FOXK2 suppresses the malignant phenotype and induces apoptosis through inhibition of EGFR in clear-cell renal cell carcinoma

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Forkhead box K2 (FOXK2) belongs to the forkhead box transcription factor family. Recent studies have revealed that FOXK2 plays essential roles in cancer cell proliferation and survival. However, the biological function of FOXK2 in renal cell carcinoma remains unexplored. In our study, we demonstrated that FOXK2 mRNA and protein levels were decreased in clear-cell renal cell carcinoma (ccRCC) tissues compared to those in corresponding non-tumor renal tissues, and decreased FOXK2 levels were associated with poor prognosis in ccRCC patients after nephrectomy. FOXK2 suppressed proliferation, migration and invasion capabilities of ccRCC cells and induced cellular apoptosis in vitro. Moreover, we found that FOXK2 overexpression inhibited xenograft tumor growth and promoted apoptosis in vivo. Genome-wide transcriptome profiling using FOXK2 overexpressed 769-P cells revealed that the epidermal growth factor receptor (EGFR) was a potential downstream gene of FOXK2. Overexpression of EGFR is able to rescue the inhibited proliferation capacity and the enhanced apoptosis capacity due to the overexpression of FOXK2 in 769-P cells. Collectively, our results indicate that FOXK2 inhibits the malignant phenotype of ccRCC and acts as a tumor suppressor possibly through the inhibition of EGFR.

Introduction

Renal cell carcinoma (RCC) is the most common solid tumor of kidney and accounts for approximately 2–3% of all human malignancies. Clear-cell renal cell carcinoma (ccRCC), which accounts for 80–90% of all RCCs, is the most common pathological subtype. ccRCC patients benefit very little from chemotherapy and radiotherapy, therefore, the most effective therapeutic approaches are surgery and targeted therapy. However, approximately 20–30% of patients with localized ccRCCs have metastases after partial or radical nephrectomy, and the efficacy of targeted therapies is limited. Given that ccRCC is a highly aggressive cancer with a concomitant poor prognosis, the intensive study on the molecular mechanism of ccRCC tumorigenesis and metastasis is urgently required. We previously demonstrated that a Forkhead box (FOX) transcription factor family member, FOXO3a, was crucial for the metastasis of ccRCC. However, the roles other FOX family members play in ccRCC are largely unknown.

FO XK2 (FOXK2) is a forkhead box transcription factor, which was initially recognized as a regulator of IL-2 transcription. Previous studies have shown that FOXK2 was involved in many molecular mechanisms, including starvation-induced autophagy, cell adhesion and motility, chromatin structure modification, G/T-mismatch repair and cell cycle regulation. FOXK2 was also reported to play important roles in the carcinogenesis of breast cancer and colorectal cancer. Despite all these advances, the clinical relevance, biological function and molecular mechanism of FOXK2 in ccRCC remain unknown.

In our study, we analyzed FOXK2 mRNA and protein levels in ccRCC tissues and corresponding non-tumor tissues, as well as the prognostic outcomes between FOXK2 expression and clinicopathological features. Through a series of in vitro and in vivo experiments, we investigated the effects of the change in FOXK2 activity on ccRCC proliferation, migration, invasion and apoptosis. Finally, we demonstrated that FOXK2 suppresses the malignant phenotype and sensitizes ccRCC cells to apoptosis may through inhibition of a potential downstream gene, epidermal growth factor receptor (EGFR); and FOXK2 may serve as a novel tumor suppressor in ccRCC.

Key words: clear-cell renal cell carcinoma, FOXK2, apoptosis, EGFR

Additional Supporting Information may be found in the online version of this article.

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What's new?
Forkhead box K2 (FOXK2) is involved in various molecular processes and is reported to play a role in breast and colorectal carcinogenesis. In addition, the related factor, FOXO3a, is critical to metastasis in clear-cell renal cell carcinoma (ccRCC). Whether FOXK2 also has a role in ccRCC, however, is unknown. Here, FOXK2 expression is shown to be downregulated in ccRCC, with reduced levels of FOXK2 mRNA associated with poor prognosis. In ccRCC cells, FOXK2 overexpression inhibited ccRCC cell proliferation, migration, and invasion and induced apoptosis, possibly through EGFR inhibition. The findings suggest that FOXK2 functions as a tumor suppressor in ccRCC.

Materials and Methods

Ethics statement
Our study was approved by the Ethics Committee of Chinese People’s Liberation Army (PLA) General Hospital. Each enrolled patient signed a written informed consent before sample collection. Animal experiments were approved by the Experimental Animal Ethical Committee of Chinese PLA General Hospital and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

Patients and tissue samples
A total of 42 ccRCC tissues, paired with adjacent non-tumor renal tissues, were recruited randomly from patients who underwent nephrectomy at the Urology Department of Chinese PLA General Hospital between August 2013 and October 2014. All specimens were pathologically confirmed to be ccRCC by senior pathologists. The TNM stages and nuclear grades were determined according to the 2009 TNM classification system and the Fuhrman nuclear grading system, respectively.

