MiR-182-5p Inhibits the Tumorigenesis of clear cell renal cell carcinoma by Repressing UBE2T

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Primary research

Keywords: clear cell renal cell carcinoma, UBE2T, miR-182-5p, Apoptosis, Migration and invasion

Posted Date: April 12th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-353835/v1>

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Version of Record: A version of this preprint was published at Human Cell on February 7th, 2022. See the published version at <https://doi.org/10.1007/s13577-021-00661-6>.

Abstract

Background: 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 already been clarified. MicroRNAs (miRNAs) participate in tumorigenesis by binding to genes and proteins that regulate cell proliferation or cell apoptosis. The aim of this research was to determine the role of UBE2T and the relationship between miR-182-5p and UBE2T in ccRCC.

Methods: UBE2T expression levels were assessed by real-time quantitative PCR (RT-qPCR) and western blot in ccRCC tissues and cells. Protein expression of UBE2T was assessed in a total of 93 ccRCC patients from Peking University First Hospital (PKU) by immunohistochemistry (IHC). The effects of UBE2T knockdown on ccRCC cells were assessed by 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 by dual luciferase reporter gene assay.

Results: UBE2T was highly expressed in ccRCC cells and tissues. High expression of UBE2T was positively correlated with advanced pathological stage, histological grade, maximum diameter and distant metastasis of the tumor. Multivariate analysis revealed that the 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 proliferation, migration and invasion of RCC. Flow cytometry analysis showed that knocking down UBE2T could promote RCC cell cycle arrest at G2/M phase and increase cell apoptosis. A xenograft model also confirmed that suppression of UBE2T significantly delayed tumor formation and growth in vivo. In addition, miR-182-5p inhibited the expression of UBE2T protein through targeting UBE2T mRNA, and then inhibited the proliferation, migration and invasion of ccRCC.

Conclusions: Our research reveals that UBE2T probably play a critical role in ccRCC progression, and it may be a potential therapeutic target for ccRCC treatment.

Introduction

Renal cell carcinoma (RCC) accounts for 90% of renal cancer cases, and is the most common carcinoma of the adult kidney[1]. Rate of RCC has increased by 1.6% per year for the past 10 years, making it the 6 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, about 20%-30% of RCC patients are diagnosed with metastatic disease, and for those with localized RCC, nearly 20% show recurrence and develop metastatic RCC after nephrectomy[3]. The main histologic type of RCC is ccRCC, accounting for 80–90% of cases[4, 5].

Therefore, further research into the underlying molecular mechanisms of tumorigenesis of ccRCC and the development of novel treatments aimed at specific molecular targets are required.

UBE2T (also known as HSPC150) is a member of E2 the 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 at1q32.1, has been reported in certain cancer types and overexpression of UBE2T could promote mammary carcinogenesis[8, 9]. However, little is known with regard to the association between UBE2T and renal cancer. MiRNAs are a class of endogenous small RNA with a length of approximately 22 nt. Functional studies have confirmed that miRNAs are involved in development and diseases by targeting mRNAs for cleavage or translational repression[10]. Specifically, the role of miR-182-5p in the modulation of malignant phenotypes of renal cancer cells has already been investigated[11, 12]. Furthermore, overexpression of miR-182-5p could inhibit renal cancer cell proliferation, colony formation, 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 of ccRCC cells were assessed. Then we aimed to elucidate the dominant role of miR-182-5p and UBE2T in ccRCC carcinogenesis. These 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 the Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>). TCGA-KIRC transcriptome and clinical dataset were downloaded from UCSC Xena (<http://xena.ucsc.edu/>). R software was applied to process the RNA sequencing data.

Tissue samples

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

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 conditions specified by the provider. All media contained 10% fetal bovine serum (GIBCO), penicillin G (100 U/ml), and streptomycin (100 µg/ml) (Sigma-Aldrich). The cell cultures 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 the transfected cells using the 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 Fluorescent Quantitative PCR System (Applied Biosystems, CA, USA), according to the manufacturer's instructions, and normalized to β-actin. All experiments were repeated at least three times. Information on sequence of PCR primers was listed in Table S1.

