Original Article

Downregulation of ubiquitin-specific protease 2 possesses prognostic and diagnostic value and promotes the clear cell renal cell carcinoma progression

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Background: Clear cell renal cell carcinoma (ccRCC), characterized by high mortality, invasion, metastasis, recurrence and drug resistance, is the most common malignant tumor of the urinary system. A clear understanding of the underlying molecular mechanisms and its role during tumorigenesis of RCC can contribute to development of prognostic and targeted therapies.

Methods: We analyzed datasets from the public database, TCGA, Oncomine, for differential expression of ubiquitin-specific protease 2 (USP2), and further investigated its relationship with the clinical stage, pathological grade and prognosis of renal cancer. We used real-time quantitative PCR and western blot analysis to validate USP2 expression in clinical samples and renal cancer cell lines. Finally, we used CCK-8 and transwell assays to determine its effects on biological functions in cells.

Results: We observed significantly lower levels of USP2 mRNA in renal cancer, relative to normal, tissues across the four datasets from the Oncomine database (P<0.001), 533 cases from TCGA database (P<0.0001) and 30 pairs of clinical samples (P<0.0001). Similarly, a decreased USP2 protein expression in ccRCC was detected following immunohistochemical (IHC) and western blot analyses. Furthermore, the aberrant expression of USP2 resulted in significant relationship with clinical stage, pathological grade and lower USP2 mRNA expression was interrelated to poor prognosis of renal cell carcinoma. USP2 acted as an independent factor for ccRCC diagnosis, with an AUC of 0.8888 (95% CI: 0.8529 to 0.9246; P<0.0001). Exogenous restoration of USP2 in ccRCC cells resulted in repression of cell proliferation, migration, and invasion.

Conclusions: Overall, these results show that USP2 acts as an anti-oncogene and an independent factor for ccRCC prognosis. Positive modulation of USP2 might lead to development of a novel strategy for ccRCC treatment.

Keywords: Ubiquitin-specific protease 2 (USP2); clear cell renal cell carcinoma (ccRCC); deubiquitination; biomarker; prognosis; targeted therapy

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Introduction

Each year, more than 400,000 new cancer diagnoses globally, accounting for more than 175,000 deaths, were afflicted by renal cell carcinoma (RCC) (1,2). Among all RCC subtypes, the clear cell renal cell carcinoma (ccRCC) accounts for the highest proportion (approximately 70–80%), mortality rate, invasion, and metastasis (3). Moreover, reports indicate that approximately 30% of ccRCC patients still develop recurrence after surgical treatment (4). It is, therefore, important to discover more effective molecular biomarkers to aid early detection and diagnosis, monitor metastasis and recurrence, and promote postoperative adjuvant targeted therapy.

Deubiquitination, a post-translational modification, is known to highly regulate mechanisms of cell progression by inhibiting ubiquitination and degradation of protein substrates (5). To date, several deubiquitinating enzymes (DUBs) have been implicated in human benign and malignant diseases, and their substrates and functions have been extensively studied over the past couple of decades (6). Ubiquitin-specific protease 2 (USP2), initially detected in rat testis (7), has been reported to regulate various cellular events, including cell-cycle and proliferation (8), DNA repair (9), modulation of the circadian rhythm (10-13), inflammatory responses (14-16), regulation of lipoprotein clearance (17), as well as maintenance of normal sodium balance and blood pressure (18). Moreover, USP2 has shown oncogenic properties in many cancer types, particularly breast cancer and prostate cancer, by stabilizing fatty acid synthase (19), EGFR (5), MDM2 (20) and MDM4 (21). However, it has been shown that ectopic expression of USP2a in the breast cancer cell line, MCF7, inhibited the NF-κB signaling pathway and thus exhibited a unique anticancer characteristic (22,23). These results suggest that USP2 has a more complicated function in human carcinoma, beyond carcinogenesis and anti-apoptotic effects (21). Despite accumulating evidence on the role of USP2 in several types of cancer, its underlying effects and specific mechanisms in ccRCC remain uncharacterized. Previous studies have shown that the different functions of USP2 may depend on the cellular context (24), emphasizing the necessity for further exploration into its function in ccRCC.

In this study, we focused on the role of USP2 in tumor development biology in human ccRCC. Our results revealed that USP2 was downregulated in ccRCC tissues and renal cancer cell lines. Moreover, we identified low-expression of USP2 possesses crucial prognostic and diagnostic value in ccRCC. Furthermore, USP2 acted as an anti-oncogene in ccRCC, with its overexpression inhibiting proliferation, migration, and invasion of ccRCC cells.

Methods

Tissue samples

A total of 30 pairs of clinical renal cancer samples, collected between 2017–2018, were obtained from the Department of Urology, Union Hospital, Tongji Medical College (Wuhan, China). The resected samples were divided into two parts. The first set of samples was immediately frozen in liquid nitrogen for use in RNA extraction and Western blotting experiments. The second set was fixed in formalin and embedded in paraffin, then used for immunohistochemistry assays. No adjuvant anticancer therapy was received before surgery. Prior to the study, every patient's fully informed consent was obtained, and the study approval by the Institutional Review Board of Huazhong University of Science and Technology.

Immunohistochemical staining assays

The second batch of sampled tissues, described above, was incubated with rabbit USP2 polyclonal antibody (ABclonal, A10399, 1:1,000) overnight at 4 ℃, tissue sections washed with PBS then incubated with anti-rabbit secondary antibodies at room temperature for 2 hours. Color change was monitored by 3,3-N-Diaminobenzidine Tertrahydrochloride (DAB), with samples showing a dark brown coloration regarded as positive.

Cell culture

A normal (HK2) and ccRCC cell lines (ACHN, 786-O, CAKi-1, and A498) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in high-glucose DMEM. The high-glucose DMEM was supplemented with 10% FBS and 1% penicillin-streptomycin into. All cells were cultured in an incubator at 37 ℃, and 5% CO_2.

Transient transfection assays

We cultured Caki-1 and A498 cells in 6-well plates, at approximately 60–70% confluence, then transfected them with 4 μg of expression plasmids harboring USP2

(Genechem, China), using Lipofectamine 2000 (Invitrogen, CA, USA). Cells were collected 48 hours after transfection. Subsequent assays of total RNA and protein expression levels were performed by western blot and qRT-PCR.

RNA Isolation and qRT-PCR

Total RNA was first isolated from cell lines and tissues using the TRizol reagent (Thermo; Massachusetts, USA), then 2 μg of the RNA reverse transcribed to cDNA. The SYBR Green mix (Thermo, Massachusetts, USA) was used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis of USP2, with GAPDH included as an internal amplification control. Oligonucleotide primers used in the analysis were purchased from Tianyi Huiyuan, and are listed as follows:

GAPDH: left primer 5'-CCAGAACAGCATCCCTGCCT-3'; right primer 5'-CCTGCTTCACCACCTTCTTG-3';

USP2: left primer 5'-CCGCGCTTTGTTGGCTATAA-3'; right primer 5'-CCCGATCCTACTGTCTTCCC-3'.

Western blot analysis

Firstly, cell and tissue proteins were pyrolysed in RIPA protein lysis buffer (Beyotime Institute of Biotechnology; Haimen, China) in a mixture containing 1 mM protease inhibitor cocktail and PMSF. Secondly, 30 μg of protein per hole was subjected to 10% SDS-PAGE gel electrophoresis then transferred onto PVDF membranes at 250 mA for 90 min. The membranes were blocked in PBST, containing 5% nonfat milk, at room temperature and after 2 hours, they were incubated with primary antibodies against USP2 (ABclonal, A10399, 1:1,000), GAPDH (Proteintech, 60004-1-Ig, 1:1,000) at 4 ℃ for at least 12 hours. Finally, they were washed three times, and then incubated with a secondary antibody for 2 hours before detection by ChemiDoc-XRS+ (Bio-Rad, USA).

Cell proliferation assays

Briefly, 2000 cells were added to each hole in the 96-well plates, 48 hours after transfection. Cell proliferation capacity was then assessed using the CCK-8 assay at 0, 24, 48, 72 and 96 hours following treatments, respectively, according to the manufacturer's instructions.

Migration and invasion assays

These assays were performed on cells 48 hours after transfection and 24 hours after starvation. Briefly, cells were resuspended with serum-free medium, then 3×10^4 A498 and 20×10^4 Caki-1 cells added to the upper chamber of the transwell for analysis of migration. To assess invasion, 6×10^4 A498 and 40×10^4 Caki-1 cells were seeded in chambers where Matrigel (Thermo Fisher Scientific; Waltham, USA) had been added in advance. After 24 hours (A498 cells) or 48 hours (Caki-1 cells) incubation, the invaded cells were fixed in 100% methanol, then stained with 0.05% crystal violet before counting of 5 randomly selected fields.