Cell culture
Human ccRCC cell lines 769-P, 786-O, Caki-1 and Caki-2 and human renal proximal tubular epithelial cell line HKC were purchased from the Institute of Basic Medical Sciences Chinese Academy of Medical Sciences, China Infrastructure of Cell Line Resources (Beijing, China). The cells were cultured in RPMI-1640 medium (HyClone, USA) or Dulbecco’s modified Eagle’s medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and incubated in a humidified atmosphere of 5% CO₂ at 37°C.

Quantitative real-time PCR (qRT-PCR)
Total RNA were extracted using TRIzol reagent (ComWin Biotech, Beijing, China). Reverse transcription of mRNA was performed using Transcript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech, Beijing, China) according to the manufacturer’s instructions. Quantitative real-time PCR (qRT-PCR) was performed with TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China) on an Applied Biosystems 7500 Detection system. The relative mRNA levels were normalized to peptidylprolyl isomerase A (PPIA). The primer sequences are listed in Supporting Information Table S1.

Western blot analysis
Total proteins were extracted using RIPA lysis buffer (Solarbio, Beijing, China) mixed with EDTA-free Protease Inhibitor (Roche Applied Science, Mannheim, Germany). The proteins were separated by 12% SDS-PAGE, then transferred onto PVDF membranes (Millipore, Billerica, MA). The membranes were blocked with 5% non-fat milk for 1 hr at 37°C, then incubated with primary antibodies overnight at 4°C, and followed by incubation with the corresponding secondary antibodies (ZSGB-BIO, Beijing, China) for 1 hr at room temperature. Immunoreactive bands of target proteins were normalized to β-actin and visualized using enhanced chemiluminescence detection reagent (Thermo Fisher Scientific, Waltham, MA). The relative protein levels were quantitatively analyzed by gray value method. The antibodies are listed in Supporting Information Table S2.

Immunohistochemistry (IHC)
IHC assay was performed as previously described. All human tissues and xenograft tumor tissues were fixed in 10% neutral formalin and embedded in paraffin. Sections (4 μm thick) were cut serially from paraffin-embedded blocks and mounted on APES-coated glass slides. Paraffin section that without adding primary antibody during IHC assay was used as negative control. The product of the staining intensity and range scores was used as the index for the result analysis and named as histoscore. The staining intensity was scored 0 (negative staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining); the staining range was scored 0 (0% staining), 1 (1–25% staining), 2 (26–50% staining) and 3 (51–100% staining). Immunostaining analyses were performed blindly by two-independent pathologists. The antibodies are listed in Supporting Information Table S2.

Immunofluorescence
The cells were seeded on cover slips 24 hr prior to the experiment. After fixation with 4% paraformaldehyde–PBS for 15 min, the cells were permeabilized with 0.5% Triton X-100 for 15 min at room temperature and then blocked with 3% bovine serum albumin for 30 min at 37°C. The cover slips were incubated with primary anti-FOXK2 antibody (Abcam, Cambridge, UK, ab5298) at 1:100 dilution for 1 hr at 37°C and then incubated with Rhodamine (TRITC)-conjugated rabbit anti-goat IgG (ZSGB-BIO, Beijing, China) at 1:50.
dilution for 1 hr at 37°C. Nuclei staining was performed with 0.2 mg/ml DAPI.

**RNAi knockdown**

Small interfering RNA (siRNA) sequences targeting FOXK2 (si-FOXK2), EGFR (si-EGFR) and negative control (NC) were chemically synthesized by GenePharma Co. (Shanghai, China). Transfection was performed using Lipofectamine® 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The sequences of si-FOXK2, si-EGFR and NC are listed in Supporting Information Table S3.

**Plasmid construction and transfection**

The full-length fragment of FOXK2 coding sequence was inserted into lentiviral vector pLV-EGFP-(2A) Puro (Inovogen Tech. Co., Beijing, China) (plv-FOXK2). The full-length fragment of EGFR coding sequence was inserted into lentiviral vector pLVX-RES-Hyg (YouBio Tech. Co., Changsha, China) (plv-EGFR). Empty vector (EV) was used as control. The constructed sequence was verified through DNA sequencing. After transfecting plasmid into 293T cells for 48 hr, the collected viral supernatant was filtered. After filtration, the supernatant was used to infect target cells. The FOXK2/EGFR overexpressed and control cells were cultured in corresponding medium containing puromycin/hygromycin to select stable cell lines.

**MTS assay**

The cells were preseeded into 96-well plates (1 × 10³ cells/well). At 0, 24, 48, 72, and 120 hr after seeding, 20 μl CellTiter 96® AQeuous One Solution (Promega, Madison, WI) was added to the cells and then co-incubated for 2 hr at 37°C prior to absorbance measurement. All assays were performed in triplicates.

**Colony formation assay**

The cells were seeded on six-well plates at a density of 1 × 10⁵ cells per well in triplicates. After culturing for 14 days, the cells were fixed with 100% methanol and stained with 1% crystal violet before the colony numbers were counted. All assays were performed in triplicate.

**Cell migration and invasion assays**

Cell migration and invasion assays were performed in uncoated and Matrigel-coated Transwell® chambers (Corning, NY) containing polycarbonate membrane filters with a pore size of 8 μm. A total of 1 × 10⁵ cells in 200 μl of serum-free medium were seeded in the upper chamber, and 500 μl of 10% FBS medium was added into the lower chamber. After culturing for 12 (migration) or 24 (invasion) hr at 37°C, the cells that adhered to the lower surface of the transwell chambers were fixed with 100% methanol and stained with 1% crystal violet. The cells were counted in five random fields under a microscope (OLYMPUS, 200×), and the mean values were then calculated. All assays were repeated thrice.

