MiR-182-5p inhibits the tumorigenesis of clear cell renal cell carcinoma by repressing UBE2T

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Abstract
Ubiquitin-conjugating enzyme E2T (UBE2T), a member of the E2 family, has been reported to be overexpressed in certain tumor types and to have an important role in the Fanconi anemia pathway. However, the role of UBE2T in clear cell renal cell carcinoma (ccRCC) has not been clarified. MicroRNAs (miRNAs) participate in tumorigenesis by binding to genes and proteins that regulate cell proliferation or cell apoptosis. The aim of this study was to determine the role of UBE2T and the relationship between miR-182-5p and UBE2T in ccRCC. In the present study, UBE2T expression levels in ccRCC tissues and cells were assessed using real-time quantitative PCR (RT-qPCR) and western blotting. UBE2T protein expression was assessed in a total of 93 ccRCC patients from Peking University First Hospital (PKU) via immunohistochemistry (IHC). The effects of UBE2T knockdown on ccRCC cells were assessed with MTS assays, wound healing assays, Transwell invasion assays and flow cytometry. The effects of in vivo treatment were evaluated through xenograft experiments. The relationship between miR-182-5p and UBE2T was verified with a dual-luciferase reporter gene assay. We found that UBE2T was highly expressed in ccRCC cells and tissues. High UBE2T expression was positively correlated with advanced pathological stage, histological grade, maximum tumor diameter and distant metastasis. Multivariate analysis revealed that UBE2T expression was an independent risk factor for overall survival (OS) and recurrence-free survival (RFS) in patients with ccRCC. Knockdown of UBE2T significantly suppressed RCC cell proliferation, migration and invasion. Flow cytometry analysis showed that UBE2T knockdown promoted RCC cell cycle arrest at G2/M phase and increased cell apoptosis. The xenograft model confirmed that suppression of UBE2T significantly delayed tumor formation and growth in vivo. In addition, miR-182-5p inhibited UBE2T protein expression by targeting UBE2T mRNA and then inhibited the proliferation, migration and invasion of ccRCC cell. Our research reveals that UBE2T likely plays a critical role in ccRCC progression and may be a potential therapeutic target for ccRCC.

Keywords Clear cell renal cell carcinoma · UBE2T · miR-182-5p · Apoptosis · Migration and invasion

Abbreviations
ccRCC  Clear cell renal cell carcinoma
GEO  Gene expression omnibus

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Introduction

Renal cell carcinoma (RCC) accounts for 90% of renal cancer cases, and is the most common carcinoma of the adult kidney [1]. The rate of RCC has increased by 1.6% per year for the past 10 years, making it the 6th and 8th most common cancer in the USA among males and females, respectively [2]. Surgery is the only curative treatment for RCC, and commonly used molecular targeted drugs have improved over the past several decades. However, approximately 20–30% of RCC patients are diagnosed with metastatic disease, and among those with localized RCC, nearly 20% show recurrence and develop metastatic RCC after nephrectomy [3]. The main histologic type of RCC is clear cell renal cell carcinoma (ccRCC), accounting for 80–90% of cases [4, 5]. Therefore, further research into the underlying molecular mechanisms of ccRCC tumorigenesis and the development of novel treatments aimed at specific molecular targets are required.

UBE2T (also known as HSPC150) is a member of the E2 family in the ubiquitin–proteasome pathway [6]. It has been shown to bind the E3 ubiquitin-protein ligase Fanconi anemia complementation group L (FANCL), the ubiquitin ligase subunit of the Fanconi anemia core complex, and to catalyze the monoubiquitination of Fanconi anemia complementation group D2 [7]. Importantly, the UBE2T gene, located at 1q32.1, has been reported in certain cancer types, and overexpression of UBE2T can promote tumor growth [8, 9]. However, little is known with regard to the association between UBE2T and RCC. miRNAs are a class of endogenous RNAs with a length of approximately 22 nt. Functional studies have confirmed that miRNAs are involved in disease development by targeting mRNAs for cleavage or translational repression [10]. Specifically, the role of miR-182-5p in the modulation of malignant phenotypes of renal cell carcinoma has already been investigated [11, 12]. Furthermore, overexpression of miR-182-5p inhibited RCC cell proliferation, colony formation, and apoptosis and led to G2/M-phase cell cycle arrest [13].

In the present study, UBE2T expression in RCC cell lines and ccRCC tissues was evaluated. In addition, the effects of lentivirus-mediated shRNA knockdown and overexpression of UBE2T on the proliferation, migration, invasion and cell cycle distribution of ccRCC cells were assessed. Then we aimed to elucidate the dominant role of miR-182-5p and UBE2T in ccRCC carcinogenesis. The findings indicated that UBE2T may be a potential therapeutic target for ccRCC.