Bioinformatics analysis

We analyzed USP2 mRNA expression in four renal statistics, including Jones, Eroukhim, Gumz and Lenburg renal statistics using datasets downloaded from Oncomine (https://www.oncomine.org). A total of 533 ccRCC cases, including 72 paired cases, and clinical data on age, gender, T, N, G, and TNM stages, as well as metastasis, recurrence, overall survival (OS) and disease-free survival (DFS) of patients in TCGA-KIRC database, were retrieved from the cBioPortal (http://www.cbioportal.org/public-porta). The paired normal cases were derived from normal or adjacent renal tissues of corresponding ccRCC patients and used as a control for ccRCC tissues. The gene set enrichment analysis (GSEA) platform was used to assess pathways enriched in the gene set, based on the pathway Enrichment Score (ES).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., USA) and SPSS Statistics 22.0 software (IBM SPSS, Chicago, IL). Data on paired cases were analyzed using a paired sample *t*-test, while analysis of unpaired cases was performed using a one-way analysis of variance (ANOVA) or *t*-test. In addition, a Pearson correlation coefficient was used to assess the relationship between two factors. The Kaplan-Meier analysis was performed to estimate the correlation between USP2 expression with overall survival (OS) and disease-free survival (DFS) times with the log-rank test. To evaluate the diagnostic value of USP2 mRNA expression in ccRCC patients, we generated receiver operating characteristic (ROC) curves and area under the curve (AUC). Finally, prognostic significance of USP2 in ccRCC was analyzed by univariate and multivariate Cox proportional hazard regressions. All *in vitro* experiments

were performed in triplicates and all data represented as mean ± SEM. A confidence threshold, P<0.05, was used to analyze statistical significance. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Results

USP2 is significantly downregulated in ccRCC clinical samples and cell lines

Differential expression of USP2 mRNA, in ccRCC, was first evaluated by analyzing four data sets from the Oncomine database. Results revealed reduced expression levels of USP2 in tumor tissues compared to adjacent renal tissues (*Figure 1A*,*B*,*C*,*D*). This reduction in expression was confirmed by the TCGA-KIRC database, in which 533 cases, including 72 paired cases (the paired normal cases were derived from normal kidney tissue or adjacent renal tissue of the corresponding ccRCC patients as a control for ccRCC tissue) were analyzed (*Figure 1E*,*F*). Moreover, the aberrant expression of USP2 mRNA in other tumors, including breast invasive carcinoma, brain lower grade glioma, liver hepatocellular carcinoma, prostate adenocarcinoma and bladder urothelial carcinoma, were also evaluated by analyzing datasets from the TCGA database (*Figure S1A*,*B*,*C*,*D*,*E*). To further verify the results from bioinformatics analysis, we performed a quantitative real-time polymerase chain reaction (qRT-PCR) analysis on 30 pairs of clinical samples collected between 2017–2018. Here, we found significantly lower levels of USP2 mRNA in ccRCC tissues relative to those in paired adjacent renal tissues (*Figure 1G*,*H*). To further confirm this aberrant expression, we constructed ROC curves based on USP2 expression in 30 pairs of clinical tissues. Results from clinical data showed that USP2 expression was able to differentiate ccRCC from normal tissues with an area under the curve (AUC) of 0.8144 (95% CI: 0.7071 to 0.9218, P<0.0001) (*Figure S1F*). An immunohistochemistry (IHC) assay performed to evaluate USP2 expression showed a similar profile (*Figure 1I*). In addition, we investigated USP2 protein expression using western blot analysis in ccRCC and adjacent renal tissues, and found low expression of the gene in tumor tissues (*Figure 1J*). As expected, USP2 protein expression in all the ccRCC cells (786-0, A498, ACHN, CAKI) was reduced relative to the control cell line (HK2), consistent with the previous results (*Figure 1K*). Furthermore, we conducted quantitative analysis of the results of immunohistochemistry and western blot (*Figure S1G*,*H*,*I*). In summary, USP2 expression dramatically decreased in ccRCC tissues and cells compared to adjacent tissues and immortalized renal epithelial cells.

Low USP2 mRNA level is associated with various clinicopathological variables and poor prognosis in ccRCC patients

To understand the relationship between levels of USP2 expression and clinicopathological variables in ccRCC patients, we performed a more in-depth analysis of the TCGA database (*Table 1*). The resulting data showed a lower mRNA expression in male than in female patients (*Figure 2A*). Similarly, USP2 downregulation correlated with overall survival status, Grade stage, renal tumor size, weight and malignancy in ccRCC (*Figure 2B*,*C*,*D*,*E*). On the other hand, USP2 mRNA levels were negatively correlated with the T, TNM and G stages after a one-way ANOVA (P<0.05, *Figure 2F*,*G*,*H*). In addition, differences between cancer and adjacent renal tissues with metastasis or non-metastasis were also exhibited (*Figure 2I*). However, no association was found between levels of USP2 mRNA expression with age (*Figure S2A*).

To explore the role of USP2 in differentiating benign and malignant prognosis, we performed individual analysis based on clinical outcomes from the TCGA database. Clinical outcomes of 533 cases, including 72 paired cases, were classified as OS-good (≥5 years, alive) and OS-poor (≤2 years, dead) according to the overall survival (OS) time and overall survival status, and then the USP2 mRNA levels in normal and tumor tissues of corresponding patients were analyzed. Similarly, the classification of DFS-good (≥5 years, disease-free) and DFS-poor (≤2 years, recurred/progressed) was based on disease-free survival (DFS) time and disease-free survival status. Results revealed a significant decline in USP2 expression in cancer and normal tissues of OS-poor patients compared to OS-good patients (*Figure 2J*). Similarly, DFS-poor patients showed much lower USP2 levels in cancer tissues compared to DFS-good ones (*Figure 2K*), and ccRCC patients with or without recurrence in cancer tissues could be well classified by the mRNA levels (*Figure 2L*). In summary, these results suggest that low USP2 levels are indicative of a significant risk of high stage. Consequently, USP2 could play a role as a potential biomarker and an independent prognosis- or clinical outcome-related classifier for ccRCC.

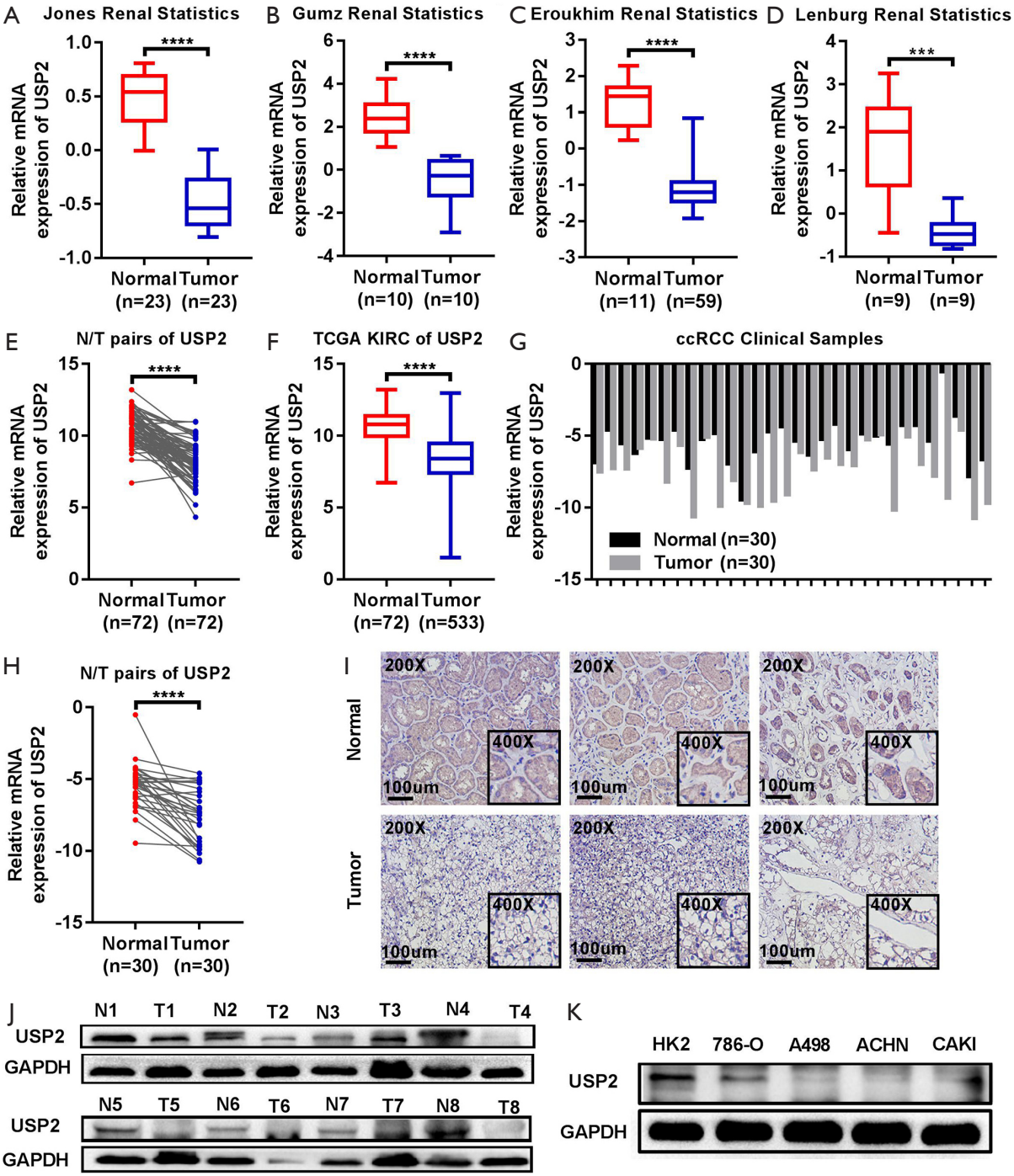

Figure 1 USP2 is significantly downregulated in ccRCC clinical samples and cell lines. (A,B,C,D) Expression profiles of USP2 mRNA in four renal statistics, including Jones, Gumz, Eroukhim and Lenburg renal statistics, were downloaded from Oncomine datasets and analyzed. (E,F) The mRNA levels in ccRCC downloaded from the TCGA-KIRC database consisting of 533 cases, including 72 paired cases. (G,H,I) Analysis of USP2 expression in clinical samples using qRT-PCR and IHC. (J,K) The protein expression of USP2 in ccRCC clinical samples and cell lines. ***P<0.001; ****P<0.0001.

Table 1 Correlation between USP2 mRNA expression and clinicopathological parameters of ccRCC patients.