Immunohistochemistry

After fixed 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. 10% normal goat serum was applied to reduce nonspecific binding. Subsequently, the slides were incubated with primary rabbit anti-human UBE2T polyclonal antibody at 4°C overnight, and the PowerVision™ two-step histostaining reagent and the 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) and 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%). At last, 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 onto SDS-PAGE. After electrophoresis, proteins in the gel were transferred to polyvinylidene difluoride membranes (PVDF, Millipore, USA). After blocked for 1 h with 5% nonfat milk, the membranes were incubated overnight at 4°C with primary antibodies, followed with horseradish peroxidase-labeled secondary antibody (sc-2004/sc-2005, Santa Cruz Biotechnology). Signals were detected by chemiluminescence (ECL Western Blotting Detection Reagents, GE Healthcare) and visualized using

G:BOX Chemi Gel Documentation System (Frederick, MD, USA). Information on primary antibodies included in this study was listed in Table S2.

Lentivirus Vector Construction

The sequence coding human UBE2T gene (NM_014176) was obtained from GenBank. The lentivirus vector system for knock down was composed of the vectors pGCSIL-GFP which stably expressed shRNA, pHelper1.0 and Helper2.0. The vectors pHelper1.0 and pHelper2.0 were vital elements for virus package. The most effective double-stranded UBE2T-targeted shRNA sequences, PscSI14026 (5'-GTACACAACCTCAACACAGAAA-3'), were synthesized and cloned into the pGCSIL vector by GeneChem Corporation (Shanghai, China). Psc-NC (5'-TTCTCCGAACGTGTCACGT-3') was used as the negative control lentiviral vectors and had no homology to any known human genes. For overexpression of UBE2T, recombinant pGC-LV-GV287 vectors with the UBE2T mRNA or with a scrambled control sequence were constructed by the 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 manufacture. The interference efficiency of gene expression was detected by RT-qPCR and western blot analysis.

Cell viability assay by MTS

The metabolic activity of 786-O and Caki-1 cells was assessed using 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 FACSCalibur flow cytometer (BD Biosciences, NJ, USA). 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 manufacturer's instructions. Briefly, cells were collected by trypsinization without EDTA, then washed twice with PBS. After cell suspension, Annexin V-FITC and propidium iodide were added at the 1:1 ratio, and then incubated for 15 min at room temperature in the dark. The FACS was performed to detect the cell apoptosis within 1 hour.

Wound healing assay and transwell assay

For the wound-healing assay, approximately 4×10^5 cells were seeded in 6-well plates at equal densities and grown to 80%~ 90% confluency. Then scratches were generated by the 1 mL sterile pipette tip. Wounded areas were marked and photographed with a microscope (Leica DMIL, Leica Microsystems, Germany) equipped with a digital camera (Leica DFC300FX). For the transwell assay, 10000 cells were plated into the upper chambers (24-well insert, pore size 8 μ m, Corning) coated with Matrigel (diluted 1:8 in PBS, product #354234, Corning, USA). The upper chambers were 300 μ L serum-free PRIM-1640 while the lower chambers were filled with 700 μ L PRIM-1640 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/Mut were constructed using the psi-CHECK 2 plasmid (Promega, USA) by restriction endonuclease digestion and T4 DNA ligase, then they were co-transfected with miR-182-5p mimics/miR-NC into ccRCC cells using Lipofectamine 3000 (Invitrogen, USA) and incubated for 48h. Finally, the luciferase activities were measured on a microplate reader by 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). The 4-weeks-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 μ L PBS were mixed with Matrigel (1:1, Product #354234, Corning Inc., NY, USA). Then 200 μ L mixtures were injected subcutaneously into the right flank of 6-week BALB/c nude mice. Tumor size was measured every fourth day and calculated using the formula: (length $\times$ width²)/2.

