



Knockdown of carboxypeptidase A4 (CPA4) inhibits gastric cancer cell progression via cell cycle arrest and apoptosis

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Background: Gastric cancer is one of the most prevalent cancers, with a low survival rate at the later stages. Carboxypeptidase A4 (CPA4) is associated with the aggressiveness and growth in cancer. However, its regulatory role in gastric cancer remains unknown. Therefore, we investigated the role of CPA4 in gastric cancer progression *in vitro*.

Methods: The human gastric adenocarcinoma cell line (AGS cell line) was used in the present study. CPA4 knockdown lentiviruses were constructed. Western blot analysis was performed to evaluate the protein expression levels of epithelial-mesenchymal transition (EMT) transcription factors, EMT biomarkers, and proteins involved in the Wnt signaling pathway, mitogen-activated protein kinase (MAPK) signaling pathway, and phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was carried out to evaluate the mRNA expression level of CPA4. The String database was employed for protein-protein interaction (PPI) network analysis. Cell colony formation, proliferation, migration, invasion, apoptosis, and cell cycle analyses were performed using corresponding kits.

Results: CPA4 is highly expressed in gastric cancer cell lines. Overexpressed CPA4 was associated with the induction of EMT. Knockdown of CPA4 inhibited cell colony formation, proliferation, migration, and invasion of gastric cancer cells. Knockdown of CPA4 also promoted cell apoptosis of gastric cancer cells.

Conclusions: Knockdown of CPA4 inhibited cell progression via arresting the cell cycle and inducing EMT in gastric cancer.

Keywords: Gastric cancer; carboxypeptidase A4; cell cycle; progression

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Introduction

Gastric cancer is one of the most prevalent cancers, with more than one million cases diagnosed worldwide annually (1). It was reported that males are more susceptible to developing gastric cancer from birth to age 74 compared with females (1). Gastric cancer accounts for 8.3% of all cancer-related deaths (2). The 5-year survival rate was shown to vary in patients with different stages during surgical intervention, with 94% at stage IA, 88% at stage IB, and only 18% at stage IIIC (3). Therefore, it is of great importance to identify key biomarkers for evaluating gastric cancer progression, as well as exploring potential therapeutic targets for gastric cancer therapy.

Carboxypeptidase A4 (*CPA4*) is a member of the Zn-containing metalloprotease (MCP) family. It catalyzes the release of carboxy-terminal amino acids. It has been confirmed that *CPA4*, an important regulator of inflammation, plays an important role in tumor microenvironment (TME) formation and distant metastasis in tumor (4,5). *CPA4* has been shown to be upregulated in several cancers, and is speculated to be involved in regulating the progression of several cancers, including non-small cell lung cancer (6), pancreatic cancer (7), bladder cancer (8), colorectal cancer (9), hepatocellular carcinoma (10), and breast cancer (11). For example, *CPA4* expression was associated with poor prognoses by promoting cell proliferation in hepatocellular carcinoma (10). Upregulated *CPA4* expression was an independent prognostic factor for patients with liver cancer (12). Sun *et al.* (13) demonstrated that *CPA4* is associated with poor prognosis in patients with gastric cancer. However, the regulatory role of *CPA4* in gastric cancer is still unknown.

In the present study, we explored the effects of *CPA4* on gastric cancer progression *in vitro*. This study may provide a foundation for further study into therapeutic targets for gastric cancer. We present the following article in accordance with the MDAR reporting checklist (available at <https://jgo.amegroups.com/article/view/10.21037/jgo-22-987/rc>).

Methods

Cell culture

Human gastric adenocarcinoma cell line (AGS cell line) were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in RPMI-1640 culture medium (Thermo Fisher Scientific, Waltham, MA,

USA) at 37 °C with 5% CO₂.

