

Original Article

The downregulation of choline kinase alpha inhibits clear cell renal cell carcinoma proliferation and metastasis via the MAPK and PI3K/AKT pathways

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Abstract: The abnormal expression of choline kinase alpha (CHKA) is associated with the development and progression of a variety of human malignancies. This study found that in patients with clear cell renal cell carcinoma (ccRCC), the overall survival of the CHKA high expression group was significantly shorter than the CHKA low expression group. The expression of CHKA in the ccRCC metastatic cell line was significantly higher than the expressions in the non-metastatic cell lines. The results of CCK-8, colony formation, Transwell and Matrigel assays showed that the knockdown of CHKA had a significant effect on the cell proliferation, migration and invasion of ccRCC. In addition, flow cytometry results showed that CHKA knockdown induced G0-G1 arrest and cell apoptosis. Mechanistic studies suggest that CHKA can promote the progression of ccRCC by altering the expression of Phospho-ERK, Phospho-AKT (Ser473), Cyclin D1, and caspase-3. Overall, our study suggests that CHKA contributes to the progression and metastasis of ccRCC and may serve as a new prognostic biomarker and potential therapeutic target.

Keywords: CHKA, renal cell carcinoma, prognosis, proliferation

Introduction

Renal cell carcinoma (RCC) is the most common type of malignant kidney neoplasm in adults [1, 2]. In 2018, it was estimated that there were 403,262 new cases of kidney cancer and 175,098 deaths globally [3]. Among the subtypes of RCC, clear cell renal cell carcinoma (ccRCC) is most frequent (75%-80%) and best studied [4]. Because ccRCC is poorly sensitive to conventional radiation and chemotherapy, surgical resection is the main treatment for local renal tumors [5, 6]. However, nearly 30% of patients have local progression or multiple metastasis at the time of diagnosis, and 20-40% of patients experience disease relapse after surgical removal of the primary tumor [7-9]. Despite advances in treatments, such as molecular targeted therapy [10-12] and immunotherapy [13], the long-term prognosis is still poor in ccRCC patients. Tumor progression and metastasis are the leading causes of death in ccRCC patients. Nevertheless, the underlying mechanisms giving rise to ccRCC progression

and metastasis are not completely known. Studying the molecular mechanisms of cancer progression is particularly critical for the diagnosis and treatment of ccRCC. Therefore, it is necessary to search for new prognostic markers and therapeutic targets.

Choline kinase Alpha (CHKA) belongs to the choline kinase protein family and is a key enzyme in the CDP-choline pathway. CHKA can phosphorylate choline to phosphorylcholine and participate in the biosynthesis of phosphatidylcholine, which is a crucial component of cell membranes and various organelle membranes [14-17]. In addition, as the second messenger, phosphocholine plays an important role in the DNA synthesis and mitosis of cells, thereby participating in physiological activities such as cell proliferation [18].

Studies have shown that CHKA has a relationship with the occurrence and progression of tumors [19-21]. CHKA is highly expressed and activated in a variety of tumors [22], and plays

a key role in maintaining the survival, proliferation and malignant phenotype of tumor cells, such as breast cancer [23-27], endometrial cancer [28], ovarian cancer [29], bladder cancer [30], T cell lymphoma [31], prostate cancer [32], colorectal cancer [33], and hepatocellular carcinoma [34]. In addition, CHKA interacts with several tumor-associated signaling pathways and molecules [35-39]. Therefore, detecting CHKA expression and enzyme activity is important for the early diagnosis of tumors, the prediction of local progression and distant metastasis, and prognosis [28, 40-44]. Moreover, targeted inhibition of CHKA is effective in inhibiting tumor growth and provides a possible option for the treatment of tumors [29, 31, 32, 42, 45-57].

However, the biological functions and underlying mechanisms of CHKA in ccRCC remain unknown. In the present study, we explored the role and related molecular mechanisms of CHKA in ccRCC. We discovered that high CHKA expression was related to a poor survival rate. We also found that siRNA-mediated CHKA knockdown notably suppresses cell proliferation, migration and invasion capability and that CHKA knockdown significantly induces cell cycle arrest and cell apoptosis in the ACHN cell line. The downregulation of CHKA causes increased expressions of phospho-PTEN and caspase-3 and reduced phospho-AKT (Ser473), phospho-ERK, and cyclinD1 expressions. Hence, CHKA may act as a potential biomarker for diagnosis and prognosis and has become a promising therapeutic target in ccRCC patients.