In silico analysis of UBE2T in ccRCC

The 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 co-expression genes of UBE2T in ccRCC from website cBioPortal (<https://www.cbioportal.org/>). The Pearson correlation coefficients was calculated to determine the co-expression relationships (|Pearson correlation coefficient|>0.40 and p-value < 0.05). Gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed by R package "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 the UBE2T expression in TCGA-KIRC database by GSEA software (downloaded from <http://software.broadinstitute.org/gsea/index.jsp>) [15].

The high expression group and the low expression group were divided corresponding to the median mRNA level of UBE2T. $|\text{Normalized Enrichment Score (NES)}| > 1$, false discovery rate (FDR) $< 25\%$ and nominal p-value < 0.05 were regarded as the cut-off criteria. ENCORI (The 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 was download 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 $\pm$ SD of these observations. The difference between two sets of data was analyzed by Student's t-test or Mann-Whitney U test. Pearson χ^2 or Fisher exact test was conducted to test the association between UBE2T mRNA expression and clinicopathological features. Cox regression was conducted for univariate and multivariate analysis. Statistical significance was considered at p-value < 0.05 .

Results

UBE2T was up-regulated in renal cancer tissues

In order to clarify the expression of UBE2T in ccRCC, we first analyzed UBE2T expression in the tumor samples in TCGA database. As shown in the Fig. 1, UBE2T expression was higher in ccRCC tumor tissues than in normal renal tissues in TCGA database (Fig. 1a). Increased UBE2T expression was associated with high-stage ccRCC (Fig. 1b). Besides, UBE2T was up-regulated 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, $p < 0.01$). To verify the expression of UBE2T in renal cancer tissues, we also performed RT-qPCR assay to explore the mRNA expression of UBE2T in renal cancer cell lines (OSRC-2, 786-O, 769-P, ACHN, A498 and Caki-1) as well as 24 pairs renal cancer samples. The results showed that UBE2T mRNA expression was higher in renal cancer cell lines compared with normal human renal cell line HK-2 (Fig. 1d) and expressed higher in renal cancer tissues compared with tumor-adjacent renal tissues (Fig. 1e). Furthermore, the protein level of UBE2T was also higher in renal cancer cell lines (Fig. 1f) and cancer tissues (Fig. 1g) compared with the paraneoplastic normal tissues.

High expression of UBE2T predicted poor prognosis of ccRCC.

To explore the potential prognostic significance of UBE2T in ccRCC, we first evaluated analyzed the correlation between UBE2T protein expression and clinicopathological features of 93 patients with ccRCC in PKU-KIRC dataset. As shown in Table 1, UBE2T protein expression was related to the size of 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 patients with high UBE2T expression group

than those with low UBE2T expression group (Fig. 2a-2d). In addition, more patients with high UBE2T expression died compared to the low UBE2T expression group (Fig. 2e). Univariate analysis followed by a multivariate analysis defined that the expression of UBE2T was an independent risk factor for OS and RFS of ccRCC in PKU-KIRC dataset (Table 2 and Table 3). Similar results were observed in TCGA database (Table S4 and Table S5). Moreover, higher UBE2T expression was related to ccRCC patients' shorter OS ($p < 0.0001$) and RFS ($p < 0.001$) in PKU-KIRC dataset (Fig. 2f-2g).

Table 1
Correlation between UBE2T expression and clinicopathological features of

Features	UBE2T expression		Pearson x2	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)	0.666	0.415
Female	13(28.3)	17(36.2)		
Laterality, no (%)				
Left	18(39.1)	28(59.6)	3.887	0.059
Right	28(60.9)	19(40.4)		
Size (longest dimension), no (%)				
≤ 5cm	38(82.6)	18(38.3)	19.053	< 0.001
> 5cm	8(17.4)	29(61.7)		
Pathological-T, no (%)				
T1	43(93.5)	32(68.1)	9.604	0.002
T2-T4	3(6.5)	15(31.9)		
Histologic grade, no (%)				
G1	11(23.9)	8(17.0)	6.801	0.033
G2	34(73.9)	31 (66.0)		
G3	1(2.2)	8(17.0)		
Postoperative metastasis, no (%)				
No	42(91.3)	35(74.5)	4.626	0.031
Yes	4(8.7)	12(25.5)		
clinicopathological features of 93 ccRCC patients in PKU-KIRC dataset.				

