Original Article

CENPF promotes the proliferation of renal cell carcinoma *in vitro*

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Background: Metastasis and drug resistance are the main causes of renal cell carcinoma (RCC) mortality. Currently, there are still a limited number of targeted therapies against advanced RCC. It is critical to develop new effective clinical biomarkers and drug targets in RCC. Several studies have shown that centromere protein F (CENPF), a microtubule binding protein, promotes cancer progression in various types of cancer. The purpose of this study was to explore the role of CENPF in RCC.

Methods: Peripheral blood and corresponding tissue samples of 23 RCC patients and 23 normal physical examination patients who were treated in our hospital from 2018 to 2020 were collected, and CENPF expression was detected by quantitative real-time polymerase chain reaction (qRT-PCR), western blot, and immunohistochemical (IHC) methods. The expression of CENPF was downregulated by small interfering RNA (siRNA) transfection, and the proliferation of the corresponding RCC cells and the corresponding cell cycle were detected.

Results: According to The Cancer Genome Atlas (TCGA) data analysis, CENPF is highly expressed in RCC, and its expression level is significantly related to the overall survival (OS) and recurrence-free survival (RFS) of RCC. In addition, high expression of CENPF was found in the tissues of RCC patients in our hospital. Knockdown of CENPF significantly reduced the proliferation of RCC cells in vitro, and knockdown of CENPF regulated the cell cycle by inhibiting the expression of cyclins such as CDK4, CDK6, and CyclinD1.

Conclusions: CENPF can be used as an independent prognostic factor of RCC and regulate the proliferation ability and cell cycle of RCC cells. CENPF is a potential oncogene and prognostic marker in RCC.

Keywords: Renal cell carcinoma (RCC); centromere protein F (CENPF); cell proliferation; cell cycle regulation

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Introduction

Renal cell carcinoma (RCC) is the ninth most common cancer worldwide. It is estimated that there were more than 337,000 new cases diagnosed in 2012. An estimated 121,000 new cases of RCC were diagnosed in 2012 in Europe, of which more than 75,000 affected men (1). Clear cell renal carcinoma is the most common type of RCC. Although the prognosis of RCC patients has been remarkably improved due to nephrectomy, ablative therapies, and targeted therapies, a large proportion of stage III and IV disease still leads to mortality and lower quality of life. Therefore, it is necessary to develop new targeted therapies against RCC.

Centromere protein F (CENPF), a microtubule binding protein, is a component of the nuclear matrix during the G2 phase of the cell cycle. It localizes to the spindle midzone

and the intracellular bridge in late anaphase and telophase, respectively, and CENPF mutation may lead to Stromme syndrome (2). A recent study showed that CENPF also serves as a centriolar disease-related gene implicated in severe human ciliopathy, namely, Stromme syndrome, and microcephaly-related phenotypes (3). In mouse embryos, CENPF depletion causes developmental failure (4).

Notably, a study has reported the function and dysregulation of CENPF in various types of cancer. For instance, reduced CENPF remodels prostate cancer cells and regulates cellular metabolism (5). CENPF contributes to prostate cancer aggressiveness, and its interaction with FOXM1 drives prostate cancer malignancy (6,7). Another study found that CENPF regulated cancer metabolism by pyruvate kinase M2 phosphorylation signaling (8). Among hepatocellular carcinoma (HCC) patients, CENPF is one of the frequently amplified genes (9). The overexpression of CENPF has been shown to predict shorter overall survival (OS) and higher cumulative recurrence in HCC and breast cancer (10-12). Taken together, CENPF has been shown to promote proliferation and metastasis in different types of cancer and to predict poor prognosis.

However, the functions of CENPF in RCC remain elusive. Microarray dataset analysis has indicated that MT2A, MYC, CENPF, and NEK2 have a high degree of participation in RCC (13). The expression level of CENPF has been significantly correlated with OS and recurrence-free survival (RFS) in clear cell RCC (14,15). In this study, we aimed to elucidate the clinical significance and potential roles of CENPF in RCC. We present the following article in accordance with the MDAR reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-22-797/rc).

Highlight box

Key findings

- This study found that CENPF was highly expressed in RCC tissues. Moreover, down-regulation of CENPF can significantly inhibit the proliferation of RCC cells and change the distribution of cell cycle. CENPF plays a tumorigenic role in RCC.

What is known and what is new?