Construction of lentiviruses for *CPA4* knockdown

The target insert sequence of *CPA4* (5'-TTCTCCGAACG TGTCACGT-3') was designed by Shanghai GeneChem Co. Ltd. Two single-chain DNA sequences were designed by Shanghai Generay Biotech Co., Ltd.: sh*CPA4*S1, 5'-CCGGGCGTATGACAACGGCATCAAATTC AAGAGA TTTGATGCCGTTGTCATACGCTTTTGG-3'; and sh*CPA4*S2, 5'-AATTC AAAAAGCGTATGACAACGGC ATCAAATCTCTTGAATTTGATGCCGTTGTCATA CGC-3'. Single-chain DNA was dissolved into annealing buffer (20 μM) and incubated in a water bath at 90 °C for 15 min. After being naturally cooled to room temperature, a double strand with sticky ends was formed. The GV115 lentiviral vector was linearized by AgeI (#R3552L; New England BioLabs, USA) and EcoRI (#R3101L; New England BioLabs, USA). Then, the DNA sequences were ligated into the linearized GV115 lentiviral vector, and the resulting plasmid was used to transform top 10 *E. coli* competent cells (#CB104-03; TIANGEN, Beijing, China). The plasmid was extracted from the top 10 *E. coli* competent cells and verified by restriction endonuclease digestion followed by Sanger sequencing (Genscript Co., Ltd.; Nanjing, China). Transfection was performed using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc., USA). We collected the supernatants containing lentiviruses after 48 h of culture and purified them at 1,000 ×g and 4 °C for 2 min (Himac CT15RE; Hitachi).

Quantitative real-time PCR (qRT-PCR) analysis

The expression level of *CPA4* was evaluated in AGS and transfected cells. The total RNA in transfected cells was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA). The primers were designed and synthesized by Sangon (Shanghai, China). The primers are listed as follows: *CPA4* forward primer: 5'-ATTGGACATTCGTTTGGAAACCG-3'; *CPA4* reverse primer: 5'-GGGAGATCCACTCTCGGGA-3'. GAPDH forward primer: 5'-TGACTTCAACAGCGACACCCA-3'; GAPDH reverse primer: 5'-CACCTGTTGCTGTAGCCAAA-3'. Then, qRT-PCR analysis was performed by applying the HiScript II One Step qRT-PCR SYBR Green Kit (#Q221-01), tested on an ABI 7900 system (Foster City, CA, USA). Expression was normalized to GAPDH and calculated using the 2^{-ΔCt} method.

Western blot analysis

The proteins were isolated by RIPA lysis buffer (#R0278, Sigma). The standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method was applied to separate the proteins (14). Then, the gels were transferred, blocked, and cultured with primary antibodies against the following: *CPA4* (1:300; #ab81543; Abcam); epithelial-mesenchymal transition (EMT)-related transcription factors NFκB p65 (1:2,000; #8242; CST), p-NFκB p65 (1:1,000; #3033; CST), Slug (1:1,000; #9585; CST), Fibronectin (1:500; MAB1918; R&D), N-cadherin (1:1,000; ab18203; Abcam), Vimentin (1:1,000; #3932; CST), and E-cadherin (1:1,000; #14472; CST); multiple malignancy-associated genes Twist (1:100; ab50887; Abcam), c-Myc (1:1,000; ab32072; Abcam), and Snail (1:2,000; #3879; CST); Wnt signaling pathway proteins β-catenin (1:2,000; #8480; CST) and p-β-catenin (1:1,000; #2009; CST); PI3K/AKT/mTOR signaling pathway proteins AKT (1:2,000; ab179463; Abcam), p-AKT (1:1,000; #4060; CST), mTOR (1:500; #2972; CST), and p-mTOR (1:2,000; ab109268; Abcam); MAPK signaling pathway proteins p38 (1:2,000; #8690; CST) and p-p38 (1:1,000; #4631; CST); and tumor migration and invasion-related proteins ERK (1:2,000; ab17942; Abcam), p-ERK (1:2,000; #4370; CST), MMP-2 (1:500; #40994; CST), and MMP-9 (1:500; #13667; CST). GAPDH (1:10,000; #sc-32233; Santa-Cruz) was used as the internal control. The membranes were washed. Then they were cultured with HRP-goat anti rabbit secondary antibody (1:10,000; AS1107; ASPEN) and HRP-goat anti mouse (1:10,000; AS1106; ASPEN). Finally, the bands were observed under ECL plus and X-ray film.