Table 2

Univariate and multivariate Cox regression analysis 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 ($> 5\text{cm}$ vs $\leq 5\text{cm}$)	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

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

Features	Univariate COX		Multivariate COX	
	HR (95% CI)	P	HR (95% CI)	P
Age (> 60 vs ≤ 60)	2.272(0.928, 5.563)	0.072	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 (> 5cm vs ≤ 5cm)	4.637(1.779,12.084)	0.002	4.158(1.481,11.669)	0.007
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	4.044(1.295,12.629)	0.016

Knockdown of UBE2T attenuated the proliferation of renal cancer cells.

In order to investigate the biological function of UBE2T in renal cancer, we did Lentivirus-mediated knock-down and over-expression of UBE2T in 786-O and Caki-1 cells. UBE2T mRNA and protein levels were subsequently measured by RT-qPCR and western blot. 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–3b). MTS assays were conducted to investigate the effects of UBE2T knockdown or overexpression on proliferation of renal cancer cells. The results revealed that UBE2T knockdown dramatically inhibited the growth of 786-O and Caki-1 cells (Fig. 3c–3d). The opposite results were observed after overexpressing UBE2T in 786-O and Caki-1 cells (Fig. 3e–3f).

UBE2T knockdown significantly decreased the cell migratory and invasive ability of renal cancer cells

Migration and invasion of tumor cells lead to the formation of secondary tumors in other tissues and organs and are the main cause of cancer-related mortality and morbidity. We next examined the effects of UBE2T on the migratory and invasive ability of renal cancer cells. As shown in Fig. 4a-4b, the migratory distances of the 786-O and Caki-1 UBE2T-sh group were significantly inhibited after 16 h ($p < 0.05$). However, UBE2T OE significantly increased 786-O and Caki-1 cell migration than their counterparts

(Fig. 4c-4d). Cell invasion was also strongly inhibited in 786-O and Caki-1UBE2T-sh group compared to the sh-NC groups ($p < 0.05$, Fig. 4e-4f). On the contrary, cell invasion was strongly promoted in 786-O and Caki-1UBE2T-OE group (Fig. 4g-4h). Suppression of UBE2T attenuated migration and invasion of renal cancer cells in wound healing and transwell assays.

Knockdown of UBE2T induced cell cycle arrest in renal cancer cells.

Cell proliferative alteration is usually caused by changes in the cell cycle. In order to explore how the UBE2T affected the malignant phenotypes of ccRCC, enrichment analysis was performed using the co-expression gene of UBE2T in TCGA. GO terms including organelle fission, nuclear division, DNA replication and relevant member terms in cell cycle were enriched (Fig. 5a). Genes involved in cell cycle, DNA replication, Fanconi anemia pathway and other pathways related to replication and repair were enriched by KEGG analysis (Fig. 5b). In addition, GSEA in UBE2T was performed by ranging the gene based on expression of UBE2T in TCGA. As a result, high expression of UBE2T was related to the cell cycle (Fig. 5c). Flow cytometry was performed to confirm whether the down-regulation of UBE2T has an effect on the cell cycle of renal cancer cells, and UBE2T-sh group displayed a significant increase in G2/M phase in the percentage of 786-O cells compared with the sh-NC group (33.77 ± 0.40 vs 19.76 ± 1.24 , $p < 0.001$), suggesting that cells were arrested in G2/M phase after UBE2T gene silencing and UBE2T gene was significantly correlated with cell cycle of renal cancer cells (Fig. 5d-5f). PCNA could be used as an indicator to evaluate cell proliferation status. The results of western blot revealed that PCNA was down-regulated in the UBE2T-sh group (Fig. 5g).

