

CPA4 Promotes EMT in Pancreatic Cancer via Stimulating PI3K-AKT-mTOR Signaling

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Qingliang Shao¹
Zhiqiang Zhang¹
Rongxian Cao²
Hui Zang¹
Wanting Pei²
Tian Sun²

¹Department of General Surgery, The Peoples' Hospital of Liaoning Province, Shenyang City, Liaoning Province, People's Republic of China; ²Graduate School of China Medical University, Shenyang City, Liaoning Province, People's Republic of China

Background: Carboxypeptidase A4 (CPA4), as a novel tumor biomarker, is prevalently observed in various cancers. However, the potential role of CPA4 in pancreatic cancer (PC), to our knowledge, has not been fully clarified.

Materials and Methods: We systematically explored the detailed function of CPA4 in epithelial to mesenchymal transition (EMT) stimulated PC in human clinical samples and in vitro.

Results: CPA4 was overexpressed in clinical PC samples that was positively related with tumor size ($P=0.026$), T stage ($P=0.011$), lymph-node metastasis ($P=0.026$) and a worse prognosis for PC patients ($P=0.001$). Interestingly, CPA4 was inversely correlated with E-cadherin ($r=-0.372$, $P=0.003$) in clinical samples and PC cell lines which cooperatively contributed to a worse prognosis ($P=0.005$) for PC patients. CPA4 overexpression enhanced EMT in AsPC-1 and Capan-2 cells, which promoted EMT-like cellular morphology and cell invasion and migration. Meanwhile, CPA4 overexpression activated EMT and PI3K-AKT-mTOR signaling, following with the downregulation of E-cadherin and β -catenin, and the upregulation of N-cadherin, vimentin, p-PI3K (Tyr458), p-AKT (Ser473) and p-mTOR (Ser2448). However, PI3K inhibitor LY294002 reversed CPA4 overexpression-stimulated EMT in vitro. Moreover, CPA4 was co-immunoprecipitated with AKT in two PC cells with CPA4 high expression. Conversely, CPA4 silencing inhibited EMT in PANC-1 cells. CPA4 overexpression or silencing promoted or inhibited cell proliferation and drug resistance in Capan-2 and PANC-1 cells via regulating Bcl2/Bax and cleaved-caspase3 signaling. However, LY294002 reversed CPA4 overexpression-stimulated cell proliferation and drug resistance in vitro in Bcl2/Bax and caspase3-dependent apoptosis.

Conclusion: CPA4 overexpression contributes to aggressive clinical stage of PC patients and promotes EMT in vitro via activation of PI3K-AKT-mTOR signaling.

Keywords: CPA4, EMT, pancreatic cancer, PI3K-AKT-mTOR signaling

Introduction

Pancreatic cancer (PC) is one of the most lethal cancers, which is predicted to be the second leading cancer-related death in 2030.^{1,2} Locally extended tumor invasiveness, distant metastasis and drug resistance contributed to the worst prognosis of PC, which is aggravated by epithelial to mesenchymal transition (EMT).³ The key feature of EMT is the loss of epithelial adhesion molecule markers (E-cadherin and β -catenin) and the gain of mesenchymal markers such as N-cadherin (N-cad), vimentin, fibronectin and alpha smooth muscle actin (α -SMA).⁴

Accumulating evidence shows that EMT is regulated by multiple signaling pathways, including PI3K-AKT-mTOR signaling, which plays a critical function

Correspondence: Hui Zang
Email Drzanghui0320@163.com

in regulating cellular apoptosis, survival, motility, metabolism and differentiation.⁵ Constant stimulation in PI3K-AKT and its downstream target of mTOR pathway leads to an aggressive phenotype of cancer, including sustained angiogenesis, invasion, and metastasis.⁶ Therefore, it is urgent to explore the tumor biomarker that simultaneously targets both EMT and PI3K/AKT/mTOR signaling to prevent malignant progression of PC.

Carboxypeptidase A4 (CPA4), as a member of Zn-containing metallocarboxypeptidases family, catalyzes the release of carboxy-terminal amino acids specific to cleave C-terminal residues from protein and peptide.^{7,8} It was originally detected in the mRNAs screen which was upregulated by sodium butyrate-induced differentiation. Now it is identified as an important regulator in inflammation, and it is speculated to play a critical role in the tumor micro-environment. Recently, CPA4 is found to be overexpressed in various solid cancers, such as liver,⁹ breast,¹⁰ prostate,¹¹ non-small-cell lung,¹² and colorectal cancers.¹³ However, the potential role of CPA4 in PC, to our knowledge, has not been well elaborated.

In present study, we explored the potential function of CPA4 and the target signaling in regulating EMT in PC clinical tissues and in vitro, which supplies a novel sight in revealing the malignant biology and offering a promising gene target intervention for PC.

Materials and Methods

Tissue Specimens and Cell Lines

The present study was authorized by the academic committee at The People's Hospital of China Medical University. Informed consent and the study methodology were accepted from PC patients and the committee of the China Medical University. Sixty-five paraffin-embedded PC (ductal adenocarcinoma) and paired adjacent pancreas were picked up from surgical treatment patients from 2006 to 2016. Eighteen additional PC tissues were collected for late mRNA assays. Human Aspc-1, PANC-1 and MIA PaCa-2 PC cell lines were purchased from the cell Bank of Chinese Academic Sciences in Shanghai City. Capan-2 cells were supplied from the First Hospital of China Medical University. These cell lines were authenticated for contamination prior to any experiment in vitro.

Immunohistochemistry

IHC was conducted according to the previous study.¹⁴ In detail, the fixed PC tissues were made into a paraffin block

and 4- μ m sections that were deparaffinized and dehydrated first. The slices were next covered with peroxyacetic acid, sent to antigen retrieval, blocked with 10% BSA, and then incubated with anti-CPA4 (Abcam, Cambridge, UK, dilution: 1:100) and E-cadherin (E-cad, Abcam, dilution: 1:200). Sections were washed with PBS and incubated with the secondary antibody (Streptavidin-HRP), detected with DAB, costained with hematoxylin and finally evaluated by two professional pathologists. Staining intensity was recorded as negative to strong (0–3). Stained positive area was scored as 0–4 (<10%; 10–25%; 26–50%; 51–75%; >76%). Two professional pathologists evaluated the final scores of high CPA4 and positive E-cad expression.

Western Blot and Immunoprecipitation

The total proteins extracted from PC cell lysates were implanted into 10–12% SDS-polyacrylamide gels, subjected to wet transfer, blocked with 5% skimmed milk and incubated with primary antibodies that were shown in [Supplementary Material](#). All the bands were routinely incubated with secondary antibodies (Proteintech) and detected with the ECL instrument (Bio-Rad, California, USA). Cells were pretreated with 10 μ m of PI3 kinase inhibitor LY294002 (Cell Signaling Technology, #9901) for two hours prior to WB according to previous study.¹⁵ WB was repeated at least three times.

According to previous a study,¹⁴ lysates of MIA PaCa-2 and PANC-1 cells with high CPA4 were extracted in the immunoprecipitation (IP) lysis buffer. CPA4 and AKT antibodies were first mixed with magnetic beads (Thermo scientific, Rockford, IL, USA) for at least four hours. Antibody bead complexes were next covered with the supernatants of protein lysates overnight. The final antibody-protein immunocomplex was stripped by boiling with five \times loading sample buffer for WB. IP was repeated three times.

Real-time Quantitative PC

According to a previous study,¹⁶ target genes were detected in a SYBR Premix Ex TaqTM II kit (Takara Bio, Japan) as below: 95°C for 40 seconds and 45 cycles of 95°C for 10 seconds and 55°C for 40 seconds. The primer sequences were shown in [Supplementary Material](#). Quality of amplification products was conducted with melt-curve dissociation following the $\Delta\Delta$ Ct method.