Protein-protein interaction (PPI) network

According to the significantly dysregulated proteins in sh*CPA4* compared with shCtrl cells, a PPI network of *CPA4* was generated. Analysis of the interaction network was performed using the STRING database (<https://string-db.org/>).

Cell colony formation analysis

AGS cells in logarithmic growth phase were subjected to cell trypsin digestion (#25200-720; Gibco), resuspended, then counted. About 500 cells/well were seeded in a 6-well plate, and each sample was performed in triplicate. After

culture for 14 days, the cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 30–60 min, and washed with PBS again. Then, the cells were stained with impurity-free crystal violet staining solution for 10–20 min, washed with ddH₂O, and photographed under a fluorescence microscope (XDS-100; Caikon, Shanghai, China).

Cell proliferation assay

AGS cells in logarithmic growth phase were subjected to cell trypsin digestion (#25200-720; Gibco; Thermo Fisher Scientific, Inc., USA), resuspended, then counted. Approximately 2,000 cells/well were seeded in each well of a 6-well plate and cultured at 37 °C with 5% CO₂. Celigo® Image Cytometer (Nexcelom) was used to evaluate the number of cells by scanning green fluorescence daily for 5 days at room temperature (15).

Cell migration and invasion analyses

The Transwell assays were applied to evaluate the cell migration and invasion of cells. A 24-well Transwell chamber and 8.0-μm pore membranes (Corning, USA) coated with Matrigel (BD Biosciences) were used for the cell invasion assay. Approximately 1.0×10⁵ cells/well in 100 mL serum-free Dulbecco's modified Eagle's medium (DMEM) were seeded in the top chamber for 6 h at 37 °C. Then 500 mL medium containing 30% fetal bovine serum (FBS) was placed into the lower chamber. The Transwell was washed, fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet. Eventually, 5 visual fields were randomly selected, and the cell numbers were counted under a 400× microscope. The cell migration assay was similar to that of invasion assay except that the chamber was not coated with Matrigel.

Cell apoptosis analysis

About 5.0×10⁵ cells were added into a 6-well plate and maintained at 37 °C with 5% CO₂ until the cell growth coverage reached 70%. The cells were collected, washed with pre-chilled D-Hanks, and treated with 1× binding buffer. Then, the cells were stained with Annexin V-APC for 10–15 min and observed under a fluorescence microscope (IX71, OLYMPUS, Tokyo, Japan).