Knockdown of UBE2T induces 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 sh-NC group (9.22 ± 0.26 vs. 2.52 ± 0.08 , $p = 0.001$). Furthermore, the expression of pro-apoptotic factor Bax was increased and anti-apoptotic factor Bcl-2 was decreased in the UBE2T-shRNA group compared with sh-NC group. Oppositely, high expression of Bax and low expression of Bcl-2 was also observed in UBE2T-overexpression group (Fig. 5i). This indicated 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 group. As shown in Fig. 6a, tumors growth was delayed in the UBE2T-sh group compared with the sh-NC group. Mice were sacrificed on the 56th day after injection 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-6c). The results were opposite in the UBE2T-OE groups (Fig. 6d-6f). Additionally, the protein expression of PCNA and Bcl-2 in dissected tumor samples was markedly decreased in UBE2T-sh group compared to the

sh-NC group by IHC (Fig. 6g-6h). Together, these results indicated that suppression of UBE2T inhibited the growth of ccRCC cells, and consequently inhibited tumor formation and proliferation in vivo.

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

To identify the upstream regulator of UBE2T, ENCORI (The Encyclopedia of RNA Interactomes, <http://starbase.sysu.edu.cn/>) was employed to predict the regulative miRNA of UBE2T[16]. 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 led to G2-M-phase cell-cycle arrest[13]. Thereby, 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). Dual-luciferase reporter gene assay was used to verify the binding relationship between miR-182-5p and UBE2T. The luciferase activity in cells co-transfected with UBE2T-wild-type (Wt) and miR-182-5p mimics decreased significantly ($p < 0.0001$), while there was no difference in cells co-transfected with UBE2T-mutation (Mut) and miR-182-5p mimics (Fig. 7b). Next, in order 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 mimics and miR-NC. Results from RT-qPCR and western blot analysis indicated that the expression of UBE2T in miR-182-5p mimics group was down-regulated compared with that in miR-NC group (Fig. 7c-7d). The results suggested that UBE2T was a functional target of miR-182-5p in ccRCC and overexpression of miR-182-5p could 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-OE-NC and 786-O-UBE2T-OE cells, and observed its effects on the proliferation, migration and invasion of ccRCC. We used miR-182-5p mimics and miR-NC plasmids to transfect 786-O-OE-NC and 786-O-UBE2T-OE cells, respectively. The results showed that the migration (Fig. 8a), proliferation (Fig. 8c) and invasion abilities (Fig. 8d) of 786-O-OE-NC cells were significantly decreased after transfection of miR-182-5p mimics. After overexpression of miR-182-5p in 786-O-UBE2T-OE cells, the migration (Fig. 8b), proliferation (Fig. 8c) and invasive (Fig. 8d) abilities did not change, indicating that miR-182-5p depended on UBE2T to inhibit the proliferation, migration and invasion of ccRCC.

Next, we explored whether the miR-182-5p-mediated cell proliferation and cell apoptosis changes contributed to malignant transformation. As showed in Fig. 8e, miR-182-5p mimics could inhibit the expression of PCNA and Bcl-2, while promoted the expression of Bax in ccRCC.

Discussion

RCC is one of the top ten most frequently diagnosed cancers in the world, and it remains an intractable social and medicine problem[2]. The treatment for advanced RCC evolved significantly in the past few years with the approval of immune checkpoint-blocking antibodies. However, the prognosis of most

advanced RCC still 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 a 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, apoptosis, cell cycle and DNA repair. Recent studies have revealed that its dysregulation was associated with tumor progression and some proteasome inhibitors were 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]. UPS consists of ubiquitin, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin-protein ligase (E3), proteasome and its target protein[23]. Most studies have focused on mutations in or overexpression of numerous E3 ubiquitin ligases that result in tumor suppression, while 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 cancers such as breast cancer, bladder cancer and liver cancer. Ueki et al [24] proved that UBE2T was 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 cell[26]. Hypoxia promoted malignancy and poor patient prognosis by regulating tumor microenvironment[27]. It was reported that hypoxia could rapidly down-regulate UBE2T expression which correlated with an increased sensitivity to crosslinking agents consistent with a defective Fanconi anemia pathway[28].

