CENPF Promotes Proliferation of Renal Cell Carcinoma In Vitro

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Research

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Abstract

Background: Metastasis and drug resistance are the main causes of renal cell carcinoma (RCC) mortality. Currently, there are still 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 is to explore the role of CENPF in RCC.

Materials and 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 the CENPF expression was detected by qRT-PCR, Western-blot and immunohistochemical methods. Down-regulate the expression of CENPF by siRNA transfection, and detect the proliferation of the corresponding RCC cells and the corresponding cell cycle.

Results: According to TCGA data analysis, CENPF is highly expressed in RCC, and its expression level is significantly related to the overall survival and recurrence-free survival of RCC. In addition, high expression of CENPF was found in the tissues of RCC patients in our hospital. Knockdown of CENPF can significantly reduce the proliferation of RCC cells in in vitro experiments, and knockdown of CENPF can regulate the cell cycle by inhibiting the expression of cyclins such as CDK4, CDK6 and CyclinD1.

Conclusion: 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.

Introduction

Renal cell carcinoma (RCC) is the ninth most common cancer across the world. It is estimated that there were more than 337 thousand new cases diagnosed in 2012. An estimated around 121,000 new cases of RCC occurred 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 lead to mortality and lower quality of life. Therefore, it is necessary to develop new targeted therapy 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, CENPF mutation may lead to Stromme syndrome(2). Recent study shows that CENPF also serves as a centriolar disease gene implicated in severe human ciliopathy, namely Stromme syndrome, and microcephaly related phenotypes (3). In mouse embryos, CENPF depletion causes developmental failure (4).

Notably, several studies have 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 the prostate cancer aggressiveness, and its interaction with FOXM1 drives prostate cancer malignancy(6, 7). Another study found out that CENPF regulated cancer metabolism by pyruvate kinase M2 phosphorylation signalling(8). Among hepatocellular carcinoma patients, CENPF is one of the frequently amplified genes(9). The overexpression of CENPF predicted shorter overall survival and higher cumulative recurrence in HCC and breast cancer (10–12). Taken together, CENPF has been shown to promote the proliferation and metastasis in different types of cancer and predict poor prognosis.

However, the functions of CENPF in RCC still remain elusive. Microarray dataset analysis indicated that MT2A, MYC, CENPF and NEK2 had high degree which participated RCC (13). The expression level of CENPF was significantly correlated with overall survival and recurrence-free survival in clear cell RCC(14, 15). In this study, we aim at elucidating the clinical significance and potential roles of CENPF in RCC.

Results

1. CENPF was highly expressed in RCC and predicted poor prognosis

We downloaded and analysed the RNA sequencing and clinical data from 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 ($P < 0.05$, Fig. 1A). Notably, stage IV tumours express higher CENPF compared to stage I and II ones (Fig. 1B). In addition, higher CENPF expression predicted poorer overall survival ($P = 0.0036$, HR = 1.6) and disease-free survival ($P = 0.0074$, HR = 1.7, Fig. 1C-D). These results suggested that CENPF may play a role in RCC progression.

Moreover, we validated the CENPF expression in our cell line and cancer tissue. We collected 23 paired RCC tissue and paracancerous kidney tissue. We assessed the CENPF expression by qPCR, Western-blot and immunohistochemistry (IHC). The results confirmed that CENPF was highly expressed in tumour samples compared to normal tissue on both mRNA and protein level (Fig. 2A-C). Among the four RCC cells we evaluated (HK2, 786-O, ACHN, A498), HK2 showed the lowest CENPF expression level, while A498 showed the highest (Fig. 2D-E).

2. Knockdown of CENPF inhibited the proliferation of RCC cells

We further studied the roles of CENPF in RCC cell lines in vitro. We downregulated CENPF in ACHN and A498 cells via siRNA. Both siRNAs reduced CENPF mRNA and protein effectively (Fig. 3A-B). The CCK-8 assay and clonogenic assay revealed that reduced CENPF significantly inhibited RCC proliferation in vitro (Fig. 4A-B). The clonogenic assay showed similar results. CENPF inhibition reduced the clone formation in vitro. (Fig. 4C-D). We also performed Edu staining by immunofluorescence. CENPF knockdown

significantly reduced Edu signal, which is a marker of cell proliferation (Fig. 4E-F). These results suggested that CENPF might contribute to the proliferation of RCC.

