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Ubiquitin-specific peptidase 2 inhibits epithelial-mesenchymal transition in clear cell renal cell carcinoma metastasis by downregulating the NF- κ B pathway

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ABSTRACT

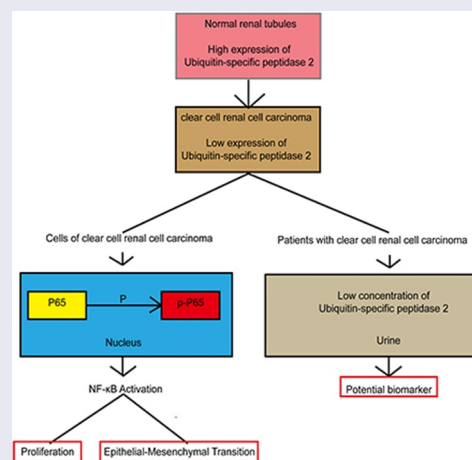
Clear cell renal cell carcinoma, the most common type of renal cancer, is associated with poor survival. Ubiquitin-specific peptidase 2 regulates the molecular mechanisms of cancer cells. However, its mechanism in clear cell renal cell carcinoma remains unclear. Quantitative real-time polymerase chain reaction, enzyme-linked immunosorbent assay, and immunohistochemistry were performed to assess ubiquitin-specific peptidase 2 expression in human clear cell renal cell carcinoma samples. Ubiquitin-specific peptidase 2 was weakly expressed in clear cell renal cell carcinoma samples and associated with poor patient outcomes. Ubiquitin-specific peptidase 2 inhibition promoted clear cell renal cell carcinoma cell proliferation, migration, and invasion. Ubiquitin-specific peptidase 2 overexpression inhibited clear cell renal cell carcinoma cell proliferation, migration, and invasion *in vitro* and *in vivo*. RNA-sequencing showed significant changes in the epithelial-mesenchymal transition-related pathways following ubiquitin-specific peptidase 2 knockdown. Western blotting was performed to detect the protein expression levels. Expression of p-nuclear factor- κ B p65, N-cadherin, Vimentin, and Snail, which were markedly increased, as well as E-cadherin, which was decreased following ubiquitin-specific peptidase 2 knockdown. Rescue experiments using the nuclear factor- κ B inhibitor BAY 11–7082 revealed that the migration and invasion abilities and the expression of epithelial-mesenchymal transition pathway proteins were inhibited in both the short hairpin RNA (shRNA) for ubiquitin-specific peptidase 2 and shRNA for negative control groups. Ubiquitin-specific peptidase 2 is a potential biomarker to distinguish clear cell renal cell carcinoma patients from healthy individuals. Ubiquitin-specific peptidase 2-mediated inhibition of epithelial-mesenchymal transition in clear cell renal cell carcinoma cells is dependent on the nuclear factor- κ B pathway.

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 Supplemental data for this article can be accessed [here](#)

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Introduction

Clear cell renal cell carcinoma is the most common type of renal cancer, making up over 70% of all cases of kidney cancer [1]. The five-year cancer-specific survival rate of clear cell renal cell carcinoma is less than 70% [2]. However, the specific mechanisms of clear cell renal cell carcinoma remain unclear. Ubiquitin-specific peptidase 2 was first described in a study on rat testis [3]. Researchers showed that upregulation of ubiquitin-specific peptidase 2 in prostate cancer and ovarian carcinoma, leads to increased levels of deubiquitinated substrates [4]. However, the mechanism of ubiquitin-specific peptidase 2 in clear cell renal cell carcinoma biological behavior has not been fully understood.

The migration and invasion abilities are important characteristics and biomarkers for malignant tumors associated with poor outcomes [5]. Migration and invasion are mainly caused by epithelial-mesenchymal transition [6], a process critical to tumorigenesis [7].

In this study, our hypothesis was whether ubiquitin-specific peptidase 2 could be served as a potential biomarker to distinguish clear cell renal cell carcinoma patients from healthy volunteers. The goals and aims of this research were to investigate the role of ubiquitin-specific peptidase 2 in clear cell renal cell carcinoma cells via *in vitro* and *in vivo* experiments and explore the mechanism behind this phenomenon.

Methods

Patient and tissue samples

A total of 26 paired cancer and paracancerous tissue specimens and 57 urine samples from patients with clear cell renal cell carcinoma were collected [8]. Moreover, we collected 27 urine samples from healthy volunteers. All samples were obtained from the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China). The first part of the paired cancer tissue specimen was used for RNA extraction. Other paired cancer tissues were stored in formalin until further use for immunohistochemistry analysis. These patients had not been administered adjuvant

anticancer therapy before surgery. All patients and volunteers provided written informed consent. The research protocol was approved by the Ethics committee of the First Affiliated Hospital of Zhengzhou University (The amendment-2019-148).

RNA sequencing

Four paired-cancer and paracancerous clear cell renal cell carcinoma tissue samples were subjected to mRNA-transcriptome sequencing using an LC-Bio Technologies instrument (Hangzhou, China) to identify differentially expressed genes. RNA-sequencing was performed to detect mutated pathways using short hairpin RNA (shRNA) for ubiquitin-specific peptidase 2 and shRNA for negative control in 786-0 cells.