Materials and methods

Cell lines and cell culture

Three RCC cell lines, ACHN, 786-O, and 769P, were purchased from the American Type Culture Collection (ATCC, USA). ACHN was cultured in MEM medium (Hyclone, USA), while 786-O and 769P were cultured in RPMI-1640 medium (Hyclone, USA), both containing 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA). All cell lines were grown at 37°C in humidified air with 5% CO₂.

Transient transfection

Small interference RNA (si-RNA) were obtained from GenePharma (GenePharma, China). The

sequences were demonstrated as follows: si-RNA1, 5'-GCAAGGUUUGAUGCCUUAUUTT-3' (sense) and 5'-AAUAGGCAUCAAACCUUGCTT-3' (antisense); si-RNA2, 5'-CAUGCUGUUCAGUCGUCCTT-3' (sense) and 5'-GGAGCACUGGAACAGCAUGTT-3' (antisense); and negative control (si-NC) 5'-UUCUCCGAACGUGUCACGUTT-3' (sense) and 5'-ACGUGACACGUUCGGAGAATT-3' (antisense). The si-RNAs were transfected into ACHN cells using Lipofectamine 3000 Reagent (Invitrogen, USA) according to the manufacturer's instructions. Afterwards, the effects of the CHKA knockdown were measured utilizing qRT-PCR and western blot.

RNA isolation, reverse transcription, and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were isolated from cells using a Total RNA Kit (Tiangen, China). Then, Reverse transcription was performed using a FastKing RT Kit (Tiangen, China). Afterwards, qRT-PCR was performed using the iQ5 real-time detection system (Bio-Rad, USA) with PowerUp SYBR Green Master Mix (Thermo Fisher, USA). In addition, the mRNA levels of CHKA were normalized to GAPDH and the fold changes were calculated using the 2- $\Delta\Delta C_t$ method. The primers used were as follows: CHKA (forward, 5'-TGGTCCATTGTACAAGCCAA-3' and reverse, 5'-CAAGCTTCCTCTTCTGGTGG-3'), and GAPDH (forward, 5'-CACCCACTCCTCCACCTTTG-3' and reverse, 5'-CCACCACCCTGTTGCTGTAG-3').

Protein extraction and western blot

Total cellular proteins were lysed using RIPA (SolarBio, China) containing a protease inhibitor mixture (SolarBio, China) and a phosphatase inhibitor cocktail (Bimake, China). The protein concentration was quantified using a BCA Protein Assay kit (SolarBio, China).

Equal weights of proteins were separated using 10% SDS-PAGE gels, then they were transferred to nitrocellulose membranes. The membranes were blocked in 5% skim milk for 90 minutes at room temperature and incubated with primary antibodies at 4°C overnight. After being incubated with a secondary antibody for 1 hour at room temperature, the signals were measured using a chemiluminescent and fluorescent imaging systems (Sagecreation, China). The primary antibodies used were as follows: CHKA rabbit polyclonal antibody (1:250, Sigma-

Aldrich, USA), GAPDH mouse monoclonal antibody (1:5000, ProteinTech, USA) and PTEN/Phospho-PTEN/Akt/Phospho-AKT(Thr308)/Phospho-AKT (Ser473)/ERK/Phospho-ERK/cyclinD1/Caspase-3 rabbit monoclonal antibodies (1:1000, Cell Signaling Technology, USA). The secondary antibodies were goat anti-rabbit IgG/HRP (1:2000, Liankebio, China) and goat anti-mouse IgG/HRP (1:2000, Liankebio, China).

Cell proliferation and colony formation assay

Cell proliferation was evaluated using Cell Counting Kit-8 (Dojindo, Japan). After transfection for 24 hours, 1500 cells were seeded into 96-well plates with 100 μ l medium per well and cultured for 5 days under normal conditions. The experiment was conducted every 24 hours after seeding. 10 μ l CCK-8 reagent was added to each well and incubated at 37°C for 2 hours, and then the absorbance at 450 nm was measured using a microplate reader. Each experiment was performed in triplicate.

