KLK4 Silencing Inhibits the Growth of Chromophobe Renal Cell Carcinoma through ERK/AKT Signaling Pathway

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Abstract
Introduction: Renal cell carcinoma (RCC) generally has a poor prognosis because of late diagnosis and metastasis. Despite its abundance in RCC cells, the functions of kallikrein-related peptidase 4 (KLK4) in RCC cells remain unknown. The results of this investigation were examined to discover if KLK4 gene silencing influences the development of RCC cells. Methods: The mRNA levels of KLK4 and the relationship between KLK4 and tumor stage in patients with RCC were analyzed from the GEPIA database. Real-time PCR and Western blotting were used to measure the mRNA and protein levels of KLK4. Cell Counting Kit 8 (CCK-8), colony formation, wound healing, and Transwell assays were used to examine the proliferation, invasion, and migration of RCC cells after KLK4 suppression. Finally, xenograft experiments in a mouse model helped understand the in vivo effects of KLK4 knockdown. Results: Our research found that KLK4 expression was upregulated in the kidney chromophobe (KICH) specimens and cell lines. Moreover, inhibiting KLK4 growth led to a slowdown in RCC cell proliferation and colony formation. Additionally, KLK4 knockdown inhibited migration, invasion, and epithelial-mesenchymal transition (EMT) of RCC cells. AKT and ERK phosphorylation were enhanced with KLK4 silencing. In the nude mouse xenograft cancer model, KLK4 silencing also prevented the expression of Ki-67, CD105, and the growth of tumors. Conclusion: KLK4 accelerated KICH progression via the ERK/AKT signaling pathway, providing a novel regulatory mechanism for KICH pathogenesis.

Introduction
Renal cell carcinoma (RCC) is the most common type of kidney cancer in adults, with more than 431,000 new cases and more than 179,000 deaths worldwide each year [1, 2]. RCC is a diverse illness, with the most frequent pathological subgroups being kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), and kidney chromophobe (KICH) [3]. Early detection of renal cancer is challenging owing to the slow start of the disease and the lack of distinct clinical signs in
the early stages of the disease [4]. At the time of diagnosis, about 20–30% of RCC patients have already developed metastasis, which means that the treatment result is often poor [5]. Consequently, further study is required to investigate biomarkers for the early detection of kidney cancer as well as a possible treatment target for RCC.

Fifteen distinct genes on human chromosome 19q13.4 code for serine proteases which are a group of kallikreins (KLKs) [6]. KLKs often exhibit aberrant expressions in different cancers and have been shown to serve as predictive indicators for the progression of illness [7, 8]. In several malignancies, kallikrein-related peptidase 4 (KLK4) is a subunit of the KLK family and operates as an oncogene, such as triple-negative breast cancer [9], oral squamous cell carcinoma [10], and prostate cancer [11]. It is linked to proliferation, metastasis, and worse survival [12]. Yet, it is not understood exactly how and why KLK4 contributes to the development of RCC. We conducted research in which we examined the public databases and validation tests were conducted outside the public databases to confirm the highly-correlated relationship between KLK4 and RCC. Additionally, RCC cell proliferation, migration, invasion, and apoptosis were examined alongside the KLK4-mediated pathway. Our research has shown that KLK4 may influence RCC via ERK/AKT signaling.

Materials and Methods

RCC Cell Lines and Reagents

The human renal epithelial cell line HK-2 and seven human RCC cell lines (786-O, CaKi-1, Caki-2, A-704, 769-P, A498, and ACHN) were obtained from the American Type Tissue Collection. The human renal epithelial cell line HK-2 and seven human RCC cell lines (786-O and ACHN) were obtained from the American Type Tissue Collection. The cells were grown in Eagle’s Minimal Essential Media supplemented with 10% fetal bovine serum (FBS; Hyclone), and the cells were incubated at 37°C and in 5% CO₂. We purchased SCH772984 (an ERK-selective inhibitor) from Sigma.

KLK4 Expression

In this study, we used the GEPIA (Gene expression profiling interactive analysis) to obtain box plots of the expression difference between these tumor tissues and the corresponding normal tissues of the GTEx (Genotype-tissue expression) database. The default parameters ([Log2FC] cutoff of 1, and p value cutoff of 0.01, and log2 (transcripts per million + 1) for log-scale) were employed.