**Wound healing assay**

A total of 2 × 10⁵ cells were seeded on six-well plates. After culturing overnight, the confluent monolayer of cells was scratched using a sterile 200 μl pipette tip and washed with PBS thrice. Photographs of the same position were taken at 0, 6 and 12 hr after scratching. The coverage of the scratching area was measured at three positions for each well. All assays were performed in triplicate.

**Cell cycle and apoptosis analysis**

Cell cycle and apoptosis were analyzed through flow cytometry. The cells were fixed in 70% precool ethanol at 4°C overnight, and then RNase A and propidium iodide (Solarbio, Beijing, China) were added prior to cell cycle analysis. For apoptosis analysis, the cells were treated with Annexin V-FITC and propidium iodide (Solarbio, Beijing, China) prior to flow cytometry. All analyses were performed on FACS-Calibur (BD Biosciences, San Jose, CA).

**In vivo growth assay of xenograft tumor**

A total of 1 × 10⁷ ccRCC cells stably expressing plv-FOXK2 or EV were suspended in 100 μl PBS, then subcutaneously injected into the left armpit of male BALB/c nude mice (4 weeks old, 10 mice per group). After tumor formation, the tumor volume was calculated weekly by using the following formula: V (mm³) = 0.5 × length (mm) × width² (mm²). All mice were sacrificed for weight measurement and IHC staining of xenograft tumors 56 days after the injection.

**Microarray profiling and bioinformatics analysis**

Microarray profiling was performed using Affymetrix GeneChip® Human Transcriptome Array 2.0. The quantification and quality check of total RNA were performed through NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA labeling, microarray hybridization, array scanning, data extraction and result analysis were performed by Shanghai Biotechnology Corporation (Shanghai, China). Differentially expressed genes (DEGs) with statistical significance (fold change > 2.0 or < 0.5, p < 0.05) were identified by comparing the normalized expression levels in plv-FOXK2 and EV groups. Gene ontology (GO) and pathway analyses based on DAVID and KEGG databases were performed to identify the molecular functions and biological processes involved in these DEGs.

**Statistical analysis**

SPSS 19.0 (SPSS, Inc., USA) and Prism 5.0 (GraphPad, USA) software were used for all statistical analyses. Normally distributed variables were expressed as means ± SD, and compared using Student’s t-test. Univariate and multivariate analyses were performed using the Cox proportional hazards model. Disease-free survival (DFS), was used for prognostic evaluation of ccRCC patients, which was defined as the time...
from the date the patient underwent radical or partial nephrectomy to the date of local recurrence, distant metastasis or death. Prognostic analysis was performed using the Kaplan-Meier method with log-rank test. A p-value < 0.05 was considered statistically significant.

Results

Downregulation of FOXK2 correlates with poor prognosis in ccRCC

Of all the 42 ccRCC patients, 36 patients underwent radical nephrectomy and 6 patients underwent partial nephrectomy. The average age was 55.38 ± 12.13 years. The average BMI was 24.74 ± 2.50 kg/m². The median tumor diameter was 6 cm with a range of 2–13 cm. T1N0M0 ccRCCs were found in 24 patients, T2N0M0 ccRCCs were found in 14 patients, T3N0M0 ccRCCs were found in 4 patients, and T4N0M0 ccRCCs were found in 4 patients. FOXK2 expression was analyzed through qRT-PCR, western blot and IHC staining. The mRNA levels of FOXK2 were significantly downregulated in ccRCC tissues ($p < 0.01$, Fig. 1a) compared to that in adjacent non-tumor renal tissues. Next, we compared the FOXK2 mRNA levels between the different subgroups of age, body mass index (BMI), gender, Overall TNM staging, Fuhrman grade and tumor diameter of the 42 ccRCC tissues. The relative lower levels of FOXK2 mRNA were compared the FOXK2 mRNA levels between the different sub-parameters of ccRCC patients and FOXK2 mRNA levels are shown in Table 1. The significantly decreased protein levels of FOXK2 were also detected in ccRCC tissues compared to adjacent non-tumor renal tissues through western blot (Fig. 1g) and the quantitative analysis of band gray values ($p < 0.001$, Fig. 1e). IHC staining demonstrated that FOXK2 protein mainly expressed in the cell nuclei of both ccRCC tissues and normal renal tissues. The positive staining intensity and range were significantly attenuated in ccRCC tissues compared to that in adjacent non-tumor renal tissues (Fig. 1h). Quantitative analysis of IHC staining using histoscore confirmed such result ($p < 0.001$, Fig. 1f). To investigate whether FOXK2 expression affects the prognosis of ccRCC patients, we followed up 42 ccRCC patients for 3.0–39.7 months (median, 32.8 months). Thirteen patients developed local recurrence, distant metastasis or death by the end of the follow-ups. Logarithmic transformation was performed on the ratio of FOXK2 mRNA levels in ccRCC tissues (T) to those in normal tissues (N). We selected the median FOXK2 level as the cutoff point to divide patients into high and low FOXK2 groups ($n = 21$ per group, Fig. 1i). Kaplan-Meier analysis with log-rank test showed that ccRCC patients with low FOXK2 levels had significantly poorer DFS than patients with high FOXK2 levels (log-rank test $p = 0.010$, Fig. 1j). Univariate Cox regression analysis showed that Overall TNM staging (HR = 2.849, $p = 0.067$, 95%CI = 0.929–8.731) and FOXK2 level (HR = 4.702, $p = 0.019$, 95%CI = 1.288–17.169) were two prognostic factors for ccRCC patients. Multivariate analysis using these two factors revealed that FOXK2 mRNA level was an independent prognostic factor (HR = 4.115, $p = 0.034$, 95%CI = 1.110–15.254) for ccRCC patients (Table 2). These results indicate that FOXK2 may be closely related to ccRCC malignant progression and may functioned as a tumor suppressor in ccRCC.