3. Inhibition of CENPF regulated cell cycle distribution in RCC cells

Since CENPF participates in chromosome segregation during mitosis, we investigated whether CENPF regulated cell cycle in RCC. Our results indicated that CENPF-depleted cells exhibited lower proportion in G1 phase, while higher in G2 phase in ACHN and A498 cells (Fig. 5A-B). This suggested that CENPF inhibition promoted cells to enter into G1 phase. Furthermore, we studied the mRNA expression of cell cycle regulator in G1 phase. CENPF inhibition by siRNA significantly reduced the mRNA level of CDK4, CDK6 and CyclinD1 ($P < 0.05$, Fig. 5C-E). These results confirmed that CENPF promoted RCC proliferation via cell cycle regulation in vitro.

Methods And Methods

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

Total RNA was extracted with RNeasy® kit (Qiagen) according to the manufacturer's instructions. 500 ng RNA was used to generate cDNA for each sample. the PCR reaction was preceded by 94°C for 5 min, then 30 cycles of 94°C for 45 sec, 55°C for 45 sec, and 72°C for 1 min followed by 72°C for 7 min. The Ct values were normalized to the expression levels of 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:TGCTGGATGTCATTACACAGA

CDK4-R:TTGATGAGGGGAAGAGGAATGC

CDK6-F:GCCTTGCCCGCATCTATAGT

CDK6-R:GCAGCCAACACTCCAGAGAT

CyclinD1-F:TTCGTGGCCTCTAAGATGAAGG

CyclinD1-R:GTTCCACTTGAGCTTGTTCAAC

GAPDH-F:TGGTCTCCTCTGACTTCAACAG

GAPDH-R:CCCTGTTGCTGTAGCCAAATTC

2. Cell culture and siRNA transfection

HK2, 786-O, ACHN and A498 cell line were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in the presence of 5% CO₂. siCENPF was transfected to ACHN and A498 cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The siRNA sequences are as follows:

siCENPF-1:GGGUUCUCUUACCCUGAGAAUGA

siCENPF-2:CGUCGUACUUAUGUCUGUUAGA

3. Protein extraction and Western-blot analysis

Cell lysates were extracted with M-PER lysis buffer supplemented with protease inhibitor and phosphatase inhibitors. The protein concentrations were measured by Pierce BCA protein assay kit (Thermo). 30 µg proteins were loaded onto the SDS-PAGE gel and run with 1X SDS running buffer. Proteins were transferred onto a 0.45 µm PVDF membrane which was later blocked with 5% milk in TBS-0.1% Tween-20. Then, the membrane was incubated in anti-CENPF (1:1000, Proteintech) or anti-β-actin (1:5000, Proteintech) primary antibody overnight in 4°C and secondary antibody for 1h. Finally, proteins were detected using ECL.

4. Immunohistochemistry (IHC)

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

5. Proliferation assay

In CCK-8 assay, ACHN and A498 cells were plated in 96-well-plate at the density of 2000 cells/well. On day 1, 2, 3 and 4, 10 µL CCK-8 was added in each well. After incubation for 3 hours, the absorbance (OD value) was measured at 450nm. In clonogenic assay, ACHN and A498 cells were plated in 6-well-plate at the density of 400 cells/well. On day 7, the clones were stained with crystal violet.

6. Immunofluorescence (IF)

The cells were seeded on coverslips in 6-well plates. After siRNA transfection, cells were fixed in 4% paraformaldehyde for 30 min, then blocked by 0.3% BSA and 0.3% Triton X-100 to block nonspecific binding. The coverslips were incubated with primary antibody of Edu (RiboBio) at 4°C overnight, followed by secondary antibody of Apollo488 for 1h at room temperature. Images were captured under a fluorescence microscope.