CPA4 Overexpression Construction and CPA4siRNA

The pCMV2 flag-tagged CPA4 plasmid (CPA4-FLAG) and corresponding empty plasmid (FLAG) was purchased by GenePharma in Shanghai City. Two verified sequences (OPF) of CPA4siRNA were shown in [Supplementary Material](#). The CPA4siRNA were purchased from GenePharma corporation. CPA4-FLAG and CPA4siRNA transfections were mixed with Oligofectamine 3000 (Invitrogen) for the transient transfection according to the protocol. One of the most effective siRNA sequences was used in vitro. AsPC-1 and Capan-2 cells with low CPA4 were used for CPA4 overexpressing experiment, while PANC-1 cells with high CPA4 were used for silencing experiments. The silencing and overexpressing effect of CPA4 was detected by WB as shown in the result section.

EMT Construction

Two percent FBS growth media were used to better induce EMT in CPA4-FLAG and FLAG (scramble) transfected AsPC-1 and Capan-2 (exchanged three times within four and a half days). CPA4siRNA and siRNActrl (scramble) transfected PANC-1 cells were cultured with normal growth medium. The change involving EMT-like cellular morphology, EMT-related signaling and cell motility was regarded to reflect the EMT construction model. Prior to cell morphology assays, we pretreated PC cells with LY294002 (10 μ m) for two hours daily within four days in parallel cultured with 2% FBS medium. DMSO was used as the control regarding it as the dilution of LY294002.

Cell Motility Assays

Based on a previous study,¹⁴ CPA4siRNA and siRNAcontrol (siRNActrl) transfected PANC-1 cells and CPA4-FLAG and FLAG transfected AsPC-1 cells pretreated with LY294002 (10 μ m for two hours, twice) were implanted into 12.0- μ m pore size membrane inserts (BD Biosciences, MD, USA) coated with matrigel (BD Bioscience) in 24-well plates containing serum-free media. Media containing 10% FBS was put at the bottom of the wells as a stimulus. Cells were invaded under condition in 5% CO₂ at 37°C for 24 h. The migrated cells from the underside of the inserts were fixed and costained with crystal violet (Sigma-Aldrich Co., St Louis, MO, USA). The final migrated cell numbers were calculated in at least five random fields/each well. The invasion assay was

conducted in a similar way other than the matrigel. Cell motility assays were repeated at least three times.

MTT Assays

MTT assay was first conducted to investigate cell proliferation in CPA4 silencing or overexpressing PC cells at different time points (one to five days) combining with or without LY294002 treatment. CPA4siRNA transfected PANC-1 cells and CPA4-FLAG transfected Capan-2 cells (with or without 10 μ m LY294002 treatment three times) were seeded into 96-well plates at the density of 5000 viable cells per well and incubated for one to five days. At the end of each time point, 15 μ L of MTT (5 mg/mL in PBS, Sigma) was added for four hours and the medium was then incubated with 100 μ L of dimethyl sulphoxide (Sigma) was added to each well for 20 min. Drug resistance assays were done in a similar way with various concentrations of gemcitabine (GEM, Abcam) as shown in results section. Per experiment group at a wavelength of 570 nm in an ELISA 96-well microtiter plate reader (BIORAD680, USA). Experiments were performed in triplicate, and data were presented as a percentage of treated cells compared with control cells.

Statistical Analysis

Paired nonparametric, chi-squared and Spearman correlation tests were used to calculate the statistical analyses in IHC data. The log-rank and Cox's regression tests were used for patients' survival. WB, qRT-PCR and cell motility were identified as means \pm SE and were compared through independent *t*-test. *P*-value is identified statistically significant as: *P*<0.05; *P*<0.01.

Results

CPA4 Overexpression in PC Tissues was Associated with the Clinicopathological Characters of PC Patients

CPA4 exhibited cytoplasmic expression in normal pancreas (Figure 1A) and PC tissues (Figure 1B). E-cad, as a key hallmark and regulator in EMT, showed membrane expression in PC which was regarded as positive expression (Figure 1D and F). IHC showed CPA4 was overexpressed in human PC tissues (Figure 1A) that was significantly higher in contrast with corresponding pancreas (36/65, 55.3% vs 15/65, 23%, *P*<0.01) (Figure 1B). PC tissues with CPA4 overexpression was related with E-cad negative expression in most serial slices (Figure 1C and D), and vice

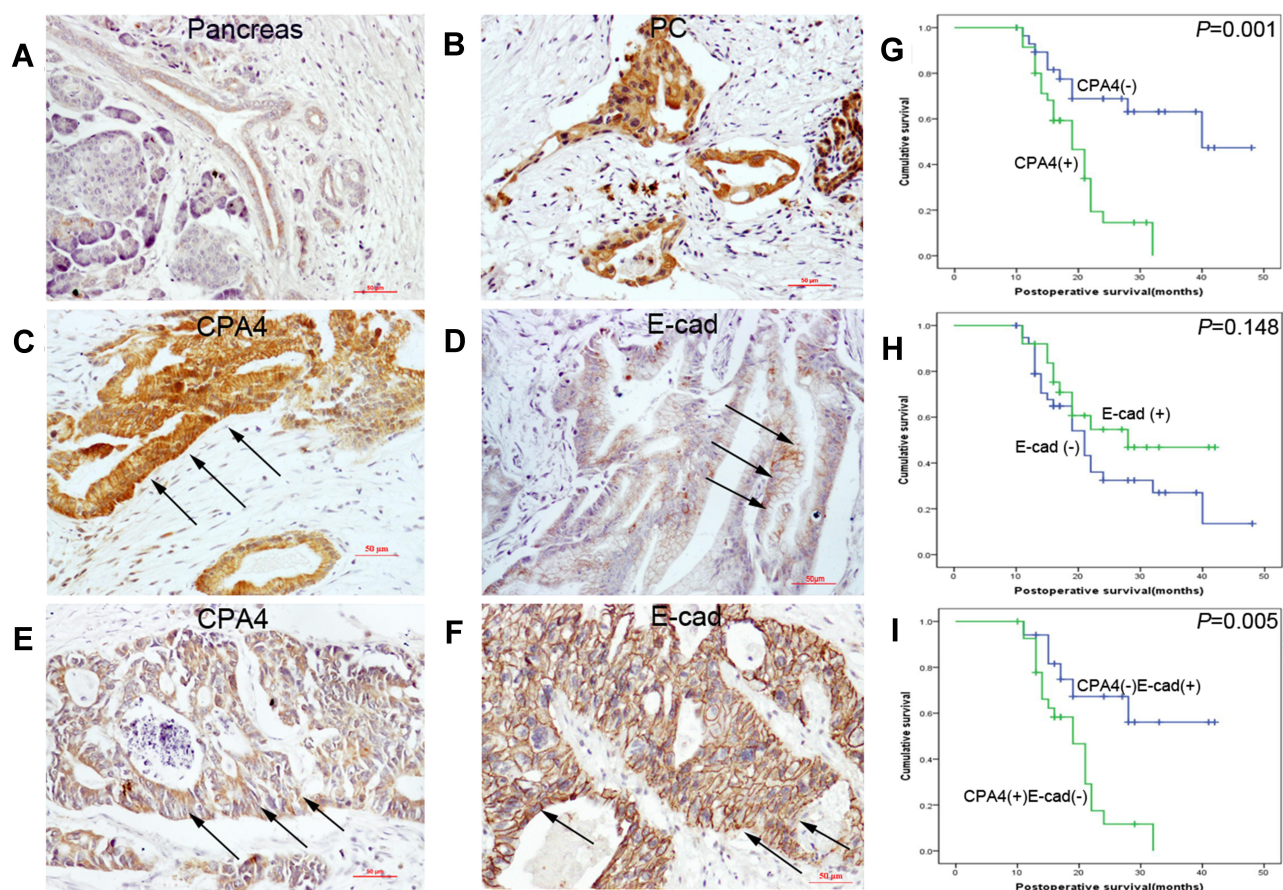


Figure 1 The expression of CPA4 and E-cad in PC. (A and B) CPA4 expression in pancreas (A) and corresponding PC (B). (C and D) CPA4 high expression (C) and E-cad negative expression (D) in one PC tissue as arrows shown. (E and F) CPA4 low expression (E) and E-cad positive expression (F) in another sample as arrows shown. (G) High (+) and low (-) expression of CPA4 in Kaplan-Meier analysis. (H) Positive (+) and negative (-) expression of E-cad in Kaplan-Meier analysis. (I) Cooperative expression of CPA4 and E-cad in Kaplan-Meier analysis.