Materials and methods

Bioinformatics data mining

Microarray profiling data of ccRCC (GSE36895, GSE46699, GSE53000, GSE53757, GSE68417, GSE76351) were included in this study and obtained from Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/). TCGA-KIRC transcriptome and clinical datasets were downloaded from UCSC Xena (http://xena.ucsc.edu/). R software was applied to process the RNA sequencing data.

Tissue samples

ccRCC tissues and adjacent renal tissues were obtained from ccRCC patients who underwent nephrectomy at the Department of Urology, Peking University First Hospital (Beijing, China). Informed consent was obtained from the patients involved in this study, and protocols were approved by the Medical Ethics Committee of Peking University First Hospital. The pathological types of the samples were evaluated by hematoxylin–eosin staining and confirmed by experienced pathologists. Fresh samples were fixed in 4% paraformaldehyde for 12–24 h and then paraffin-embedded for immunohistochemistry or snap frozen immediately after resection and stored in liquid nitrogen for western blotting.

Cell culture

The cell lines (HK-2, OSRC-2, 786-O, 769-P, ACHN, Caki-1 and A498) were obtained from the American Type Culture Collection (Rockville, MD, USA). Cell lines were cultured according to the conditions specified by the provider. The cells were maintained as a monolayer culture at 37 °C in a humidified atmosphere containing 5% CO₂.

RT-qPCR and reverse transcription PCR (RT-PCR)

Total RNA was extracted from tissue samples or transfected cells using TRIzol reagent (Invitrogen; Thermo
Fisher Scientific Inc.), according to the manufacturer’s instructions. cDNA was generated using reverse transcription (TansGEN, Beijing, China). RT-qPCR was performed using the ABI PRISM 7000 quantitative fluorescence PCR system (Applied Biosystems, CA, USA) according to the manufacturer’s instructions, and the results were normalized to β-actin expression. All experiments were repeated at least three times. Information on the sequence of PCR primers is listed in Table S1.

**Immunohistochemistry**

After fixation with 4% formalin and embedded in paraffin wax, tissues were cut into 5 μm sections using a microtome. The sections were deparaffinized in xylene and rehydrated with gradient concentrations of alcohol. Then the slides were heated (120 °C) for 20 min in citrate buffer (10 mmol/L; pH 6.0) for antigen retrieval. Normal goat serum (10%) was applied to reduce nonspecific binding. Subsequently, the slides were incubated with primary rabbit anti-human UBE2T polyclonal antibody at 4 °C overnight, and a PowerVisionTM two-step histostaining reagent and 3,3-diaminobenzidine tetrahydrochloride substrate kit (Zhongshan Golden Bridge Biotechnology, Beijing, China) were used to visualize the localization of the antigen according to the manufacturer’s instructions. Two experienced pathologists evaluated UBE2T immunostaining by examining three random fields of view using a light microscope at 400X magnification. The intensity of cellular staining was assigned a score of 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). The proportion of stained tumor cells was assigned a score of 0 (0–5%), 1 (6–25%), 2 (26–50%), 3 (51–75%) or 4 (> 75%). Finally, the total score was calculated by multiplication of the above two parts, and the median of the total score was used as a cutoff to distinguish low versus high levels of expression.

**Western blotting**

Total protein from renal cancer samples and cell lines was prepared with ice-cold radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck Millipore), quantified, and loaded into an SDS-PAGE gel. After electrophoresis, proteins in the gel were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). After blocking for 1 h with 5% nonfat milk, the membranes were incubated overnight at 4 °C with primary antibodies, followed by horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology). Signals were detected by chemiluminescence (ECL western blotting Detection Reagents, GE Healthcare) and visualized using a G:BOX Chemi Gel Documentation System (Frederick, MD, USA). Information on the primary antibodies included in this study is listed in Table S2.

**Lentivirus vector construction**

The sequence coding the human UBE2T gene (NM_014176) was obtained from GenBank. The lentivirus vector system for knockdown was composed of the vector pGCSIL-GFP, which stably expressed shRNA, pHelper1.0 and Helper2.0. The vectors pHelper1.0 and pHelper2.0 were vital elements for the virus package. The most effective double-stranded UBE2T-targeted shRNA sequence, PscSI14026 (5’-GTA CAC AAC TCA ACA CAG AAA-3’), was synthesized and cloned into the pGCSIL vector by GeneChem Corporation (Shanghai, China). Psc-NC (5’-TTC TCC GAA CGT GTC ACG T-3’) was used as the negative control lentiviral vector and had no homology to any known human genes. For overexpression of UBE2T, recombinant pGC-LV-GV287 vectors with UBE2T mRNA or with a scrambled control sequence were constructed by Genechem Corporation (Shanghai, China). Cancer cells were plated in six-well plates (5 × 10^4 cells/well) until cell fusion reached 60%, and then, appropriate volumes of lentivirus were added to the cells according to the MOI value (number of lentiviruses per number of cells) recommended by the manufacturer. The interference efficiency of gene expression was detected by RT-qPCR and western blotting analysis.