7. Cell cycle analysis

Cell cycle was analysed by flow cytometry. Briefly, after fixation, the cells were prepared for propidium iodide (Sigma) staining according to manufacturer's protocol. DNA content was determined using FACSCaliber Analyzer and analysed by FlowJo.

8. Statistical Analysis

All data were analysed by SPSS 19.0. Statistical analysis of significance was performed by one-way ANOVA with LSD *Post hoc* multiple comparisons. Student t-test was used to compare the differences between two groups. $P < 0.05$ indicated significance.

Discussion

Patients with RCC recurrence or metastasis show poor prognosis. There are already several potential new biomarkers of RCC progression and treatment (16–18). VEGF pathway inhibitor and mTOR inhibitor are the main targeted therapies against RCC. However, the prognosis of advanced RCC patients are still limited if the tumours 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 one of the promising drug targets for cancer treatment. It has been shown that CENPF plays important roles in 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 pro-tumour activity of CENPF in RCC cell lines for the first time.

Our results may provide new insights into the RCC proliferation and malignancy. In this study, we revealed that CENPF is highly expressed in RCC tissue and downregulation of CENPF can significantly inhibit RCC cell proliferation and change cell cycle distribution. These results are consistent with the previous studies (13, 14). Interestingly, many studies reported that several genes and non-coding RNAs (ncRNAs) can promote cancer cell proliferation or metastasis via modulating CENPF. For instance, LncRNA MCM3AP-AS1 contributes to breast cancer cell progression through miR-28-5p/CENPF axis (20). Er-Bao Chen et al. report that the HnRNPR-CCNB1/CENPF axis promotes gastric cancer proliferation and metastasis (21). These evidence may further confirm the pro-tumour 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 checkpoint. Specifically, the G1/S transition begins in early G1, triggering an increase of D-type cyclins (D1, D2, and D3), which bind to CDK4 or CDK6. This cyclin-CDK complexes translocate to the nucleus where they are phosphorylated. In turn, activated CDK4/6 complexes phosphorylate the retinoblastoma (RB) tumour suppressor protein. 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. In our study, we found out that CENPF inhibition significantly reduced CDK4/6 and Cyclin D1 expression, which may partially explain the

phenotype. Our results provide evidence that CENPF downregulation or mutation may sensitizes RCC tumors to cell cycle inhibitors.

In summary, this project found that the expression of CENPF is up-regulated in RCC, and down-regulation 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.

Declarations

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Bing Zheng designed the research, Ji Zhang and Shushu Yuan performed research, Ji Zhang and Hua Zhu wrote the paper, Zhan Chen, Zhenmin Liu performed research and modified the paper, Yuhang Ji analyzed data.

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Ethical approval and consent to participate

This study was approved by the Institutional Review Board of Nantong First People's Hospital. The approval date is 2017-12-06 and the code is LI2017-059

Consent for publication

Written, informed consent was obtained from the patients for published images of this article. A copy of the written consent is available for review by the Editor of this journal.

Competing interests

The authors declare that they have no competing interests.

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Figures

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 overall survival (C) and disease-free survival (D) among RCC patients with low and high CENPF expression.

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 figures of IHC staining of CENPF in RCC tumor and normal tissue samples. D-E CENPF mRNA (D) and protein (E) expression level in HK2, 786-O, ACHN and A498 cell lines. * $P < 0.05$. ** $P < 0.001$. Error bars represent SD.

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.

Figure 4

CENPF inhibition reduced RCC cell proliferation A CCK-8 proliferation assay in ACHN and A498 cells. B Clonogenic assay in ACHN and A498 cells. C Edu staining quantification in ACHN and A498 cells. *P < 0.05.

Figure 5

Downregulation of CENPF regulated cell cycle and inhibited CDK4, CDK6 and CyclinD1 A Cell cycle analysis of CENPF-depleted cells in ACHN and A498 cells. B Fold changes of 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.