In this study, we first examined UBE2T expression in ccRCC and para-carcinoma tissues. UBE2T expression was increased in ccRCC compared with paracarcinoma tissues. Similar results were obtained using samples from TCGA and GEO database. 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. These results indicated that suppression of UBE2T inhibited cell proliferation in ccRCC cells. Next, wound 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 distribution of cell cycle[9, 29]. In order to explore the possible mechanisms regarding specific action of UBE2T in ccRCC, *in silico* analysis was performed using the co-expression 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, suggesting that UBE2T knockdown also attenuated renal cancer formation and growth *in vivo*.

Recent studies have revealed that miR-182-5p played an important role in tumorigenesis. For example, miR-182-5p overexpression repressed colon cancer tumorigenesis, and triggered G1 arrest and apoptosis[30]. LncRNA SNHG10 can maintain the activation of Wnt/ β -catenin signaling pathway in osteosarcoma cells by sponging miR-182-5p[31]. In addition, decreased expression of miR-182-5p increased the cell proliferation and cisplatin resistance of lung adenocarcinoma cells via targeting GLI2[32]. Most of the potential targets of miR-182-5p involved in DNA repair[33]. Our study also demonstrated that UBE2T was a functional target of miR-182-5p in ccRCC, and miR-182-5p depended on UBE2T to inhibit the proliferation, migration and invasion of ccRCC.

In summary, we confirmed UBE2T was high 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-1305 could target UBE2T and impair overall growth of ccRCC. However, mechanisms of UBE2T in regulating renal cancer tumorigenesis and progression, and the potential relationship between UBE2T and downstream molecules for example BRCA1 and Fanconi anemia pathway factors would also need further studies. Those studies will undoubtedly provide new insights for renal cancer therapy regarding the role of UBE2T in both cell cycle regulation and tumorigenesis.

Abbreviations

ccRCC, clear cell renal cell carcinoma; GEO, Gene Expression Omnibus; IHC, Immunohistochemistry; KEGG, Kyoto encyclopedia of Genes and Genomes; KIRC, Kidney renal clear cell carcinoma; MiRNA, MicroRNA; OS, Overall survival; PCNA, Proliferating cell nuclear antigen; RCC, Renal cell carcinoma; RFS, Recurrence-free survival; RT-PCR, Reverse Transcription- Polymerase Chain Reaction; RT-qPCR, Real-Time quantitative Polymerase Chain Reaction; TCGA, The Cancer Genome Atlas; UBE2T, Ubiquitin-conjugating enzyme E2T; UPS, Ubiquitin Proteasome System.

Declarations

Acknowledgements

Not applicable.

Authors'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 and WZ collected the clinical samples and performed the statistical analysis. All authors read and approved the final manuscript.

Funding

The present study was funded by the National Key R&D Program of China (grant no. 2019YFA09006001) and the National Natural Science Foundation of China (grant nos. 81772703 and 81972380).

Availability of data and materials

All data generated or analysed during this study are included in this article.

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of Peking University First Hospital. All patients had read and signed the informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 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. KIRC. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2

Enhanced UBE2T protein expression was associated with poor prognosis of ccRCC. (a-e) High UBE2T expression was related to advanced T stage (a), advanced histological grade (b), increased maximum diameter of tumor values (c), common postoperative metastasis (d) and increased mortality (e). (f-g) High UBE2T expression was related to low OS (f) and RFS (g).

