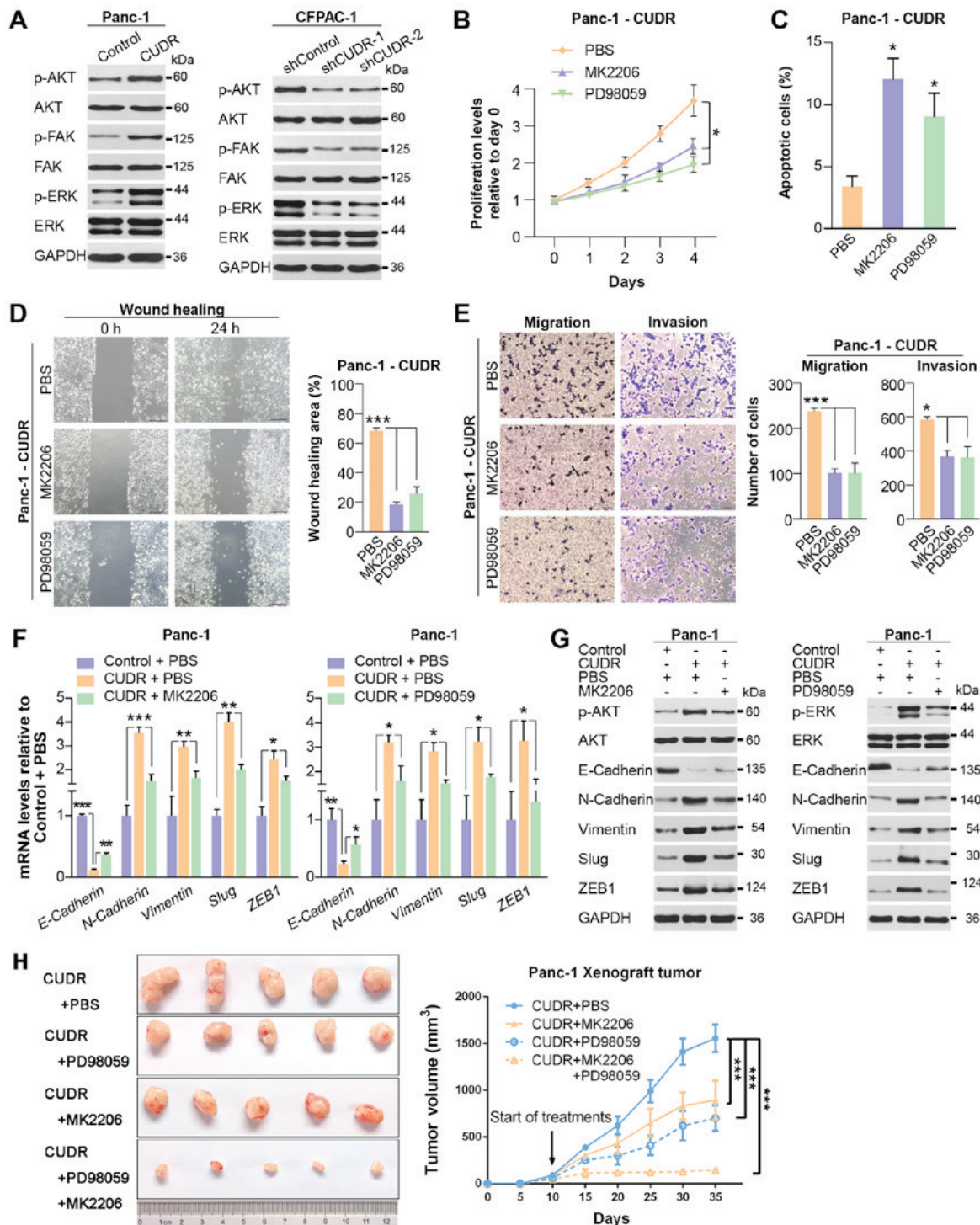


Figure 6. CUDR regulates pancreatic ductal adenocarcinoma development via activating AKT and ERK pathways. (A) Western blot analysis of p-AKT, total AKT, p-FAK, total FAK, p-ERK and total ERK expression in Panc-1 and CFPAC-1 cells stably transfected with CUDR cDNA or CUDR shRNAs (shCUDR-1 and shCUDR-2) compared with controls. The expression levels of p-AKT, total AKT, p-FAK, total FAK, p-ERK and total ERK were normalized to that of GAPDH. (B) MTT proliferation assay of CUDR-overexpressing Panc-1 cells following treatment with MK2206 (2 μ M) for 24 h, or with PD98059 (10 μ M) for 48 h compared with controls. (C) Apoptosis analysis of overexpressing CUDR Panc-1 following treatment with MK2206 (2 μ M) for 24 h, or with PD98059 (10 μ M) for 48 h compared with controls. (D) Wound-healing assay of overexpressing CUDR Panc-1 cells following treatment with MK2206 (2 μ M) for 24 h, or with PD98059 (10 μ M) for 48 h compared with controls. (E) Migration and invasion Transwell assays of CUDR-overexpressing Panc-1 cells following treatment with MK2206 (2 μ M) for 24 h, or with PD98059 (10 μ M) for 48 h compared with controls. (F) Reverse transcription-quantitative polymerase chain reaction analysis and (G) western blot analysis of p-AKT, total AKT, E-cadherin, N-cadherin, vimentin, Slug and ZEB1 expression in Panc-1 cells following treatment with MK2206 (2 μ M) for 24 h, and p-ERK, total ERK, E-cadherin, N-cadherin, vimentin, Slug and ZEB1 expression in Panc-1 cells following treatment with PD98059 (10 μ M) for 48 h. The expression of total AKT, p-AKT, total ERK, p-ERK, N-cadherin, vimentin, E-cadherin, Slug and ZEB1 was normalized to that of GAPDH. (H) In vivo xenograft tumor size formation assays were performed using Panc-1 cells (2x10⁶) stably transfected with CUDR cDNA, subcutaneously injected into the right posterior flanks of 4-week-old female BALB/c nude mice, followed by treatment with MK2206 (20 mg/kg) or PD98059 (10 mg/kg) administered