FOXK2 expression and subcellular localization in ccRCC cell lines

To verify the above results derived from ccRCC tissues and to further explore the function of FOXK2 in vitro, cell lines 786-O, 769-P, Caki-1, Caki-2 and HKC were subjected to qRT-PCR and western blot for the detection of FOXK2 expression. The mRNA and protein levels of FOXK2 were significantly downregulated in all ccRCC cell lines compared to that in HKC cell line (Fig. 2a). To achieve FOXK2 overexpression, 769-P ($p < 0.001$, Fig. 2b) and 786-O ($p < 0.001$, Fig. 2d) cells were transfected with plv-FOXK2. The same cells were transfected with EV as controls. Knockdown of FOXK2 was performed in 786-O cells ($p < 0.001$, Fig. 2c) through transfection of si-FOXK2. Simultaneously, 786-O cells transfected with NC were used as controls. The efficiencies of knockdown and overexpression were verified through qRT-PCR and western blot. Nuclear localization of FOXK2 proteins in both 769-P and 786-O cells was detected through immunofluorescence (Fig. 2e). The intranuclear fluorescence intensity was significantly elevated in 769-P cells transfected with plv-FOXK2 compared to that in EV, whereas a reversed effect was observed in 786-O cells.

FOXK2 inhibits the proliferation of ccRCC cells in vitro

MTS assays showed that the proliferation was significantly inhibited in both FOXK2 overexpressed 769-P and 786-O cells compared to those in EV cells (Figs. 3a and 3c). A stronger proliferative ability was noted in 786-O cells transfected with si-FOXK2 than 786-O cells transfected with NC (Fig. 3b). Similar results were observed in colony formation assay: the colony-forming ability was remarkably decreased in 769-P and 786-O cells transfected with plv-FOXK2 compared to the same cells transfected with EV ($p < 0.001$ and $p < 0.01$, Figs. 3d and 3e). By contrast, the colony-forming ability was enhanced in 786-O cells transfected with si-FOXK2 ($p < 0.001$, Fig. 3f) compared to those in NC cells.

FOXK2 suppresses the migration and invasion of ccRCC cells in vitro

Transwell assays revealed that the migration and invasion were significantly inhibited in FOXK2 overexpressed 769-P cells compared to those in EV cells ($p < 0.01$, Fig. 3g). Inverse
Figure 1. Expression and prognostic value of FOXK2 in ccRCC. (a) The mRNA levels of FOXK2 in 42 pairs of primary non-metastatic ccRCC tissues and corresponding adjacent normal renal tissues were analyzed through qRT-PCR. (b–d) Comparison of FOXK2 mRNA levels between subgroups of tumor diameter, Fuhrman grade and Overall TNM staging. (e) Quantitative analysis of FOXK2 protein levels in ccRCC tissues and corresponding normal renal tissues based on western blot. Protein levels were normalized to β-actin expression. (f) Histoscores of FOXK2 in IHC-stained ccRCC tissues and corresponding normal renal tissues. (g) Representative western blot images showing the FOXK2 protein levels in ccRCC tissues (T) and corresponding normal renal tissues (N). (h) Representative IHC staining images showing the FOXK2 protein expression in ccRCC tissues and corresponding normal renal tissues. (i) Ratios between FOXK2 mRNA levels in ccRCC tissues and that in corresponding normal renal tissues. All data were logarithmically transformed. Red arrow shows the cutoff point. (j) Kaplan-Meier survival curve for ccRCC patients with high (n = 21) and low (n = 21) FOXK2 mRNA levels (*p < 0.05, **p < 0.01, ***p < 0.001). [Color figure can be viewed at wileyonlinelibrary.com]
effects were found in FOXK2 knocked-down 786-O cells ($p < 0.001$, Fig. 3h). The wound healing assay showed that the scratching area was covered at a relatively slower speed in FOXK2 overexpressed 769-P cells at the time points 6 hr ($p < 0.001$) and 12 hr ($p < 0.001$) compared to those in EV cells (Fig. 3i). The scratching area was covered faster in FOXK2 knocked-down 786-O cells at the time points 6 hr ($p < 0.001$) and 12 hr ($p < 0.001$) than those in NC cells (Fig. 3j). These results suggest that FOXK2 suppresses the malignant phenotype and serves as a tumor suppressor of ccRCC cells in vitro.