After transfection for 24 hours, 1500 cells per well were seeded into 6-well plates and cultured for 10 days under normal conditions. Then, the colonies were washed with phosphate buffer saline (PBS) twice, fixed in paraform for 15 min and stained with 0.1% crystal violet for 15 min.

Transwell migration assay and Matrigel invasion assay

After transfection for 48 hours, the cells resuspended in the serum-free medium were seeded into the upper chamber of each transwell at a density of 4×10^4 /well. Transwells were coated with or without Matrigel (BD Biosciences, USA) for the invasion and migration assays respectively. 700 μ L of medium containing 10% FBS was added to the bottom chamber as a attractant. After incubation under normal condition for 24 hours, the cells were fixed in paraform for 15 min and stained with 0.1% crystal violet for 15 min. After being carefully washed with PBS, the cells on the upper surface of the membrane were wiped off gently. The migratory and invasive cells which moved to the lower surface of membrane were observed and counted in five random fields per chamber using a microscope. The experiments were repeated three times.

Cell cycle assay

A cell cycle assay was performed using a Cell Cycle Staining Kit (Liankebio, China). After transfection for 48 hours, 1×10^6 cells were collected, washed with PBS, then incubated with 1 ml DNA staining solution and 10 μ l permeabilization solution at room temperature in the dark for 30 min. Subsequently, the cell cycle was quantified using flow cytometry (Beckman Coulter, USA) and analyzed using CytExpert software 2.1.

Cell apoptosis assay

A cell apoptosis assay was performed using an Annexin V-FITC Apoptosis Detection Kit (Dojindo, Japan). After transfection for 48 hours, 1×10^6 cells were collected, washed with PBS, resuspended with 1 ml Annexin V binding solution and then 100 μ l cell suspension was moved into new tubes. Subsequently, 5 μ l Annexin V-FITC and 5 μ l PI solution were added to the cell suspension and incubated at room temperature in the dark for 15 min. Afterwards, 400 μ l Annexin V binding solution was added to the cell suspension. The cell apoptosis was measured using flow cytometry (Beckman Coulter, USA) and analyzed using CytExpert software 2.1.

Statistical analysis

The statistical analysis was performed using a rank-sum test and Student's *t* test using SPSS 20.0. The results are presented as the mean \pm SD from three different independent experiments. The overall survival curve was drawn using the Kaplan-Meier method and compared with a log-rank test. $P < 0.05$ was considered statistically significant.

Results

The expression level of CHKA is significantly correlated with overall survival in ccRCC patients

By analyzing the transcriptome data of *The Cancer Genome Atlas* (TCGA), we assessed the relationship between CHKA expression and patient outcome. The results showed that the overall survival of the ccRCC patients in the CHKA high expression group was shorter than the overall survival of the patients in the CHKA low expression group (**Figure 1**).

The downregulation of CHKA inhibits ccRCC

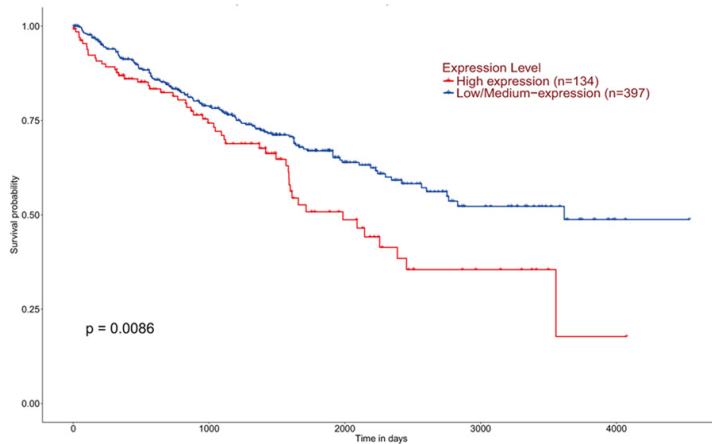


Figure 1. A Kaplan-Meier curve for the relationship between the overall survival rate and CHKA expression in ccRCC patients. Compared to the low CHKA expression group, the overall survival rate of the patients in the high CHKA expression group is lower.

Expression level of CHKA in ccRCC cell lines

By using qRT-PCR and WB, we evaluated the expression of CHKA in ccRCC cell lines, ACHN, 786-O, and 769P. The results showed that the CHKA expression of ACHN was significantly higher than the expressions of 786-O and 769P (Figure 2A, 2B).