**Cell viability assay by MTS**

The metabolic activity of 786-O and Caki-1 cells was assessed using a CellTiter 96™ AQueous Nonradioactive Cell Proliferation Assay (Promega, WI, USA). The optical density of the wells was measured at 450 nm using a Multiscan microplate spectrophotometer (Thermo LabSystems, MA, USA).

**Cell cycle analysis**

Cells infected with UBE2T-shRNA lentivirus or NC lentivirus were collected, washed with ice-cold phosphate-buffered saline (PBS), and fixed with 70% ice-cold ethanol at 4 °C overnight. Subsequently, the samples were washed with PBS and stained for 30 min at room temperature in the dark with 50 g/ml propidium iodide (PI; Sigma-Aldrich, P4170) containing 125 U/ml RNase and then tested using a FACSCalibur flow cytometer (BD Biosciences, NJ, USA). The fluorescence signal was recorded with excitation at 488 nm. Cell cycle analysis was carried out using ModFit 2.0 software (Becton Dickinson).
Cell apoptosis analysis

Cell apoptosis was assayed by staining with Annexin V-FITC and PI (KeyGEN BioTECH) following the manufacturer’s instructions. Briefly, cells were collected by trypsinization without EDTA, and then washed twice with PBS. After cell suspension, Annexin V-FITC and propidium iodide were added at a 1:1 ratio and then incubated with the cells for 15 min at room temperature in the dark. FACS was performed to detect cell apoptosis within 1 h.

Wound healing assay and transwell assay

For the wound-healing assay, approximately 4 × 10^5 cells were seeded in six-well plates at equal densities and grown to 80 ~ 90% confluency. Then scratches were generated by a 1 mL sterile pipette tip. Wounded areas were marked and photographed using a microscope (Leica DMIL, Leica Microsystems, Germany) equipped with a digital camera (Leica DFC300FX). For the Transwell assay, 10,000 cells were plated into the upper chambers (24-well insert, pore size 8 μm, Corning Inc., NY, USA) coated with Matrigel (diluted 1:8 in PBS; product #354234; Corning, USA). The upper chambers contained 300 μL serum-free PRIM-1640 medium, while the lower chambers were filled with 700 μL PRIM-1640 medium containing 10% fetal bovine serum. After 16 h, the Transwells were washed with PBS, and cells under the surface of the lower chamber were stained with 0.5% crystal violet for 30 min.

Dual-luciferase reporter assay

Dual-luciferase reporter assays were conducted using a Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer’s instructions. Briefly, UBE2T-Wt and UBE2T-Mut were constructed via restriction endonuclease digestion and T4 DNA ligase using a psi-CHECK 2 plasmid (Promega, USA) and then, ccRCC cells were co-transfected with the constructs along with miR-182-5p mimic or miR-NC using Lipofectamine 3000 (Invitrogen, USA) and incubated for 48 h. Finally, luciferase activities were measured with a microplate reader using a luciferase assay kit (KeyGEN BioTECH, China).

Mouse model experiments

Animal experiments were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals with the approval of the Review Board of Peking University First Hospital (Beijing, China). Four-week-old male BALB/c nude mice were purchased from Vitalriver (Beijing, China). Approximately 5 × 10^6 786-O-UBE2T-sh /786-O-sh-NC or 786-O-UBE2T-OE /786-O-OE-NC cells suspended in 100 μL PBS were mixed with Matrigel (1:1, Product #354234, Corning Inc., NY, USA). Then 200 μL of the mixtures was injected subcutaneously into the right flank of 6-week-old BALB/c nude mice. Tumor size was measured every fourth day and calculated using the formula: (length × width^2)/2.