- CENPF, a microtubule binding protein, has been highlighted the correlation with cancer progression in various types of cancer, which is significantly correlated with OS and RFS in clear cell RCC;
- Our new finding is that knockdown CENPF can significantly inhibit the proliferation of RCC cells and change the distribution of cell cycle.

What is the implication, and what should change now?

- CENPF is a potential oncogene and prognostic marker in RCC, which can be used as an independent prognostic factor.

Methods

Quantitative real-time polymerase chain reaction (qRT-PCR)

Peripheral blood and corresponding tissue samples of 23 RCC patients and 23 normal physical examination patients who were treated in our hospital from 2018 to 2020 were collected. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Institutional Review Board of Nantong First People's Hospital (No. LI2017-059) and informed consent was taken from all individual participants. Total RNA was extracted with an RNeasy® kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A total of 500 ng RNA was used to generate complementary DNA (cDNA) for each sample. The polymerase chain reaction (PCR) was preceded by 94 ℃ for 5 minutes, followed by 30 cycles of 94 ℃ for 45 seconds, 55 ℃ for 45 seconds, and 72 ℃ for 1 minute followed by 72 ℃ for 7 minutes. The cycle threshold (Ct) values were normalized to the expression levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the relative amount of mRNA specific to each of the target genes was calculated via the $2^{-\Delta\Delta Ct}$ method. The sequences of primers are as follows:

- CENPF-F: AAACAAGATTCCCGAGGGTCTC;
- CENPF-R: GCCTGAAGCTTTATTTTGGCCA;
- CDK4-F: TGCTGGATGTCATTCACACAGA;
- CDK4-R: TTGATGAGGGGAAGAGGAATGC;
- CDK6-F: GCCTTGCCCGCATCTATAGT;
- CDK6-R: GCAGCCAACACTCCAGAGAT;
- CyclinD1-F: TTCGTGGCCTCTAAGATGAAGG;
- CyclinD1-R: GTTCCACTTGAGCTTGTTCACC;
- GAPDH: TGGTCTCCTCTGACTTCAACAG
- GAPDH-R: CCCTGTTGCTGTAGCCAAATTC.

Cell culture and siRNA transfection

The HK2, 786-O, ACHN, and A498 cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 supplemented with 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 ℃ in the presence of 5% CO_2. siCENPF

was transfected into ACHN and A498 cells by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The siRNA sequences are as follows:

- siCENPF-1: GGGUUCUCUUACCCUGAGAAUGA;
- siCENPF-2: CGUCGUACUUAAUGUCUGUUAGA.

Protein extraction and Western blot analysis

Cell lysates were extracted with mammalian protein extraction reagent (M-PER) lysis buffer supplemented with a protease inhibitor and phosphatase inhibitors. The protein concentrations were measured by a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher, Waltham, MA, USA). A total of 30 μg of protein was loaded onto a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and run with 1× SDS running buffer. Proteins were transferred onto a 0.45 μm polyvinylidene fluoride (PVDF) membrane, which was later blocked with 5% milk in tris-buffered saline (TBS)-0.1% Tween-20. Then, the membrane was incubated with anti-CENPF (1:1,000; Proteintech, Rosemont, IL, USA) or anti-β-actin (1:5,000; Proteintech) primary antibody overnight at 4 ℃ and secondary antibody for 1 hour. Finally, proteins were detected using enhanced chemiluminescence (ECL).

Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded (FFPE) tissue samples were cut into 4 μm thick sections and dried at 60 ℃ for 30 minutes. After blocking endogenous peroxidase activity using blocking reagent (Dako, Santa Clara, CA, USA) for 5 minutes at room temperature, epitopes were retrieved using pH 9.0 Tris-ethylenediamine tetraacetic acid (EDTA) buffer for 40 minutes at 95 ℃ followed by incubation with anti-CENPF (1:200, Proteintech) antibody at room temperature for 30 minutes. Subsequently, the slides were incubated with peroxidase/diaminobenzidine (DAB)-10 min K5007 from a DAKO EnVision™ Detection Kit (Dako) for 30 minutes.

Proliferation assay

In the Cell Counting Kit-8 (CCK-8) assay, ACHN and A498 cells were plated in 96-well plates at a density of 2,000 cells/well. On days 1, 2, 3, and 4, 10 μL CCK-8 was added to each well. After incubation for 3 hours, the absorbance [optical density (OD) value] was measured at 450 nm. In the clonogenic assay, ACHN and A498 cells were plated in 6-well plates at a density of 400 cells/well. On day 7, the clones were stained with crystal violet.