CHKA knockdown significantly suppresses cell proliferation and colony formation abilities

In order to determine the role of CHKA in tumor proliferation, we transfected two si-RNAs to knockdown the expression of CHKA in ACHN, and then we used qRT-PCR and WB to determine knockdown effects at the mRNA and protein levels respectively (Figure 3A, 3B). CCK-8 and colony formation assays were utilized to detect the proliferative capacity of cells after the knockdown of CHKA. Compared with the si-NC group, the proliferation ability of the si-RNA groups was significantly inhibited (Figure 3C, 3D).

Knockdown of CHKA inhibits cell migration and invasion

To investigate the role of CHKA in cell movement, after the knockdown of CHKA, we used Transwell and Matrigel assays to determine the migration and invasion abilities of ACHN cells respectively. The results showed that the cell migration ability of the si-RNA groups was significantly impaired compared to the si-NC group

(Figure 4A). Similarly, the cell invasive ability of the si-RNA groups was also significantly inhibited (Figure 4B).

CHKA knockdown induces cell cycle arrest in ACHN

After 48 hours of si-RNAs transfection of ACHN, we used flow cytometry to detect changes in the cell cycle after the knockdown of CHKA. The results showed that the cell cycle of ACHN was blocked, and the proportion of cells in the G0-G1 phase was increased (Figure 5).

Knockdown of CHKA induces cell apoptosis in ACHN

After 48 hours of si-RNA transfection of ACHN, we used flow cytometry to detect apoptosis after the knockdown of CHKA. The results showed that the proportion of apoptotic cells in the si-RNA groups increased (Figure 6).

CHKA knockdown up-regulated phospho-PTEN and caspase-3 expression and repressed phospho-AKT (Ser473), phospho-ERK1/2, and cyclinD1 expressions

To explore the underlying mechanism of CHKA in ccRCC, we used WB to analyze several altered molecules after the knockdown of CHKA at the protein level. The results showed that the expression of phospho-PTEN and Caspase-3 in the si-RNA groups was significantly up-regulated compared to the si-NC group. In addition, the expressions of phospho-AKT (Ser473), phospho-ERK1/2, and cyclinD1 were significantly decreased (Figure 7).

Discussion

ccRCC remains one of the most terrible malignant diseases in humans due to tumor recurrence and metastasis after surgical resection. Therefore, a better understanding of the molecular mechanisms of disease pathogenesis is necessary to identify biomarkers for its prediction and intervention.

In the present study, we found that patients with a high expression of CHKA in ccRCC had shorter overall survival than patients with a

The downregulation of CHKA inhibits ccRCC

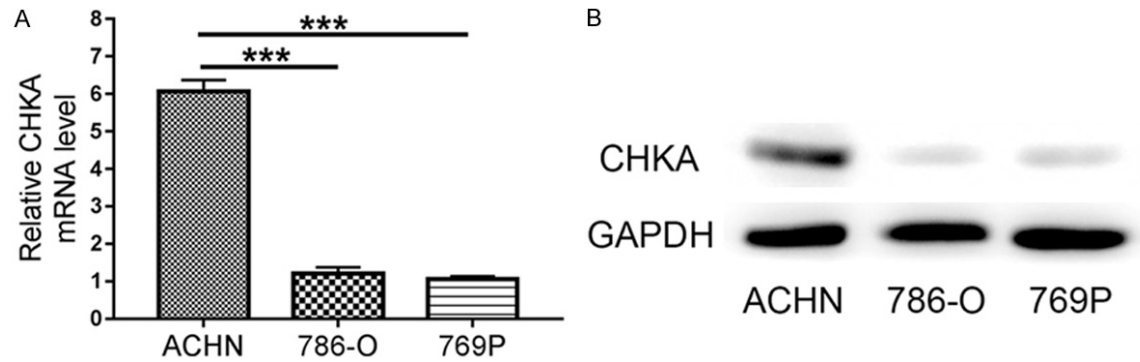


Figure 2. CHKA expression in ccRCC cell lines. The expression of CHKA was detected by qRT-PCR and western blotting at the mRNA and protein levels respectively. The CHKA expression of ACHN is higher than the expressions of 786-O and 769P at both the mRNA and protein levels (** $P < 0.001$).