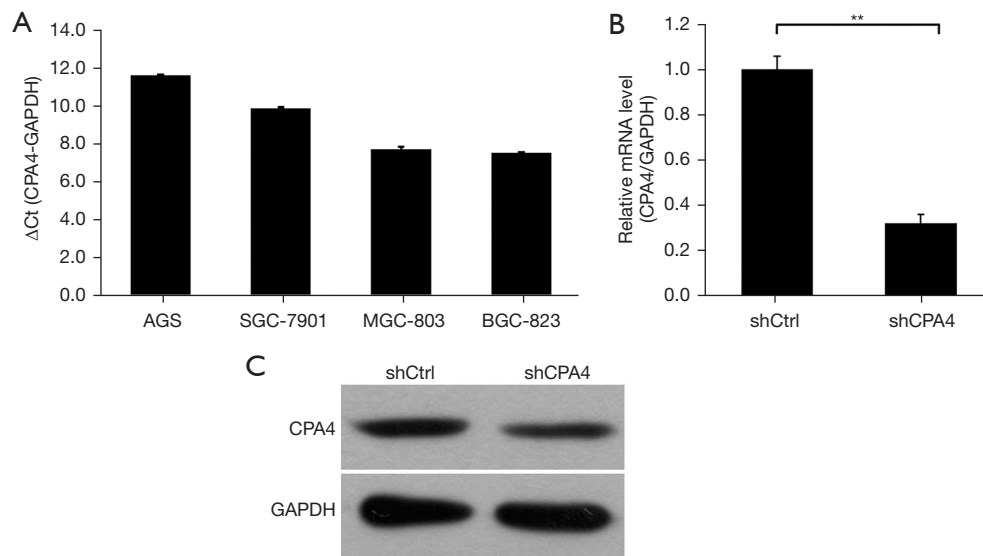


Figure 1 The expression levels of *CPA4* in gastric cancer cell lines. (A) The mRNA expression levels of *CPA4* in gastric cancer cell lines. (B) The mRNA expression levels of *CPA4* in sh*CPA4* and shCtrl gastric cancer cells. (C) The protein expression levels of *CPA4* in sh*CPA4* and shCtrl gastric cancer cells. **, $P < 0.01$ compared with shCtrl group. *CPA4*, carboxypeptidase A4; AGS, human gastric adenocarcinoma cell line.

Cell cycle assay

About 2.0×10^6 cells were seeded into a 6-cm dish after lentiviral infection for 5 days. The dishes were maintained at 37 °C for 40 h until the confluence of cells reached about 80%. The cells were washed with pre-chilled D-Hanks (pH 7.2–7.4) and fixed with 70% ethanol overnight at 4 °C. After digestion with RNaseA, the cells were labeled with a solution of 40× PI, 100× RNase, and 1× D-Hanks at a ratio of 25:10:1,000 and were analyzed with Cell Lab Quanta Beckman Coulter to measure fluorescence. The cell cycle was analyzed by ModFit LT 3.2 (Verity Software House).

Statistical analysis

GraphPad Prism 8 (San Diego, CA, USA) was used for the statistical analysis and graph drawing. Student's *t*-test or one-way ANOVA was performed for comparative analyses depending on the number of groups. Data are exhibited as mean ± standard deviation (SD). $P < 0.05$ indicated that the difference was statistically significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results

CPA4 is highly expressed in gastric cancer cell lines

The expression of *CPA4* was significantly upregulated in

the gastric cancer cell lines AGS, SGC-7901, MGC-803, and BGC-823, with the highest expression level in AGS cells and the lowest in MGC-803 cells (Figure 1A). After lentivirus transfection with sh*CPA4*, the mRNA and protein expression levels of *CPA4* were significantly decreased ($P < 0.01$; Figure 1B,1C), which demonstrated the successful construction of knockdown samples.

Overexpressed *CPA4* is associated with induction of EMT

The role of *CPA4* in gastric cancer was assessed by testing the expression levels of EMT-related markers in transfected cells. In the western blot analysis, we found that E-cadherin was upregulated, while N-cadherin and Vimentin were downregulated in sh*CPA4* cells (Figure 2A), indicating that overexpressed *CPA4* was associated with the induction of EMT. As transcription factors of EMT, p-NFκB-p65, Slug, Twist, and Snail protein expression levels were significantly downregulated in sh*CPA4* compared with shCtrl cells (Figure 2B). These results demonstrated that *CPA4* promotes EMT processes in gastric cancer cells.

PPI network analysis

The regulatory relationship among *TWIST1*, *FN1*, *SNAI1*, *SNAI2*, *VIM*, *MMP9*, *RELA*, *CDH1*, *CDH2*, and *CPA4* was carried using STRING database (Figure 3). *CPA4* was

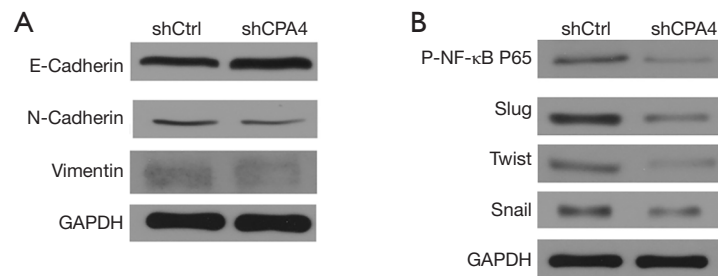


Figure 2 *CPA4* promotes the process of EMT in gastric cancer cells. (A) Western blot results showed that the effect of knockdown of *CPA4* on expression levels of EMT-related markers in gastric cancer cells. (B) Western blot results demonstrated that the effect of knockdown of *CPA4* on expression levels of transcription factors of EMT in gastric cancer cells. *CPA4*, carboxypeptidase A4; EMT, epithelial-mesenchymal transition.