Abbreviations: CPA4, carboxypeptidase A4; E-cad, E-cadherin.

versa (Figure 1E and F). Interestingly, CPA4 was inversely associated with E-cad expression by Spearman's correlation test ($r=-0.372$, $P=0.003$) (Table 1).

CPA4 was overexpressed in clinical PC specimens that were positively associated with tumor size ($P=0.026$), T stage ($P=0.011$) and lymph-node metastasis ($P=0.026$) of PC patients shown in Table 2 ($P>0.05$). Patients with CPA4 high expression showed a poor survival compared with CPA4 low expression ($P=0.001$) (Figure 1G). Moreover, CPA4 was an independent risk prognostic indicator ($P=0.042$) in the multivariate model (Table 3). E-cad was not associated with the prognosis ($P=0.148$) (Figure 1H). However, patients with high CPA4 and negative E-cad expression cooperatively promoted a worse overall survival ($P=0.005$) (Figure 1I).

High mRNA level of CPA4 were observed in 18 additional PC tissues compared with the adjacent pancreas ($P=0.014$) (Figure 2A). In four PC cell lines, the level of

CPA4 protein (Figure 2B–D) and mRNA (Figure 2E and F) was significantly increased in PANC-1 and MIA PaCa-2 cells in contrast in AsPC-1 and Capan-2 cells which was inversely associated to E-cad expression. We also detected CPA4 and E-cad expression in liver cancer cell lines: Hep G2, HuH-7 and RH-35 by WB (Supplemental Figure 1). However, no significant relationship between their expression was observed in liver cancer cell lines, which indicated

Table 1 CPA4 was Inversely Associated with E-Cad in Human PC Samples

Character		CPA4			r	P
		Negative	Positive			
E-cad	Abnormal	12	28	40	-0.372	0.003
	Normal	17	8	25		
		29	36			

Abbreviations: PC, pancreatic cancer; CPA4, carboxypeptidase A4; E-cad, E-cadherin.

Table 2 The Clinical Significance of CPA4 Expression in Human PC Samples

Characters	No. of Patients	CPA4		P
		Low	High	
Cases	65	29	36	
Gender				
Male	43	18	25	0.532
Female	22	11	11	
Age (years)				
<60	37	18	19	0.452
≥60	28	11	17	
Tumor location				
Head	46	21	25	0.794
Body-tail	19	8	11	
Tumor size (cm)				
<4	42	23	19	0.026
≥4	23	6	17	
Differentiation				
Well	29	15	14	0.301
Moderate/poor	36	14	22	
T stage				
T1+T2	29	18	11	0.011
T3+T4	36	11	25	
Lymph nodes metastasis				
N0 (negative)	42	23	19	0.026
N1 (positive)	23	6	17	
Vascular permeation				
Absent	35	18	17	0.223
Present	30	11	19	
Pretherapeutic CA19-9 level				
<37 U/mL	24	12	12	0.504
≥37 U/mL	41	17	24	

Abbreviation: PC, pancreatic cancer.

a specific association of CPA4 and E-cad in PC cells. Based on the tightly inverse relationship of CPA4 with E-cad in clinical PC specimens and cell lines, we reasoned that CPA4 might play a significant role in PC EMT in vitro.

CPA4 Mediated EMT and PI3K-AKT-MTOR Signaling in PC Cells

As shown in the methods section, Capan-2 cells with low CPA4 and PANC-1 cells with high CPA4 was used for CPA4 overexpressing and silencing construction, respectively. CPA4 protein expression in CPA4siRNA group was obviously lower compared with that in the siRNActrl group (Figure 3A and B), whereas CPA4 was overexpressed in

CPA4-FLAG group (Figure 3C and D). CPA4 silencing upregulated E-cad and β -catenin and downregulated N-cad and vimentin. Some EMT markers, such as fibronectin (FB) and α -SMA, showed no change (Figure 3A and B). Meanwhile, CPA4 silencing inhibited PI3K-AKT-mTOR signaling that p-PI3K (Tyr458), p-AKT (Ser473) and p-mTOR (Ser2448) was significantly decreased in the CPA4siRNA group compared with the siRNActrl group (Figure 3A and B). Conversely, CPA4 overexpression upregulated N-cad, vimentin, MMP9, p-PI3K (Tyr458), p-AKT (Ser473) and p-mTOR (Ser2448) but downregulated E-cad and β -catenin protein expression in Capan-2 cells (Figure 3C and D). Based on the above results, CPA4 simultaneously regulated both EMT and PI3K-AKT-mTOR signaling in PC cells in vitro.

CPA4 Promoted EMT in PC Cells via Activating PI3K-AKT-mTOR Signal Pathway

LY294002 is a selective inhibitor toward PI3K-dependent AKT phosphorylation and kinase activity.^{17,18} Firstly, CPA4 overexpression stimulated EMT-like cellular morphology in Capan-2 cells: most cells (70–85%) lost their epithelial features (the loss of tight and adhesive junction), and exhibited a spindle-shaped/fibroblast-like and fusiform morphology (Figure 4A and B). However, LY294002 restored CPA4 overexpression-induced EMT-like cellular morphology. Little spindle-shaped/fibroblast-like (fusiform) cell morphology (30–40%) was observed in CPA4-FLAG plus LY294002 group compared with that in CPA4-FLAG alone group (Figure 4C). We also showed the similar results in CPA4 overexpressing AsPC-1 cells (Figure 4D–F), which indicated that CPA4 overexpression stimulated EMT-like cellular morphology in vitro via activating PI3K-AKT-mTOR signaling.

WB next showed that CPA4 overexpression-induced the downregulation of E-cad and β -catenin and the upregulation of N-cad, vimentin and p-AKT (Ser473) in Capan-2 cells, which was significantly inhibited by LY294002 (Figure 5A and B). Moreover, CPA4 was co-immunoprecipitated with AKT in normal MIA PaCa-2 (Figure 5C) and PANC-1 cells (Figure 5D) with CPA4 overexpression, which indicated a close interaction between CPA4 and PI3K-AKT-mTOR signaling in vitro.