Parameter	Number	USP2 mRNA expression		P value
		Low (n=262)	High (n=262)	
Age (years)				0.430
<60	242	126	116	
≥60	282	136	146	
Gender				0.006
Male	339	185	154	
Female	185	77	108	
T stage				0.036
T1 + T2	336	156	180	
T3 + T4	188	106	82	
N stage				0.007
N0 + NX	509	249	260	
N1	15	13	2	
M stage				0.390
M0 + MX	446	219	227	
M1	78	43	35	
G stage				0.255
G1 + G2 + GX	244	115	129	
G3 + G4	280	147	133	
TNM stage				0.025
I + II	318	146	172	
III + IV	206	116	90	

Downregulation of USP2 correlated with a shorter prognosis time of ccRCC patients

We generated Kaplan-Meier curves to investigate the correlation between mRNA levels of USP2 and prognosis time of patients. Based on the median value from USP2 expression, 532 ccRCC patients from the TCGA-KIRC database were divided into 'high' and 'low' groups. We found a shorter overall survival time in patients in the group with low USP2 levels (*Figure 3A*, log-rank test, P=0.0009). Subsequently, we performed an overall survival analysis to understand USP2 mRNA levels in subgroups of ccRCC patients with results providing further evidence that low expression is important in prognosis of ccRCC in male patients (*Figure 3B*, P=0.0062), age ≥60 years (*Figure 3C*, P=0.0019), T1+T2 stage (*Figure 3D*, P=0.0003), N0 stage (*Figure 3E*, P=0.0370), non-metastasis (*Figure 3F*, P=0.0023), TNM (I + II) (*Figure 3G*, P=0.0005), G1+G2 stage (*Figure 3H*, P=0.0285) and G3 + G4 stage (*Figure 3I*, P=0.0227), but not with M1 stage, N1 stage, TNM (III + IV) , age <60 years, T3 + T4 stage (*Figure S2B,C,D,E,F*).

To further verify the prognostic value of USP2, we used Kaplan-Meier curves to analyze the relationship between USP2 mRNA levels and disease-free survival (DFS) time. Here, a total of 434 ccRCC patients were divided into 'low' and 'high' group using a similar classification method described above. Results indicated a significantly higher DFS in the group with high USP2 expression compared to that with low expression (*Figure 3J*, log-rank test, P=0.0041). In addition, we analyzed the DFS in subgroups of ccRCC patients, and found that low USP2 expression can also be a prognostic factor for patients with male (*Figure 3K*, P=0.0006), age ≥60 years (*Figure 3L*, P=0.0002), non-metastasis (*Figure 3M*, P=0.0031), T1 + T2 stage (*Figure 3N*, P=0.00901), TNM (I+II) (*Figure 3O*, P=0.0096) in ccRCC. However, this was not the case with M1, T3 + T4 stage, age <60 years, N0 and N1, TNM (III + IV), G1 + G2 and G3 + G4 stages, as well as in females (*Figure S3*).

Additionally, the prognostic value of USP2 in ccRCC was further evaluated by the Cox regression model in which OS time and OS status, or DFS time and DFS status, were considered as dependent variables. The status of USP2 expression in ccRCC risk factors were assessed by univariate and multivariate analysis, which revealed that USP2 was an independent prognostic factor for ccRCC patients (*Tables 2* and *3*). And multivariate models were adjusted for age, gender, T stage, N stage, M stage, G grade and USP2 mRNA level.

Diagnostic value of USP2 mRNA expression in ccRCC patients

To test whether USP2 has diagnostic value in ccRCC, receiver operating characteristic (ROC) curves for the clinicopathological variables were analyzed. Results showed that ccRCC could be sensitively differentiated from normal tissues by USP2 expression, with an AUC of 0.8888 (95% CI: 0.8529 to 0.9246; P<0.0001) (*Figure 4A*). A further analysis of USP2 mRNA levels in subgroups of ccRCC patients, including gender, T/N/G/TNM stage, metastasis/OS/DFS status, OS-good/poor and DFS-good/poor, showed that low mRNA expression had an effective diagnostic value in ccRCC patients with male *vs.* female (AUC =0.5985, P=0.002), TNM (I + II) *vs.* (III + IV) stage (AUC =0.5888, P=0.006), (G1 + G2) *vs.* (G3 + G4) stage

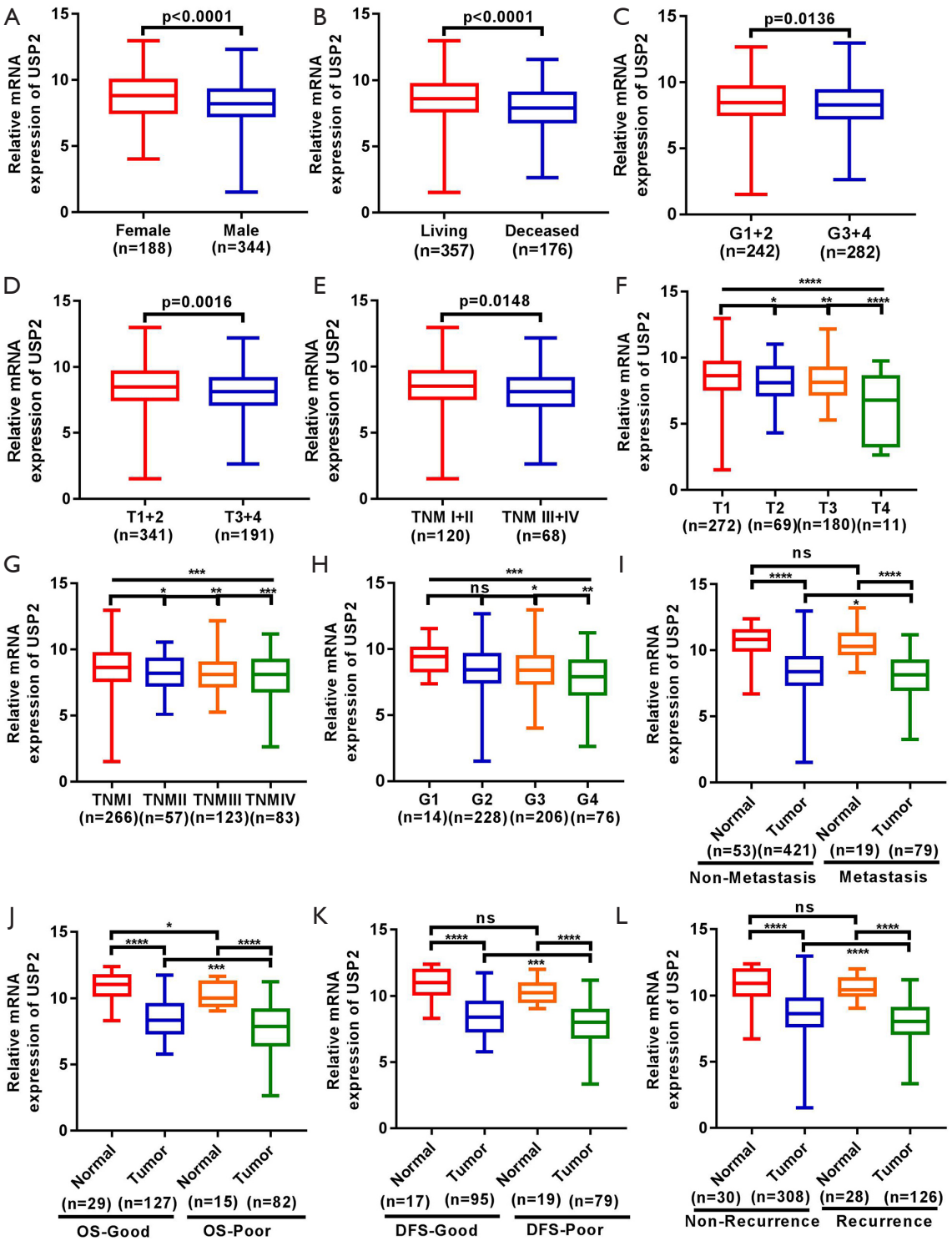

Figure 2 Low USP2 mRNA level is associated with various clinicopathological variables and negative prognosis in ccRCC patients. USP2 mRNA levels were compared in different clinicopathological variables: (A) male *vs.* female, (B) deceased *vs.* living, (C) G3 + G4 *vs.* G1 + G2, (D) T3 + T4 *vs.* T1 + T2, (E) TNM (III + IV) *vs.* TNM (I + II), (F) T stage, (G) TNM stage, (H) G stage, (I) non-metastasis *vs.* metastasis, (J) OS-good *vs.* OS-poor, (K) DFS-good *vs.* DFS-poor, and (L) non-recurrent *vs.* recurrent. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