Bioinformatics analysis

Ubiquitin-specific peptidase 2 mRNA expression in clear cell renal cell carcinoma was analyzed to determine the stage, overall survival and disease-free survival for clear cell renal cell carcinoma patients using Gene Expression Profiling Interactive Analysis software (www.cancer-pku.cn) [9]. Further, the Human Protein Atlas was used to search for data on the protein expression of ubiquitin-specific peptidase 2 [10,11]. STRING (functional protein association networks (string-db.org)) was used to identify relevant proteins and pathways involving ubiquitin-specific peptidase 2 [12].

RNA isolation and quantitative real-time polymerase chain reaction

According to the research method of Cao, et al [13], we extract RNA from tissue and cell line samples using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was prepared using the Prime Script RT reagent kit with gDNA Eraser (Takara, Shiga, Japan). The quantitative real-time polymerase chain reaction was carried out by SYBR Green (Takara) to analyze the mRNA expression of ubiquitin-specific

peptidase 2. We used *GAPDH* as the internal amplification control. Primer sequences were as follows: *ubiquitin-specific peptidase 2* forward: 5'-AGTAGGATCGGGGATCTCTTTG-3' and reverse: 5'-GAAGACCGTAGAACAGTAACCAC-3'; *GAPDH* forward: 5'-GTCTCCTCTGACTTCAACAGCG-3' and reverse: 5'-ACCACCCTGTTGCTGTAGCCAA-3'.

Western blot analysis

As described by Xie et al [14], all proteins were isolated by the RIPA buffer (Solarbio, Beijing, China) and the concentration of protein was tested using a BCA Protein Assay Kit (Solarbio). The proteins were subjected to 10% SDS-PAGE, followed by transfer of the separated proteins onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The main antibodies were ubiquitin-specific peptidase 2 (Proteintech, Rosemont, IL, USA, 1:1000), *GAPDH* (Proteintech, 1:5000), nuclear factor (NF)- κ B p65 (Cell Signaling Technology, Danvers, MA, USA, 1:1000), phospho-NF- κ B p65 (Cell Signaling Technology, 1:1000), N-cadherin (Proteintech, 1:5000), E-cadherin (Proteintech, 1:25,000), Vimentin (Proteintech, 1:5000), and Snail (Cell Signaling Technology, 1:1000). Secondary antibodies used were horseradish peroxidase-conjugated Affinipure Goat Anti-Rabbit IgG(H + L) and horseradish peroxidase-conjugated Affinipure Goat Anti-Mouse IgG(H + L) (Proteintech, 1:8000). Incubations with the antibodies were performed per the manufacturer's instructions.

Enzyme-linked immunosorbent assay

The concentration of ubiquitin-specific peptidase 2 in the urine samples from the clear cell renal cell carcinoma patients and healthy volunteers was measured using a Human ubiquitin-specific peptidase 2 enzyme-linked immunosorbent assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) [15]. The urine samples were first centrifuged at 3000 rpm for 10 min, and the upper layer of the urine samples was collected. The upper layer was divided into 100 μ l aliquots and then stored at -80°C until the

experiments. For the enzyme-linked immunosorbent assay experiment, the upper layer was added to wells that have been pre-coated with primary antibodies. For each experimental well, recognition antigens labeled with horseradish peroxidase were added. After incubating for 30 min at 37°C, phosphate-buffered saline was used to wash each well. We used Varioskan Lux (Thermo Fisher Scientific) to measure the absorbance at 450 nm.

Immunohistochemistry analysis

As described by Li et al [16], embedded tissues from clear cell renal cell carcinoma patients were incubated with the primary ubiquitin-specific peptidase 2 antibody described above (Proteintech, 10,392-1-AP, 1:200) overnight at 4°C and then washed with phosphate-buffered saline. Furthermore, the samples were incubated at 23°C with anti-rabbit secondary antibodies for 2 h. For the color reaction, 3,3'-N-diaminobenzidine tetrahydrochloride was used. The development of dark brown coloration was defined as a positive result.

Cell culture

The kidney cell line HK2 and three clear cell renal cell carcinoma cell lines 769-P, 786-0, and Caki-1 were obtained from the China Center for Type Culture Collection (Wuhan, China). The four cell lines were cultured in Roswell Park Memorial Institute 1640 (HyClone, Logan, UT, USA) containing 10% heat-inactivated fetal bovine serum (HyClone) as well as 1% penicillin and streptomycin. The cells were cultured at 37°C with 5% CO₂ in the incubator. Roswell Park Memorial Institute 1640 containing 10% serum and 2 μ g/mL puromycin (Sigma, St. Louis, MO, USA) was used to culture the clear cell renal cell carcinoma cells transduced with lentivirus.