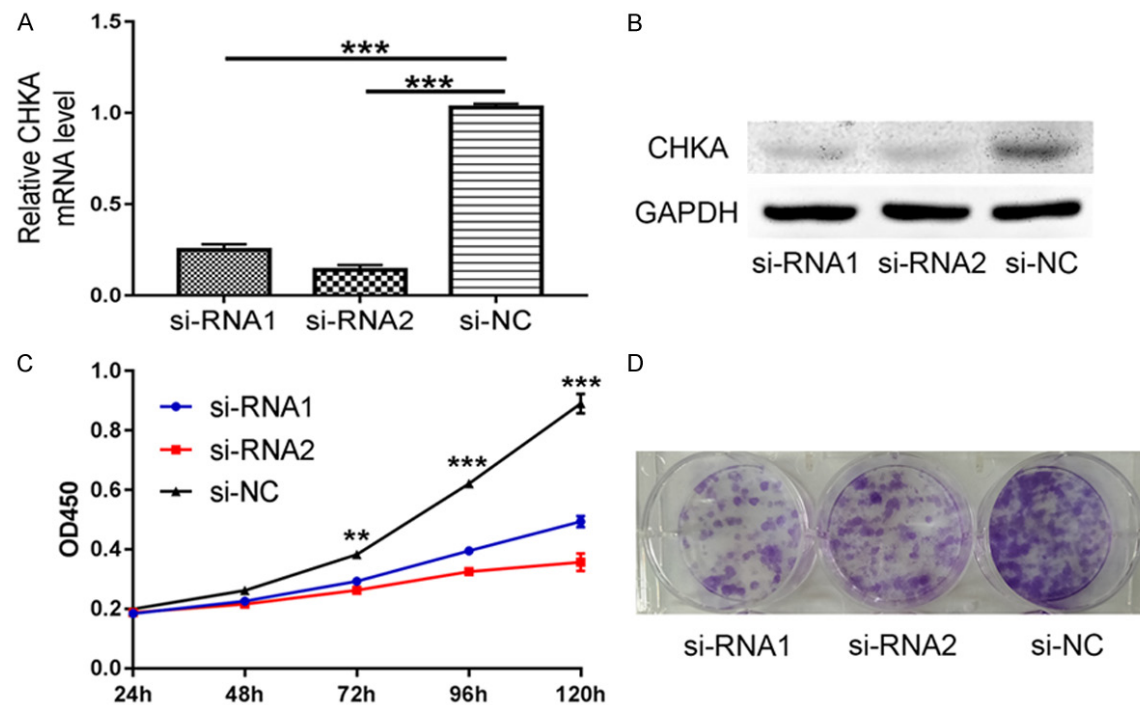


Figure 3. The proliferation and colony formation abilities were inhibited after the knockdown of CHKA in ACHN. A. The efficiency of CHKA knockdown by si-RNA was evaluated by qRT-PCR at the mRNA level. B. The efficiency of CHKA knockdown by si-RNA was detected by Western blot at the protein level. C. The knockdown of CHKA significantly suppressed the cell growth rate in ACHN, showing that reduced CHKA expression impaired cell proliferation in RCC. D. CHKA knockdown notably inhibited colony formation ability in ACHN (** $P < 0.01$, *** $P < 0.001$).

lower expression of CHKA. Therefore, we speculate that CHKA may play a role in promoting disease progression in ccRCC. In order to prove our conjecture, we did a series of functional experiments using ccRCC cell lines. First, we used qPCR and WB to detect CHKA expression in several ccRCC cell lines. The results showed that the expression of CHKA in ACHN was significantly higher than it was in 786-O and 769P

at both the mRNA and protein levels. Next, we knocked down the CHKA of the ACHN cell line using si-RNAs and confirmed the knockdown effect using qPCR and WB. Then, by utilizing CCK-8, colony formation, Transwell, and Matrigel assays, we found that the proliferation, migration and invasion of ACHN cells were significantly inhibited after CHKA knockdown. In addition, using flow cytometry, we found that