Transfection

For short hairpin RNA transfections, cells were grown on a 6-well plate (1 × 10⁵ cells per well) for 24 h and transfected with KLK4 short hairpin RNA plasmids (2 μg) or an empty vector (2 μg), using Lipofectamine 2000 (Invitrogen) transfection reagent for 48 h.

Quantitative Real-Time PCR

Total RNA was extracted from tissues and cells according to the instructions of the total RNA extraction test kit, and its concentration and purity were determined. Then, all extracted RNA was reversed into cDNA according to the reverse transcription kit, and PCR amplification was performed to determine gene expression level. The quantitative real-time PCR (qRT-PCR) system was 20 μL and programmed at 94°C for 3 min. 94°C, 15 s; 58–61°C, 15 s; 72°C, 40 s; 40 cycles; The internal parameter is GAPDH.

Western Blot

The cells were washed in an ice bath of 1 × PBS, and an appropriate amount of cell lysate was added to the ice to lyse the cells, and the cellular proteins were collected for the quantitative determination of BCA protein. Then mix with 5× loading buffer and boil for 5 min. Using 10% SDS-PAGE glue, 100 V constant pressure electroswimming, and then at a continual pressure 110 V in the ice water bath transfer film. After that, the membrane was sealed in 50 g/L skim milk powder for 60 min, and the primary antibody was diluted in an appropriate proportion and gently shaken at 4°C overnight. On the second day, the film was washed 4 times with 1 × TBS/T and incubated with a secondary antibody for 30 min. The film was washed 4 times with 1 × TBST. The ECL luminescent reagent was coated on the film, and then the film was put into the darkroom for luminescence, development, and fixing.

Cell Proliferation Assays

The CCK-8 kit (Solarbio, China) was used to perform the cell proliferation assay on the cells. 96-well plates were infected with 1 × 10⁵ cells per well, with various treatments applied to each well. It was discovered that cell multiplication had occurred after 24 h of adding CCK-8 reagent to wells using a microplate reader that measured the absorbance at 450 nm.

Colony Formation Assay

The cell colony formation test was used to assess whether tumor cells could grow. The short version is that the cells were put onto six-well plates after being treated and then seeded at a low density. All of the work was completed within 10 days, including the fact that the colonies were cleaned, dyed, photographed, and numbered.

Wound Healing Assay

786-O and ACHN cells from the sh-KLK4 group and sh-NC group were seeded at a density of 5 × 10⁵ cells/well in 6-well plates and incubated for 24 h. A scratch was then created using a 200 μL pipette tip and the shed cells were washed off with PBS. The width of the scratch was observed, and the area of the scratch was calculated by using an inverted microscope at 0 and 24 h, and the migration distance of the scratch inward within 24 h was calculated.

Migration and Invasion Assays

786-O and ACHN cells (4 × 10⁴ cells of each condition in 200 μL serum-free medium) were initially seeded onto the upper surfaces of “Transwell” chambers. The lower compartments were always filled with complete medium (containing 10% FBS). Immigrated cells on the bottom surface were fixed, stained, and counted after a 24-h incubation period. Matrigel (Sigma) was applied to the inside surfaces of the cell chambers to assess the level of cell invasion.
**Fig. 1.** KLK4 expression in RCC. 

**a** Differential expression of KLK4 between RCC tissues and normal tissues. The log2 (TPM + 1) was applied for the log-scale. **b** The relationship between the level of KLK4 genes and pathological stages (stage I, stage II, stage III, and stage IV) of KICH, KIRC, and KIRP. The log2 (TPM + 1) was applied for the log-scale. qRT-PCR (c) and Western blot (d) were performed to analyze the mRNA and protein expression of KLK4 in human normal kidney cell line HK-2 and RCC cell lines. *p < 0.05, **p < 0.01, ***p < 0.001. TPM, transcripts per million.
Fig. 2. KLK4 silencing suppressed the proliferation and colony formation of RCC cells. 

a) The expression of KLK4 was detected after transfection with sh-KLK4 by qRT-PCR and Western blot.

b) CCK-8 assays were performed at 0, 24, 48, and 72 h.

c) Alterations in cellular morphology by sh-KLK4 were observed microscopically via Giemsa staining.