Knockdown and overexpression of ubiquitin-specific peptidase 2

The small interfering (Si) RNAs Si-ubiquitin-specific peptidase 2-1, Si-ubiquitin-specific peptidase 2-2, and Si-negative control were transfected into 769-P and 786-0 clear cell renal cell

carcinoma cells using JetPRIME (Polyplus-transfection, New York, NY, USA) per the manufacturer's instructions. All three small interfering RNAs were synthesized by GenePharma (Shanghai, China) [17]. The two Si-ubiquitin-specific peptidase 2 sequences were: Si-ubiquitin-specific peptidase 2-1 (5'-3'): UCGCUGACGUGUACAGAUUUU and Si-ubiquitin-specific peptidase 2-2 (5'-3'): CCAGCAAGCUCACAACAUUUU. Additionally, a plasmid for overexpressing ubiquitin-specific peptidase 2 was transfected into the three clear cell renal cell carcinoma cell lines using JetPRIME. The plasmid vector and ubiquitin-specific peptidase 2 plasmid were synthesized by Genechem (Shanghai, China). To evaluate the function of ubiquitin-specific peptidase 2 *in vivo*, we constructed Caki-1 cells stably overexpressing ubiquitin-specific peptidase 2 by infection with lentivirus synthesized by Genechem. To explore the mechanism of ubiquitin-specific peptidase 2 in clear cell renal cell carcinoma cells, shRNA ubiquitin-specific peptidase 2 (sequence as ubiquitin-specific peptidase 2-1) for 769-P and 786-0 cells were constructed by Genechem.

Cell proliferation, invasion, and migration assays

As described by Shen et al [18], the Cell counting kit-8 and clone formation experiments were conducted to evaluate the proliferative abilities. For cell counting kit-8 experiments, 5,000 cells were seeded into each well of 96-well plates with six replicates for 769-P, 786-0, and Caki-1. Absorbance at 450 nm was measured using cell counting kit-8 assay (Dojindo, Kumamoto, Japan) at 12, 24, 48, and 72 h post-seeding. To analyze clone formation, 150 cells/well were seeded into each well of the 6-well plates. The cells were cultured for 2 weeks. According to the research method of Zhang, et al [19], wound-healing experiments were performed to determine migratory ability of the cell. The cells

were grown in a 6-well plate until reaching greater than 90% confluence. Images were captured under 200× magnification at 0, 24, and 48 h using the microscope. As described by Hong et al [20], transwell chambers, coated with Matrigel (BD Bioscience, Franklin Lakes, NJ, USA), were used for the invasion assay. Cells (1×10^5) were seeded into the upper chamber with Roswell Park Memorial Institute 1640. The lower chamber contained 10% fetal bovine serum in Roswell Park Memorial Institute 1640. We randomly selected five images and counted the number of cells under 200× magnification with an IX71 microscope after 24 h.

Animal model

Animal experiments were conducted in accordance with the National Institutes of Health's guide for the care and use of laboratory animals as well as with ARRIVE guidelines [21]. The procedures were approved by the Animal Ethics Committee of the First Affiliated Hospital of Zhengzhou University. As described by Shen et al [22], BALB/c nude mice (male, 5 weeks old) were randomly divided into two groups for *in vivo* experiments ($n = 6$ in each group). A subcutaneous injection in the right flank with 1×10^7 Caki-1(Lentivirus-vector and Lentivirus-Overexpression- ubiquitin-specific peptidase 2) cells was performed to investigate the tumor growth ability *in vivo*. The tumor size was measured every 3 days, and the tumor volume was calculated as $[\text{length} \times \text{width}^2]/2$. Mice were sacrificed at 9 weeks after inoculation. The size and weight of tumors were measured.

Rescue experiments

The NF- κ B inhibitor BAY 11-7082 from Peprotech (Rocky Hill, NJ, USA), was used for the rescue experiments [23]. The migratory and

Table 1. The prediction of ubiquitin-specific peptidase 2 between clear cell renal cell carcinoma patients and healthy volunteers.

Group	No.	Group	No.
True positive †	16	True negative ‡	54
False positive	3	False negative	13
Positive prediction rate	84.21%	Negative prediction rate	80.60%