In silico analysis of UBE2T in ccRCC

Prognostic analyses were performed by gene expression profiling interactive analysis (GEPIA, http://gepia.cancer-pku.cn/) [14]. To explore the mechanism of UBE2T in ccRCC, we obtained the coexpression data of UBE2T genes in ccRCC from the cBioPortal website (https://www.cbioportal.org/). Pearson correlation coefficients were calculated to determine the coexpression relationships (|Pearson correlation coefficient| > 0.4 and p value < 0.05). Gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed using the R packages “org.Hs.eg.db”, “clusterProfiler” and “ggplot2”. Gene set enrichment analysis (GSEA) was conducted to assess whether a predefined set of genes showed statistically significant, concordant differences according to UBE2T expression in the TCGA-KIRC database using GSEA software (downloaded from http://software.broadinstitute.org/gsea/index.jsp) [15]. The high-expression group and the low-expression group were divided according to the median mRNA level of UBE2T expression. |Normalized Enrichment Score (NES)| > 1, false discovery rate (FDR) < 25% and nominal p value < 0.05 were regarded as the cut-off criteria. ENCORI (Encyclopedia of RNA Interactomes, http://starbase.sysu.edu.cn/) was used to predict the miRNAs that regulated UBE2T[16]. All sequence and annotation data of miRNAs were downloaded from miRBase (http://www.mirbase.org/) [17].

Statistical analysis

SPSS version 22.0 (SPSS, Chicago, IL, USA) was applied for statistical analyses. All values in the text and figures are expressed as the mean ± SD of the observations. The difference between two sets of data was analyzed using Student’s t test or Mann–Whitney U test. Pearson χ^2 or Fisher’s exact test was conducted to test the association between UBE2T mRNA expression and clinicopathological features. Cox regression was conducted for univariate and multivariate analyses. Statistical significance was considered at a p value < 0.05.

Results

UBE2T was upregulated in renal cancer tissues

To clarify the expression of UBE2T in ccRCC, we first analyzed UBE2T expression in tumor samples in TCGA
database. As shown in Fig. 1, the mRNA expression of UBE2T was higher in ccRCC tumor tissues than in normal renal tissues in TCGA database (Fig. 1a). Increased mRNA expression of UBE2T was associated with high-stage ccRCC (Fig. 1b, $p < 0.001$). Besides, UBE2T was upregulated in ccRCC gene microarray profiling data: GSE36895, GSE46699, GSE53000, GSE53757, GSE68417 and GSE76351 (Fig. S1a). Meta-analysis revealed that UBE2T mRNA expression was still obviously increased in the union dataset (Fig. S1b, $p < 0.01$). In addition, high expression of UBE2T was related to poor survival of ccRCC patients (Fig. 1c, $p < 0.001$). To verify the expression of UBE2T in RCC, we also performed RT-qPCR assays to explore the mRNA expression of UBE2T in RCC cell lines (OSRC-2, 786-O, 769-P, ACHN, A498 and Caki-1) as well as in 24 pairs of RCC samples. The results showed that UBE2T mRNA expression was higher in renal cancer cell lines than in the normal human renal cell line HK-2 (Fig. 1d) and was higher in renal cancer tissues than in tumor-adjacent renal tissues (Fig. 1e, $p < 0.001$). Furthermore, the protein level of UBE2T was also higher in renal cancer cell lines (Fig. 1f) and cancer tissues (Fig. 1g) than in paraneoplastic normal tissues.

**Fig. 1** The expression level of UBE2T in KIRC. **a** The mRNA level of UBE2T in TCGA clinical samples. **b** UBE2T expression among KIRC patients with different pathological stages. **c** The mRNA level of UBE2T was related to survival of KIRC patients. **d** The mRNA level of UBE2T in RCC cell lines. **e** The mRNA level of UBE2T in RCC tissues and adjacent normal tissues. **f** Protein expression of UBE2T in RCC cell lines. **g** The protein level of UBE2T in RCC tissues and adjacent normal tissues. All experiments were repeated three times independently ($n=3$). Data are reported as means±standard deviation. *$p<0.05$, **$p<0.01$, ***$p<0.001$
High UBE2T expression predicted poor prognosis in ccRCC

To explore the potential prognostic significance of UBE2T in ccRCC, we first evaluated the correlation between UBE2T protein expression and the clinicopathological features of 93 patients with ccRCC in the PKU-KIRC dataset. As shown in Table 1, UBE2T protein expression was related to the size of the tumor, pathological T stage, histologic grade and postoperative metastasis of ccRCC. Similar results were observed in TCGA database (Table S3). Advanced pathological T stage and histologic grade, large tumor size and postoperative metastasis were more common in the high UBE2T expression group than in the low UBE2T expression group (Fig. 2a–d). In addition, more patients with high UBE2T expression died than those with low UBE2T expression (Fig. 2e). Univariate analysis followed by a multivariate analysis defined UBE2T expression as an independent risk factor for OS (HR = 19.475, 95% CI 2.565, 147.873, \(p = 0.004\), Table 2) and RFS (HR = 4.044, 95% CI 1.295, 12.629, \(p = 0.016\), Table 3) of ccRCC patients in the PKU-KIRC dataset. Similar results were observed in TCGA database (Table S4 and Table S5). Moreover, higher UBE2T expression was related to shorter OS (\(p < 0.0001\)) and RFS (\(p < 0.001\)) in ccRCC patients in the PKU-KIRC dataset (Fig. 2f–g).