**Overexpression of FOXK2 induces apoptosis in ccRCC cells**

We performed flow cytometry to explore the possible mechanism underlying the suppressive function of FOXK2 in ccRCC. Cell cycle profiling demonstrated that both FOXK2 overexpressed 769-P (Supporting Information Fig. S1a) and FOXK2 knocked-down 786-O cells (Supporting Information Fig. S1b) exhibited no significant change in the percentage of G0/G1 phases cells and S phase cells. Annexin V-FITC and propidium iodide were used in the apoptosis analysis to stain the early and late apoptotic cells, respectively. Immunofluorescence with DAPI staining was used to show the change in nuclear morphology of apoptotic cells. The apoptosis analyses demonstrated that the percentage of early and late apoptotic cells were significantly increased in both FOXK2 overexpressed 769-P ($p < 0.001$) and 786-O ($p < 0.001$) cells (Fig. 4a). Immunofluorescence images showed that nuclear condensation and DNA fragmentation could be visualized in both FOXK2 overexpressed 786-O and 769-P cells through DAPI staining (Fig. 4a). The percentage of apoptotic cells in si-FOXK2 group had no significant difference as compared to that in NC group, but it showed a decreasing trend following FOXK2 knockdown in 786-O cells (Supporting Information Fig. S2). Next, we quantitatively analyzed several key apoptotic proteins through western blot. As shown in Figure 4b, FOXK2 overexpression significantly decreased the protein levels of Bcl-2 and caspase-3, and increased the protein levels of cleaved caspase-3, in both 769-P and 786-O cells. These data indicate that FOXK2 exerts a tumor-suppressive function probably through the induction of apoptosis in ccRCC cells.

**Overexpression of FOXK2 inhibits xenograft tumor growth and promotes apoptosis in vivo**

To verify whether FOXK2 affects tumor growth in vivo, we subcutaneously injected FOXK2 overexpressed 769-P or 786-O cells into the left armpits of immunodeficient BALB/c mice to build the xenograft tumor models. The mice in control group were simultaneously injected with corresponding EV cells. In 786-O models, the tumor volumes (Figs. 5a and 5b) were remarkably reduced in plv-FOXK2 group as compared to that in EV group; statistically significant differences were observed at 42 ($p < 0.001$), 49 ($p < 0.001$) and 56 ($p < 0.001$) days after injection (Fig. 5c). The tumor weights in plv-FOXK2 group were lower than that in EV group ($p < 0.001$, Fig. 5d). The similar results were obtained from 769-P models (Supporting Information Figs. S3a–S3c). The xenograft tumors were then paraffin-embedded for IHC staining and quantitative analysis of apoptotic proteins. The results confirmed that the protein levels of Bcl-2 were decreased ($p < 0.001$), whereas the protein levels of caspase-3 were increased ($p < 0.01$) in plv-FOXK2 group compared to that in EV group (Figs. 5e and 5f). The above results are consistent with that from in vitro studies: FOXK2 suppresses xenograft tumor growth by inducing apoptosis.

**EGFR is a downstream gene and its expression is regulated by FOXK2**

To further investigate the potential downstream targets of FOXK2, we conducted the genome-wide transcriptome profiling of 769-P cells transfected with plv-FOXK2 and EV. The whole datasets have been deposited in the GEO database (GSE92777). By using twofold change as the cutoff point, 1,432 DEGs were identified between the two groups; 656 DEGs were upregulated, whereas 776 DEGs were downregulated (Fig. 6a). For further verification of the microarray profiling result, we randomly selected 15 upregulated (Fig. 6b) and 15 downregulated (Fig. 6c) genes from the whole datasets and analyzed the expression of these genes by using qRT-PCR. The variation tendency of these genes following FOXK2 overexpression is in accordance with that in the microarray profiling result. Among all the DEGs, 660 genes

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**Table 1. FOXK2 mRNA levels in relation to clinical-pathological parameters of ccRCC patients**

| Characteristics       | No. $\bar{n} = 42$ | FOXK2 levels $\pm$ Value | p-Value |
|-----------------------|---------------------|--------------------------|---------|
| Age (year)            |                     |                          |         |
| <60                   | 23                  | 0.097 ± 0.027            | 0.924   |
| ≥60                   | 19                  | 0.101 ± 0.017            |         |
| BMI (kg/m$^2$)        |                     |                          | 0.814   |
| <25                   | 27                  | 0.102 ± 0.022            |         |
| ≥25                   | 15                  | 0.094 ± 0.023            |         |
| Gender                |                     |                          | 0.452   |
| Male                  | 30                  | 0.091 ± 0.013            |         |
| Female                | 12                  | 0.119 ± 0.050            |         |
| Overall TNM staging   |                     |                          | 0.011   |
| Stage I               | 24                  | 0.135 ± 0.027            |         |
| Stages II+III         | 18                  | 0.051 ± 0.007            |         |
| Fuhrman grade         |                     |                          | 0.025   |
| ≤2                    | 27                  | 0.126 ± 0.024            |         |
| >2                    | 15                  | 0.050 ± 0.008            |         |
| Diameter (cm)         |                     |                          | 0.017   |
| ≤7                    | 25                  | 0.131 ± 0.026            |         |
| >7                    | 17                  | 0.052 ± 0.007            |         |
were enriched for 226 functional annotation clusters according to the GO enrichment analysis; 323 genes were enriched for 24 pathways, including 11 cancer-related (Fig. 6d) and 4 RCC-related pathways. By overlapping the DEGs enriched for RCC-related pathways (Fig. 6e), we selected EGFR, VEGFA and IGF1R as candidate target genes of FOXK2. We finally excluded VEGFA and IGF1R and focused on EGFR by using qRT-PCR. By performing the qRT-PCR and western blot assays, we observed that overexpression of FOXK2 decreased the mRNA and protein levels of EGFR in 769-P cells; whereas knockdown of FOXK2 increased mRNA and protein levels of EGFR in 786-O cells (Supporting Information Figs. S4a and S4b). These findings suggest that EGFR is a potential downstream gene of FOXK2.