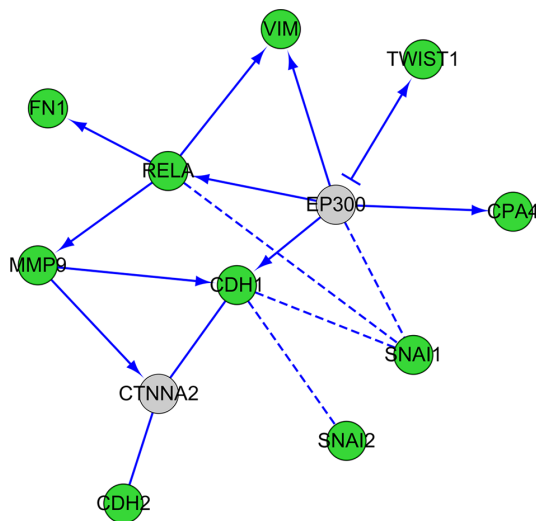


Figure 3 The PPI network of *CPA4*. *CPA4* was activated by *EP300*. The green circles represent the genes we selected. The gray circles represent related genes in the STRING database. The blue arrow represents the activation relationship. The termination arrow represents an inhibitory relationship. The dotted line represents the predicted regulatory relationship. The solid line represents the established control relationship. PPI, protein-protein interaction; *CPA4*, carboxypeptidase A4.

activated by *EP300*, and it also activates *VIM*, *RELA*, *SNAI1* and *CDH1*. The results provide insights into the potential regulatory relationship of *CPA4* in gastric cancer.

Knockdown of *CPA4* inhibits cell colony formation, proliferation, migration, and invasion of gastric cancer cells

The colonies of *shCPA4* cells were significantly lower than

those of *shCtrl* cells ($P < 0.01$; *Figure 4A*). The cell count and cell count/fold ratio in the *shCPA4* group were significantly lower than those in *shCtrl* cells ($P < 0.01$; *Figure 4B*). The results indicated that *CPA4* promoted the proliferation of gastric cancer cells. The numbers of migrating and invading cells were significantly repressed by *shCPA4* ($P < 0.01$; *Figure 4C,4D*). Overall, the results demonstrate that knockdown of *CPA4* inhibits cell colony formation, proliferation, migration, and invasion of gastric cancer cells.

Knockdown of *CPA4* promotes cell apoptosis in gastric cancer

ShCPA4 significantly increased the number of apoptotic cells compared with *shCtrl* ($P < 0.01$; *Figure 5A*), demonstrating that *shCPA4* promotes cell apoptosis. Compared with *shCtrl*, *shCPA4* significantly decreased the cells in S (25.88% vs. 18.66%; $P < 0.05$) and G2/M phase (12.83% vs. 10.57%; $P < 0.05$), and increased those in G1 phase (60.39% vs. 69.43%; $P < 0.05$; *Figure 5B*). These results demonstrated that knockdown of *CPA4* promoted the cell apoptosis of gastric cancer cells due to its arrest of cell cycle in G1 phase.

Discussion

Gastric cancer is a common cancer with a high survival rate at the early stage. However, the 5-year overall survival rate significantly decreases when patients are diagnosed at the later stage. Therefore, exploring useful biomarkers for gastric cancer progression and therapeutic targets are of great importance. In the present study, we investigated the role of *CPA4* in gastric cancer progression *in vitro*.