d) Colony formation assay. *p < 0.05, **p < 0.01, ***p < 0.001.
Fig. 3. Knockdown of KLK4 suppresses RCC cell migration, invasion, and EMT. a Cell migration by wound healing assay. Cell migration (b) and invasion (c) were examined by the Transwell assay. d Western blot analysis was used to detect the effects of KLK4 silencing on EMT-associated markers (N-cadherin and E-cadherin) in 786-O and ACHN cells. *p < 0.05, **p < 0.01, ***p < 0.001.
Tumor Xenograft Implantation

Six-week-old BALB/c nude mice were randomly divided into two groups of five each. Subcutaneous injection of ACHN cells overexpressing KLK4 into the dorsal flank of mice was used to induce tumor development, and the animals were anesthetized with 50 mg/kg pentobarbital sodium on day 28 post-injection. The mice were then killed, and the tumors were collected, weighed, and prepared for further examination.

Immunohistochemistry

Paraffin sections (5 μm-thick) from nude mice were dewaxed, dehydrated with gradient ethanol, EDTA (PH 9.0) was repaired with high pressure for 10 min, cooled to room temperature, repaired under light protection, and sealed in a warm box with goat serum. Then the sections were incubated with primary anti-KLK4/Ki-67/CD105 (1:200, Abcam) at 4°C overnight, and the fluorescent secondary antibody was incubated the next day. The stained sections were observed under a microscope and stained with light yellowish brownish to yellowish brown as positive cells.

**Fig. 4.** KLK4 silencing inhibited the activation of the ERK/AKT signaling pathway. **a** Representative Western blots of phospho-specific and total ERK, AKT, p38, and JNK among the two groups. **b** CCK-8 assays were performed among four groups. **c** KLK4 activates ERK/AKT signaling to regulate the activity of RCCs. *p < 0.05, **p < 0.01, ***p < 0.001.
Statistical Analysis
The data are provided as the mean ± SD with three separate experiments. GraphPad Prism 8.0 was used to run one-way ANOVA and Tukey’s post hoc tests. A statistically significant difference was defined as $p < 0.05$.

Results

KLK4 Was Upregulated in KICH Tissues and Cell Lines
We first analyzed the data in the GEPIA database, and the results showed that compared with normal tissues, KLK4 was highly expressed in KICH, but there was no significant change in KIRC and KIRP (Fig. 1a). Violin-like plots showed that KLK4 mRNA expression decreased with tumor stage in KICH, while there was no linear association in KIRC and KIRP. We also measured the expression level of KLK4 in RCC cell lines using qRT-PCR. Among the six tested RCC cell lines, 786-O and ACHN cells expressed the highest level of KLK4, so they were selected for the following experiments (Fig. 1c).

Silence of KLK4 Inhibited the Proliferation of RCC Cells
Transfection of sh-KLK4 into 786-O and ACHN cells resulted in a significant decrease in the mRNA and protein expression of KLK4 (Fig. 1d).

Fig. 5. KLK4 silencing inhibited RCC growth in vivo. Schematic representation (a) in the vivo growth curve (b), and weight (c) at the end points of xenograft tumors formed by subcutaneous injection of ACHN cells stably transfected with sh-NC or sh-KLK4 into nude mice. d IHC staining for KLK4, Ki-67, and CD105 in tumor xenografts. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. IHC staining, immunohistochemical staining.
tein levels of KLK4, as shown by qRT-PCR and Western blot analysis (Fig. 2a). Figure 2b showed that KLK4 knockdown inhibited the growth of 786-O and ACHN cells. Morphological observations showed that sh-KLK4 significantly altered the morphology of 786-O and ACHN cells (Fig. 2c). Sh-KLK4 group proliferation rates were much lower than those of the sh-NC groups, as shown by colony formation assays (Fig. 2d).

**Knockdown of KLK4 Suppresses RCC Cell Migration, Invasion, and EMT**

Subsequently, we examined the roles of KLK4 in migration and invasion. Transwell assays were implemented to evaluate the migration and invasion abilities of RCC cells, and reduced migration and invasion abilities of RCC cells with stable KLK4 silencing were observed (Fig. 3a–c). The EMT is often a step in the process of tumor development. Figure 3c illustrates that the expression of E-cadherin was boosted by KLK4 silencing, while N-cadherin, vimentin, and Snail displayed the opposite trend (Fig. 3d).