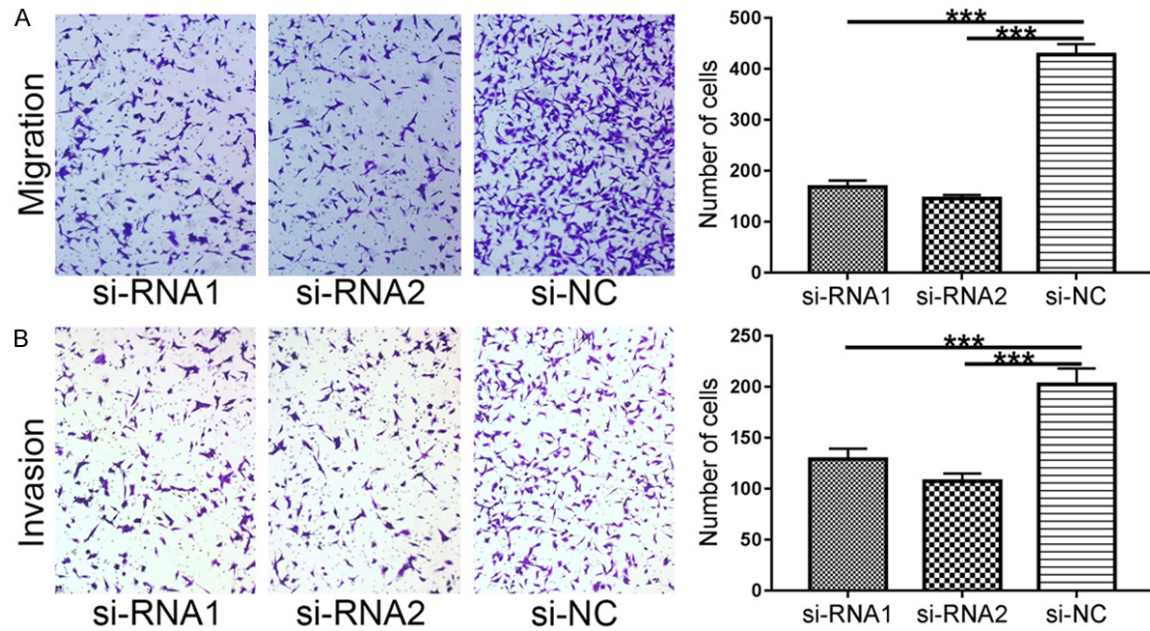


Figure 4. The knockdown of CHKA impaired cell migration and invasion ability in ACHN. A. CHKA knockdown notably inhibited cell migration in ACHN. B. CHKA knockdown significantly suppressed cell invasion ability in ACHN (** $P < 0.001$).

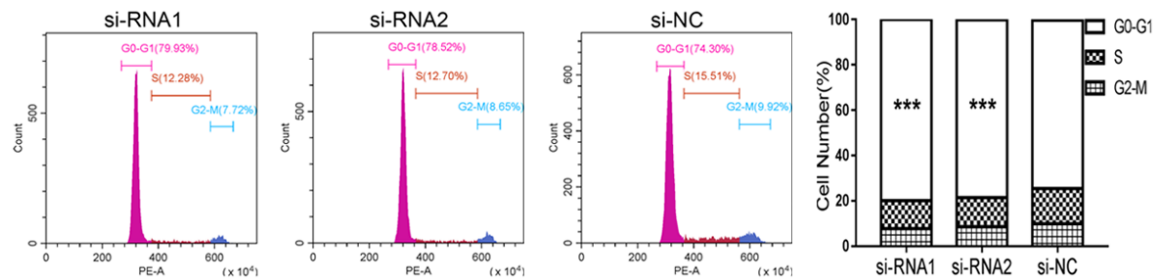


Figure 5. CHKA knockdown induced cell cycle arrest at the G0-G1 period (** $P < 0.001$).

after the knockdown of CHKA, the cell cycle was blocked and the proportion of apoptotic cells was increased. These results confirmed our initial conjecture that CHKA may contribute to the progression and metastasis of ccRCC. Finally, in order to further explore the molecular mechanism of CHKA in ccRCC, we used WB to detect alterations of several molecules at the protein level after the knockdown of CHKA. The results showed that the expressions of phospho-PTEN and caspase-3 were up-regulated, but the expressions of phospho-AKT (Ser473), phospho-ERK, and cyclinD1 were down-regulated. Taken together, our research suggests that CHKA may be an oncogene and may play a key role in facilitating the progression of ccRCC.