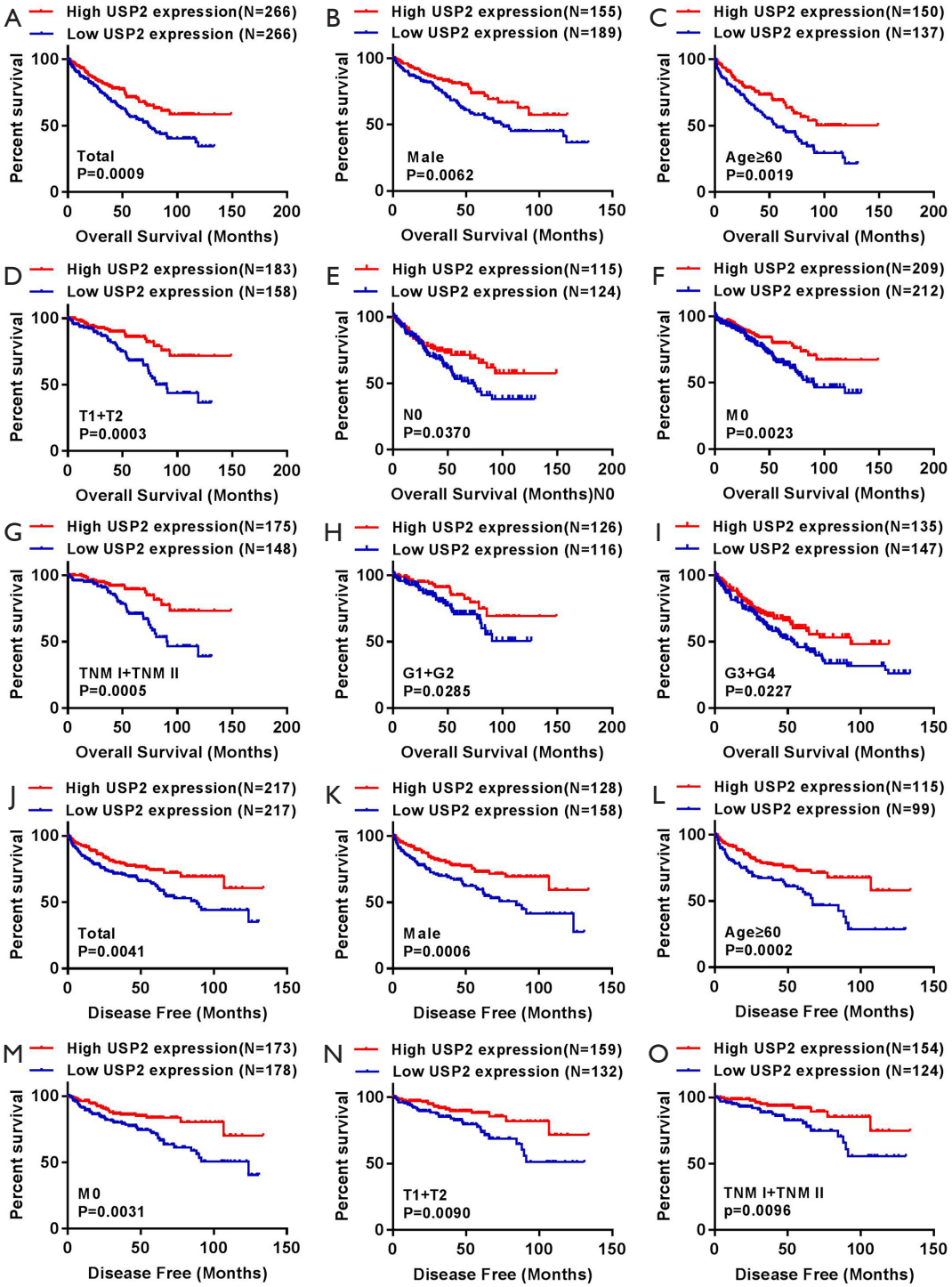

Figure 3 Downregulation of USP2 correlated with overall survival and disease-free survival times of ccRCC patients. (A) A correlation between USP2 expression and OS times of 532 ccRCC patients analyzed by the Kaplan-Meier method. Overall survival analysis toward the expression of USP2 mRNA was performed in subgroups of ccRCC patients: (B) male, (C) age ≥60, (D) T1 + T2, (E) N0, (F) non-metastasis, (G) TNM I + TNM II, (H) G1 + G2, (I) G3 + G4. (J) The correlation between USP2 expression and DFS time for 434 ccRCC patients analyzed by Kaplan-Meier. DFs analysis toward the expression of USP2 mRNA was performed in subgroups of ccRCC patients: (K) male, (L) age ≥60 years, (M) non-metastasis, (N) T1 + T2, and (O) TNM I + TNM II.

Table 2 Univariate and multivariate analysis of USP2 mRNA level and patient overall survival

Variable	Univariate analysis			Multivariate analysis		
	HR[a]	95% CI[b]	P	HR	95% CI	P
Overall survival (n=524)						
Age (years)						
≥60 (n=282)						
<60 (n=242)	1.797	1.312–2.460	0.000	1.537	1.112–2.125	0.009
Gender						
Male (n=339)						
Female (n=185)	0.944	0.693–1.285	0.714			
T stage						
T3 or T4 (n=188)						
T1 or T2 (n=336)	3.115	2.302–4.217	0.000	1.580	1.101–2.266	0.013
N stage						
N1 (n=15)						
N0 or NX (n=509)	3.914	2.119–7.228	0.000	2.081	1.094–3.961	0.026
M stage						
M1 (n=78)						
M0 or MX (n=446)	4.300	3.153–5.863	0.000	2.693	1.880–3.860	0.000
G grade						
G3 or G4 (n=280)						
G1 or G2 or GX (n=244)	2.610	1.863–3.657	0.000	1.700	1.181–2.447	0.004
USP2						
High (n=262)						
Low (n=262)	0.572	0.419–0.781	0.000	0.618	0.449–0.850	0.003

[a]Hazard ratio, estimated from Cox proportional hazard regression model. [b]Confidence interval of the estimated HR. [c]Multivariate models were adjusted for T, N, M, G classification, age, gender and USP2 mRNA level.

(AUC =0.5604, P=0.1771), (T1 + T2) *vs.* (T3 + T4) stage (AUC =0.5804, P=0.0021), OS living *vs.* deceased status (AUC =0.6372, P<0.0001), non-recurrence *vs.* recurrence (AUC =0.6241, P<0.0001), OS-good *vs.* OS-poor (AUC =0.6157, P=0.0048), DFS-good *vs.* DFS-poor (AUC =0.6014, P=0.0214) (*Figure 4B,C,D,E,F,G,H,I*).

In order to further investigate the clinical value of USP2 in ccRCC, we compared it with existing markers. The three main predictive and prognostic markers validated in RCC are Von Hippel Lindau (VHL), vascular endothelial growth factor (VEGF) and carbonic anhydrase IX (CAIX) (25,26). We analyzed datasets from the public database, TCGA, for differential expression of VHL, VEGFA, CAIX, and assessed their prognostic and diagnostic value by Kaplan-Meier curves and ROC curves (*Figure S4A,B,C,D,E,F,G,H,I*). Results showed USP2 expression had similar diagnostic value, with an AUC of 0.8888, compared with VHL (AUC =0.8649, P<0.0001), VEGFA (AUC =0.9642, P<0.0001), CAIX (AUC:0.9688, P<0.0001). However, no association was found between OS time and mRNA levels of VHL (P=0.5597), VEGFA (P=0.6210), CAIX (P=0.1329). Therefore, in comparison to these existing genes, USP2 has a comparative advantage in predicting the prognosis of ccRCC patients by its mRNA levels. Moreover, USP2 was found to be related to CAIX (P<0.0001) and VHL (P=0.0214), but not with VEGFA (P=0.2961) (*Figure S4J,K,L*). In conclusion, quantification of USP2 gene expression, coupled with a crucial diagnostic

Table 3 Univariate and multivariate analysis of USP2 mRNA level and patient disease-free survival

Variable	Univariate analysis			Multivariate analysis		
	HR[a]	95% CI[b]	P	HR	95% CI	P
Disease-free survival (n=425)						
Age (years)						
<60 (n=216)						
≥60 (n=209)	1.398	0.980–1.996	0.065	1.506	1.030–2.203	0.035
Gender						
Male (n=281)						
Female (n=144)	1.428	0.961–2.122	0.078			
T stage						
T3 or T4 (n=139)						
T1 or T2 (n=286)	4.594	3.180–6.636	0.000	1.952	1.280–2.975	0.002
N stage						
N1 (n=12)						
N0 or NX (n=413)	5.981	3.002–11.913	0.000	3.385	1.611–7.109	0.001
M stage						
M1 (n=51)						
M0 or MX (n=374)	8.616	5.935–12.506	0.000	5.370	3.523–8.184	0.000
G grade						
G3 or G4 (n=214)						
G1 or G2 or GX (n=211)	3.446	2.282–5.203	0.000	2.421	1.580–3.709	0.000
USP2						
High (n=212)						
Low (n=213)	0.597	0.414–0.861	0.006	0.670	0.460–0.977	0.037

[a]Hazard ratio, estimated from Cox proportional hazard regression model. [b]Confidence interval of the estimated HR. [c]Multivariate models were adjusted for T, N, M, G classification, age, gender and USP2 mRNA level.

and prognostic value, could be used as an important diagnostic marker and an independent factor for ccRCC patients, in addition to VHL, VEGFA and CAIX.

Overexpression of USP2 significantly represses proliferation, migration, and invasion of ccRCC

To investigate the role of USP2 in carcinogenesis and progression of ccRCC, we used Gene Set Enrichment Analysis (GSEA), based on the TCGA database, and revealed the biological pathways regulated by the gene. We found a high association between USP2 with signatures of cellular respiration signaling (NSE =2.3989558, NOM P=0, FDR q=0), cyclin-related signaling (NES =1.5381801, NOM P=0.029, FDR q=0.131) and energy homeostasis (NES =1.4738845, NOM P=0.035, FDR q=0.173), which proved to be the basis of cell growth and survival (*Figure 5A*,*B*,*C*). Based on these findings, we speculated that dysregulation of USP2 in ccRCC might have an important impact on ccRCC progression. To test this hypothesis, we overexpressed USP2 by transfecting plasmids into CAKi-1 and A498 cell lines (*Figure 5D*,*E*). We observed that USP2 overexpression significantly repressed the proliferation rates of A498 and CAKi-1 cells (*Figure 5F*,*G*). We then assessed migration and invasion abilities of ccRCC cells, hallmarks of tumor progression, using Transwell assays and found a significant inhibition of these processes in cells overexpressing USP2 (*Figure 5H*,*I*). Overall, proliferation, migration, and invasion of ccRCC cells *in vitro* were repressed by USP2 overexpression.