Knockdown of UBE2T attenuated the proliferation of renal cancer cells

To investigate the biological function of UBE2T in renal cancer, we performed lentivirus-mediated knockdown and overexpression of UBE2T in 786-O and Caki-1 cells. UBE2T mRNA and protein levels were subsequently measured via RT-qPCR and western blotting. shRNA infection dramatically decreased UBE2T gene expression in 786-O and Caki-1 cells, and ccRCC cell lines (786-O and Caki-1) stably overexpressing UBE2T were established (Fig. 3a, b). MTS assays were conducted to investigate the effects of UBE2T knockdown or overexpression on the proliferation of renal cancer cells. The results revealed that UBE2T knockdown dramatically inhibited the growth of 786-O and Caki-1 cells (Fig. 3c, d). The opposite results were observed after UBE2T overexpression in 786-O and Caki-1 cells (Fig. 3e, f).

| Features | UBE2T expression | Pearson × 2 | \(p\) |
|----------|------------------|-------------|------|
|          | Low High         |             |      |
| Age (years), no (%) |                |             |      |
| ≤ 60     | 28 (60.9) 26 (55.3) | 0.294       | 0.588 |
| > 60     | 18 (39.1) 21 (44.7) |             |      |
| Gender, no (%) |                |             |      |
| Male     | 33 (71.7) 30 (63.8) |             |      |
| Female   | 13 (28.3) 17 (36.2) | 0.666       | 0.415 |
| Laterality, no (%) |            |             |      |
| Left     | 18 (39.1) 28 (59.6) |             |      |
| Right    | 28 (60.9) 19 (40.4) | 3.887       | 0.059 |
| Size (longest dimension), no (%) | |             |      |
| ≤ 5 cm   | 38 (82.6) 18 (38.3) |             |      |
| > 5 cm   | 8 (17.4) 29 (61.7) | 19.053      | < 0.001 |
| Pathological-T, no (%) |            |             |      |
| T1       | 43 (93.5) 32 (68.1) |             |      |
| T2–T4    | 3 (6.5) 15 (31.9) | 9.604       | 0.002 |
| Histologic grade, no (%) |            |             |      |
| G1       | 11 (23.9) 8 (17.0) |             |      |
| G2       | 34 (73.9) 31 (66.0) |             |      |
| G3       | 1 (2.2) 8 (17.0) | 6.801       | 0.033 |
| Postoperative metastasis, no (%) | |             |      |
| No       | 42 (91.3) 35 (74.5) |             |      |
| Yes      | 4 (8.7) 12 (25.5) | 4.626       | 0.031 |

Bold values implies that the data are statistically different.
replication, Fanconi anemia pathway and other pathways related to replication and repair were enriched according to KEGG analysis (Fig. 5b). In addition, GSEA of UBE2T was performed by ranging the genes based on the expression of UBE2T in TCGA. As a result, high expression of UBE2T was found to be related to the cell cycle (Fig. 5c). Flow cytometry was performed to confirm whether downregulation of UBE2T has an effect on the cell cycle distribution.

Table 2 Univariate and multivariate Cox regression analyses of UBE2T level with overall survival in PKU-KIRC dataset

| Features          | Univariate COX                  | Multivariate COX            |
|-------------------|--------------------------------|------------------------------|
|                   | HR (95% CI)                    | p                            | HR (95% CI)                      | p        |
| Age (> 60 vs ≤ 60) | 3.312 (1.150, 9.539)           | 0.026                        | 2.941 (1.002, 8.632)             | 0.050    |
| Gender (male vs female) | 0.685 (0.221, 2.123)       | 0.512                        |                                 |          |
| Laterality (left vs right)     | 0.413 (0.145, 1.175)       | 0.097                        |                                 |          |
| Longest dimension (> 5 cm vs ≤ 5 cm) | 5.754 (1.853, 17.869)  | 0.002                        |                                 |          |
| Pathological-T (T2–T4 vs T1)   | 1.491 (0.480, 4.625)        | 0.490                        |                                 |          |
| Histologic grade (G3 vs G2 vs G1) | 2.480 (1.013, 6.072)   | 0.047                        |                                 |          |
| UBE2T level (high vs low)       | 20.778 (2.749, 157.063)  | 0.003                        | 19.475 (2.565, 147.873)          | 0.004    |

Bold values implies that the data are statistically different.
MiR-182-5p inhibits the tumorigenesis of clear cell renal cell carcinoma by repressing UBE2T