**Overexpression of EGFR is able to rescue the inhibited proliferation capacity and the enhanced apoptosis capacity in FOXK2 overexpressed cells**

We next performed rescue experiments in 769-P cells to verify whether the phenotypic change following FOXK2 overexpression could be rescued by overexpressing EGFR. The overexpression efficiencies of plv-FOXK2 and plv-EGFR in 769-P cells were verified through qRT-PCR (Figs. 2b and 6f) and western blot (Fig. 6g). MTS assay showed that overexpression of FOXK2 can inhibit proliferation capacity in 769-P cells, whereas overexpression of EGFR can promote such capacity. The inhibited proliferation capacity in FOXK2 overexpressed 769-P cells could be rescued by overexpressing EGFR ($p < 0.001$, Fig. 6h). Apoptosis analyses showed that the percentage of early and late apoptotic cells were increased in FOXK2 overexpressed 769-P cells and decreased in EGFR overexpressed 769-P cells compared to 769-P cells transfected with EV ($p < 0.001$). When compared to 769-P cells transfected with plv-FOXK2, the enhanced apoptosis capacity could be reversed following overexpression of EGFR ($p < 0.01$, Figs. 6i and 6j). We also performed rescue experiments by simultaneously co-transfecting 786-O cells with siRNAs targeting FOXK2 and EGFR. The results suggested that simultaneous knockdown of FOXK2 and EGFR can partly rescue the enhanced proliferation capacity and the attenuated apoptosis capacity in FOXK2 single knocked-down 786-O cells (Supporting Information Figs. S5a–S5e). All above rescue experiments indicate that FOXK2 suppresses the proliferation and induces apoptosis possibly through the inhibition of EGFR in ccRCC cells.

**Discussion**

FOX transcription factor family, which is named after the forkhead winged-helix DNA-binding domain, has been studied extensively in various cancers. The members of this family, including FOXK1 and FOXK2, have been shown to be involved in cell proliferation, apoptosis, and drug resistance in RCC. In this study, we investigated the molecular mechanisms underlying the roles of FOXK2 in RCC, focusing on the interaction between FOXK2 and EGFR.
Figure 2. Expression and subcellular localization of FOXK2 in ccRCC cell lines. (a) mRNA and protein levels of FOXK2 in 786-O, 769-P, Caki-1, Caki-2 and HKC cells. (b–d) The change of FOXK2 mRNA and protein levels in 786-O and 769-P cell lines after knockdown or overexpression. (e) Representative immunofluorescent staining images showing the variation of intranuclear fluorescence intensity in FOXK2 overexpressed 769-P cells and FOXK2 knocked-down 786-O cells (***p < 0.001). [Color figure can be viewed at wileyonlinelibrary.com]
Figure 3. FOXK2 inhibits the proliferation, migration and invasion of 786-O and 769-P cell lines in vitro. (a) MTS assay showing that overexpression of FOXK2 reduced the proliferation of 769-P cells. (b) MTS assay showing that knockdown of FOXK2 promoted the proliferation of 786-O cells. (c) Overexpression of FOXK2 reduced the proliferation of 786-O cells. (d–f) Representative images and quantitative analysis of colony formation assay showing that overexpression of FOXK2 decreased the clone number in both 769-P and 786-O cell lines, whereas knockdown of FOXK2 increased the clone number in 786-O cell line. Each experiment was performed in triplicate. (g, h) Representative images and quantitative analysis showing the suppression of migration and invasion in FOXK2 overexpressed 769-P cell line, whereas the reverse effect was detected in FOXK2 knocked-down 786-O cell line. (i, j) Photos of the wound healing assay were taken at different time points (0, 6 and 12 hr after scratching). FOXK2 overexpression inhibited cell mobility in 769-P cell line. Knockdown of FOXK2 enhanced the cell mobility in 786-O cell line. Each experiment was performed in triplicate (**p < 0.01, ***p < 0.001). [Color figure can be viewed at wileyonlinelibrary.com]
Figure 4. Influence of FOXK2 overexpression on apoptosis in ccRCC cell lines. (a) Flow cytometry and quantitative analysis showing that overexpression of FOXK2 increased the percentage of early and late apoptotic cells in both 769-P and 786-O cell lines. The percentage of early and late apoptotic cells were showed on the lower right (LR) and upper right (UR) of the flow cytometry analysis diagram. The histogram was used to compare the percentage of apoptotic cells between groups. DAPI-stained immunofluorescence showing nuclear condensation and DNA fragmentation (white arrows). (The 769-P EV in Fig. 4a and Fig. 2e were taken from the same immunofluorescence experiment.) (b) Western blot and quantitative analysis of Bcl-2, caspase-3 and cleaved caspase-3 protein levels in FOXK2 overexpressed 769-P and 786-O cell lines. (The FOXK2 and β-actin western blot bands of 786-O cells in Fig. 4b and Fig. 2d were taken from the same western blot experiment.) (*p < 0.05, **p < 0.01, ***p < 0.001). [Color figure can be viewed at wileyonlinelibrary.com]
Figure 5. Overexpression of FOXK2 attenuates xenograft tumor growth in vivo. (a) Representative images of nude mice with xenograft tumors derived from subcutaneous implantation of 786-O cells treated with plv-FOXK2 or EV. (b) Representative images of xenograft tumors excised from nude mice. (c) Comparison of tumor growth curves between plv-FOXK2 group and EV Group (10 mice per group). (d) Comparison of tumor weight between plv-FOXK2 group and EV group. (e) Histoscores of FOXK2, Bcl-2 and caspase-3 in IHC-stained xenograft tumors from plv-FOXK2 group and EV group. (f) Representative IHC staining images of FOXK2, Bcl-2, caspase-3 and negative control in paraffin-embedded xenograft tumors from plv-FOXK2 group and EV group (**p < 0.01, ***p < 0.001). [Color figure can be viewed at wileyonlinelibrary.com]
FOXK2 suppresses the malignant phenotype in ccRCC