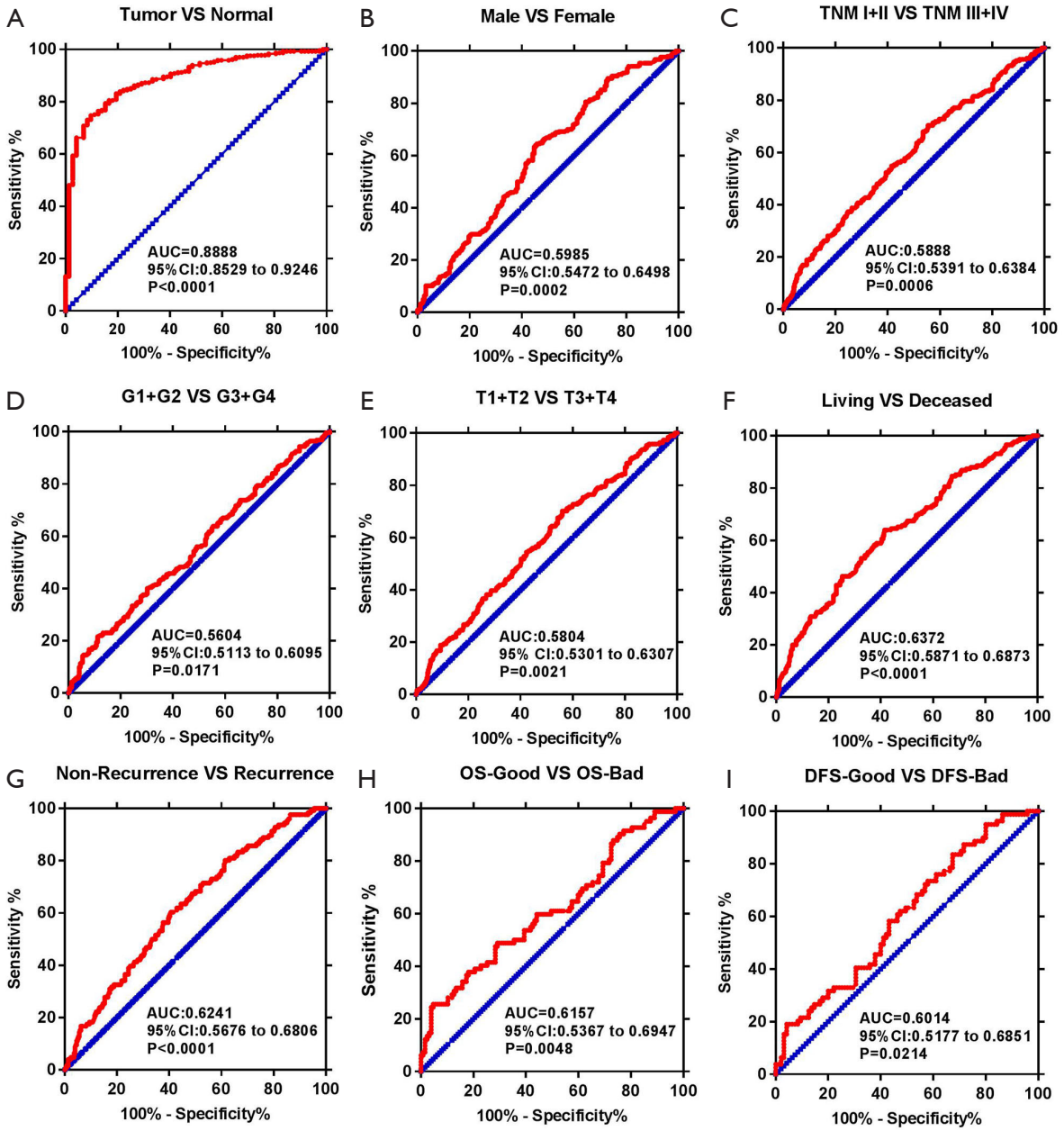

Figure 4 Diagnostic value of USP2 mRNA expression in ccRCC patients. (A) ROC curve showing that USP2 could effectively distinguish ccRCC from para-cancer tissues. ROC curve analysis towards the expression of USP2 mRNA in subgroups of ccRCC patients against: (B) male *vs.* female, (C) TNM I + II *vs.* TNM III + IV, (D) G1 + G2 *vs.* G3 + G4, (E) T1 + T2 *vs.* T3 + T4, (F) living *vs.* deceased, (G) non-recurrent from recurrent, (H) OS-good from OS-poor, (I) DFS-good from DFS-poor.

Discussion

Numerous efforts have been devoted to the research of genomics, transcriptomics, and proteomics in the malignant tumors (27), especially RCC. Increased understanding of the underlying molecular pathways involved in pathogenesis of RCC has contributed to development of targeted therapies (28). Currently, molecular-targeted therapies are becoming a possible option for patients with unresectable RCC (29). For instance, the targeted agents mainly refer to inhibition of VEGFR, mTORC1, c-MET

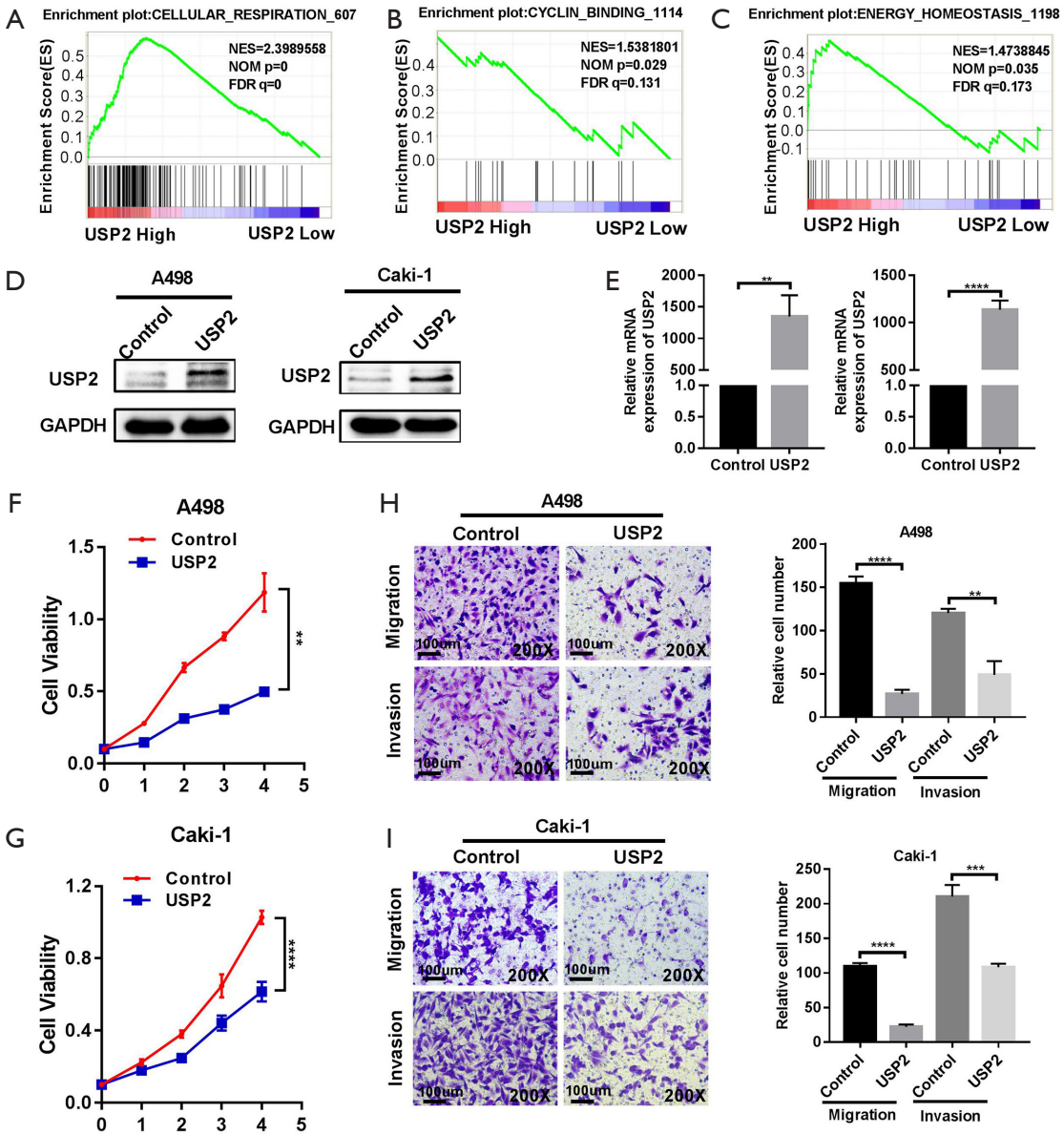

Figure 5 Overexpression of USP2 represses proliferation, migration, and invasion of ccRCC *in vitro*. (A,B,C) GSEA was performed to investigate the biological pathways involved in USP2 regulation based on the TCGA database. (D,E) USP2 mRNA and protein expressions were successfully overexpressed in A498 and caki-1 cells. (F,G) CCK-8 assays showing cell growth curves of A498 and CAKi-1 cells. (H,I) Representative images of migration and invasion assays performed using A498 and CAKi-1 cells; the number of cells was counted in ten randomly selected images from each group. **P<0.01; ***P<0.001; ****P<0.0001.

and FGFR, cytokines and PD1/PDL1 immune checkpoint inhibition (27). However, most ccRCC patients still develop poor clinical progression due to low complete response rates and high drug resistance. Meanwhile, despite advanced imaging techniques have greatly improved the early detection of ccRCC patients, characterization of small renal masses and residual tumor remains a diagnostic conundrum (30). Unfortunately, reliable biomarkers and prognostic indicators for better predicting disease course are still lacking. USP2 has emerged as an oncology target in many solid malignancies, and its therapeutic potential is attracting increasing interest. However, the role of this

target in renal cell carcinoma has not been elucidated. In the current work, therefore, we focused this crucial cell-growth-related gene, which is also closely correlated to cellular respiration and energy homeostasis in ccRCC cell lines. We explored its role in occurrence and progression of ccRCC, through bioinformatics analysis, and assessed its importance as a diagnostic biomarker and an independent prognosis factor for ccRCC patients. In addition, we also analyzed the GeneCards and HPA RNA-seq normal tissues from PubMed in order to evaluate the value of USP2 in liquid biopsy. Results showed that the systemic expression of USP2, including in blood and other body fluids, might represent a potential biomarker in liquid biopsy (*Figure S5*).