It is well known that cancer cell motility and invasiveness is significantly boosted by EMT.¹⁹ In this study, cell invasion (Figure 6A) and migration (Figure 6B) were significantly increased in CPA4-FLAG transfected PANC-1

Table 3 Survival Data in Univariate and Multivariate Model

Characters	Median Survival (Months)	Univariate Analysis P (Log Rank)	Multivariate Analysis Hazard Ratio (95% CI ^a)	P
Gender (male/female)	24/28	0.738	–	0.299
Age (<60/≥60 years)	25/29	0.424	–	
Tumor location (head/body-tail)	29/20	0.065	–	
Tumor size (<4/≥4 cm)	30/22	0.080	–	
Well/poor and moderate differentiation	32/21	0.019	1.484 (0.704–3.125)	
T stage (T1+T2/T3+T4)	28/25	0.094	–	0.012
Lymph nodes metastasis (N0/N1)	33/18	0.001	2.541 (1.224–5.275)	
Vascular permeation (absent/present)	28/24	0.075	–	
CA19-9 level (<37 U/mL/≥37 U/mL)	32/24	0.148	–	0.042
CPA4 (high/low)	19/35	0.001	2.380 (1.032–5.493)	
E-cad (positive/negative)	29/25	0.148	–	

Note: ^aConfidence interval.

Abbreviations: CPA4, carboxypeptidase A4; E-cad, E-cadherin.

cells compared with that in the siRNActrl group. However, LY294002 significantly inhibited CPA4 overexpression-enhanced cell invasion (Figure 6A) and migration (Figure 6B) in vitro. Conversely, CPA4 silencing inhibited cell invasion (Figure 6C) and migration (Figure 6D) in PANC-1 cells. Taken together, CPA4 promoted cell motility in PC cells via activating PI3K-AKT-mTOR signaling.

CPA4 Promoted Cell Proliferation and Drug Resistance in PC Cells via Activating PI3K-AKT-mTOR Signal Pathway in Bcl2/Bax and Caspase3-dependent Apoptosis

Targeting EMT has been considered a novel opportunity to overcome cancer drug resistance.⁴ Thus, we next investigated the potential role of CPA4 in cell proliferation and drug resistance in vitro. MTT showed that CPA4 silencing inhibited cell proliferation in PANC-1 cells when cultured for four and five days (Figure 7A), whereas CPA4 overexpression promoted cell proliferation in Capan-2 cells (Figure 7B). Moreover, LY294002 reversed CPA4 overexpression-promoted cell proliferation in vitro after four to five days of culturing (Figure 7B). Regarding little effect of CPA4 in cell proliferation within one to three days shown above, we treated the above cell lines with various concentrations of GEM for two days. CPA4 silencing inhibited drug resistance in PANC-1 cells, especially in 50 and 100 μm of GEM treatment (Figure 7C), whereas CPA4 overexpression promoted drug resistance in Capan-2 cells (Figure 7D). Moreover, LY294002 reversed

CPA4 overexpression-promoted drug resistance in vitro with 50 and 100 μm of GEM treatment (Figure 7D).

In addition, under GEM (50 μg/mL) treatment in PANC-1 cells, CPA4 silencing downregulated Bcl2, but upregulated Bax and cleaved caspase3 protein expression (Figure 7E). Conversely, CPA4 overexpression upregulated Bcl2, but downregulated Bax and cleaved caspase3 protein expression in Capan-2 cells, which was reversed by LY294002 (Figure 7F). Taken together, CPA4 promoted cell proliferation and drug resistance in PC cells via activating PI3K-AKT-mTOR signaling in Bcl2/Bax and caspase3-dependent apoptosis.

Discussion

CPA4 is a newly discovered oncogene which is prevalently observed in solid cancers. However, the definite function of CPA4 in PC is poorly understood. Our present study, for the first time, provides reliable evidence to verify that overexpression of CPA4 contributes to advanced clinical stage of PC patients in coordination with E-cad. Meanwhile, CPA4 promotes EMT in PC cells via activating PI3K-AKT-mTOR signaling, which is rarely reported, to our knowledge.

We first showed that CPA4 was overexpressed in PC tissues that was closely related with tumor size, T stage, lymph-node metastasis, and poor prognosis of PC patients. In previous studies, overexpression of CPA4 was significantly associated with grade, invasion depth, clinical stage, and poor prognosis in liver cancer.⁹ In esophageal squamous cell carcinoma, CPA4 overexpression, as an independent

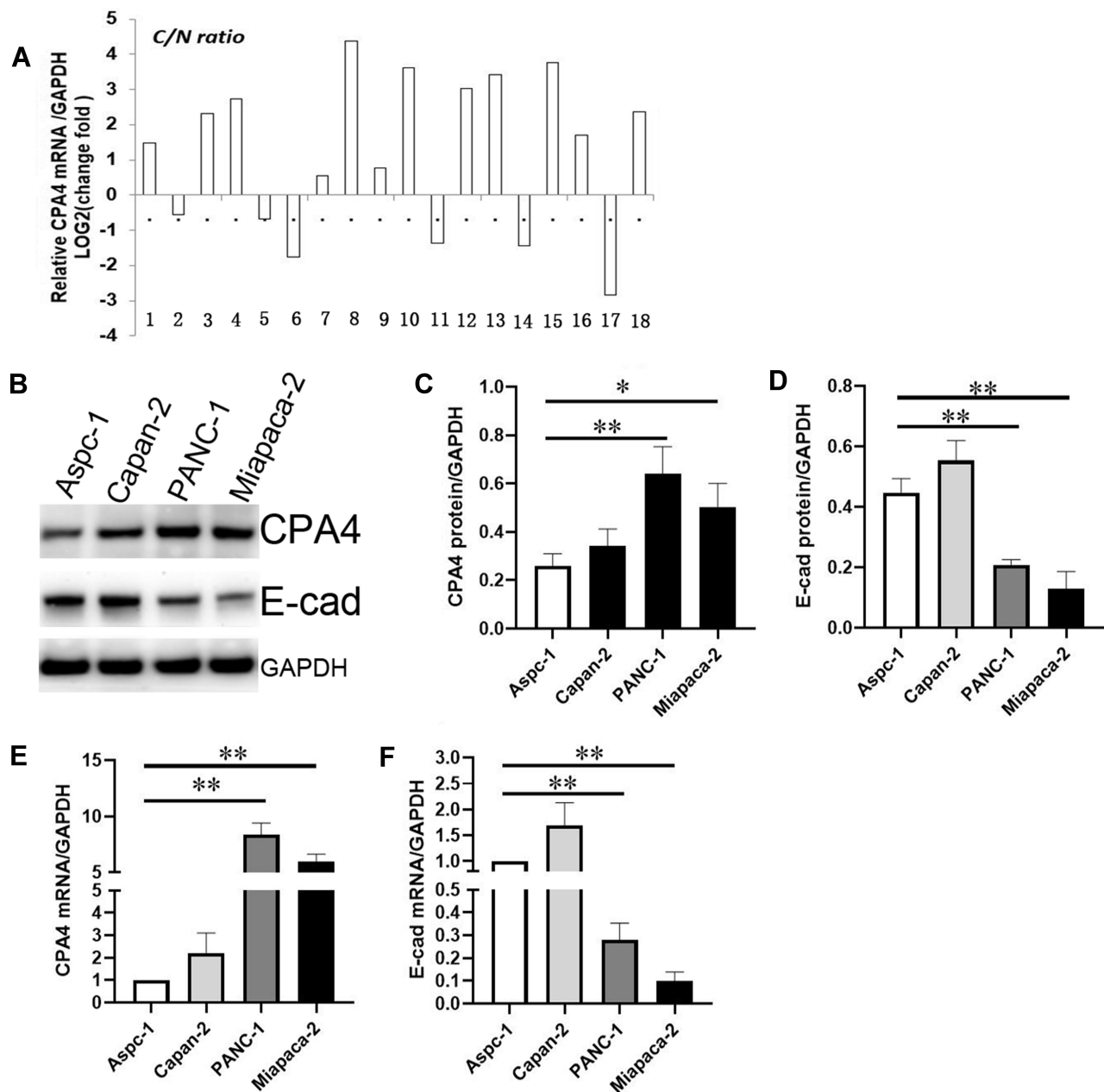


Figure 2 CPA4 was inversely related with E-cad expression in PC. (A) CPA4 mRNA level in 20 PC and adjacent pancreas (N: paired adjacent pancreas; (C) PC). (B) CPA4 and E-cad protein levels in PC cells. (C and D) the quantification of CPA4 (C) and E-cad (D) in WB. (E and F) CPA4 (E) and E-cad (F) mRNA levels in PC cells. Bars indicate \pm SE. * $P < 0.05$; ** $P < 0.01$ compared with the control.