Figure 6.
reported to act as important regulators of numerous biological processes, including cell cycle regulation, proliferation, differentiation, senescence, survival, metabolism and apoptosis. A series of FOX genes, such as FOXC2, FOXJ1, FOX1, FOXL1, FOXM1, FOXO9, and FOXPs have been reported to play vital roles in the carcinogenesis, metastasis and prognosis of ccRCC. A previous study reported that FOXK2 expression was elevated in human colorectal cancer and is associated with intestinal tumorigenesis; two other studies reported that FOXK2 was downregulated in breast cancer and suppressed the carcinogenesis of breast cancer. However, the role that FOXK2 plays in ccRCC remains unknown.

We demonstrated in our study that the mRNA and protein levels of FOXK2 were decreased in ccRCC tissues compared to those in adjacent non-tumor renal tissues. The longer-diameter (diameter > 7 cm), higher-Fuhrman-grade (Fuhrman III and IV) and later-TNM-stage (T2N0M0 and T3N0M0) ccRCCs had lower levels of FOXK2 mRNA. Moreover, the decreased FOXK2 mRNA levels predicted poor prognosis in patients with ccRCC, which was in accordance with the result of prognosis analysis on low FOXK2 expression in breast cancer. Proliferation, migration and invasion are three main kinds of phenotype in malignant tumors and are mainly linked to the biological functions of cancer cells. We found that overexpression of FOXK2 inhibited the proliferation, migration and invasion of ccRCC cells, whereas knockdown of FOXK2 promoted these aspects. Previous studies on breast cancer illustrated that knockdown of FOXK2 led to a significant decrease in the percentage of G0/G1 phase cells and a increase in the percentage of S phase cells, whereas overexpression of FOXK2 decreased the percentage of S phase cells. Unlike previous studies, our results showed that in both FOXK2 knocked-down and overexpressed cells, no statistically significant changes were detected in the percentage of G0/G1 phases and S phase cells. These results agree with our previous finding that FOXK2 mRNA level has no correlation with ccRCC tumor size, which imply that other potential mechanisms may be involved in the tumor-suppressive function of FOXK2. Apoptosis is a predominant molecular mechanism involved in programmed cell death and is linked to several human pathologies, including cancer, aging and infectious disease. Evasion of apoptosis has been recognized as a crucial event during cancer cell survival, growth and malignant transformation. Our apoptosis assays showed that overexpression of FOXK2 remarkably increased the percentage of apoptotic ccRCC cells, whereas knockdown of FOXK2 did not significantly decrease the percentage of these cells. A possible reason for the latter is that the basal percentage of apoptotic ccRCC cells is very low, and the reduction in the percentage may exceed the detection threshold. Another evidence for cell apoptosis associated with FOXK2 overexpression is that the typical changes in nuclear morphology, including the emergence of nuclear condensation and DNA fragmentation. The Bcl-2 family including a series of pro-apoptotic and anti-apoptotic proteins, the balance among these proteins plays a crucial role in the cellular apoptosis. Bcl-2 protein downregulation results in loss of the inhibitory effect on Bax, which is the most common trigger of the intrinsic apoptotic pathway, and finally induces apoptosis. Intrinsic apoptosis is executed by three caspase family members, namely, caspase-3, −6 and −7. Caspase protein is initially synthesized as inactive pro-enzyme and finally cleaved to form active enzyme, which is cleaved caspase, during the demolition phase of apoptosis. We seriatim analyzed the above apoptosis-related proteins through western blot. The results indicated that FOXK2 overexpression in ccRCC cells increased the level of apoptotic protein cleaved caspase-3, whereas it decreased the protein levels of Bcl-2 and caspase-3. Similar results were obtained from the in vivo assays. Taken together, these data suggested that FOXK2 suppresses the ccRCC malignant phenotype and induces cell apoptosis may through the regulation of apoptosis-related proteins. Marais et al. reported that the lack of two cyclin-dependent kinase (CDK) phosphorylation sites, serines 368 and 423, causes mutation of FOXK2 and leads to cellular apoptosis, but the wild-type and other CDK site-mutant FOXK2 may also contribute to a similar effect. In the following experiments, we will further analyze whether the mutation of CDK phosphorylation sites occurs in ccRCCs during FOXK2-induced apoptosis.

