Ubiquitin-specific protease 44 inhibits cell proliferation and migration via inhibition of JNK pathway in clear cell renal cell cancer

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Abstract

Background: Clear cell renal cell carcinoma (ccRCC) is the most common form of adult kidney cancer. USP44 has been reported to be involved in various cancers. This study aimed to investigate the function role and molecular mechanism of USP44 in ccRCC.

Methods: Data obtained from TCGA data portal and GSO database were analyzed to uncover the clinical relevance of USP44 expression and tumor development. The function of USP44 in cell proliferation and migration was assessed by cellular and molecular analysis.

Results: USP44 was lowly expressed in the ccRCC cancer tissues compared to the normal tissue. Further, USP44 expression was negatively correlated with tumor stage, tumor grade, and patient survival. USP44 overexpression significantly inhibited tumor cell proliferation and migration of 786-O cell as well as Caki-1 cell. In addition, USP44 overexpression also prohibited cell proliferation by up-regulating P21, down-regulating Cyclin D1 expression, and inhibited cell migration by up-regulating MMP2 and MMP9 expression. In contrast, USP44 knockdown enhances ccRCC cell proliferation and migration. Furthermore, the USP44 function in inhibiting ccRCC cell proliferation and migration is associated with the phosphorylation level of JNK.

Conclusion: In summary, this study showed that USP44 may be a marker in predicting the ccRCC progression and USP44 inhibits ccRCC cell proliferation and migration dependent on the JNK pathway.

Background
Renal cell cancer is the most common form of adult kidney cancer, representing between 80 and 90% of kidney cancers. The incidence of renal cell cancer varies geographically, with the highest incidence in developed countries [1]. Based on the recent guideline, the most effective treatment for ccRCC in early stage is surgical operation and targeted therapy [2]. Unfortunately, the major cause of death for most ccRCC patients is tumor metastasis and recurrence [3]. At this stage, many new biomarkers have been explored to diagnose and predict the occurrence and development of ccRCC[4-6].

Chromosomal instability, leading to aneuploidy, is one of the hallmarks of human cancers [7]. Ubiquitin-specific protease 44(USP44), which is located at 12q22 and encodes a 712 amino acid, is a member of the deubiquitinating enzyme family and plays an important role in human tumor diseases[8]. USP44 is an important regulator of the mitotic checkpoint by regulation of centrosome separation, positioning and mitotic spindle geometry[9]. USP44 can stabilize the protein level of the protectin in the normal cell cycle process until all the chromosome match correctly with the spindle fibers and prevent immature mitosis. By inhibiting the expression of USP44 in mouse, the proportion of aneuploid cells and the chromosomal instability could be significantly increased, making it more prone to malignant transformation [10, 11]. However, recent study raised a controversy about the effect of USP44 in tumor, some study showed that USP44 overexpression promotes the malignancy of glioma by stabilizing tumor-promoter securing [12].

However, the function and mechanism of USP44 in ccRCC have not been clarified. In order to better illustrate the role of USP44 in ccRCC and its mechanism, we have demonstrated it through many cell experiments and database research methods.
Methods

Reagents

The antibodies against Flag (M185–3L) and β-actin (M177–3) were purchased from Medical Biological Laboratories. The antibodies against MMP2 (A11144) and MMP9 (A0289) were purchased from ABCLONAL. The antibodies against P21(2947), Cyclin D1(2978), p-JNK (4668), JNK (9252), p-AKT (4060), AKT (4691), p-p38 (4511), p38(9212), p-ERK (4370) and ERK (4695) were purchased from Cell Signaling Technology.

JNK inhibitor(iJNK): JNK-IN–8 (HY–13319, MCE), dissolved in DMSO and diluted into 0.5 μM work solution with complete culture medium, and the same amount of DMSO was set as the control.