Immunofluorescence

The cells were seeded on coverslips in 6-well plates. After siRNA transfection, cells were fixed in 4% paraformaldehyde for 30 minutes and then blocked with 0.3% bovine serum albumin (BSA) and 0.3% Triton X-100 to block nonspecific binding. The coverslips were incubated with a primary antibody against EdU (RiboBio, Guangzhou, China) at 4 ℃ overnight, followed by a secondary antibody against Apollo488 for 1 hour at room temperature. Images were captured under a fluorescence microscope.

Cell cycle analysis

Cell cycle was analyzed by flow cytometry. Briefly, after fixation, the cells were prepared for propidium iodide (PI; Sigma, St. Louis, MO, USA) staining according to the manufacturer's protocol. DNA content was determined using a FACSCaliber Analyzer [Becton, Dickinson, and Co. (BD), Franklin Lakes, NJ, USA] and analyzed by FlowJo (BD, USA).

Statistical analysis

All data were analyzed by SPSS 19.0 (IBM Corp., Chicago, IL, USA). Statistical analysis of significance was performed by one-way analysis of variance (ANOVA) with least significant difference (LSD) *post-hoc* multiple comparisons. Student's *t*-test was used to compare the differences between 2 groups. A P value <0.05 indicated significance.

Results

CENPF was highly expressed in RCC and predicted poor prognosis

We downloaded and analyzed the RNA sequencing and clinical data from The Cancer Genome Atlas (TCGA) cancer database (https://portal.gdc.cancer.gov). Our results showed that CENPF was highly expressed in kidney renal clear cell carcinoma (KIRC) compared to normal kidney tissue (P<0.05, *Figure 1A*). Notably, stage IV tumors expressed higher CENPF than stage I and II tumors (*Figure 1B*). In addition, higher CENPF expression