by gastrogavage three times weekly when the tumor reached a volume of ~100 mm³. Tumor growth was determined every 5 days. At day 35, mice were sacrificed and images of tumors were captured. Results are presented as the mean \pm standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001. Scale bar, 100 μ m. CUDR, cancer upregulated drug resistance; AKT, protein kinase B; ERK, extracellular-signal-regulated kinase; p-, phosphorylated; shRNA/sh, short interfering RNA; E-cadherin, epithelial cadherin; N-cadherin, neuronal cadherin; ZEB1, zinc finger E-box-binding homeobox 1.

cytometric analysis, wound-healing and Transwell assays were repeated. The results indicated that CUDR-induced PDAC cell proliferation, migration, invasion and EMT progression were all markedly decreased, and apoptosis was significantly increased in Panc-1 cells treated with MK2206 (Fig. 6B-G). Following treatment of CUDR-overexpressing Panc-1 cells with PD98059 (10 μ M), a specific ERK-activation inhibitor, for 48 h, CUDR-induced proliferation, migration, invasion and EMT progression were decreased, and CUDR-induced apoptosis was increased (Fig. 6B-G). Together, these results indicated that CUDR regulated PDAC cell proliferation, migration, invasion, apoptosis and EMT progression via the AKT and ERK signaling pathways.

Immunodeficient mice xenografted with CUDR-overexpressing Panc-1 cells or controls were used to investigate these results. At 10 days after injection of Panc-1 cells, palpable tumors were present in all mice, and cohorts of mice were treated with MK2206, PD98059 or PBS. As presented in Fig. 6H, treatment of mice with either MK2206 or PD98059 resulted in marginal growth inhibition on the cohorts of CUDR-overexpressing Panc-1 tumors, and this inhibition was more obvious when treated with the two agents. It suggested that the growth promotion of CUDR-overexpressing Panc-1 tumors is via the AKT and ERK signaling pathways, which was consistent with the results for the *in vitro* assays.