†Positive: healthy volunteers; ‡Negative: clear cell renal cell carcinoma

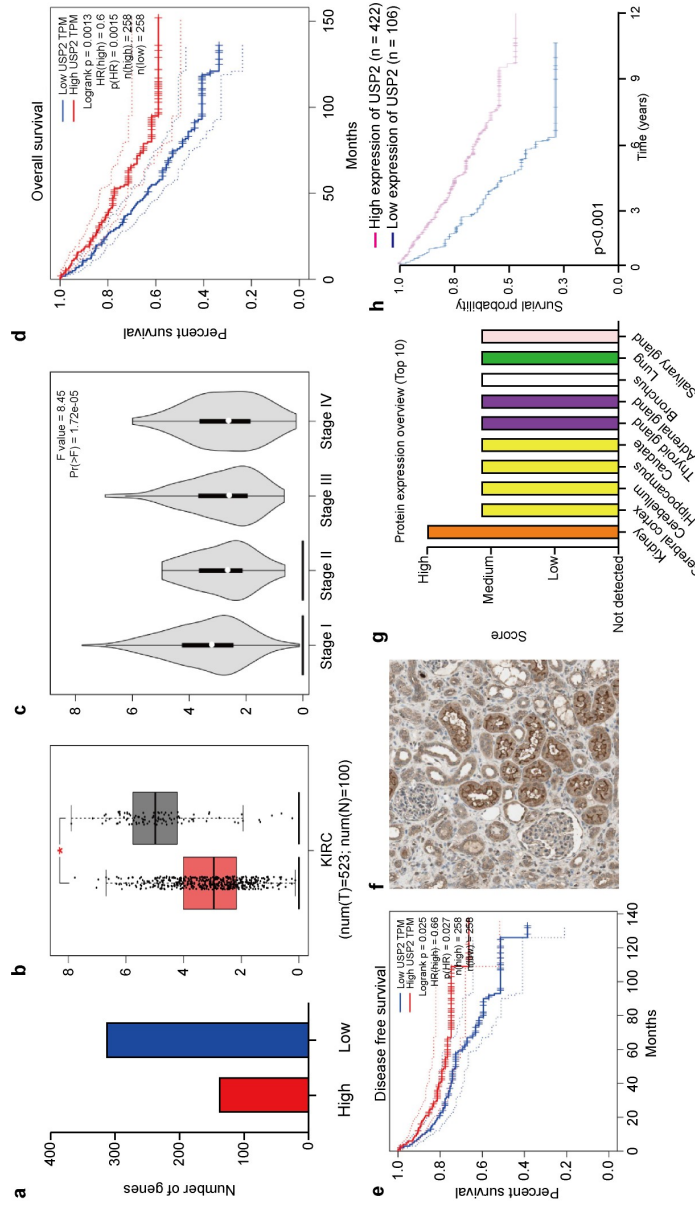


Figure 1. Ubiquitin-specific peptidase 2 downregulation in clear cell renal cell carcinoma is associated with better clinical stage and prognosis. (a) The mRNA-transcriptome sequencing was used to identify mRNAs important in clear cell renal cell carcinoma between tumor tissues and normal tissues. The numbers of highly expressed genes (red) and lowly expressed genes (blue) were shown. (b) Gene Expression Profiling Interactive Analysis showed that the expression of ubiquitin-specific peptidase 2 in paraneoplastic tissues (gray) was higher than in tumor tissue (red). (c) The expression of ubiquitin-specific peptidase 2 significantly differed between patients with stage I and stage II shown using Gene Expression Profiling Interactive Analysis. (d), (e) Furthermore, Gene Expression Profiling Interactive Analysis revealed that higher expression of ubiquitin-specific peptidase 2 was positively correlated with a better clinical clear cell renal cell carcinoma prognosis including overall survival and disease-free survival. (f) Human Protein Atlas showed that ubiquitin-specific peptidase 2 was mainly enriched in the renal tubules. (g) The Human Protein Atlas showed that ubiquitin-specific peptidase 2 protein expression was higher in the kidney than in other normal tissues. (h) The group that showed a high expression of ubiquitin-specific peptidase 2 tended to have a higher survival probability for Human Protein Atlas. * $p < 0.05$. **Abbreviations:** KIRC, kidney renal cell carcinoma; HR, hazard ratio.