Bioinformatics analysis

The experiment was performed in accordance with previous studies [13]. ccRCC’s gene sequence tertiary count data samples and clinical information are obtained through TCGA data portal. R-package DESeq2 is mainly used to standardize counting data and analyze differentially expressed genes between cancer samples and normal samples. Standardized data are mainly used to analyze the visual expression level, stage, grade and survival correlation of USP44 in ccRCC and adjacent non-cancerous tissues. According to the expression level of USP44, the clinical samples of ccRCC were divided into two groups for analysis and discussion. Kaplan-Meier survival curves were used to show the overall survival differences between USP44 higher-expression-level patients and USP44 lower-expression-level patients. At the same time, we correspondingly calculated the correlation between USP44 expression level and patient’s age, gender, tumor stage and grade through T-
text, and the obtained data were visualized through R-package ggplot2.

Cells

Human renal clear cell carcinoma 786-O cell line was purchased from BeNa culture collection (ATCC CRL–1932), and the cells were cultured in DMEM culture medium (Gibco; C11995500BT) [14]. The Caki-1 cell line, a skin metastatic cell of human renal clear cell carcinoma was purchased from the Chinese Academy of Sciences cell bank (TCHu135) and was cultured in McCoy’s 5A culture medium (BasalMedia; L630) [15]. Both DMEM culture medium and McCoy’s 5A culture medium were added with 10% fetal bovine serum FBS (Bio-one Biotechnology, F05–001-B160216), 100 U/mL penicillin, and 100 μg/mL streptomycin. 786-O and Caki-1 were cultured in a humidified environment at 37℃ containing 5% carbon dioxide.

Lentivirus of overexpression USP44 and shRNA USP44 lentivirus construction and production

The overexpression vector with flag tag and shRNA vectors of USP44 (homo) gene were designed and constructed according the classical method [13]. The gene registration number is NM_001347937.1. The pHAGE–3xflag carrier name was used as the carrier. The primers were h-USP44-NF, AAACGATTCAGGTGGTCAGG, h-USP44-NR, AGTGTACCCAGAACCCTCCT. The sequence of pLKO.1-h-USP44-shRNA1 is CGGATGATGAACTTGTGCAAT, and pLKO.1-h-USP44-shRNA2 is GCACAGGAGAAGGATACTAAT.

CCK8 assay

Cell viability was examined using a CCK–8 kit following the manufacturer’s protocol [13]. 786-O cells and Caki-1 cells were inoculated in 96-well plates. After cells were adhered to the plate, cells were further cultured for 0h, 12h, 24h, 36h, 48h, 60h, respectively. After that, 10μL CCK8 reagents (Dojindo; 44786) were added. Then,
the absorbance value at 450nm was measured.

*BrdU experiment*

BrdU experiment was performed according the manufacturer’s instruction [13]. 786-O cells and Caki-1 cells were inoculated in 96-well plates (Thermo; 167008). After 24h and 48h, BrdU experiment was performed with BrdU kit (Roche; 11647229001).

*Wound healing test*

786-O cells and Caki-1 cells were inoculated into 6-well plates with $3 \times 10^5$ per well (Thermo; 140675) and both cells were incubated overnight for wounding. After that, the original culture medium was replaced with DMEM culture medium with 10 ug/mL mitomycin. Then cells were cultured for 12 hours. Wounds were made with a pipette tip and photographs taken immediately (0h) and 6h and 12h, after wounding for 786-O cell and Caki-1 cell, respectively. The cell migration index was measured. Migration index = (width of the wound at 0 hour-width of the wound at 6 or 12 hours) *100/width of the wound at 0 hour.

*Transwell migration assay*

The 786-O cells and Caki-1 cells in good condition were resuspended with DMEM or McCoy’s 5A culture medium and plated with $3 \times 10^4$ cells/well or $5 \times 10^4$ cells/well in the upper compartment of the Transwell chamber (corning;3421), meanwhile DMEM culture medium containing 2% 600μL FBS or 10% 600μL FBS was added in the lower chamber, respectively. The cells were cultured for 2h–3h(786-O cell) or 10h–24h(Caki-1 cell), with PBS cleaning twice. 600μL 4% paraformaldehyde solution were used to fixed cells at room temperature for 15 min, and 0.1% of the 600μL crystal violet (characters; 548–62–9) were used to dyed cells for 2h at 37 °C. The image was acquired under microscope. The number of positive stain cells was to
reflect the cell migration ability.