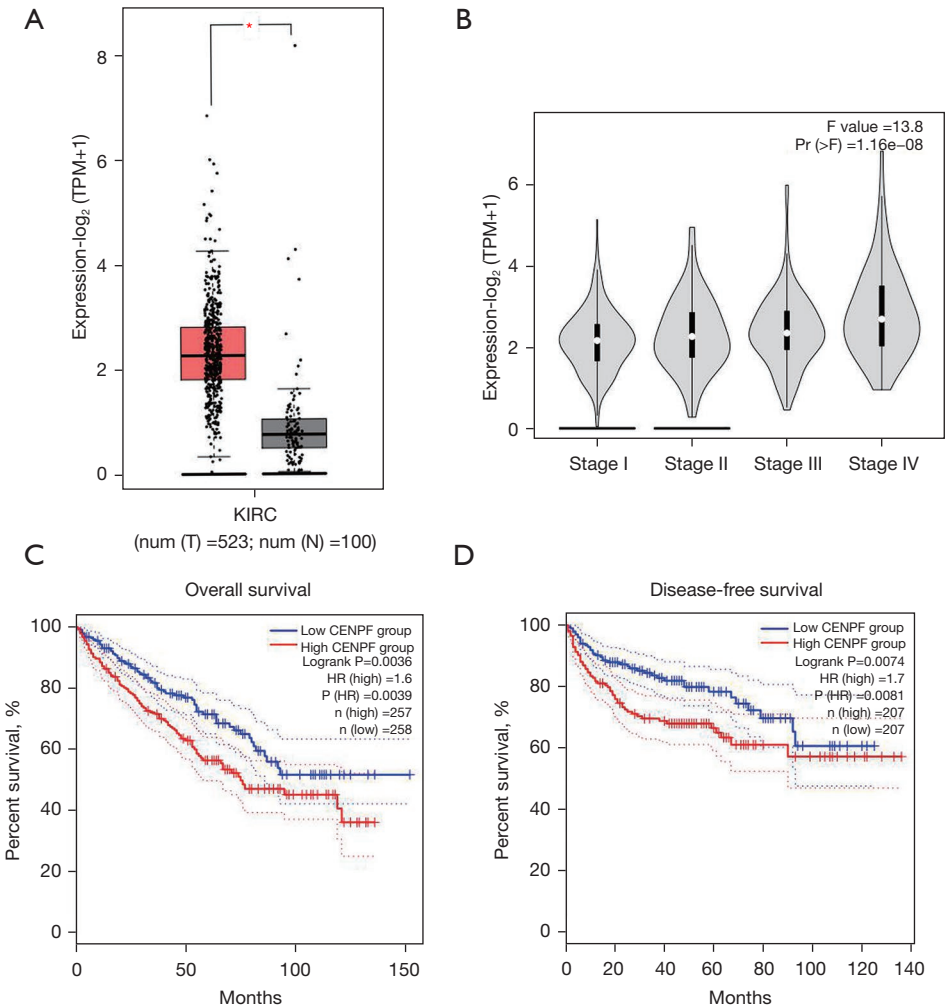

Figure 1 CENPF was highly expressed in RCC and predicted poor prognosis based on TCGA data. (A) Comparison of CENPF mRNA expression between KIRC tumor samples and normal tissue from TCGA database. (B) Comparison of CENPF mRNA expression among patients with different stages. (C,D) Kaplan-Meier analysis of OS (C) and DFS (D) among RCC patients with low and high CENPF expression. *, P<0.05. N, normal; T, tumor; TPM, transcript per million; KIRC, kidney renal clear cell carcinoma; HR, hazard ratio; CENPF, centromere protein F; RCC, renal cell carcinoma; TCGA, The Cancer Genome Atlas; mRNA, messenger RNA; OS, overall survival; DFS, disease-free survival.

predicted poorer OS [P=0.0036, hazard ratio (HR) =1.6] and disease-free survival (DFS) (P=0.0074, HR =1.7, *Figure 1C*,*1D*). These results suggested that CENPF may play a role in RCC progression.

Moreover, we validated CENPF expression in our cell line and cancer tissue. We collected 23 paired RCC tissues and paracancerous kidney tissues. We assessed CENPF expression by quantitative polymerase chain reaction (qPCR), Western blotting, and IHC. The results confirmed that CENPF was highly expressed in tumor samples compared to normal tissue at both the messenger RNA (mRNA) and protein levels (*Figure 2A-2C*). Among the 4 RCC cell lines we evaluated (HK2, 786-O, ACHN, and A498), HK2 showed the lowest CENPF expression level, whereas A498 showed the highest (*Figure 2D*,*2E*).