Abbreviations: CPA4, carboxypeptidase A4; E-cad, E-cadherin.

prognostic indicator, was closely associated with histologic grade, lymph-nodes metastasis, and TNM stage.²⁰ The similar results were also observed in breast,¹⁰ prostate,¹¹ gastric,²¹ and colorectal cancers.¹³ Taken together, CPA4 is a promising tumor biomarker in cancers. It was noteworthy that an inverse correlation between CPA4 and E-cad was observed in PC specimens and cell lines which cooperatively promoted a worse prognosis for PC patients. E-cad-mediated adhesive junctions is a key hallmark of the EMT process,²²

especially in PC. For example, MBD1 overexpression promotes PC cells EMT by downregulating of E-cad.²³ Based on the above results and literature, we further investigated the role of CPA4 in the initiation of EMT in vitro.

CPA4 overexpression stimulated EMT in PC cells which promoted EMT-like cellular morphology, enhanced cell motility, and induced EMT signaling in vitro, and vice versa. The PI3K/AKT signaling is now identified as a potential target for treating metastatic tumors via mediating the EMT process.²⁴

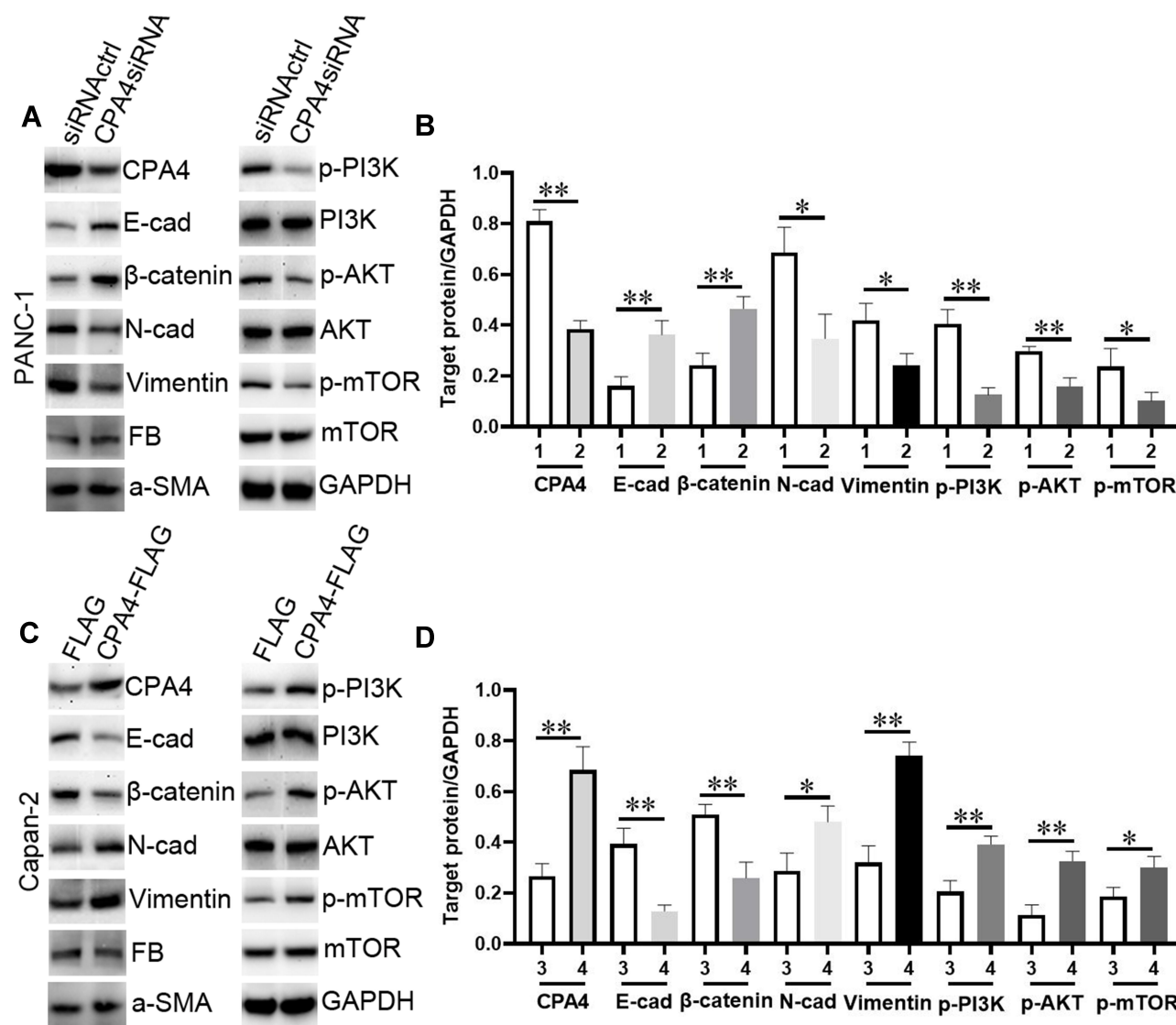


Figure 3 CPA4 mediated EMT and PI3K/AKT/mTOR signaling in vitro. **(A)** The protein level of CPA4, E-cad, β -catenin, N-cad, vimentin, fibronectin, a-SMA, p-PI3K (Tyr458), p-AKT (Ser473) and p-mTOR (Ser2448) in CPA4siRNA and siRNActrl transfected PANC-1 cells. **(B)** The quantification of VWB in **(A)**. **(C)** The expression of target proteins in CPA4-FLAG and FLAG transfected Capan-2 cells. **(D)** The quantification of VWB in **(C)**. E-cad: E-cadherin; N-cad: N-cadherin; FB: fibronectin. 1: siRNActrl; 2: CPA4siRNA; 3: FLAG; 4: CPA4-FLAG. Bars indicate \pm SE. * $P < 0.05$; ** $P < 0.01$ compared with the control.

Abbreviations: CPA4, carboxypeptidase A4; E-cad, E-cadherin; N-cad, N-cadherin; a-SMA, alpha smooth muscle actin; p-PI3K (Tyr458), phosphorylated PI3K (phosphorylated site Tyr458); p-AKT (Ser473), phosphorylated AKT (phosphorylated site Ser473); p-mTOR (Ser2448), phosphorylated mTOR (phosphorylated site Ser2448).