Discussion

The lncRNA CUDR, also known as urothelial cancer-associated 1, was identified to be overexpressed in various neoplasms, and it serves key functions in numerous biological progresses. CUDR is upregulated in bladder cancer and promotes tumorigenesis, progression and cisplatin resistance through different mechanisms, and it also represents a potential marker for the diagnosis and prognosis of bladder cancer (16,17). CUDR also serves as an oncogene in a number of types of human cancer, including gastric cancer, colorectal cancer, tongue squamous cell carcinoma, hepatocarcinoma and breast cancer (18-22). Chen *et al* (23) identified that CUDR expression in pancreatic cancer is significantly correlated with malignant factors, including tumor size, depth of invasion, cancer antigen 19-9 level and tumor stage. Fu *et al* (24) identified that CUDR is involved in PDAC progression and is associated with the overall survival of patients with PDAC. Zhang *et al* (25) suggested that microRNA-135a may be a target of CUDR in regulating the growth and metastasis of pancreatic cancer. Cheng *et al* (26) indicated that CUDR may activate AKT/mammalian target of rapamycin and ERK signaling pathways, and EMT in epidermal growth factor receptor-mutant non-small cell lung cancer. Wang *et al* (27) identified that CUDR promotes cell proliferation via recruiting enhancer of Zeste homolog 2 and activating AKT in gastric cancer. However, the further function and underlying molecular mechanism of CUDR in regulating PDAC development and progression remain unknown.

In the present study, microarrays of PDAC tissues and matched adjacent non-tumor tissues were used to profile different lncRNA expression, and 883 lncRNAs and 949 mRNAs were obtained that were differentially expressed. Among these lncRNAs, it was confirmed that CUDR is

overexpressed in PDAC tissues and 8 PDAC cell lines, and increases PDAC cell progression, migration and invasion, and inhibits apoptosis; in contrast, CUDR knockdown had the opposite effects, indicating a marked oncogenic function. The results of the present study also indicated that aberrant CUDR expression inhibited PDAC cell apoptosis induced by GEM and 5-FU, whereas CUDR knockdown had the opposite effect. As GEM and 5-FU are traditional chemotherapy drugs for PDAC, these results indicated that CUDR may decrease tumor sensitivity to GEM and 5-FU chemotherapy, inducing drug resistance in patients with pancreatic cancer and contributing to a poor prognosis. In addition, CUDR-induced progression and drug resistance of 5-FU was confirmed *in vivo*.

As migration and invasion are crucial for metastasis, which is responsible for the mortality of >90% of patients with PDAC, the focus of the present study was on the underlying molecular mechanism of how CUDR promotes PDAC cell migration and invasion. Previous study has indicated that EMT serves a pivotal and intricate function in tumor metastasis, and also serves an important function in pancreatic cancer invasion and metastasis (28). To the best of our knowledge, only a limited number of lncRNAs have been associated with EMT in PDAC: LOC389641 (8), lincRNA-ROR (29), MALAT-1 (30), MEG3 (31), HOTAIR (32) and H19 (33). The results of the present study provide the first evidence that CUDR also promotes EMT in PDAC. Ectopic CUDR expression upregulated the expression level of the mesenchymal markers N-cadherin and vimentin, and downregulated the expression level of the epithelial marker E-cadherin. CUDR also upregulated the expression of Slug and ZEB1, which are vital transcription factors for inducing EMT (29,34). Conversely, the loss of CUDR had the opposite effect.