Previous studies have described USP2's aberrant expression in different tumors as well as the modification functions it plays in regulation of signaling pathways (9,31). Particularly, USP2 is highly expressed in prostate (19), and breast cancer (32), gliomas (33), and low lowly in pancreas, colon and bladder cancer (34). For instance, compared with non-muscle-invasive bladder cancer, the expression of USP2a gene in muscle-invasive bladder cancer was found to be decreased by 36.3% (34). To date, this is the first time USP2 has been detected, shown to be lowly expressed and playing a tumor-suppressive effect in kidney cancer. Previous studies show that USP2 mainly involved in the regulation of the p53 and TNF-α/NF-κB signaling pathways. It is universally acknowledged that tumor suppressor protein, p53, which is mutated in more than 50% of solid tumors (35), plays an important role in tumor inhibition. By stabilizing the activity of MDM2 and MDMX, USP2a remarkably regulated the p53 pathway, thereby significantly influencing cell survival (20,36). In addition, NF-κB has been characterized as a crucial activator of anti-apoptotic genes during TNF-α signaling and is a vital regulator during cellular processes (37,38). Targeting the NF-κB signaling pathway, therefore, may be an effective method for treatment of carcinomas and inflammatory diseases (39). USP2 was identified to positively regulate TNF-α/NF-κB signaling, with its downregulation dramatically inhibiting NF-κB activity in breast carcinomas (40). Therefore, we hypothesized that downregulation of USP2 in clinical samples as well as cell lines, was likely to affect cell migration, invasion, and proliferation through regulation of the aforementioned signaling pathways in ccRCC cases.

The human genome encodes approximately 100 deubiquitinating enzymes (DUBs), 79 of which are predicted to be active (41). To date, DUBs have been shown to be highly important in regulating cell survival and apoptosis, and are therefore regarded as candidate target strategies for medical treatment (5). USP2, a DUB that removes Ub-chains thereby stabilizing a range of substrates such as cyclin A1 (8), cyclin D1 (9), Aurora-A (42), RIP1 and TRAF2 (22,23), has been implicated in various neoplastic and non-neoplastic diseases. In addition, ccRCC is well known for mutation of the von Hippel-Lindau (VHL) tumor suppressor gene (43) and subsequent disorder of hypoxia-inducible factor (HIF) (44). Targeting the VHL/HIF pathway in advanced ccRCC, is therefore, the new therapeutic strategy and has partially been clinically successful (45). According to the findings of this study, we hypothesized that USP2 might play a regulatory role in the VHL/HIF pathway by regulating deubiquitination in ccRCC. However, the specific mechanism of action between USP2 and the pathway in ccRCC, as well as *in vivo* studies and knockout experiments, were not investigated and therefore forms the basis of our further research.

Since DUBs regulate numerous key pathways in cancer cells and are potentially druggable, interests in developing DUB and their inhibitors as antitumor drugs have substantially increased (24,46). The crystal structures of USP2 have been resolved in previous studies, providing a basis for future works that aim to elucidate the molecular recognition of its activities (47,48) and the potential druggable values. Despite considerable progress in the study of ubiquitin conjugation, research on DUBs is still in its infancy. Nowadays, increasing attention on DUB activator have been gained (49). In conclusion, an understanding of the functions and mechanisms of USP2 action might contribute to the discovery of novel molecular-based target therapies and that could develop a cure for ccRCC patients.

Acknowledgments

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of Science and Technology.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Review Board of Huazhong University of Science and Technology (S065).

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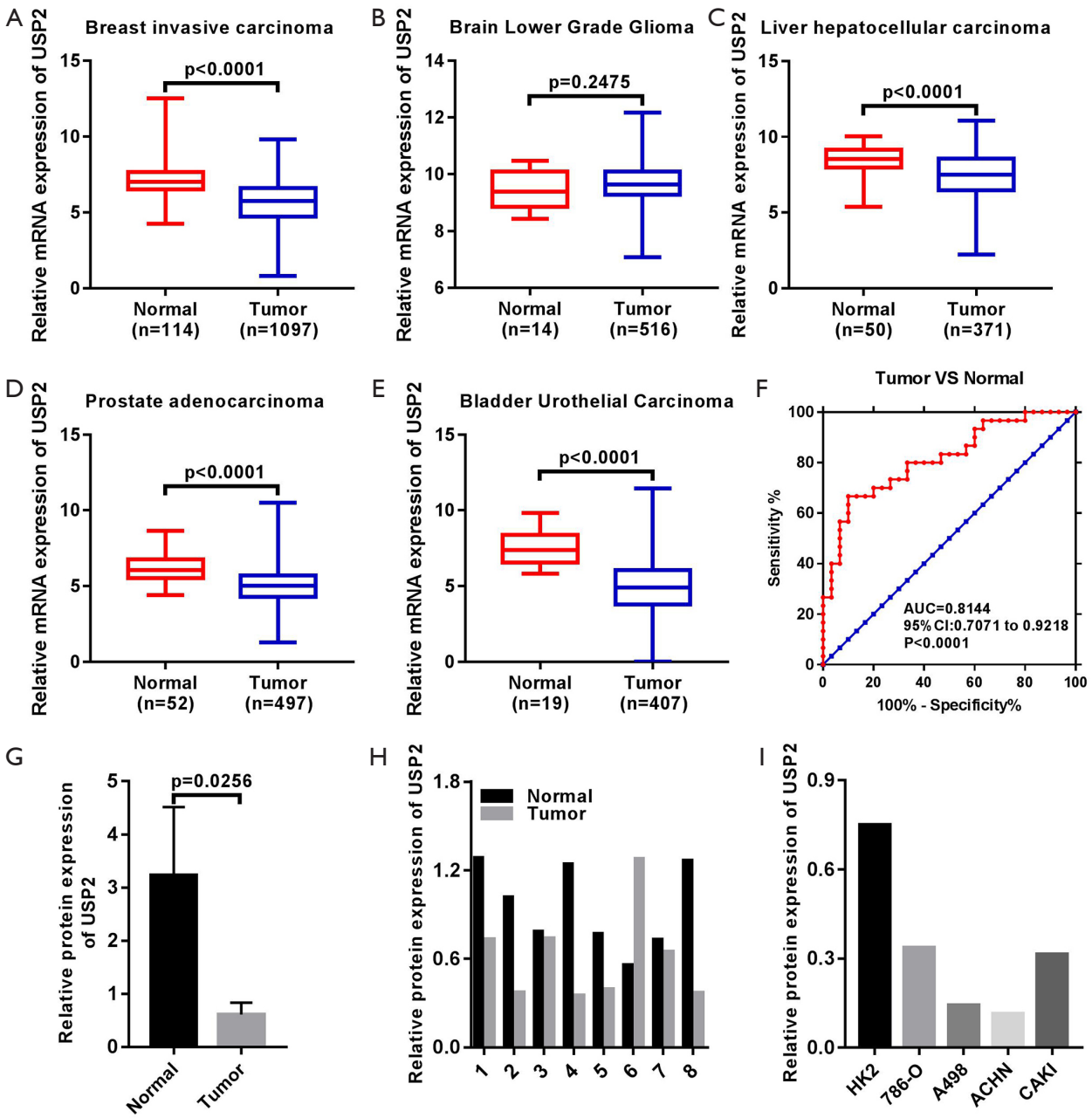


Figure S1 Expression profiles of USP2 mRNA in other tumors and quantitative analysis on USP2 in ccRCC. (A,B,C,D,E) Expression profiles of USP2 mRNA in other tumors, including breast invasive carcinoma, brain lower grade glioma, liver hepatocellular carcinoma, prostate adenocarcinoma and bladder urothelial carcinoma, were downloaded from TCGA datasets and analyzed. (F) ROC curve based on USP2 mRNA expression in 30 pairs of clinical tissues was analyzed. (G,H,I) Quantitative analysis on the results of immunohistochemistry and western blot.