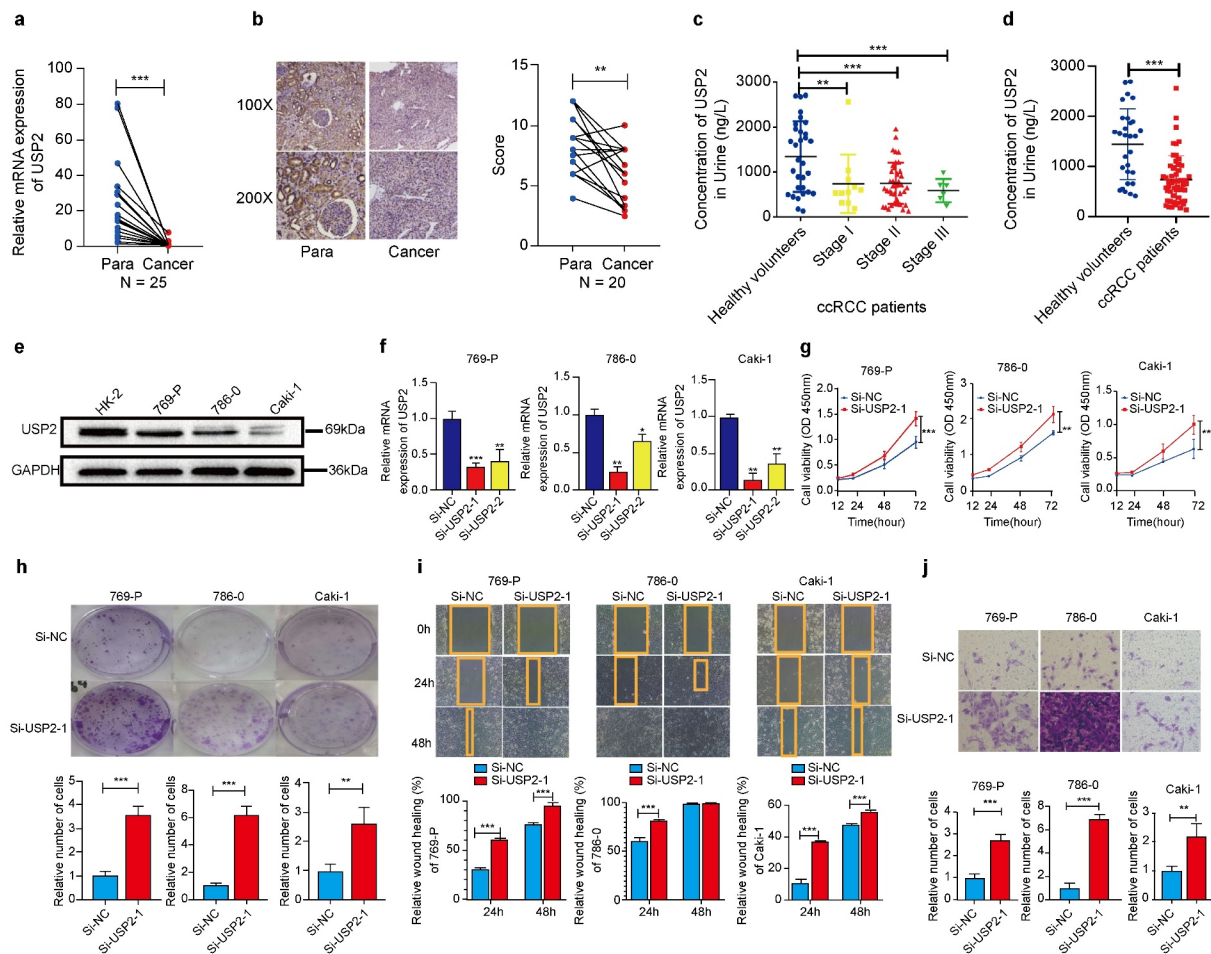


Figure 2. Ubiquitin-specific peptidase 2 knockdown promoted clear cell renal cell carcinoma cell proliferation, migration, and invasion *in vitro*. (a) The expression of ubiquitin-specific peptidase 2 was low in clear cell renal cell carcinoma tissues detected using quantitative real-time polymerase. (b) The immunohistochemistry results showed that ubiquitin-specific peptidase 2 was richer in renal proximal tubule. (c) The concentrations of ubiquitin-specific peptidase 2 in urine samples of patients with different stages of clear cell renal cell carcinoma were lower than those of healthy volunteers. (d) The concentration of ubiquitin-specific peptidase 2 in urine samples of patients with clear cell renal cell carcinoma was significantly lower than that in healthy volunteers. (e) Protein expression of ubiquitin-specific peptidase 2 in 769-P, 786-0, and Caki-1 cells detected using Western blot. (f) The knockdown efficiency of ubiquitin-specific peptidase 2 in clear cell renal cell carcinoma cells shown using quantitative real-time polymerase chain reaction. (g), (h) The results of Cell Counting Kit-8 and clone formation assays showed that ubiquitin-specific peptidase 2 knockdown enhanced the proliferation rate of clear cell renal cell carcinoma cells. (i) Wound healing assays demonstrated that ubiquitin-specific peptidase 2 knockdown promoted greater migration of clear cell renal cell carcinoma cells. Three groups, Si-negative control and Si-ubiquitin-specific peptidase 2-1 for 786-0, Si-ubiquitin-specific peptidase 2-1 for 769-P were healed in 48 hours. (j) Invasion assays revealed that ubiquitin-specific peptidase 2 knockdown promoted the invasive ability of clear cell renal cell carcinoma cells. ** $p < 0.01$, and *** $p < 0.001$.

invasive abilities were tested *in vitro*, with or without BAY 11-7082, for 786-0 and 769-P cells transfected with shRNA ubiquitin-specific peptidase 2 or shRNA Negative Control.

Statistical analysis

Statistical Product and Service Solutions software version 22.0 (SPSS, Inc., Chicago, IL, USA) was used to analyze the data. The data are presented as

the mean \pm standard deviation or mean rank. Group differences were analyzed using Student's *t*-test or Mann-Whitney U test for nonparametric data. Significance was determined at a p -value < 0.05 .

Results

In this study, we hypothesized that ubiquitin-specific peptidase 2 could be a potential biomarker