**KLK4 Silencing Inhibited the Activation of the ERK/AKT Signaling Pathway**

As shown in Figure 4a, KLK4 knockdown significantly induced AKT and ERK phosphorylation, while other MAPKs (JNK and p38) were only slightly affected (no significant difference). We thus examined the role of Erk in RCCs function using the Erk inhibitor SCH772984 and found the proliferation of RCCs were inhibited by SCH772984. Furthermore, the combination of sh-KLK4 and SCH772984 resulted in highly significant growth inhibition of RCCs (Fig. 4b). Here, the data indicated that silencing KLK4 inhibited cell proliferation, migration, colony, and invasion by blockade of the ERK/AKT signaling pathway (Fig. 4c).

**KLK4 Silencing Inhibited RCC Growth in vivo**

To study the effect of KLK4 on tumor growth in vivo, KLK4 downregulated ACHN cells or controlled shRNA-treated ACHN cells were subcutaneously injected into nude mice. After 4 weeks, the mice were killed, and the xenograft tumors were extracted (Fig. 5a). In addition, the efficient knockdown of KLK4 significantly slowed tumor growth (Fig. 5b) and decreased tumor weight (Fig. 5c) compared with the sh-NC group. Immunohistochemical analysis of nude mice bearing tumors showed that the KLK4 knockdown group had lower KLK4, proliferation-specific gene Ki-67, and CD105 protein levels (Fig. 5d). The above results were identical to those of the in vitro experiments.

**Discussion**

RCC is one of the most common and deadly forms of cancer, and it claims more than 100,000 lives each year [13]. Given this, an important goal of RCC research should be to discover new biomarkers and treatment targets for this disease [14]. As one of the major roles involved in epithelial-mesenchymal transition, KLK4 has been proved to be related to the proliferation, migration, and invasion of tumor cells [15]. Our study was conducted to investigate the impact of KLK4 on the development of RCC cells. Our research showed that inhibiting KLK4 in RCC cells reduced cell proliferation, migration, invasion, and epithelial-to-mesenchymal transition and resulted in smaller subcutaneous tumors in nude mice. Additionally, we demonstrated that ERK/AKT signaling was implicated in the KLK4-induced growth-inhibitory property.

Inhibition of KLK4 expression caused a decrease in RCC cell proliferation and colony formation, suggesting that KLK4 is a tumor oncogene. Our findings are consistent with those of previous studies, which showed that KLK4 is upregulated in prostate cancer and a proliferative factor for the disease. Also, knocking down KLK4 in vitro and in vivo reduces prostate cancer cell growth [16]. Also, it has been shown that depletion of KLK4 might decrease cell proliferation and growth by inactivating the Wnt/β-catenin signaling pathway [10].

ERK/AKT signaling has been shown to be important in the proliferation and activity of tumor cells [17]. RCC may be influenced by KLK4’s ability to activate and regulate various downstream signaling transduction pathways [18]. We predicted that KLK4 could regulate the Erk and Akt signal transduction pathways in order to generate the cellular state. The activation of signaling cascades by KLK4 has been shown to substantially increase the proliferation, motility, and colony formation abilities of RCC cells. Activation of the ERK/AKT signaling pathway was shown to be reduced by KLK4 silencing in the current research, and inhibition of the Erk pathway with the Erk inhibitor SCH772984 resulted in a significant reduction in the proliferation of RCCs. Together, our results indicate that KLK4 may enhance the activity of RCCs by activating the Erk and Akt pathways, meaning that these pathways may be a promising therapeutic target for treating KICH in the future.
Conclusion

In this work, we discovered that KLK4 is highly expressed in KICH specimens and RCC cell lines, and knockdown of KLK4 decreased RCC cells proliferation, colony formation, migration, invasion, and EMT progression, which was significantly mediated by modulating ERK/AKT signaling pathways. All these findings indicated that KLK4 acts as a tumor promoter and KLK4 might be a biomarker and potential target for KICH therapy.

Statement of Ethics

This study protocol was reviewed and approved by the Ethics Committee of The Fourth Hospital of Hebei Medical University, approval number (2020-10-12). GEPIA belong to public databases. The patients involved in the database have obtained ethical approval. Users can download relevant data for free for research and publish relevant articles. Our study is based on open source data, so there are no ethical issues and other conflicts of interest.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Author Contributions

Bo Fan and Zongtao Ren conceived and designed the research; Bo Fan and Yunfeng Niu performed the experiments and analyzed the data. Bo Fan wrote the manuscript. Aili Zhang, Shufei Wei, Yongliang Ma, and Jianzhi Su revised the manuscript. All the authors read, commented, and accepted the final version.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.