AKT, as a key component in PI3K/AKT/mTOR signaling, mediates EMT through suppressing E-cad via EMT transcription factors (Snail and Twist).^{25,26} AKT activation in prostate cancer leads to decreased E-cad that promotes Snail accumulation in the nucleus.²⁷ Meanwhile, mTOR complexes, as the key downstream target of PI3K-AKT signal pathway, also regulate EMT by direct activation of AKT.²⁸ mTOR signaling increases the invasiveness in renal carcinoma cell through inducing EMT.²⁹ TGF- β , as a critical cytokine, stimulates EMT through activating mTOR signaling.³⁰ Here, for the first time, CPA4 overexpression and silencing upregulated and downregulated p-PI3K (Tyr458), p-AKT (Ser473) and

p-mTOR (Ser2448) expression in vitro, respectively. Meanwhile, PI3K inhibitor LY294002 reversed CPA4 overexpression-promoted EMT in vitro. Briefly, CPA4 overexpression-promoted EMT-like cellular morphology, cell motility and EMT signaling was significantly inhibited by LY294002. Moreover, CPA4 was co-immunoprecipitated with AKT in CPA4 high expression PC cells. Taken together, a tight interaction of CPA4 with PI3K-AKT-mTOR signaling coordinate regulating EMT in PC cells. In previous studies, CPA4 silencing inhibited cell proliferation by suppressing the protein kinase B/c-MYC pathway in non-small-cell lung cancer.³¹ CPA4 promoted cell growth via activating STAT3 and ERK

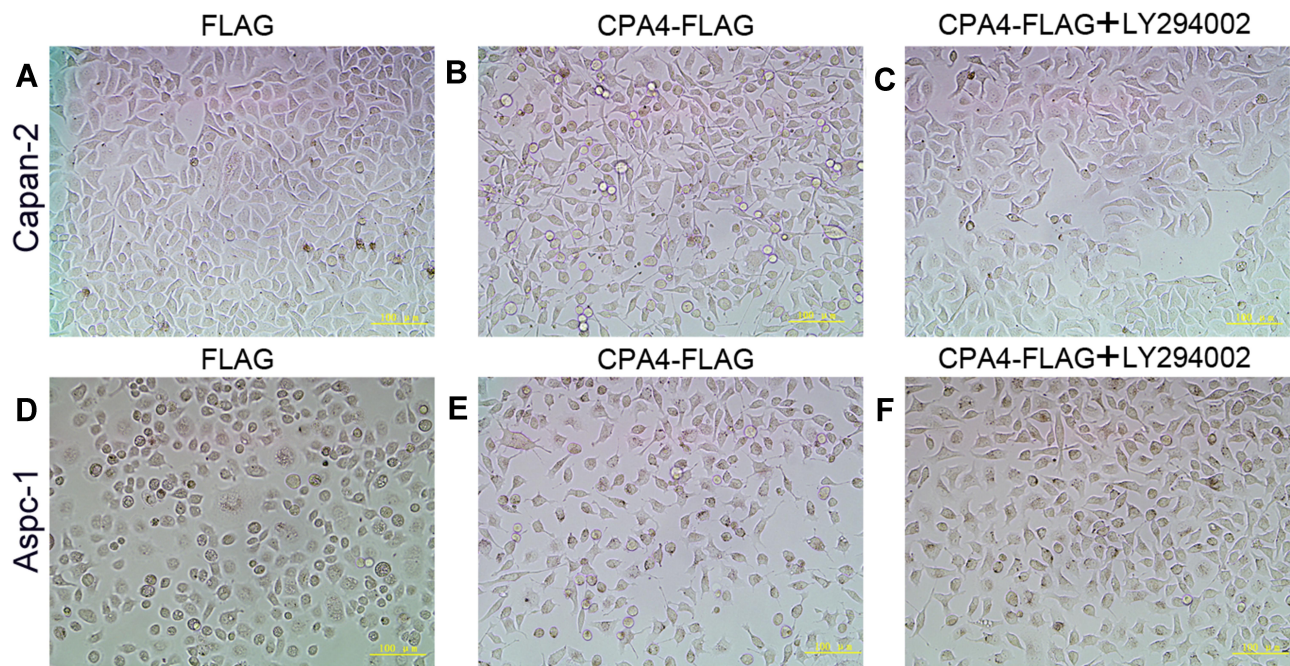


Figure 4 CPA4 promoted EMT-like cell morphology. (A–C) Cell morphology in FLAG (A), CPA4-FLAG (B) and CPA4-FLAG (C) plus LY294002 groups in Capan-2 cells (100× magnification). (D–F) Cell morphology in FLAG (D), CPA4-FLAG (E) and CPA4-FLAG (F) plus LY294002 groups in Aspc-1 cells (100× magnification).
Abbreviation: CPA4, carboxypeptidase A4.

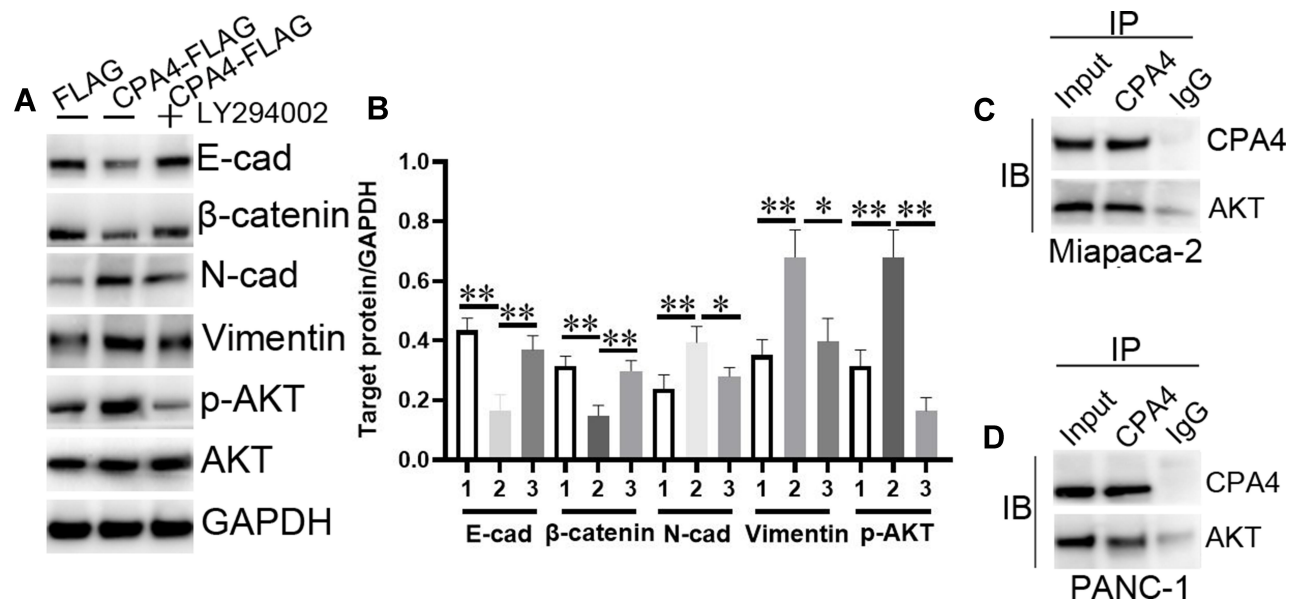


Figure 5 LY294002 inhibited CPA4 overexpression-induced EMT pathway. (A) The protein expression of EMT markers and p-AKT (Ser473) in FLAG, CPA4-FLAG and CPA4-FLAG plus LY294002 groups in Capan-2 cells. (B) The quantification of WB in (A). (C and D) CPA4 was co-immunoprecipitated with AKT in MIA PaCa-2 (C) and PANC-1 (D) cells. 1: FLAG; 2: CPA4-FLAG; 3: CPA4-FLAG+LY294002. Bars indicate \pm SE. * P <0.05; ** P 0.01 compared with the control.

Abbreviations: CPA4, carboxypeptidase A4; E-cad, E-cadherin; p-AKT (Ser473), phosphorylated AKT (phosphorylated site Ser473).

signaling pathways.¹³ Our study supplies a novel signal in CPA4-mediated EMT, which has not been reported in previous studies.