Knockdown of CENPF inhibited the proliferation of RCC cells

We further studied the roles of CENPF in RCC cell lines

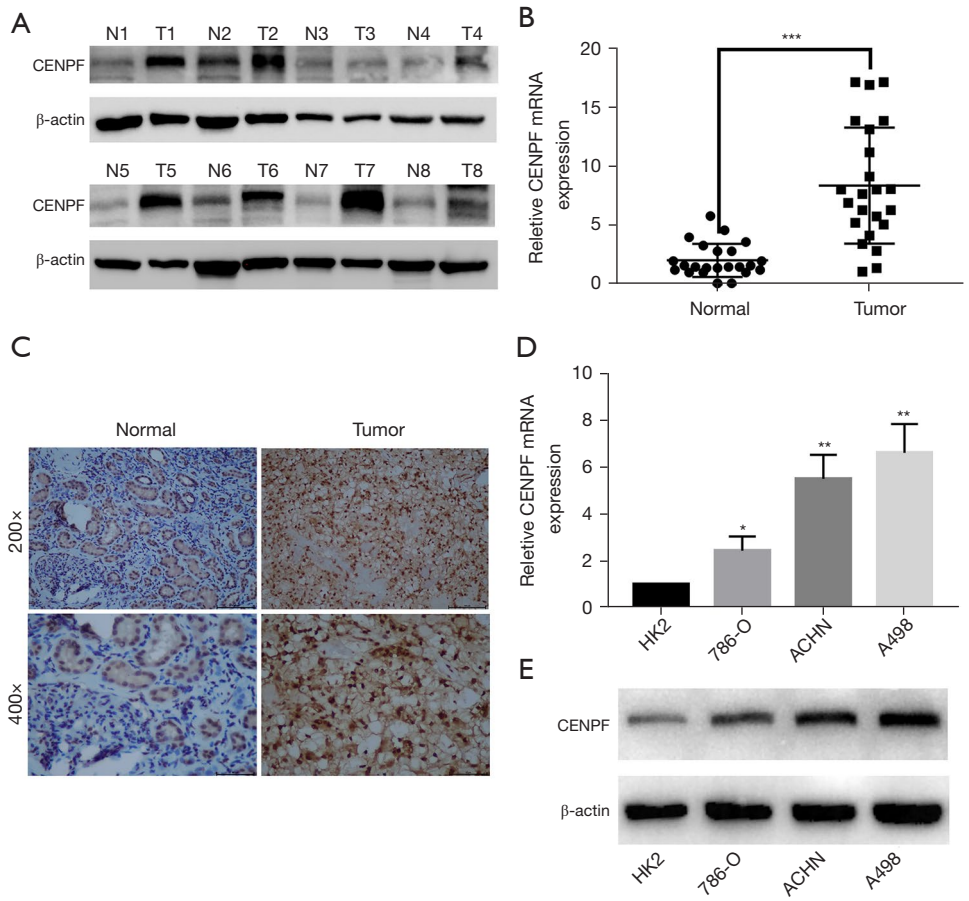

Figure 2 CENPF was highly expressed in RCC tissue. (A) Comparison of CENPF mRNA expression between RCC tumor samples and normal tissue. (B) CENPF protein expression level in paired RCC tumor and normal tissue samples. (C) Representative images of IHC staining of CENPF in RCC tumor and normal tissue samples. (D,E) CENPF mRNA (D) and protein (E) expression levels in the HK2, 786-O, ACHN, and A498 cell lines. Error bars represent SD. *, P<0.05; **, P<0.01; ***, P<0.001. N, normal; T, tumor; CENPF, centromere protein F; RCC, renal cell carcinoma; IHC, immunohistochemistry; mRNA, messenger RNA; SD, standard deviation.

in vitro. We downregulated CENPF in ACHN and A498 cells via small interfering RNA (siRNA). Both siRNAs effectively reduced CENPF mRNA and protein (*Figure 3*). The CCK-8 assay and clonogenic assay revealed that reduced CENPF significantly inhibited RCC proliferation *in vitro* (*Figure 4A,4B*). Inhibition of CENPF reduced clone formation *in vitro* (*Figure 4C,4D*). We also performed 5-ethynyl-2'-deoxyuridine (EdU) staining by immunofluorescence. CENPF knockdown significantly reduced the EdU signal, which is a marker of cell proliferation (*Figure 4E,4F*). These results suggested that CENPF might contribute to the proliferation of RCC.

Inhibition of CENPF regulated cell cycle distribution in RCC cells

Since CENPF participates in chromosome segregation during mitosis, we investigated whether CENPF regulated the cell cycle in RCC. Our results indicated that CENPF-depleted cells exhibited a lower proportion in G1 phase but a higher proportion in G2 phase in ACHN and A498 cells (*Figure 5A*). This suggested that CENPF inhibition promoted cells to enter G1 phase. Furthermore, we studied the mRNA expression of cell cycle regulators in G1 phase. CENPF inhibition by siRNA significantly reduced the