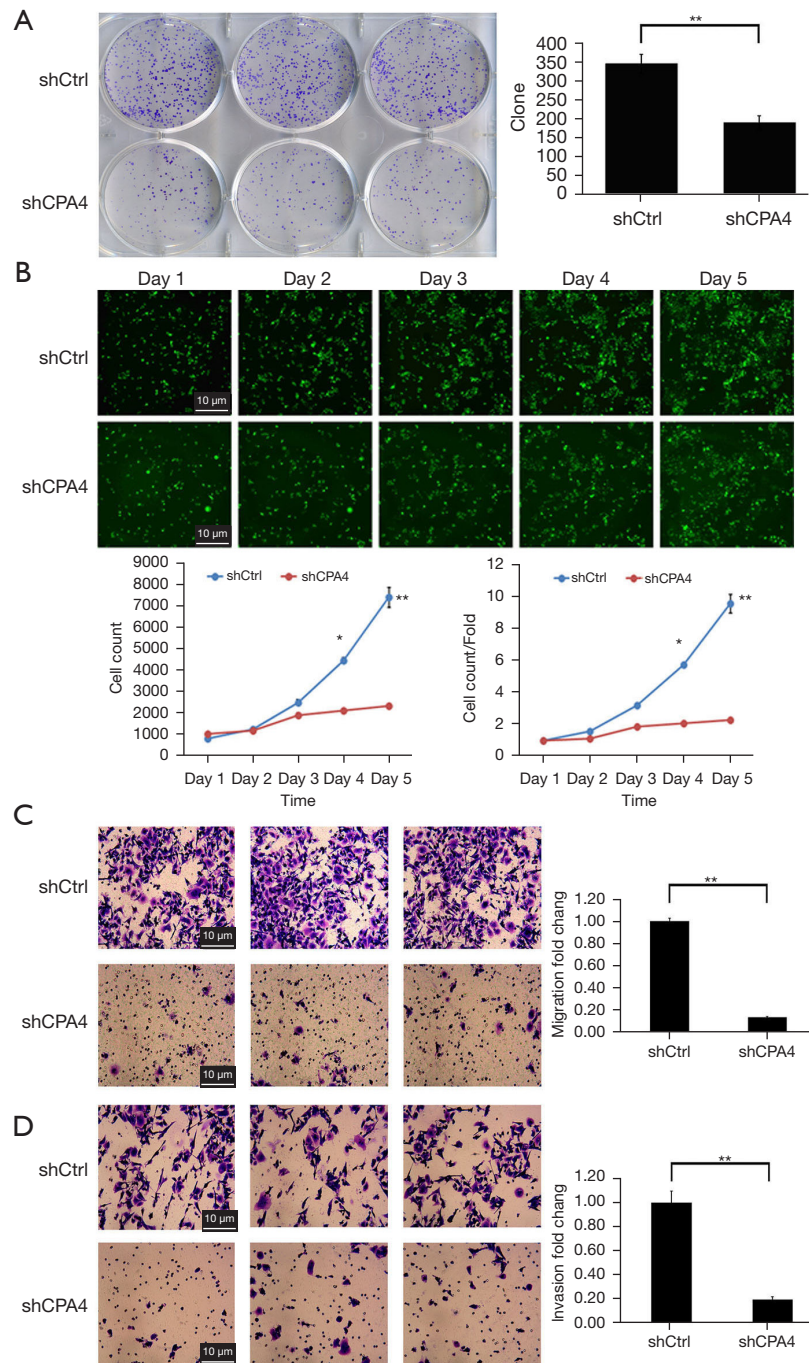


Figure 4 Knockdown of *CPA4* inhibits cell colony formation, proliferation, migration, and invasion of gastric cancer cells. (A) Colony formation was analyzed after staining with crystal violet and counted by using the ImageJ software (ImageJ Software., MD, USA). (B) Cell proliferation was analyzed in the shCtrl and shCPA4 groups. The gastric cancer cells were infected with lentiviruses expressed GFP. Cell counting was performed by using the Celigo Imaging Cytometer and the captured cell images were analyzed by using Celigo software version 1.0 (both from Nexcelom Bioscience LLC). (C) Cell migration analysis was performed in the shCtrl and shCPA4 groups. The gastric cancer cells in the lower chamber was stained with 0.1% crystal violet. (D) Cell invasion analysis was performed in the shCtrl and shCPA4 groups. The gastric cancer cells in the lower chamber was stained with 0.1% crystal violet. *, $P < 0.05$; **, $P < 0.01$ compared with shCtrl group. *CPA4*, carboxypeptidase A4; GFP, green fluorescent protein.