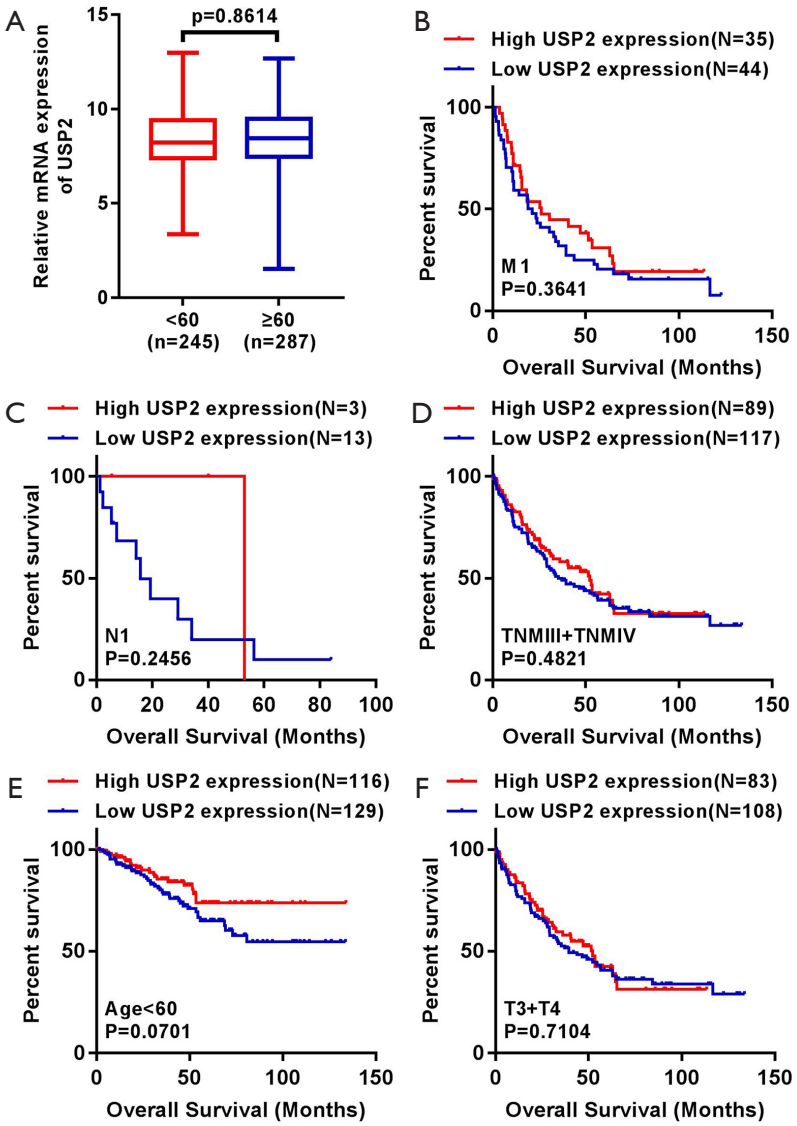


Figure S2 Downregulation of USP2 correlated with age and overall survival times of ccRCC patients. (A) USP2 mRNA levels were compared in the clinicopathological variable: Age <60 vs. Age ≥60. Overall survival analysis toward the expression of USP2 mRNA was performed in subgroups of ccRCC patients: (B) M1, (C) N1, (D) TNM III + TNM IV, (E) Age <60 years, (F) T3 + T4.

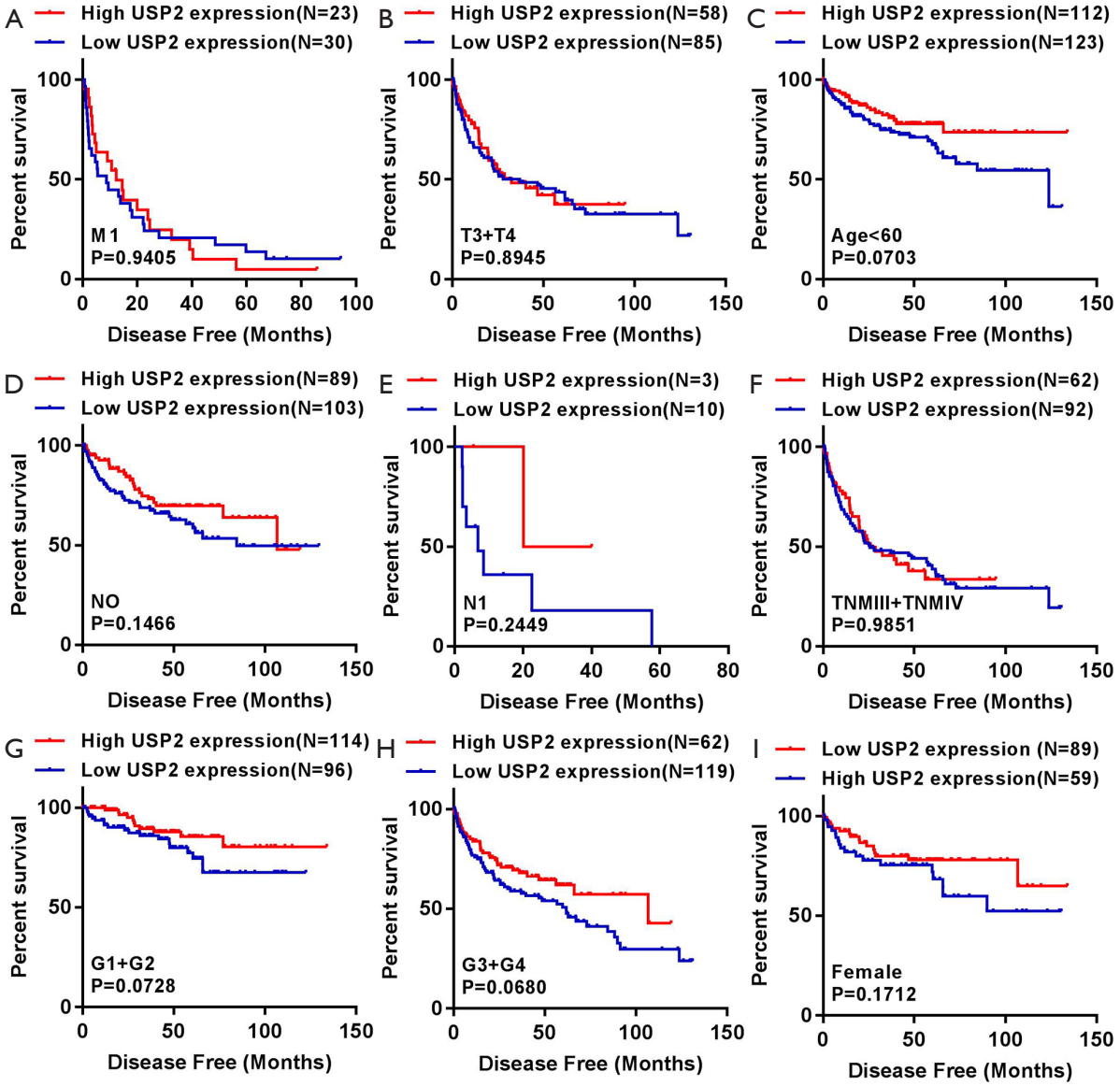


Figure S3 Downregulation of USP2 correlated with disease-free survival times of ccRCC patients. The correlation between USP2 expression and DFS time for ccRCC patients analyzed by Kaplan-Meier. DFs analysis toward the expression of USP2 mRNA was performed in subgroups of ccRCC patients: (A) M1, (B) T3 + T4, (C) Age <60 years, (D) N0, (E)N1, (F) TNM III+TNM IV, (G) G1 + G2, (H) G3 + G4, (I) female.