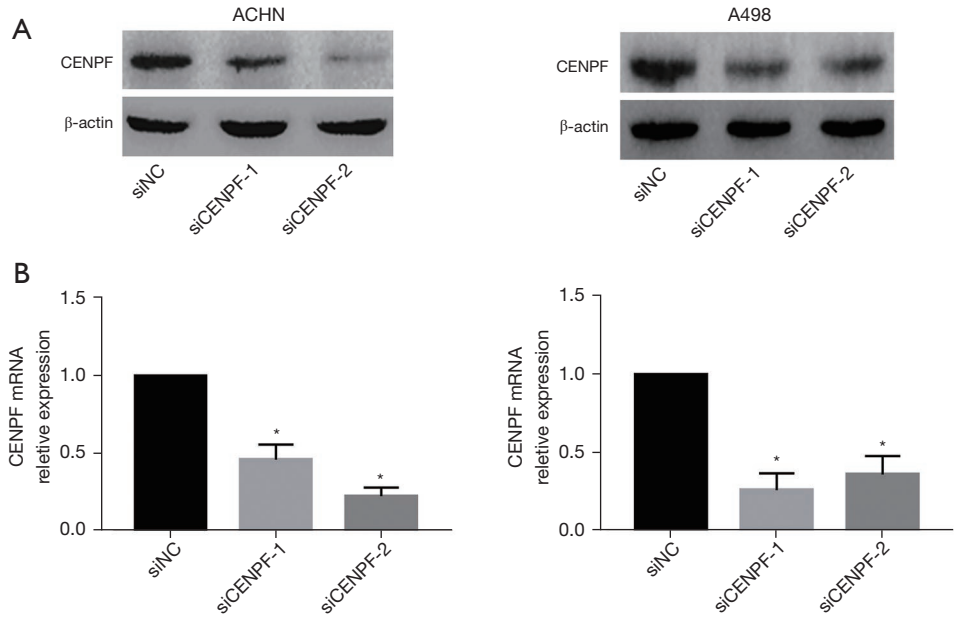

Figure 3 CENPF was significantly downregulated by siRNA in RCC cell lines. (A) Western blot analysis of CENPF knockdown by siRNA in ACHN and A498 cells. (B) Quantification of relative Western blot images. *, P<0.05. CENPF, centromere protein F; RCC, renal cell carcinoma; siRNA, small interfering RNA.

mRNA levels of CDK4, CDK6, and CyclinD1 (P<0.05, *Figure 5B*). These results confirmed that CENPF promoted RCC proliferation via cell cycle regulation *in vitro*.

Discussion

Patients with RCC recurrence or metastasis show poor prognosis. There are already several potential new biomarkers of RCC progression and treatment (16-18). Higher inflammatory indicators such as vascular endothelial growth factor (VEGF) were found in the tumor microenvironment of RCC, VEGF pathway inhibitors and mammalian target of rapamycin (mTOR) inhibitors are the main targeted therapies against RCC. However, the prognosis of advanced RCC patients is still limited if the tumors become resistant to these therapies. Cell mitosis is, to some extent, dependent on the centromere-kinetochore complex, especially cancer cells. Therefore, kinetochore activity may be a promising drug target for cancer treatment. CENPF plays important roles in the cell cycle and division. During mitosis, ataxia telangiectasia mutated and Rad3-related (ATR) kinase localizes to centromeres through Aurora A-regulated association with CENPF, allowing ATR to engage replication protein A (RPA)-coated centromeric R loops. After activation, ATR then stimulates Aurora B, preventing lagging chromosomes (19). In our study, we reported the protumor activity of CENPF in RCC cell lines for the first time. It provides a basis for the development of targeted drugs in the future.

Our results may provide new insights into RCC proliferation and malignancy. In this study, we revealed that CENPF is highly expressed in RCC tissue and that downregulation of CENPF can significantly inhibit RCC cell proliferation and change cell cycle distribution. These results are consistent with previous studies (13,14). Interestingly, many studies have reported that several genes and noncoding RNAs (ncRNAs) can promote cancer cell proliferation or metastasis by modulating CENPF. For instance, lncRNA MCM3AP-antisense 1 (MCM3AP-AS1) contributes to breast cancer cell progression through the miR-28-5p/CENPF axis (20). Chen *et al.* reported that the HnRNPR-CCNB1/CENPF axis promotes gastric cancer proliferation and metastasis (21). This evidence may further confirm the protumor activity of CENPF. However, how CENPF is overexpressed in RCC cells is still unclear.

Cell cycle progression is one of the determinants of cancer cell malignancy. Cancer cells need to pass from the G1 phase into S phase through a tightly regulated