There have been some studies on the role and mechanism of CHKA in other tumors. Previous studies have shown that the expression of CHKA in tumor tissues is closely related to patient prognosis, and that a high expression of CHKA indicates a lower survival rate [34, 40, 41, 44]. Our results were consistent with the previous findings. This suggests that CHKA may help to pre-judge the patient's prognosis and thus help the clinician adjust the treatment strategy.

Previous studies have shown that CHKA plays a key role in the promotion of multiple tumors [23-34, 48]. However, the role of CHKA in ccRCC has not been studied. We have demonstrated for the first time that CHKA may contrib-

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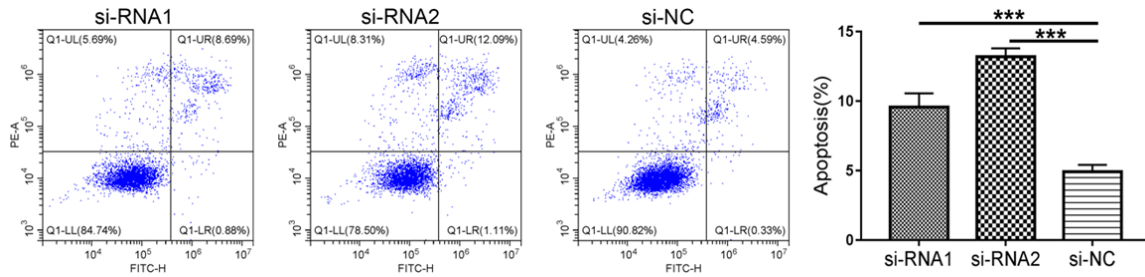


Figure 6. The knockdown of CHKA significantly induced cell apoptosis (** $P < 0.001$).

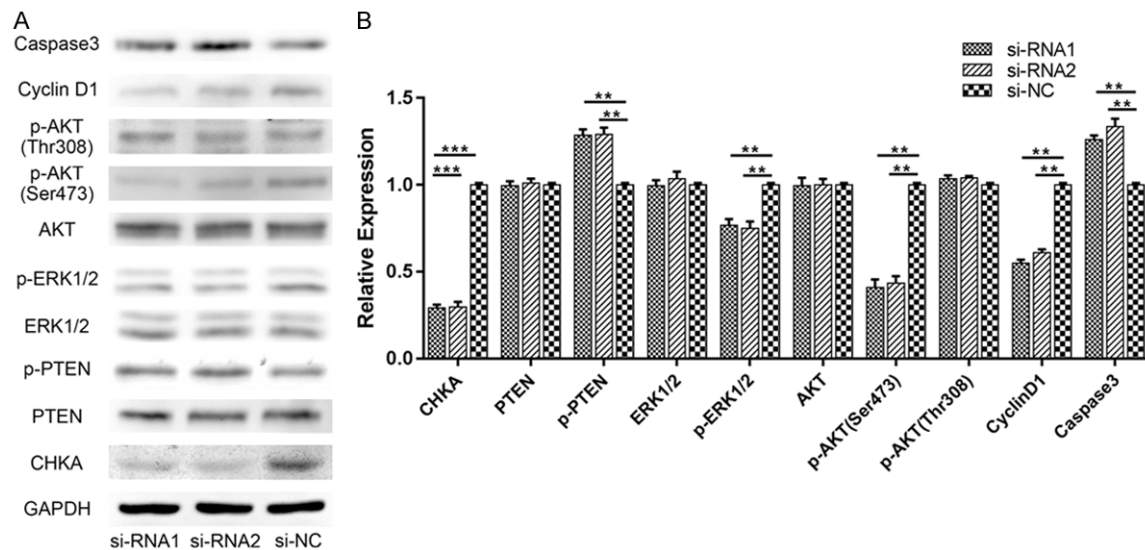


Figure 7. The expression of several proteins after CHKA knockdown in ACHN. A. Compared to the si-NC group, the phospho-ERK1/2, phospho-AKT(473) and cyclinD1 expressions were down-regulated notably in the si-RNAs groups. In addition, the phospho-PTEN and Caspase-3 expressions were clearly up-regulated in the si-RNAs groups. B. Quantification of protein levels (** $P < 0.01$, *** $P < 0.001$).

ute to the progression and metastasis of ccRCC. Next, we further explored the molecular mechanism by which CHKA plays a role in ccRCC. The results showed that the expressions of phospho-PTEN and caspase-3 was up-regulated after the knockdown of CHKA, and the expressions of phospho-AKT (Ser473), phospho-ERK and cyclinD1 were down-regulated.