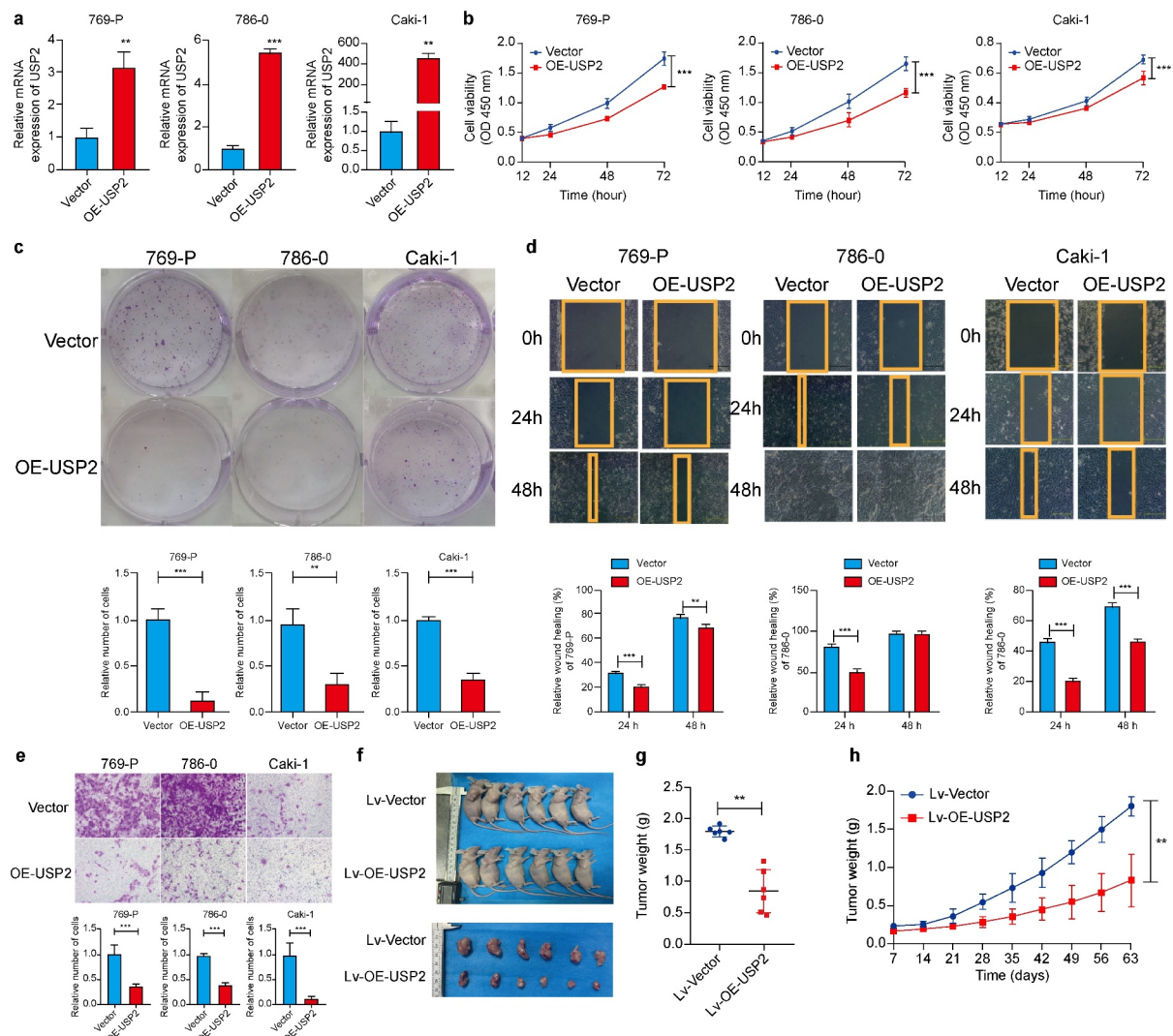


Figure 3. Ubiquitin-specific peptidase 2 overexpression suppressed clear cell renal cell carcinoma cells proliferation *in vitro* and *in vivo*. (a) The over-expression efficiency of ubiquitin-specific peptidase 2 in clear cell renal cell carcinoma cells shown using quantitative real-time polymerase chain reaction. (b, c) The results of Cell Counting Kit-8 and clone formation assays showed that ubiquitin-specific peptidase 2 overexpression suppressed the proliferation rate of clear cell renal cell carcinoma cells. (d) Wound healing assays demonstrated that ubiquitin-specific peptidase 2 overexpression inhibited migration ability of clear cell renal cell carcinoma cells. Both Vector and stably overexpressing ubiquitin-specific peptidase 2 groups for 786-0 cells healed at 48 hours. (e) Invasion assays revealed that ubiquitin-specific peptidase 2 overexpression prevented the invasive ability of clear cell renal cell carcinoma cells. (f) The size of tumor was significantly inhibited in the stably overexpressing ubiquitin-specific peptidase 2 vector group than in the lentivirus vector group. (g) Tumors in ubiquitin-specific peptidase 2 overexpression group were relatively lighter in weight. (h) Tumor growth curves for the lentivirus-Vector group and lentivirus-overexpression-ubiquitin-specific peptidase 2 group. Cells in the Vector and overexpression-ubiquitin-specific peptidase 2 groups were transfected with plasmid. The lentivirus-Vector and lentivirus-overexpression-ubiquitin-specific peptidase 2 groups were transduced with lentivirus. ** $p < 0.01$ and, *** $p < 0.001$.

for clear cell renal cell carcinoma detection and clinical diagnosis. The results from our *in vitro* and *in vivo* research demonstrated that ubiquitin-specific peptidase 2 inhibits epithelial-mesenchymal transition in clear cell renal cell carcinoma metastasis by downregulating the NF- κ B pathway.

Ubiquitin-specific peptidase 2 downregulation in clear cell renal cell carcinoma is associated with worse clinical stage and prognosis

To identify mRNAs important in clear cell renal cell carcinoma, mRNA-transcriptome sequencing was conducted. The threshold was set as \log_2 (fold-change) >1.5 or \log_2 (fold-change) <-1.5 ,

$p < 0.01$. Ubiquitin-specific peptidase 2 (ENSG00000036672) was significantly downregulated in clear cell renal cell carcinoma tissues compared with in normal tissues (Figure 1(a)).