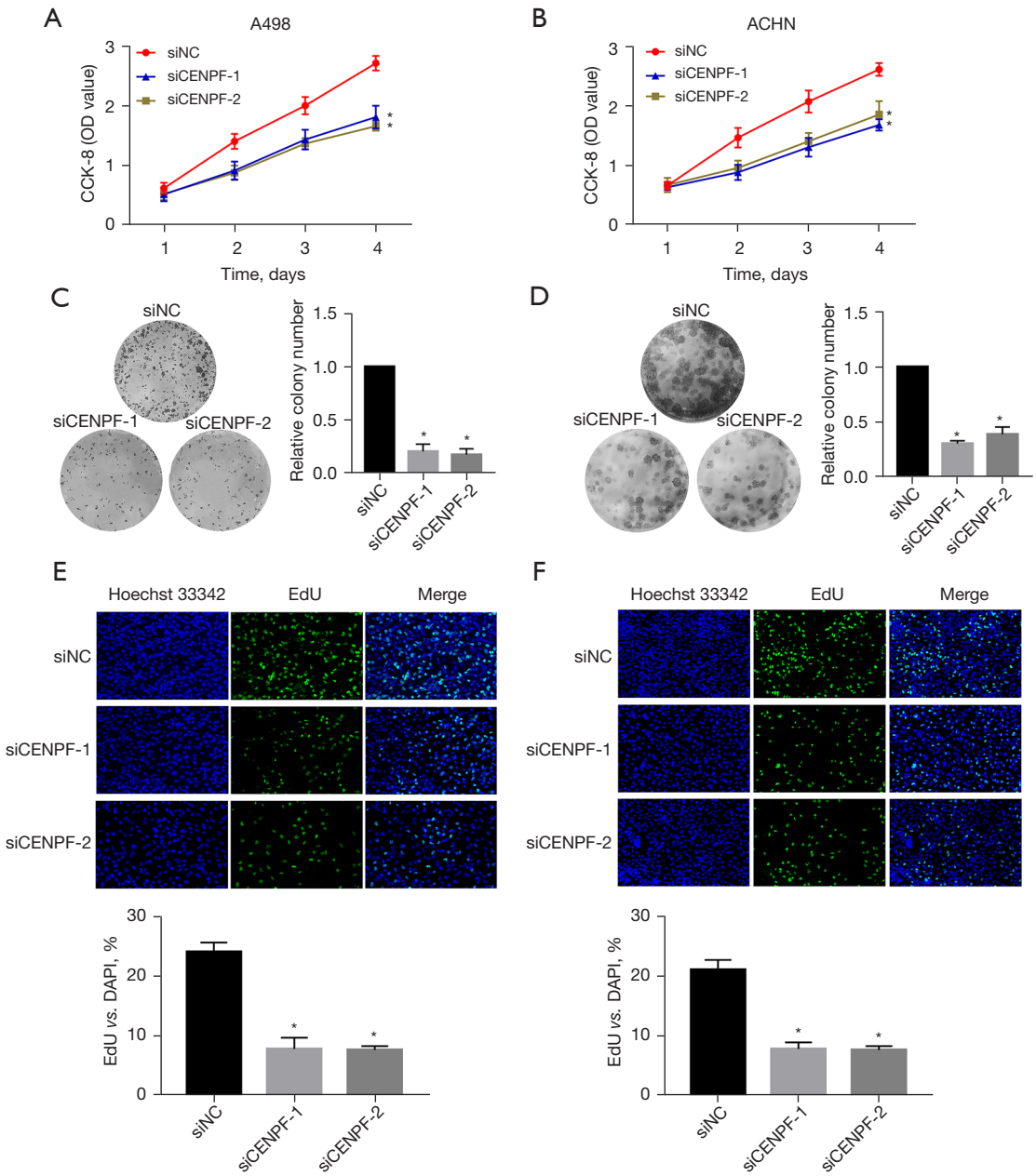

Figure 4 CENPF inhibition reduced RCC cell proliferation. (A,B) CCK-8 proliferation assay in ACHN and A498 cells. (C,D) Clonogenic assay in ACHN and A498 cells (crystal violet, ×1). (E,F) EdU staining quantification in ACHN and A498 cells (magnification, ×100). *, P<0.05. OD, optical density; NC, negative control; CENPF, centromere protein F; RCC, renal cell carcinoma; CCK-8, Cell Counting Kit-8; EdU, 5-ethynyl-2'-deoxyuridine.