Previous studies have shown that after the inhibition of CHKA expression in breast cancer, phospho-AKT (Ser473) expression is down-regulated, and phospho-AKT (Thr308) expression is unchanged [36]. Moreover, studies have shown that the inhibition of CHKA can simultaneously attenuate the MAPK and PI3K/AKT pathways [37]. In our study, after the knockdown of CHKA, phospho-PTEN expression was

up-regulated, phospho-AKT (Ser473) and phospho-ERK1/2 expressions were decreased, and phospho-AKT (Thr308) expression was unchanged, which is consistent with previous studies.

The PI3K/AKT signaling pathway is an important tumor-associated pathway involved in biological activities such as cell proliferation, differentiation, movement, and apoptosis. PTEN is an important tumor suppressor gene that inhibits the PI3K/AKT signaling pathway. In our study, phospho-PTEN expression was up-regulated and phospho-AKT (Ser473) expression was down-regulated after the knockdown of CHKA. Therefore, CHKA may activate the PI3K/AKT pathway by inhibiting the expression of PTEN, ultimately promoting the progression of ccRCC.

The MAPK pathway is also a classical tumor-associated pathway involved in biological behaviors such as cell proliferation, differentiation, movement and apoptosis. Phosphorus-ERK1/2 expression is down-regulated after the knock-down of CHKA. Therefore, CHKA may play a role in promoting ccRCC progression by activating the MAPK pathway.

Previous studies have shown that CHKA is a regulatory mediator of phospholipid metabolism and the cell cycle, promoting G1-S phase transition [35]. Our study showed that after knocking down the CHKA of ACHN, the cell cycle underwent a G1-S phase arrest, and the expression of cyclinD1 promoting G1-S phase transition was decreased, which is consistent with previous studies, demonstrating that CHKA may play a key role in maintaining the cell cycle, thereby promoting the progression of ccRCC.

Apoptosis is programmed cell death, and the progression of tumors is closely related to the inhibition of tumor cell apoptosis. Previous studies have shown that the inhibition of CHKA expression can induce apoptosis [47]. In our study, after knocking down CHKA, the percentage of apoptosis in ACHN cells increased, and the expression level of the apoptosis-related molecule caspase-3 was up-regulated, suggesting that CHKA may promote the progression of ccRCC by inhibiting the apoptosis of tumor cells.

Previous studies have shown that CHKA expression in metastatic prostate cell lines is higher than in primary prostate cancer cell lines [58]. In this study we used three ccRCC cell lines, in which the CHKA expression of ACHN was significantly higher than 786-O and 769P. ACHN is derived from pleural effusion with multiple metastasis of ccRCC, while 786-O and 769P are primary ccRCC cell lines. In addition, after knocking down the CHKA expression of ACHN, the cell migration and invasion abilities were reduced notably. These two points suggested that CHKA may play a key role in the metastasis of ccRCC. Determining the specific mechanism requires additional research.

In summary, this is the first study to explore the role of CHKA in ccRCC. A high expression of CHKA in ccRCC is significantly associated with

patient prognosis and the malignant biological behavior of tumor cells. Our study suggested that CHKA may promote the progression of ccRCC by regulating the PTEN, PI3K/AKT pathway, the MAPK pathway, and cyclinD1 and caspase-3. Therefore, CHKA is a promising biomarker for predicting ccRCC progression and may be a therapeutic target. This study reports our initial results. Next, we will use a co-immunoprecipitation assay to detect the interaction between CHKA and related molecules, establish nude mouse xenograft models to study the role and mechanism of CHKA in vivo, and complete other experimental methods to explore CHKA-related functions and molecular regulation mechanisms more accurately.

Conclusion

Taken together, our results show that CHKA expression could serve as a new prognostic indicator for ccRCC patients. CHKA plays a key role in the proliferation and metastasis of ccRCC, which provides a theoretical basis for the potential utility of CHKA in the treatment of ccRCC.

Disclosure of conflict of interest

None

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