Figure 3

The expression of UBE2T was related to the proliferation of ccRCC cells. (a) RT-qPCR analyses of 786-O cells and Caki-1 cells infected with a UBE2T shRNA or lentivirus-mediated UBE2T-overexpressing vector. (b) Western blot analyses of 786-O cells and Caki-1 cells infected with a UBE2T shRNA or lentivirus-mediated UBE2T-overexpressing vector. (c-d) The results of MTS indicated knockdown of UBE2T attenuated the proliferation of 786-O (c) and Caki-1(d). (e-f) The results of MTS indicated overexpression of UBE2T promote the proliferation of 786-O (e) and Caki-1(f). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4

UBE2T suppression significantly decreased cell migratory and invasive ability. (a-b) Wound-healing assay. Representative images of wound-induced cell migration by the UBE2T-sh 786-O (a) and Caki-1 cells (b). (c-d) Wound-healing assay. Representative images of wound-induced cell migration by the UBE2T-OE 786-O (c) and Caki-1 cells (d). (e-f) Representative images of transwell invasion assay of UBE2T-sh 786-O cells (20X) (e) and Caki-1 cells (10X) (f). (g-h) Representative images of transwell invasion assay of UBE2T-overexpressed 786-O cells (g) and Caki-1 cells (10X) (h). Scale bar, 200 μ m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5

Knockdown of UBE2T expression induced G2/M phase arrest and apoptosis. (a) Dot plot of gene ontology analysis with the co-expression genes of UBE2T. (b) Dot plot of Kyoto Encyclopedia of Genes and Genomes analysis with the co-expression genes of UBE2T. (c) GSEA analysis was performed in TCGA database according to the expression level of UBE2T. (d-e) The cell cycle of 786-O cells was analyzed by propidium iodide staining and flow cytometry. (f) The histogram showed the proportion of 786-O cells in different cell cycles after UBE2T shRNA infection. (g) The PCNA protein expression of 786-O cells with a UBE2T shRNA or lentivirus-mediated UBE2T-overexpressing vector. (h) The histogram showed the proportion of apoptosis cells after UBE2T shRNA infection. (i) The Bax and Bcl-2 protein expression of 786-O cells with a UBE2T shRNA or lentivirus-mediated UBE2T-overexpressing vector. * $p < 0.05$, ** $p < 0.01$.

Figure 6

The expression of UBE2T affected the growth of human RCC cells in vivo. (a) Tumor volume curves of the sh-UBE2T and sh-NC treatment groups were measured. (b) Tumors collected from mice injected with 786-O-sh-UBE2T cells are shown. (c) Tumor weights of the sh-UBE2T and sh-NC treatment groups were measured. (d) Tumor volume curves of the UBE2T-OE and OE-NC treatment groups were measured. (e) Tumors collected from mice injected with 786-O-OE-UBE2T cells are shown. (f) Tumor weights of the UBE2T-OE and OE-NC treatment groups were measured. (g) The protein expression of PCNA in tumor in nude mice injected with 786-O-sh-UBE2T cells, evaluated by IHC. (h) The protein expression of Bcl-2 in tumor in nude mice injected with 786-O-sh-UBE2T cells, evaluated by IHC. Scale bar, 100 μ m. * p < 0.05.

Figure 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 mimics co-transfection significantly inhibited luciferase activity, and UBE2T Mut and miR-182-5p mimics co-transfection didn't affect luciferase activity in renal cancer cell. (c) The mRNA expression of UBE2T after transfecting miR-182-5p mimics in renal cancer cell. (d) The protein expression of UBE2T after transfecting miR-182-5p mimics in renal cancer cell. * $p < 0.05$, **** $p < 0.0001$.

Figure 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 mimics in 786-O-OE-NC cells. (b) The wound healing assay was performed after transfecting miR-182-5p mimics 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 (10X). (e) The protein expression of PCNA, Bax and Bcl-2 after transfecting miR-182-5p mimics in renal cancer cell. Scale bar, 200 μ m. *p <0.05, **p<0.01.

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