It is well known that Bcl2 and the caspase family mediated apoptosis are closely associated with drug resistance in solid

cancers.^{32–35} For the first time, we found that CPA4 overexpression or silencing promoted or inhibited cell proliferation and drug resistance in Capan-2 and PANC-1 cells via PI3K-AKT-mTOR signaling in Bcl2/Bax and caspase3-dependent apoptosis, which has not been reported before, to our

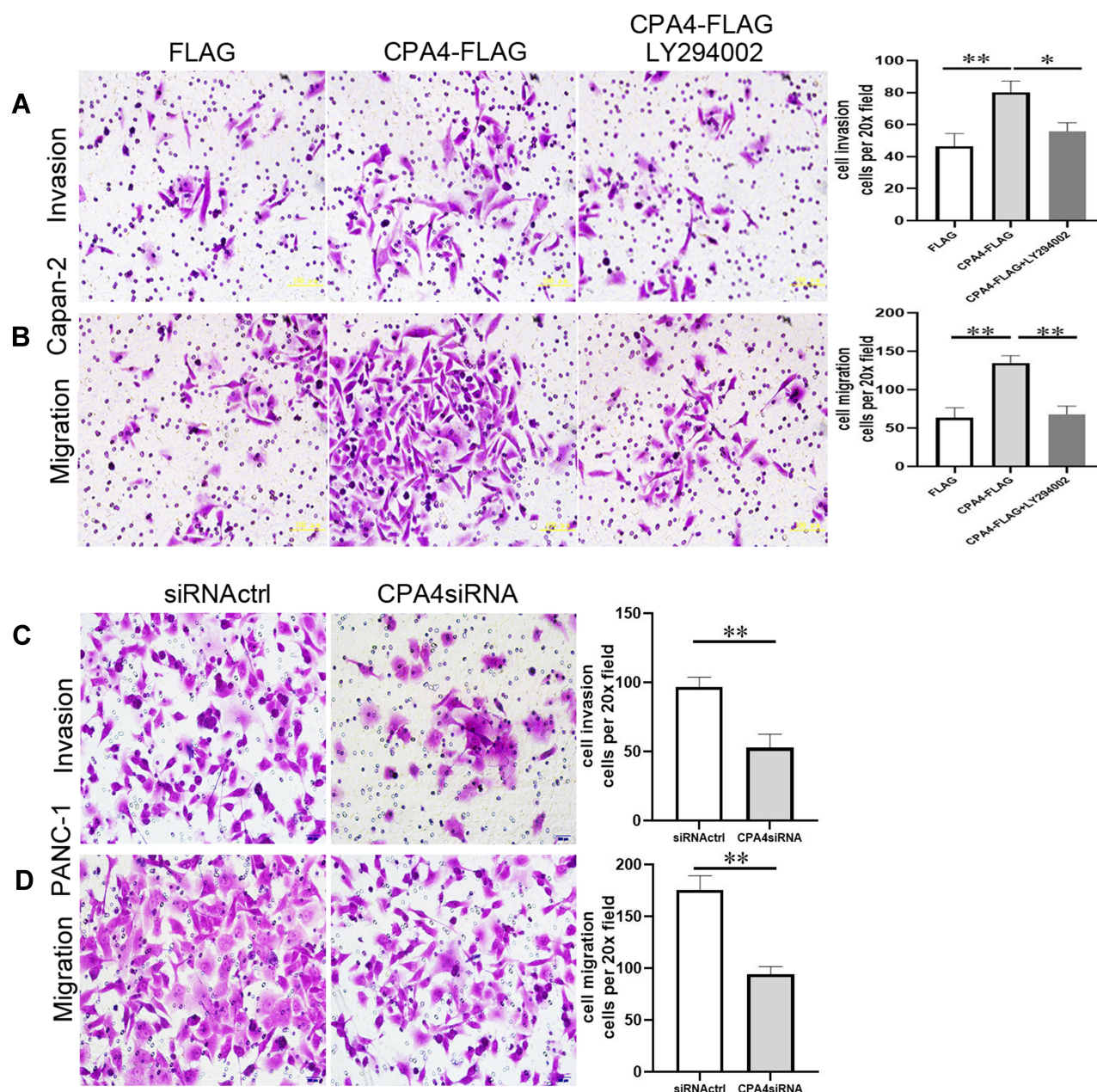


Figure 6 LY294002 inhibited CPA4 overexpression-enhanced cell invasion and migration in vitro. (**A** and **B**) Cell invasion (**A**) and migration (**B**) in FLAG, CPA4-FLAG and CPA4-FLAG combining LY294002 groups in Capan-2 cells. (**C** and **D**) Cell invasion (**C**) and migration (**D**) in siRNActrl and CPA4siRNA groups in PANC-1 cells. Bars indicate \pm SE. * $P < 0.05$; ** $P < 0.01$ compared with the control.

Abbreviation: CPA4, carboxypeptidase A4.

knowledge. As shown in Figure 8, CPA4 activates PI3K-AKT-mTOR signaling, following activating the phosphorylation of PI3K (Tyr458), AKT (Ser473) and mTOR (Ser2448). The subsequent activation of PI3K-AKT-mTOR signaling enhanced cell mobility via inducing EMT signaling and promoted drug resistance by attenuating apoptosis signaling (Bcl2/Bax and caspase3).

In conclusion, overexpression of CPA4 contributes to aggressive clinical stage of PC patients in coordination with

E-cad. Meanwhile, CPA4 promotes EMT in vitro via activating PI3K-AKT-mTOR signaling. Cooperative interaction between CPA4 and PI3K-AKT-mTOR signaling coordinately promotes EMT development in PC. However, the potential molecular mechanism remains unclear. E-cad, as the hallmark of EMT, is controlled by various transcription regulators such as Snail, Slug, ZEB1/2, and Twist. All the above transcription regulators are key regulators of EMT signaling, which are also regulated by PI3K-AKT-mTOR signaling.^{36,37} For example,