The ectopic expression of CUDR was identified to activate the AKT and ERK signaling pathways via activating FAK, AKT and ERK phosphorylation, whereas CUDR knockdown led to decreased FAK, AKT and ERK activation. FAK is known to control the mesenchymal characteristics imparting adhesion and invasiveness in cancer cells (35), and phosphorylated FAK induces downstream AKT pathway activation (36). Activation of the AKT signaling pathway is correlated with cell cycle progression, migration and invasion, and may decrease cell apoptosis in a number of types of cancer (35,37). In addition, AKT is activated and promotes invasiveness in human pancreatic cancer (38). ERK, as an important member of the mitogen-activated protein kinase signaling pathway, is a crucial kinase in regulating tumorigenesis and progression. Persistent ERK activation regulates cell cycle arrest and promote tumor cell growth, proliferation and differentiation (39,40). Consistent with the results of the present study, when treated with MK2206 and PD98059, the effect of CUDR on cell proliferation, apoptosis, migration and invasion could be inhibited. Thus, we hypothesize that CUDR promotes proliferation, migration and invasion, and inhibits cell apoptosis via activating the AKT and ERK signaling pathways. Furthermore, 90% of patients with pancreatic cancer harbor oncogenic point mutations in *KRAS*, which lead to constitutive *KRAS* activation, thus cell lines without *KRAS* mutations are possibly not representative of the majority of pancreatic cancer types. CFPAC-1 and PANC-1 cells are the most commonly used pancreatic cancer cell lines, and contain *KRAS* mutations (41). As it is known

that oncogenic KRAS activates ERK and AKT signaling pathways (42), which are also suggested to be regulated by CUDR in the present study, we hypothesized that CUDR may be associated with KRAS activation. However, no association between CUDR and KRAS was identified in an mRNA array assay (C. Shao, unpublished data), indicating that the KRAS pathway is unlikely to be involved in CUDR downstream. Further experiments are required to dissect the mechanisms by which CUDR activates AKT and ERK signaling pathways.

Activation of the AKT signaling pathway is an important feature of EMT (43). Results of our previous study had also suggested that EMT is initiated by the AKT/Snail signaling pathway in pancreatic cancer, promoting cell metastasis and invasion (44). The ERK signaling pathway has been confirmed to have another vital function in promoting EMT (45). Consistent with the results of the present study, we therefore hypothesize that CUDR overexpression promotes EMT via the activation of AKT and ERK signaling pathways. In support of this hypothesis, in the present study, when AKT phosphorylation was abolished by its specific inhibitor MK2206 or ERK phosphorylation was abolished by its specific inhibitor PD98059 in PDAC cells, CUDR-induced EMT progression was able to be restored. Thus, it is concluded that CUDR promotes EMT via activating the AKT and ERK signaling pathways in PDAC cells. In addition, EMT contributes to drug resistance (46). An inverse association between E-cadherin and ZEB1 was also identified to be associated with resistance to GEM, 5-FU and other chemotherapy drugs in pancreatic cancer (47,48). The results of the present study indicated that CUDR inhibited the apoptosis induced by GEM and 5-FU, promoted the drug resistance of GEM and 5-FU, and promoted EMT in PDAC cells; CUDR also induces the chemotherapy resistance of 5-FU in PDAC cells *in vivo*. Furthermore, the results of the present study indicated that chemosensitivity to 5-FU in PDAC cells could be decreased by CUDR through promoting EMT. Considering that lncRNAs exhibit versatility in molecular function, it should be noted that promoting EMT and activating FAK/AKT and ERK signaling pathways are only some of the mechanisms for regulating pancreatic cancer migration, invasion, metastasis and apoptosis. Alternative mechanisms of CUDR in promoting pancreatic cancer progression require investigation.

In conclusion, the present study has identified differentially expressed lncRNAs and demonstrated that CUDR is overexpressed in PDAC. CUDR is able to increase PDAC cell proliferation, migration and invasion, inhibit apoptosis, and promote drug resistance; it also regulates PDAC cell EMT. The CUDR-induced PDAC malignant phenotypes is via the AKT and ERK signaling pathways. Downregulation of CUDR may therefore provide a novel therapeutic strategy to prevent PDAC progression, metastasis and drug resistance in the future.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article. The microarray data were published in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) under accession number GSE101094 as part of this study.

Authors' contributions

XL and MQ performed all the experiments and analyzed the data. RW and LT provided support with experimental materials and techniques. AL, DC, JC and XH provided human specimens. WL analyzed the data. CS and LZ designed the research and revised the manuscript. XL wrote the manuscript. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

The patient study and the animal experiments were approved by the Ethics Committee for Biomedical Research of Second Military Medical University. Written informed consent was obtained from all patients or their relatives.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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