The results of Gene Expression Profiling Interactive Analysis showed that the ubiquitin-specific peptidase 2 expression in paracancerous tissues was higher than that in tumor tissue ($p < 0.05$; Figure 1(b)). Further, the expression of ubiquitin-specific peptidase 2 significantly differed between patients with stage I and stage II clear cell renal cell carcinoma ($p = 1.72e-05$; Figure 1(c)). Furthermore, Gene Expression Profiling Interactive Analysis revealed that higher expression of ubiquitin-specific peptidase 2 was positively correlated with a better clinical clear cell renal cell carcinoma prognosis, including overall survival and disease-free survival ($p = 0.0013$ and $p = 0.025$; Figures 1(d,e)). The results of immunohistochemistry in the Human Protein Atlas showed that ubiquitin-specific peptidase 2 was mainly enriched in the renal tubules (Figure 1(f)). The Human Protein Atlas showed that the protein expression level of ubiquitin-specific peptidase 2 was higher in the kidneys than in other normal tissues (Figure 1(g)), and the group that showed a high expression of ubiquitin-specific peptidase 2 tended to have a higher survival probability for the Human Protein Atlas ($p < 0.001$; Figure 1(h)).

The expression levels of ubiquitin-specific peptidase 2 were low in clear cell renal cell carcinoma tissues and cell lines, as detected using quantitative real-time polymerase chain reaction ($p < 0.001$; Figure 2(a)), immunohistochemistry ($p < 0.01$; Figure 2(b)), and Western blot (Figure 2(e)). These results suggested that ubiquitin-specific peptidase 2 is downregulated in clear cell renal cell carcinoma tissues and cell lines. The immunohistochemistry results also showed that ubiquitin-specific peptidase 2 is mainly expressed in the renal proximal tubule (Figure 2(b)).

The findings suggested that ubiquitin-specific peptidase 2 concentrations may differ in urine samples from clear cell renal cell carcinoma patients and healthy volunteers. Therefore, Enzyme-linked immunosorbent assay was performed to detect the concentration of ubiquitin-specific peptidase 2 in urine samples. The results

showed that the concentration of ubiquitin-specific peptidase 2 in patients with clear cell renal cell carcinoma was significantly lower than that in healthy volunteers ($p < 0.001$; Figures 2(c,d) and Table 1). The cutoff value was 1,549 ng/L, and the area under receiver operating characteristic curve was 0.784.

Ubiquitin-specific peptidase 2 inhibited the proliferation, migration, and invasion of clear cell renal cell carcinoma cells

Caki-1 exhibited the lowest expression of ubiquitin-specific peptidase 2, whereas 769-P and 786-O showed relatively higher expression levels (Figure 2(e)). These three cell lines were all used to perform loss- and gain-of-function experiments.

Two independent ubiquitin-specific peptidase 2-specific siRNAs and Si-negative control were produced and transfected into 769-P, 786-O, and Caki-1 cells to reduce ubiquitin-specific peptidase 2 expression. Si-ubiquitin-specific peptidase 2-1 showed a stronger effect in these cell lines compared with Si-ubiquitin-specific peptidase 2-2 according to quantitative real-time polymerase chain reaction (Figure 2(f)). Therefore, Si-ubiquitin-specific peptidase 2-1 was used for subsequent experiments. The results of the Cell Counting Kit-8 and clone formation assays showed that ubiquitin-specific peptidase 2 knockdown enhanced the proliferation rate of 769-P, 786-O, and Caki-1 cells (Figures 2(g,h)). Wound healing assays demonstrated that ubiquitin-specific peptidase 2 knockdown promoted greater migration ability of clear cell renal cell carcinoma cells (Figure 2(i)). Invasion assays revealed that ubiquitin-specific peptidase 2 knockdown promoted the invasive ability of clear cell renal cell carcinoma cells (Figure 2(j)).

Further, we explored the role of ubiquitin-specific peptidase 2 overexpression in the three clear cell renal cell carcinoma cell lines. Efficiency was measured using quantitative real-time polymerase chain reaction (Figure 3(a)). The proliferative abilities shown by the Cell Counting Kit-8 and clone formation assays indicated that cell proliferation was inhibited by ubiquitin-specific peptidase 2 overexpression in all three cell lines (Figures 3(b,c)). Ubiquitin-specific

peptidase 2 overexpression prevented the migration and invasion ability of clear cell renal cell carcinoma cells (Figures 3(d,e)). These data showed that overexpression of ubiquitin-specific peptidase 2 downregulates the metastatic ability of clear cell renal cell carcinoma cells.

Overexpression of ubiquitin-specific peptidase 2 suppressed metastasis of clear cell renal cell carcinoma cells *in vivo*

Caki-1 cells, stably overexpressing ubiquitin-specific peptidase 2 and lentivirus vector, were subcutaneously injected into male BALB/c nude mice. A subsequent reduction in the tumor size was observed (Figures 3(f,h)). Tumors in the ubiquitin-specific peptidase 2 overexpression group weighed relatively lower (Figure 3(g)). These results demonstrate that ubiquitin-specific peptidase 2 inhibited Caki-1 cell proliferation both *in vivo* and *in vitro*.