Table 3 Univariate and multivariate Cox regression analyses of UBE2T level with recurrence-free survival in PKU-KIRC dataset

| Features | Univariate COX | Multivariate COX |
|----------|----------------|------------------|
|          | HR (95% CI)    | HR (95% CI)      |
| Age (> 60 vs ≤ 60) | 2.272 (0.928, 5.563) | 2.794 (1.119, 6.976) | 0.028 |
| Gender (male vs female) | 0.673 (0.244, 1.851) | 0.443 |
| Laterality (left vs right) | 0.605 (0.250, 1.463) | 0.265 |
| Longest dimension (> 5 cm vs ≤ 5 cm) | 4.637 (1.779, 12.084) | **0.002** |
| Pathological-T (T2–T4 vs T1) | 2.021 (0.776, 5.265) | 0.150 |
| Histologic grade (G3 vs G2 vs G1) | 2.842 (1.262, 6.401) | **0.012** |
| UBE2T level (high vs low) | 5.547 (1.860, 16.542) | **0.002** |

Bold values implies that the data are statistically different

Knockdown of UBE2T-induced apoptosis of renal cancer cells

Flow cytometry was used to investigate the effect of UBE2T on apoptosis of 786-O cells infected with sh-NC lentivirus or UBE2T-shRNA lentivirus. As shown in Fig. 5h, the percentage of cells undergoing apoptosis was significantly increased in the UBE2T-sh group compared with the sh-NC group (9.22 ± 0.26 vs. 2.52 ± 0.08, p < 0.01). Furthermore, the expression of the pro-apoptotic factor Bax was increased and that of the anti-apoptotic factor Bcl-2 was decreased in the UBE2T-shRNA group compared with the sh-NC group. Oppositely, low expression of Bax and high expression of Bcl-2 was also observed in UBE2T-overexpression group (Fig. 5i). This indicates that knockdown of UBE2T may abrogate malignant phenotypes of ccRCC by inducing apoptosis of renal cancer cells.
Suppression of UBE2T inhibited the growth of human RCC cells in vivo

Next, we performed in vivo studies to confirm the effects of UBE2T on tumor formation and growth. We injected 786-O/UBE2T-sh and 786-O/UBE2T-OE cells into nude mice to generate the UBE2T-sh and UBE2T-OE groups. As shown in Fig. 6a, tumor growth was delayed in the UBE2T-sh group compared with the sh-NC group. Mice were killed on the 56th day after injecting 786-O/UBE2T-sh cells, and the tumors were collected. Tumors from the sh-NC group were larger and heavier than those from the UBE2T-sh group (p < 0.01, Fig. 6b, c). The results were opposite in the UBE2T-OE groups (Fig. 6d–f). Additionally, based on IHC, the protein expression of PCNA and Bcl-2 in dissected tumor samples was markedly decreased in the UBE2T-sh group compared to the sh-NC group (Fig. 6g, h). Together, these results indicate that suppression of UBET2 attenuated the growth of ccRCC cells, and consequently inhibited tumor formation and proliferation in vivo.

UBE2T is a functional target of miR-182-5p in ccRCC

To identify the upstream regulator of UBE2T, ENCORI (Encyclopedia of RNA Interactomes, http://starbase.sysu.edu.cn/) was employed to predict the regulatory miRNAs.
MiR-182-5p inhibits the tumorigenesis of clear cell renal cell carcinoma by repressing UBE2T

Then, 14 miRNAs were found to interact with UBE2T (Table S6). Among these miRNAs, overexpression of miR-182-5p was determined previously to inhibit cell apoptosis and to lead to G2-M-phase cell-cycle arrest [13]. Therefore, it was speculated that miR-182-5p might affect the development of ccRCC by targeting UBE2T. Sequence analysis revealed that miR-182-5p could target the UBE2T gene (Fig. 7a). A dual-luciferase reporter gene assay was used to verify the binding relationship between miR-182-5p and UBE2T. The luciferase activity in renal cancer cells (786-O and OSRC-2) co-transfected with UBE2T-wild-type (WT) and miR-182-5p mimic was significantly decreased ($p < 0.0001$), while there was no difference in cells co-transfected with UBE2T-mutation (Mut) and miR-182-5p mimic (Fig. 7b). Next, to further understand the effect of miR-182-5p on UBE2T expression, renal cancer cells (786-O and OSRC-2) were transfected with miR-182-5p mimic and miR-NC. Results from RT-qPCR and western blotting analysis indicated that the expression of UBE2T in the miR-182-5p mimic group was downregulated compared with that in the miR-NC group (Fig. 7c, d). These results suggest that UBE2T is a functional target of miR-182-5p in the tumorigenesis of ccRCC.
ccRCC and that overexpression of miR-182-5p can inhibit the expression of UBE2T.