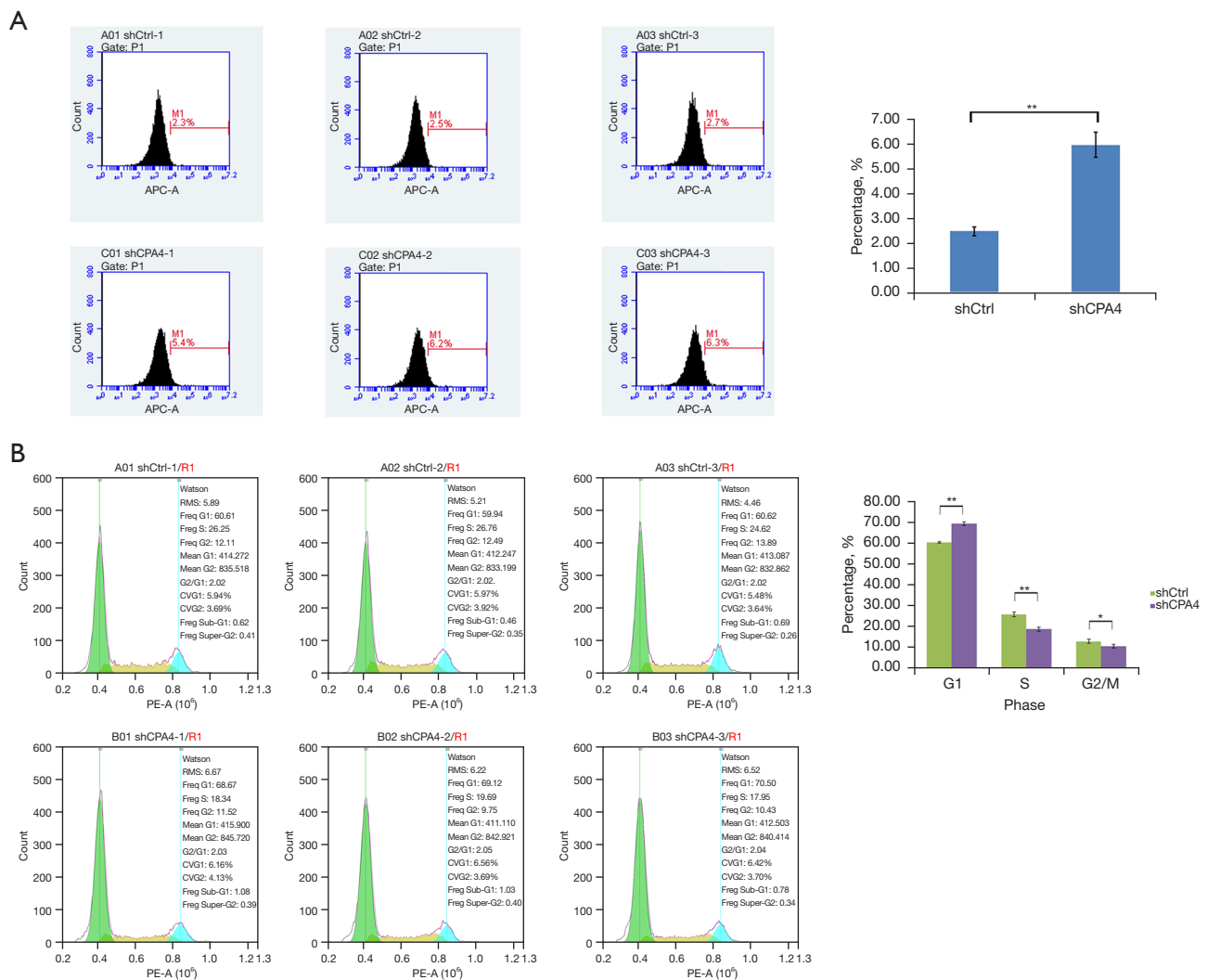


Figure 5 Knockdown of *CPA4* promotes gastric cancer cell apoptosis and cell cycle arrest at G1 phase. (A) Cell apoptosis was analyzed in the shCtrl and sh*CPA4* groups. (B) The cell cycle was assessed in the shCtrl and sh*CPA4* groups. *, $P < 0.05$; **, $P < 0.01$ compared with shCtrl group. *CPA4*, carboxypeptidase A4.