Figure 6. EGFR is a downstream molecule in FOXK2-mediated proliferation and apoptosis. (a) Heat map of differentially expressed genes from microarray profiling using Affymetrix GeneChip® Human Transcriptome Array 2.0 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE92777). Column represent differentially expressed genes, row represent 769-P cells transfected with plv-FOXK2 or EV. (b) The comparison of the variation tendency between microarray profiling and qRT-PCR results by using 15 upregulated genes randomly selected from the whole dataset. The foldchange represent for the upregulation of expression level following FOXK2 overexpression. (c) The comparison of the variation tendency between microarray profiling and qRT-PCR results by using 15 downregulated genes randomly selected from the whole dataset. The foldchange represent for the downregulation of expression level following FOXK2 overexpression. (d) DEGs enriched for cancer-related KEGG pathways due to overexpression of FOXK2 in 769-P cell line. (e) Venn diagram showing the overlap of DEGs enriched for 4 RCC-related pathways. (f) qRT-PCR result showing the mRNA levels of EGFR in 769-P cells transfected with plv-FOXK2 and/or plv-EGFR, 769-P cells transfected with EV were used as controls. (g) Western blot showing the overexpression efficiencies of plv-FOXK2 and/or plv-EGFR in 769-P cells, 769-P cells transfected with EV were used as controls. (h) MTS assay showing the proliferation curves of 769-P cells transfected with plv-FOXK2 and/or plv-EGFR and EV. (i) Flow cytometry showing the percentage of early and late apoptotic cells of 769-P cells transfected with plv-FOXK2 and/or plv-EGFR and EV. (j) Histogram showing that enhanced apoptosis capacity following FOXK2 overexpression could be reversed by overexpressing EGFR in FOXK2 overexpressed 769-P cells (**p < 0.01, ***p < 0.001, compared to EV; \( \nabla \nabla p < 0.01, \nabla \nabla \nabla p < 0.001 \) compared to plv-FOXK2). [Color figure can be viewed at wileyonlinelibrary.com]
Our in vivo assay showed that the xenograft tumor volume was reduced after FOXK2 overexpression. But previous analysis by using ccRCC tissues demonstrated no significant correlation between FOXK2 mRNA level and tumor diameter. A possible reason is that the ccRCC tumor size may be regulated by numerous genes rather than a single one such as FOXK2 in human body, any increase or decrease in tumor size may result from interaction between these molecules. The effect that FOXK2 exert on the ccRCC tumor size in human body may be neutralized by other molecules; moreover, the total number of sample that we used in our study is relatively less. Our in vivo study overexpressed FOXK2 level for 8–10 times than control, this may enhance the effect that FOXK2 exert on the tumor size, and result in the decrease in tumor size following FOXK2 overexpression in mice.

EGFR, also known as ErbB1 or HER, is a receptor tyrosine kinase that belongs to the ErbB family. EGFR overexpression has been found in a variety of human epithelial malignancies and was associated with malignant progression, poor prognosis and targeted therapy resistance.44 After binding to the ligand, EGFR is activated and lead to the activation of several downstream signaling pathways, including MAPK, PLCγ, STAT and PI3K/AKT, within the cell. All these pathways are closely linked to a broad array of tumor phenotype, including proliferation, differentiation, migration, adhesion and apoptosis.45,46 Numerous studies have demonstrated that EGFR activation could inhibit cell apoptosis44–47 and promote malignant progression of RCC.2,48 Our microarray profiling data showed that various DEGs that resulted from overexpression of FOXK2 in ccRCC cells were enriched for different kinds of pathways; some of these pathways were cancer-related, including ErbB, Rap1, PPARα, focal adhesion, adherens junction and regulation of actin cytoskeleton. This finding was consistent with the previous study based on ChiP-seq analysis that FOXK2 controls the expression of genes associated with cellular signaling pathways, transcriptional control, apoptosis and cell movement.13 Through analyzing RCC-related pathways and using quantitative verification, we focused on a downstream gene of FOXK2, namely EGFR, and found that the expression of EGFR is regulated by FOXK2. The rescue experiments indicated that FOXK2 suppresses malignant phenotype and induces apoptosis may through inhibition of EGFR in ccRCC. However, the specific regulatory mechanisms concerning the interactions between FOXK2 and EGFR need further investigation.

There are several limitations within our study; first, the relatively lower sample size with shorter follow-up time. Second, both non-tumor renal tissues and benign renal tumor tissues from patients who underwent nephrectomy during the same period should be used as controls to confirm above results. At last, the specific mechanism that how FOXK2 regulates EGFR expression remains unknown. A subsequent study will improve these limitations.

In summary, we demonstrated that FOXK2 acts as a novel tumor suppressor in ccRCC. Decreased FOXK2 mRNA level is associated with poor prognosis in ccRCC patients. FOXK2 suppresses the malignant phenotype and induces apoptosis in ccRCC probably through the inhibition of EGFR. Therefore, FOXK2 may serve as a potential therapeutic target and prognostic marker for patients with ccRCC.

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