**The inhibition of ccRCC malignant phenotypes by miR-182-5p was dependent on UBE2T**

To further elucidate the effect of miR-182-5p on the malignant phenotype of ccRCC, we first overexpressed miR-182-5p in 786-O-NC and 786-O-UBE2T-NC cells, and observed its effects on the proliferation, migration and invasion of ccRCC cells. We used miR-182-5p mimic and miR-NC plasmid to transfect 786-O-UBE2T-NC cells, respectively. The results showed that the migration ($p<0.01$, Fig. 8a), proliferation ($p<0.05$, Fig. 8c) and invasion abilities ($p<0.01$, Fig. 8d) of 786-O-UBE2T-NC cells were significantly decreased after transfection with miR-182-5p mimic. After overexpression of miR-182-5p in 786-O-UBE2T-NC cells, the migration (Fig. 8b), proliferation (Fig. 8c) and invasion (Fig. 8d) abilities did not change, indicating that miR-182-5p depended on UBE2T to inhibit the proliferation, migration and invasion of ccRCC cells.

Next, we explored whether the miR-182-5p-mediated cell proliferation and cell apoptosis changes contributed to malignant transformation. As shown in Fig. 8e, miR-182-5p mimic inhibited the expression of PCNA and Bcl-2 while promoting the expression of Bax in ccRCC.
**Fig. 7** UBE2T was a target of miR-182-5p in ccRCC. 

- **a** The predictive binding site between UBE2T 3'UTR and miR-182-5p. 
- **b** UBE2T Wt and miR-182-5p mimic co-transfection significantly inhibited luciferase activity, and UBE2T Mut and miR-182-5p mimic co-transfection did not affect luciferase activity in renal cancer cell. 
- **c** The mRNA expression of UBE2T after transfecting miR-182-5p mimic in renal cancer cell. 
- **d** The protein expression of UBE2T after transfecting miR-182-5p mimic in renal cancer cell. All experiments were repeated three times independently (n = 3). Data are reported as means ± standard deviation. *p < 0.05, ****p < 0.0001

**Fig. 8** The inhibition of ccRCC malignant phenotypes by miR-182-5p was dependent on UBE2T. 

- **a** The wound healing assay was performed after transfecting miR-182-5p mimic in 786-O-OE-NC cells. 
- **b** The wound healing assay was performed after transfecting miR-182-5p mimic in 786-O-UBE2T-OE cells. 
- **c** The proliferative ability of 786-O cells was evaluated by MTS assay in the presence of miR-182-5p mimic or UBE2T overexpression. 
- **d** The invasive ability of 786-O cells was evaluated by transwell assay in the presence of miR-182-5p mimic or UBE2T overexpression (10×). 
- **e** The protein expression of PCNA, Bax and Bcl-2 after transfecting miR-182-5p mimic in renal cancer cell. Scale bar, 200 μm. All experiments were repeated three times independently (n = 3). Data are reported as means ± standard deviation. *p < 0.05, **p < 0.01
Discussion

RCC is one of the top ten most frequently diagnosed cancers in the world, and it remains an intractable social and medical problem [2]. The treatment for advanced RCC has evolved significantly in the past few years with the approval of immune checkpoint-blocking antibodies. However, the prognosis of most advanced RCC patients remains poor due to intrinsic or adaptive resistance [18]. As the most common pathological type of RCC, it is desperately necessary to find a new therapeutic strategy for more effective ccRCC treatment. The ubiquitin–proteasome system (UPS) is responsible for the degradation of most intracellular proteins and plays an important role in regulation of cell proliferation and apoptosis, cell cycle progression and DNA repair. Recent studies have revealed that its dysregulation is associated with tumor progression, and some proteasome inhibitors are currently under preclinical and clinical investigation for solid tumors [19, 20]. Moreover, targeting the UPS can restore the sensitivity of cancer cells to conventional chemotherapeutic drugs [21, 22]. The UPS consists of ubiquitin, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin-protein ligase (E3), the proteasome and its target proteins [23]. Most studies have focused on mutations in or overexpression of numerous E3 ubiquitin ligases that result in tumor suppression, but few have examined the effects of E2 enzymes, such as UBE2T.