We found that: (I) *CPA4* was significantly upregulated in gastric cancer cells; (II) knockdown of *CPA4* inhibits cell proliferation, and cell migration and invasion, and promotes cell apoptosis and arrests the cell cycle at G1 phase; (III) *CPA4* regulates gastric cancer progression through EMT processes.

In the present study, *CPA4* was upregulated in gastric cancer cells, which was consistent with a report by Sun *et al.* (13). They analyzed the relationship between overexpressed *CPA4* and clinical factors such as tumor size, stage, lymph node metastasis, depth of invasion, and distant metastasis and demonstrated that overexpressed *CPA4* can be utilized

as an independent poor prognostic factor in gastric cancer. In our present study, we lacked clinical verification, but emphasized the role of *CPA4* in gastric cancer progression. We demonstrated that overexpressed *CPA4* promotes cancer cell progression. In other cancers, *CPA4* was also reported to be upregulated, and overexpressed *CPA4* has been shown to be involved in cancer cell progression. Zhang *et al.* (10) revealed that *CPA4* promotes the proliferation and stem cell characteristics of hepatocellular carcinoma. Downregulation of *CPA4* inhibits the cell growth of non-small cell lung cancer through the AKT/c-MYC pathway (16). Shao *et al.* (7) demonstrated that overexpressed *CPA4* promotes

pancreatic cancer via stimulating PI3K/AKT/mTOR signaling. Overall, we can conclude that *CPA4* exhibited similar functions in gastric cancer cells as in other cancers.

As mentioned above, *CPA4* regulates cancer through various signaling pathways. However, in the present study, we also found that EMT processes were induced. EMT is an important process for vertebrate embryonic development, driven by transcription factors such as *SNAIL1*, *TWIST1*, and *ZEB1* in mesenchymal programs during embryogenesis, and deletion of *SNAIL1* in mice results in embryonic lethality with impaired mesoderm development (17,18). In the present study, the expression levels of *SNAIL* and *TWIST* were significantly downregulated by sh*CPA4*. The EMT-related biomarkers E-cadherin, N-cadherin, and Vimentin were dysregulated by sh*CPA4*. These results demonstrated that *CPA4* induces EMT in gastric cancer cells. EMT is involved in the progression of many cancers. It may also be involved in gastric cancer progression by arresting the cell cycle at G1 phase. Lovisa *et al.* (19) found that transgenic expression of either *TWIST* or *SNAIL* is sufficient to promote prolonged TGF- β 1-induced G2 arrest of tubular epithelial cells, limiting the cells' potential for repair and regeneration. *SNAIL* can block the cell cycle and confers resistance to cell death (20). Yang *et al.* (21) demonstrated that the role of cell cycle state in the regulation of TGF- β -induced EMT and apoptosis, and also confirmed that TGF- β induced EMT via a cell cycle-dependent mechanism. Therefore, we can assume that EMT is regulated by cell cycle arrest caused by the dysregulation of *CPA4*. Altogether, *CPA4* was upregulated in gastric cancer cells, and overexpressed *CPA4* promotes cancer cell progression through EMT by inducing cell cycle arrest. We speculate that administering a molecule that can specifically inhibit the transcription or translation of the *CPA4* gene in tumor cells, or specifically inhibit the expression or activity of the *CPA4* protein, may inhibit the growth, proliferation, differentiation, and/or survival of tumor cells. However, the regulation process is a complex scientific issue that requires more experiments for verification, as *in vivo* experiments are lacking.

Conclusions

The present study confirmed the role and function of *CPA4* in gastric cancer *in vitro*. It has revealed that *CPA4* was significantly upregulated in AGS gastric cancer cells, and knockdown of *CPA4* was related to inhibited cell progression via arresting the cell cycle and inducing EMT.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <https://jgo.amegroups.com/article/view/10.21037/jgo-22-987/rc>

Data Sharing Statement: Available at <https://jgo.amegroups.com/article/view/10.21037/jgo-22-987/dss>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://jgo.amegroups.com/article/view/10.21037/jgo-22-987/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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