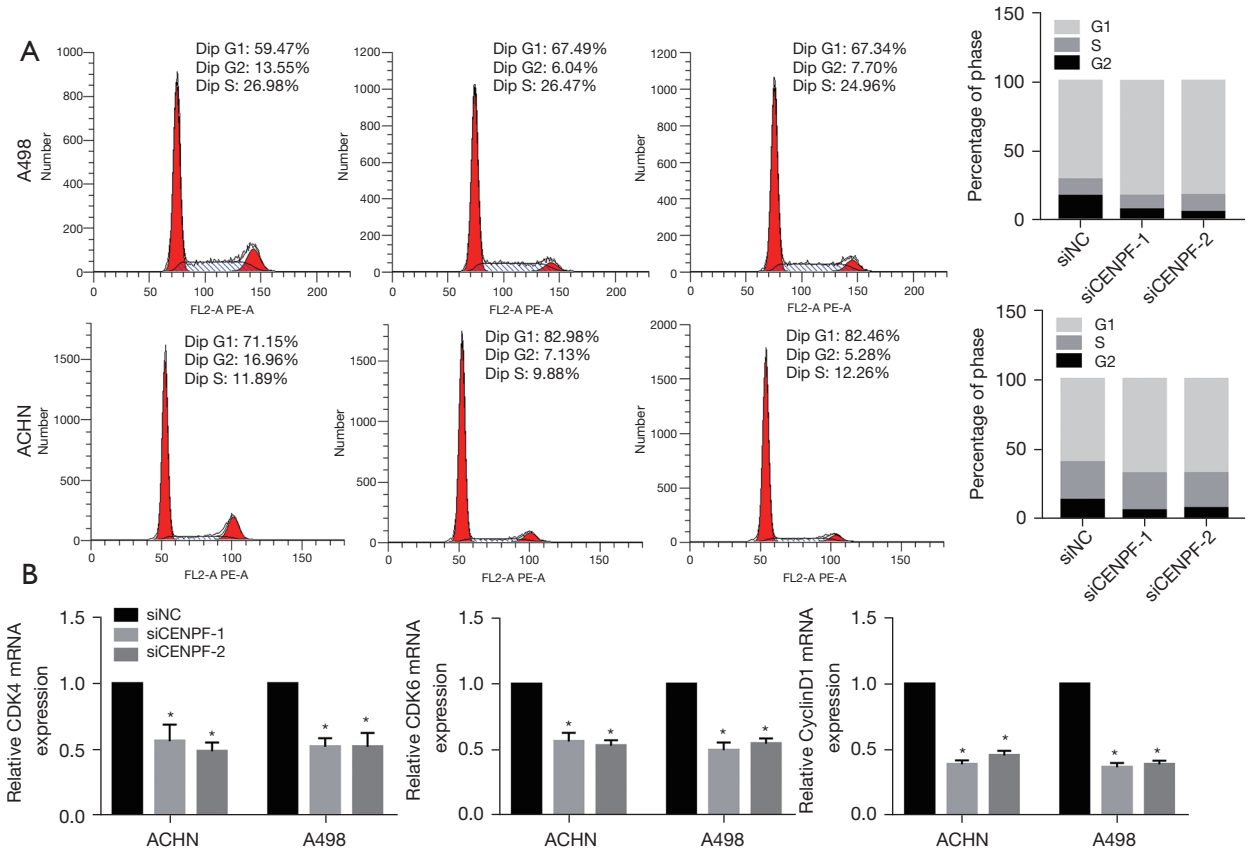

Figure 5 Downregulation of CENPF regulated the cell cycle and inhibited CDK4, CDK6, and CyclinD1. (A) Cell cycle analysis of CENPF-depleted ACHN and A498 cells. (B) Fold changes in CDK4, CDK6, and CyclinD1 mRNA expression in CENPF-depleted cells. *, P<0.05. For the mRNA levels, the control values were arbitrarily set to 1. NC, negative control; CENPF, centromere protein F; mRNA, messenger RNA.

checkpoint. Specifically, the G1/S transition begins in early G1, triggering an increase in D-type cyclins (D1, D2, and D3), which bind to CDK4 or CDK6. These cyclin-CDK complexes translocate to the nucleus, where they are phosphorylated. In turn, activated CDK4/6 complexes phosphorylate retinoblastoma (RB) tumor suppressor proteins. RB reduces the expression of S phase genes by directly inhibiting E2F transactivation (22). Therefore, the G1/S transition largely depends on the activity of CDK4/6 and D-type cyclins (23). In our study, we found that CENPF inhibition significantly reduced CDK4/6 and CyclinD1 expression (24), which may partially explain the phenotype. Our results provide evidence that CENPF downregulation or mutation may sensitize RCC tumors to cell cycle inhibitors.

In summary, this project found that the expression of CENPF is upregulated in RCC and that downregulation of CENPF can inhibit the proliferation of RCC cells. It also clarified the mechanism by which CENPF regulates the cell cycle of RCC by inhibiting the expression of cyclins such as CDK4, CDK6, and CyclinD1. This project has discovered new pathogenic genes and pathogenesis in RCC, which provides a scientific basis for exploring new therapeutic targets.

Conclusions

CENPF can be used as an independent prognostic factor for RCC. Down-regulation of CENPF can significantly inhibit the proliferation of RCC cells and change the distribution of cell cycle. CENPF is a potential oncogene and prognostic marker in RCC.

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Footnote

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Data Sharing Statement: Available at https://tau.amegroups.com/article/view/10.21037/tau-22-797/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-22-797/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. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Institutional Review Board of Nantong First People's Hospital (No. LI2017-059) and informed consent was taken from all individual participants.

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