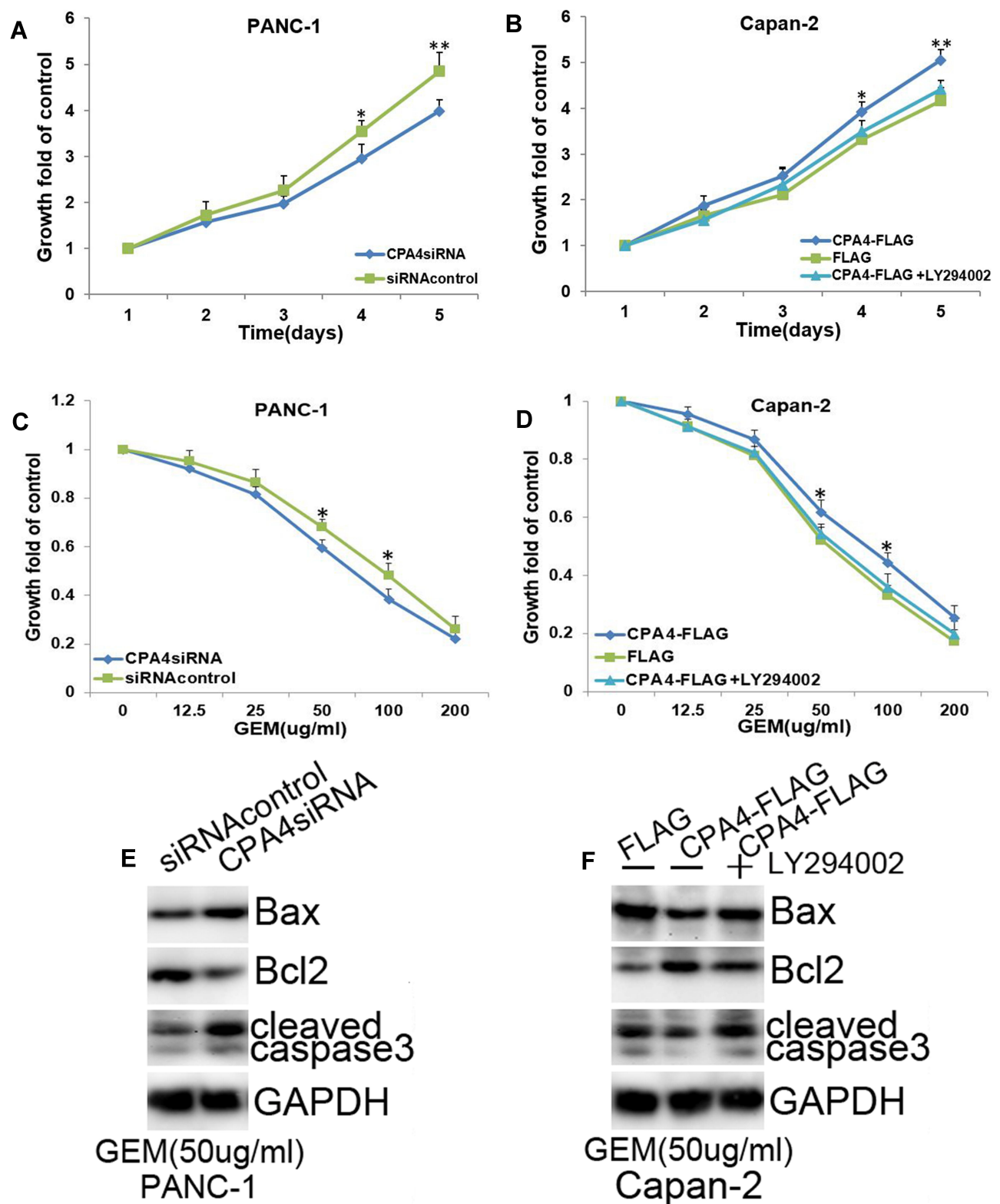


Figure 7 CPA4 promoted cell proliferation and drug resistance in PC cells via activating PI3K-AKT-mTOR signal pathway. (A and B) Cell proliferation in CPA4 silencing PANC-1 (A) and CPA4 overexpressing Capan-2 cells by MTT. (C and D) Cell proliferation in CPA4 silencing PANC-1 (A) and CPA4 overexpressing Capan-2 cells with various concentration of GEM treatment by MTT. (E) Bax, Bcl2, and cleaved caspase3 protein level in CPA4 silencing PANC-1 cells under GEM (50 μ g/mL) treatment. (F) Bax, Bcl2, and cleaved caspase3 protein level in FLAG, CPA4-FLAG and CPA4-FLAG combining LY294402 groups in Capan-2 cells under GEM (50 μ g/mL) treatment. Bars indicate \pm SE. * P 0.05; ** P <0.01 compared with the control.

Abbreviation: CPA4, carboxypeptidase A4.

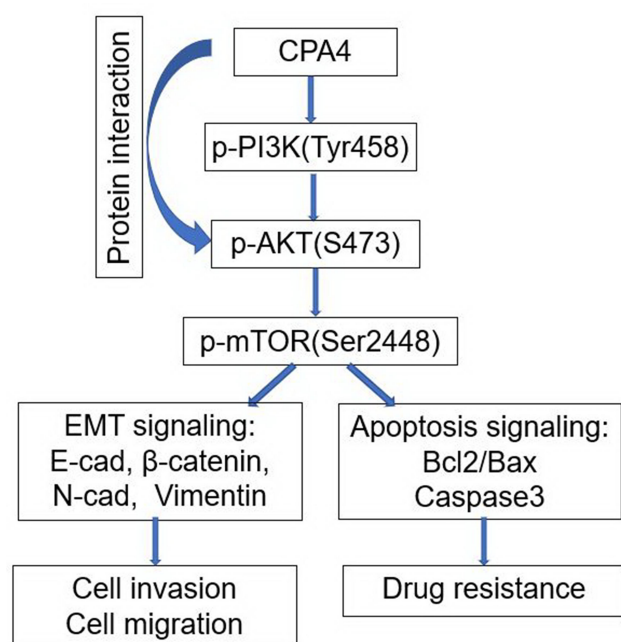


Figure 8 CPA4 activates PI3K-AKT-mTOR signaling, following activating the phosphorylation of PI3K (Tyr458), AKT (Ser473) and mTOR (Ser2448). The subsequent activation of PI3K-AKT-mTOR signaling enhanced cell mobility via inducing EMT signaling and promoted drug resistance by attenuating apoptosis signaling (Bcl2/Bax and caspase3).

Abbreviations: CPA4, carboxypeptidase A4; E-cad, E-cadherin; N-cad, N-cadherin; p-PI3K (Tyr458), phosphorylated PI3K (phosphorylated site Tyr458); p-AKT (Ser473), phosphorylated site Ser473; p-mTOR (Ser2448), phosphorylated mTOR (phosphorylated site Ser2448).

AKT inhibitor decreased Snail and Twist expression in oral squamous cell carcinoma,³⁸ and vice versa.³⁹ PI3K-AKT activation antagonizes the TGF- β /SMAD-induced cytostatic response in cancer.⁴⁰ PI3K-AKT pathway is critical in the increased expression of Twist induced by osteopontin in hepatocellular carcinoma.⁴¹ PI3K/mTORC2 regulates TGF- β /Activin signaling by modulating Smad2/3 activity via linker phosphorylation.⁴² Based on the above research, we intend to investigate if CPA4 regulates PI3K-AKT-mTOR targeted transcription regulators in a future study. In additional, PI3K/AKT/mTOR signaling acts as a critical regulator of autophagy which plays a dual role in the EMT process.^{43,44} We reason that the interaction of CPA4 and PI3K/AKT/mTOR in autophagy also plays a significant role in EMT. Finally, as we know, mTOR forms two multiprotein complexes, mTORC1 and mTORC2 which are composed of discrete protein binding partners to regulate cell growth, motility, and metabolism. mTORC1 phosphorylates several downstream targets to promote biosynthesis of proteins, lipids, and nucleotides, while mTORC2 plays seminal functions in cell metabolism and survival through the p-AKT (Ser473).⁴⁵ However, LY294002 we used in current study did not distinguish

between these two multiprotein complexes. mTOR is targeted by rapamycin (a macrolide antibiotic with critical antiproliferative properties), which showed different sensitivity on mTORC1 and mTORC2. As Mukhopadhyay et al suggest, high-dose rapamycin (middle), completely inhibits mTORC1 and cell-cycle progression, while low-dose rapamycin plus PDL inhibitor inhibits both mTORC1 and mTORC2 and cell death.⁴⁵ We will use rapamycin with various concentration to investigate the different role of mTORC1 and mTORC2 in CPA4 mediated PI3K-AKT-mTOR signaling in the future. Interestingly, AMP-activated protein kinase (AMPK), a critical sensor of energy sufficiency, acts as a central metabolic switch in cell metabolism. AICAR—a compound that activates AMP-activated protein kinase, suppresses mTORC1, but also stimulates a feedback activation of mTORC2, which activates the survival kinase AKT.⁴⁶ As we know, a critical factor regulating mTOR is phosphatidic acid (PA), a central metabolite of membrane lipid biosynthesis and the product of the phospholipase D (PLD)-catalyzed hydrolysis of phosphatidylcholine. Recently, a reciprocal regulation of PLD by AMPK and regulation of AMPK by PLD is observed in MDA-MB-231 cells. Suppressing AMPK activity led to an increase in PLD activity, and vice versa. Suppressing PLD activity resulted in elevated AMPK activity.⁴⁷ Thus, AICAR would be the next target for us to investigate additional molecular mechanisms of CPA4 mediated PI3K/AKT/mTOR signaling in PC.

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Disclosure

The authors declare no conflicts of interest in this work.

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