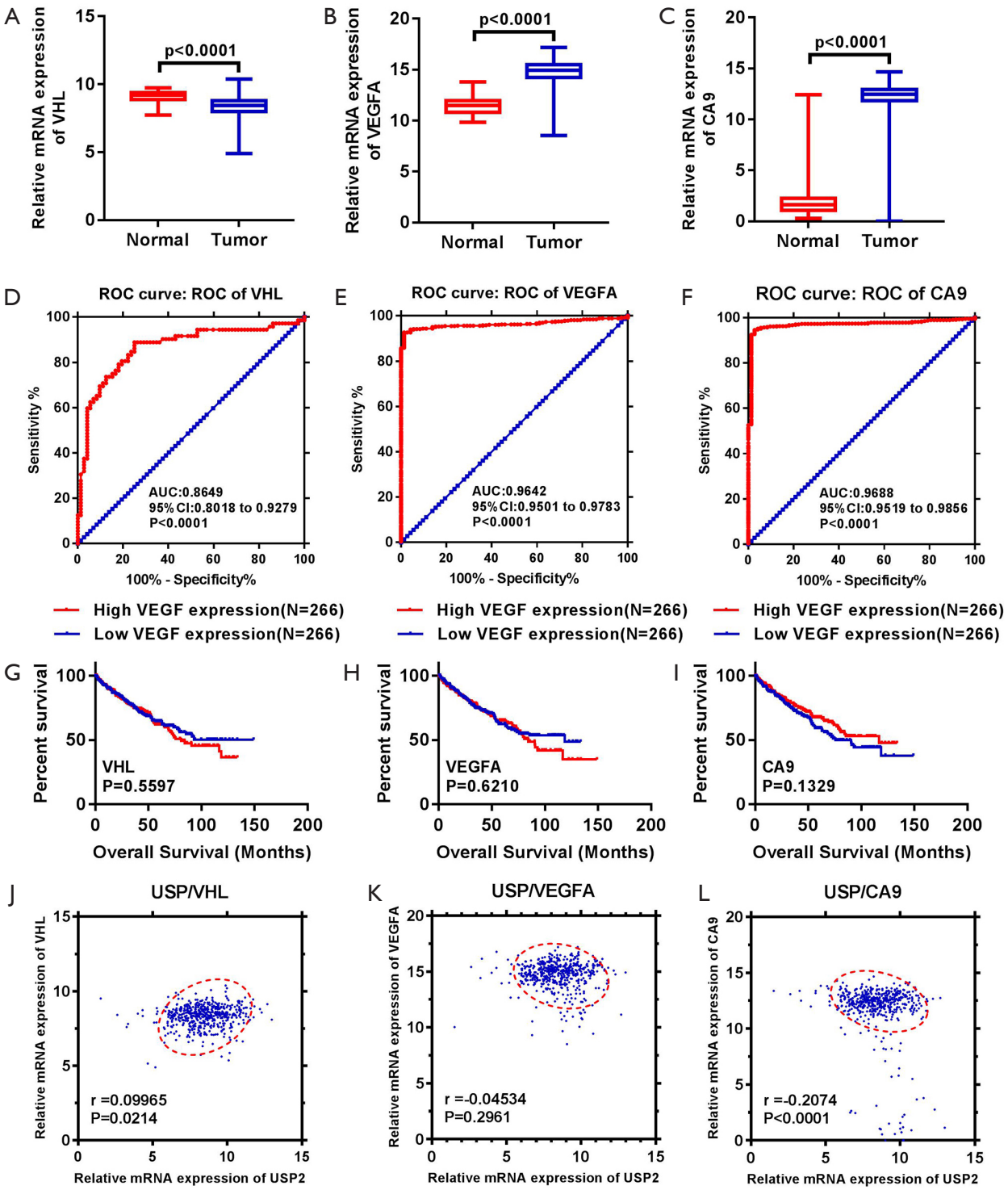
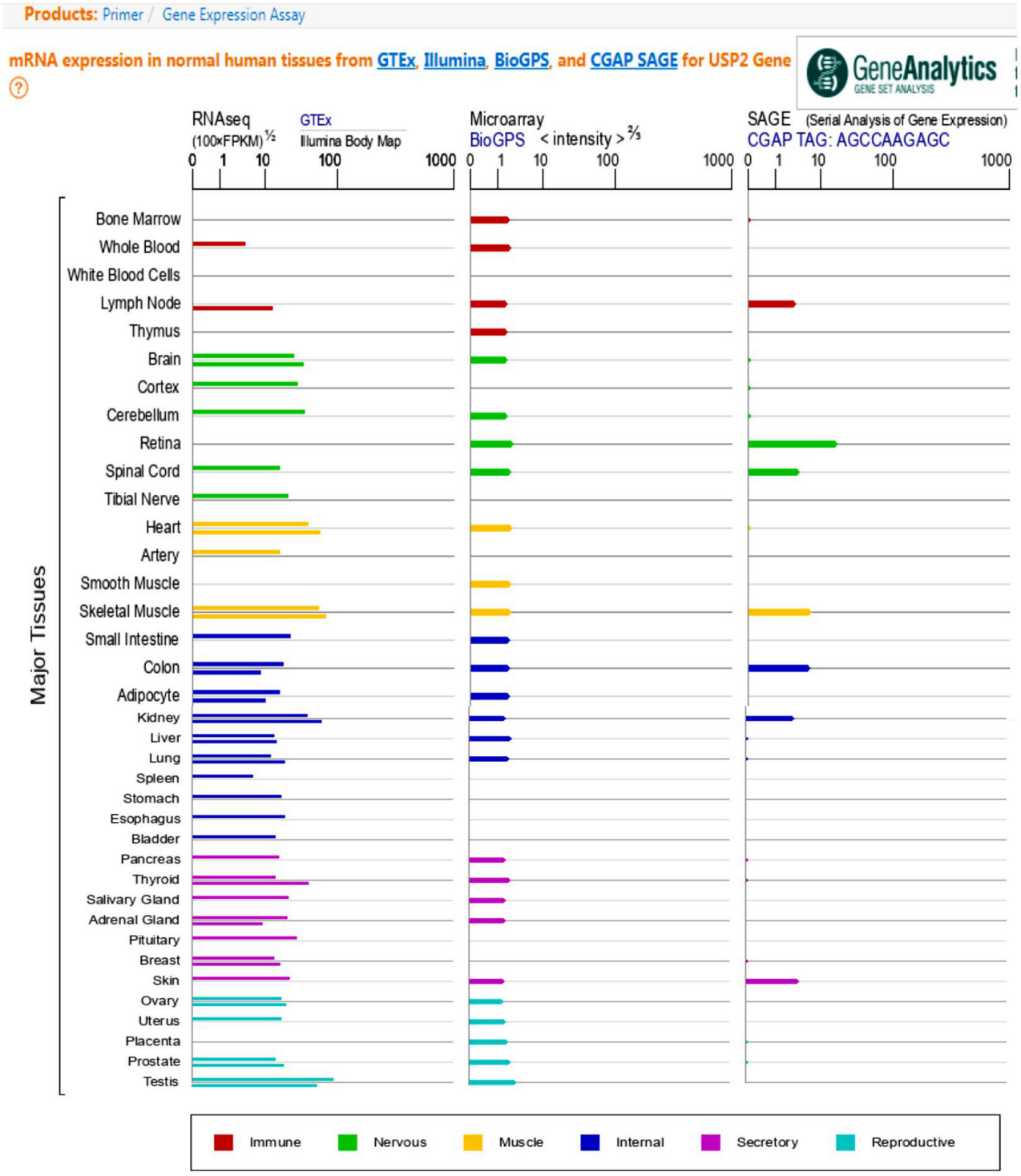


Figure S4 Comparison between USP2 with the three existing markers: VHL, VEGF and CAIX. Expression profiles of VHL, VEGFA, CAIX mRNA were downloaded from TCGA datasets and analyzed. (A,B,C) Differential expression of VHL, VEGFA, CAIX in ccRCC patients. (D,E,F) Diagnostic value of VHL, VEGFA, CAIX were investigated by ROC curves. (G,H,I) The correlation between VHL, VEGFA, CAIX mRNA expression and OS time for ccRCC patients analyzed by Kaplan-Meier. (J,K,L) The correlation between VHL, VEGFA, CAIX mRNA expression, and USP2 mRNA expression.



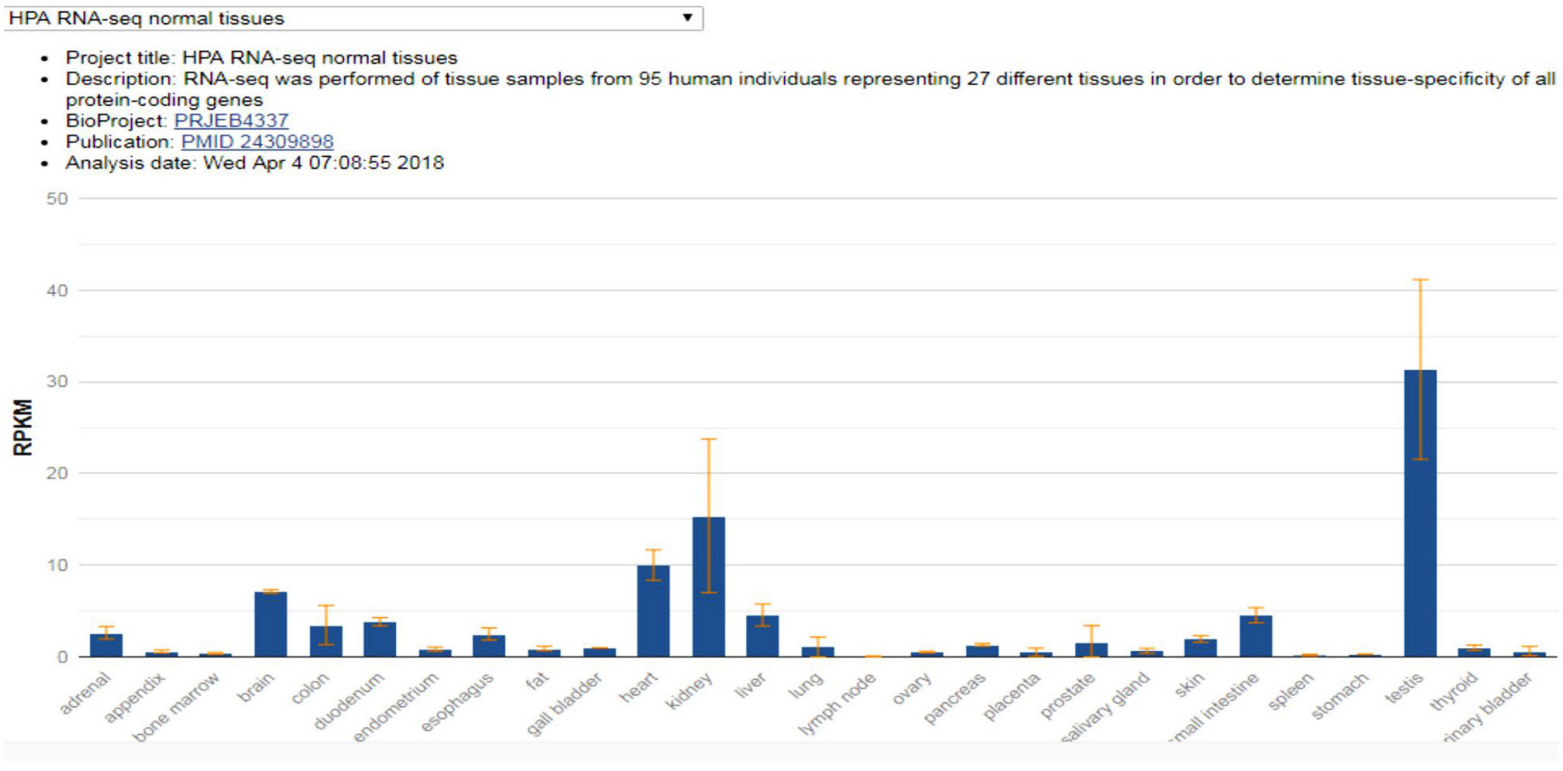


Figure S5 Potential value of USP2 in liquid biopsy. The expression of USP2 in blood and other body fluids was explored by analyzing the GeneCards and HPA RNA-seq normal tissues from PubMed.