Ubiquitin-specific peptidase 2 inhibited epithelial-mesenchymal transition in clear cell renal cell carcinoma cells

The results of RNA-seq sequencing showed that tight junction and extracellular matrix receptor interaction, epithelial-mesenchymal transition-related pathways, were changed remarkably (Figures 4(a,b)). Western blot results showed that the protein expression of N-cadherin, Vimentin, and Snail were negatively correlated with ubiquitin-specific peptidase 2 protein expression in Caki-1 cells (Figure 4(d)), 769-P and 786-O (Figure 4(e)). Protein expression of E-cadherin was positively correlated with ubiquitin-specific peptidase 2 protein expression in all three cell lines (Figures 4(d,e)).

Ubiquitin-specific peptidase 2 inhibits epithelial-mesenchymal transition in clear cell renal cell carcinoma metastasis by downregulating the NF- κ B Pathway

STRING results showed that ubiquitin-specific peptidase 2 was related to the NF- κ B pathway according to inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma (IKBKG), receptor-interacting protein kinase 1 (RIPK1), and

TNF receptor associated factors 2 (TRAF2) (Figure 4(c)). Western blot results showed that the NF- κ B pathway was activated when ubiquitin-specific peptidase 2 was knocked down in 786-O and 769-P cells (Figure 4(e)). The activation of the NF- κ B pathway was decreased when ubiquitin-specific peptidase 2 was overexpressed in Caki-1 cells (Figure 4(d)).

The rescue experiment results showed that both the migration and invasion abilities of 769-P (Figures 4(f,g)) and 786-O (Figures 4(i,j)) cells were inhibited by BAY 11-7082 treatment in the shRNA ubiquitin-specific peptidase 2 and shRNA negative control groups. Protein expression of N-cadherin, Vimentin and Snail increased, whereas E-cadherin expression decreased following BAY 11-7082 treatment in both the shRNA ubiquitin-specific peptidase 2 and shRNA negative control groups (Figures 4(h,k)). Hence, ubiquitin-specific peptidase 2 inhibits epithelial-mesenchymal transition of clear cell renal cell carcinoma by downregulating the NF- κ B pathway.

Discussion

Recently, researchers have mainly focused on the clinical features of clear cell renal cell carcinoma, such as the low rate of early-stage diagnosis and resistance to chemotherapy [24]. We showed that expression of ubiquitin-specific peptidase 2 is reduced in both clear cell renal cell carcinoma cell line as well as tissues and urine samples, which was consistent with the results of previous studies [25]. However, the potential of ubiquitin-specific peptidase 2 as a biomarker for clear cell renal cell carcinoma diagnosis and the mechanisms of this phenomenon have not been thoroughly investigated. On the other hand, we further evaluated ubiquitin-specific peptidase 2 knockdown *in vitro* and overexpressed ubiquitin-specific peptidase 2 in a xenograft mouse model to confirm the role of ubiquitin-specific peptidase 2 in clear cell renal cell carcinoma, which provided more support for our hypothesis [26].

The research of biomarker in cancer has become a promising point and more meaningful than only *in vitro* experiments [27,28]. Given that urine sample collection is noninvasive compared to the collection of blood samples [29-31].

Furthermore, since ubiquitin-specific peptidase 2 expression is mainly localized to the renal proximal tubule, we collected urine samples from both patients with clear cell renal cell carcinoma and healthy volunteers for clinical biomarker analysis [32,33]. The concentration of ubiquitin-specific peptidase 2 in urine samples from both patients with clear cell renal cell carcinoma and healthy volunteers showed a significant difference. Our results suggested that ubiquitin-specific peptidase 2 is a potential biomarker for distinguishing patients with clear cell renal cell carcinoma from healthy individuals.

Ubiquitin-specific peptidase 2 has shown promise for cancer therapy, especially in the treatment of ErbB2-positive breast cancer, targeting ubiquitin-specific peptidase 2-SKP2 axis and bladder cancer [34–36]. Through our experiments, the results showed that ubiquitin-specific peptidase 2 negatively regulated the migration and invasion abilities in clear cell renal cell carcinoma cell lines. Additionally, ubiquitin-specific peptidase 2 was found to negatively regulate the epithelial-mesenchymal transition pathway by RNA sequencing results. A previous study reported that NF- κ B is strongly involved in controlling carcinogenesis and positively regulating tumor proliferation [37,38]. Further research suggested that activation of NF- κ B can promote prostate cancer metastasis [39]. Epithelial-mesenchymal transition, which is positively regulated by NF- κ B, also regulate and affect clear cell renal cell carcinoma migration and invasion through ubiquitin-specific peptidase 2 as shown in rescue experiments [40]. The results revealed that ubiquitin-specific peptidase 2 down-regulated NF- κ B to inhibit epithelial-mesenchymal transition in clear cell renal cell carcinoma.

Conclusion

In conclusion, this research showed that ubiquitin-specific peptidase 2 was downregulated in clear cell renal cell carcinoma by RNA sequencing, bioinformatics and tissue validation. The results from both *in vitro* and *in vivo* showed that ubiquitin-specific peptidase 2 inhibited epithelial-mesenchymal transition by downregulating the NF- κ B pathway. Our findings suggest that ubiquitin-specific peptidase

2, in urine samples, might be a potential biomarker for clear cell renal cell carcinoma detection and clinical diagnosis.

Disclosure statement

No potential conflict of interest was reported by the author(s)

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