UBE2T is related to the repair of damaged DNA by interacting with the E3 ligase FANCL in the Fanconi anemia pathway [7]. As a novel therapeutic target, UBE2T has been found to be overexpressed in various cancer types, such as breast cancer, bladder cancer and liver cancer. Ueki et al. [24] proved that UBE2T is overexpressed in breast cancer cells and related to the development of breast cancer by interacting with and regulating the BRCA1/BARD1 complex. Besides, UBE2T silencing suppresses proliferation in bladder cancer cells and induces cell cycle arrest and apoptosis [25]. Inhibiting the UBE2T-dependent Akt-signaling pathway can suppress the self-renewal and tumorigenicity of liver cancer stem cells [26]. E2 largely relies on E3 ligases to target binding substrates. Sun et al. [27] identified the E3 ligase RNF8, which binds to UBE2T. When exposed to radiation, UBE2T/RNF8 regulated H2AX monoubiquitination and promoted phosphorylation of CHK1 for activation, which induced radiosensitivity in hepatocellular carcinoma. Hypoxia promotes more aggressive and metastatic tumor phenotypes and is related to poor patient prognosis by regulating the tumor microenvironment [28]. It was reported that hypoxia can rapidly downregulate UBE2T expression, which was correlated with an increased sensitivity to crosslinking agents, consistent with a defective Fanconi anemia pathway [29].

In this study, we first examined UBE2T expression in ccRCC and paracarcinoma tissues. UBE2T expression was increased in ccRCC compared with paracarcinoma tissues. Similar results were obtained using samples from TCGA and GEO databases. Next, we examined whether shRNA-mediated suppression of UBE2T expression in 786-O and Caki-1 cells inhibited renal cancer progression. After confirming that shRNA successfully suppressed UBE2T expression, we measured cell growth using MTS assays. The results indicated that suppression of UBE2T inhibited ccRCC cell proliferation. Next, healing and Transwell assay results indicated that suppression of UBE2T attenuated cell migration and invasion. UBE2T has been confirmed to promote a malignant phenotype by influencing the cell cycle distribution [9, 30]. To explore the possible mechanisms underlying the specific action of UBE2T in ccRCC, silico analysis was performed using the coexpression gene of UBE2T. Pathway enrichment indicated that UBE2T may cause cell cycle deregulation and cancer development by acting on DNA replication and repair. In our study, FCM results also revealed that suppression of UBE2T increased G2/M cell cycle arrest and induced apoptosis in renal cancer cells.

We next investigated the effect of UBE2T on tumor growth in vivo. We found that gross tumor volumes and weights were lower in 786-O sh-UBE2T mice than in the sh-NC group mice, suggesting that UBE2T knockdown also attenuated renal cancer formation and growth in vivo.

Functional studies have confirmed that the dysregulation of miRNA expression is the cause of some cancers. miRNAs, as tumor suppressor genes or oncogenes, affect the occurrence and development of tumors. Currently, miRNA mimics and miRNA-targeting molecules (AntimiRs) have shown promise in preclinical development [31]. Previous studies have found that miR-1305 inhibits the UBE2T-mediated AKT signaling pathway by directly targeting UBE2T, thereby inhibiting the stemness and tumorigenesis of hepatocellular carcinoma stem cells [26]. However, whether there are miRNAs in ccRCC that regulate UBE2T, which in turn affects the occurrence and development of tumors, has not yet been reported. Recent studies have revealed that miR-182-5p plays an important role in tumorigenesis. For example, miR-182-5p overexpression repressed colon cancer tumorigenesis and triggered G1 arrest and apoptosis [32]. LncRNA SNHG10 can maintain activation of the Wnt/β-catenin signaling pathway in osteosarcoma cells by sponging miR-182-5p [33]. In addition, decreased expression of miR-182-5p increased cell proliferation and cisplatin resistance of lung adenocarcinoma cells via targeting GLI2 [34]. Most of the potential targets of miR-182-5p are involved in DNA repair [35]. Our study demonstrated that UBE2T is also a functional target of miR-182-5p in ccRCC and that miR-182-5p is dependent on UBE2T to inhibit the proliferation, migration and invasion of ccRCC cells.
In summary, we confirmed that UBE2T is highly expressed in ccRCC. Knockdown of UBE2T with lentivirus-mediated specific shRNA significantly suppressed the proliferation of renal cancer cells, and induced cell cycle arrest and apoptosis. Upregulation of miR-182-5p targeted UBE2T and impaired the overall growth of ccRCC. However, mechanisms of UBE2T in regulating renal cancer tumorigenesis and progression, and the potential relationship between UBE2T and downstream molecules, such as BRCA1 and other factors of Fanconi anemia pathway would also need further studies. These studies will undoubtedly provide new insights into the role of UBE2T both cell cycle regulation and tumorigenesis that may aid in renal cancer.

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Author contributions LZ, XL, YG and SH contributed to the experimental design and analysed the data. YW, CZ and DP implemented the experiments, drafted the manuscript and analysed the data. CH, JQ, WZ and NF collected the clinical samples and performed the statistical analysis. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethics approval This study was approved by the Medical Ethics Committee of Peking University First Hospital (Approval No. 2015[977]). All animal experiments were followed by the Guide for the Care and Use of Laboratory Animals and approved by the Ethical Committee of Peking University First Hospital.

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