**Western blot**

The proteins were respectively extracted from the 786-O and Caki-1 cells according to standard protocols. Meanwhile, protease inhibitors (Roche; 04693132001) and phosphatase inhibitors (Roche; 4906837001) were added. Protein concentrations were determined using a BCA Protein Assay kit (Thermo Fisher Scientific; 23225). In brief, we separated the protein samples on 12.5% SDS-PAGE and then transferred them to a nitrocellulose membrane. We blocked the membrane using 5% nonfat dry milk in a TBS-T buffer and the incubated it overnight at 4°C with primary antibody. After rinsing the blots extensively with TBS-T buffer, secondary antibodies with incubated them for 1 hour. We applied Bio-rad Chemi Doc XRS+ gel imaging system to detect the target bands.

**RT-PCR**

Total mRNA of 786-O and Caki-1 cell lines were extracted with TRIzol reagent (Invitrogen; 15596-026), which were then reverse-transcribed into cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche; 04896866001) according to the manufacturer’s instructions. SYBR Green (Roche; 04887352001) was used to quantify the PCR amplification products. The mRNA expression level of target genes was normalized to β-actin expression.

**Statistical Analysis**

All data were presented as the mean ± standard error. We used SPSS 19.0 for all statistical analysis. The student’s t test was used to analyze all data, with P values less than 0.05 as the threshold for statistically significant differences.

**Results**
USP44 expression is deceased in the ccRCC tissue and is correlate with the tumor stage, grade and survival

In order to find out whether USP44 is involved in the progression of ccRCC, analysis of information from the TCGA data portal demonstrated that USP44 expression was significantly lower in ccRCC specimens than in normal tissues (Fig.1A). Data analysis from the GSE102101 database also confirmed this result (Fig.1B). Subsequently, a subgroup analysis was performed based on tumor stage and grade. The results showed that USP44 was closely related to tumor stage and grade (Fig.1C and D). With the increment of stage and grade, USP44 expression level showed a gradual decrease. Finally, we determined that USP44 expression level was closely related to patient survival (Fig.1E). Based on these results, USP44 might be a potential marker to predict ccRCC progression and play an important role in progress of ccRCC.

USP44 overexpression inhibits ccRCC cell proliferation

In this part, we intend to explore the effect of USP44 in vitro. Because 786-O cells and Caki-1 cells show different metastatic and invasive abilities in ccRCC model, we chose these two cell lines for experiments. The overexpressed stable cell lines were obtained by viral infection of USP44 in these two tumor cell lines (Fig.2A-D). The viability and proliferation potential of cells can be evaluated through CCK8 and BraU experiments. As shown in Fig.2E and 2F, in comparison with negative controls, we found that the overexpression of USP44 significantly inhibited the cell viability of the two cell lines. In order to further explore the direct influence of USP44 on ccRCC proliferation, we labeled proliferation cells with BrdU in overexpressed USP44 and control cells. As shown in the results of Fig.2G and Fig.2H, USP44 overexpression significantly reduced the BrdU absorption capacity of the two cell lines, which
confirmed that USP44 can inhibit the proliferation of ccRCC. In addition, previous studies have shown that Cyclin D1 and P21 are closely related to the occurrence of tumors and are markers of tumor proliferation [16, 17]. The main function of cyclin D1 is to promote cell proliferation by regulating cell cycle, which is closely related to the occurrence of tumors and is a marker of tumor proliferation including ccRCC [18], while P21 is closely related to tumor inhibition and can coordinate the relationship between cell cycle, DNA replication and repair by inhibiting the activity of cyclin-dependent kinases CDKs complexes[19]. Thus, we also found that USP44 expression is positively correlated with P21 gene and protein expression, and negatively correlated with Cyclin D1 gene and protein expression. (Fig.2I-L) Taken together, these results demonstrated that USP44 inhibited the proliferation of ccRCC.

**UPS44 overexpression inhibits the ccRCC cell migration**

In order to investigate whether USP44 overexpression inhibited the migration of ccRCC cells, we conducted a series of experiments. We first used Transwell experiment to evaluate USP44 overexpression on cell migration. Through Transwell experiment (Fig.3A and 3B), we found that USP44 overexpression significantly slowed down the migration of tumor cells, which was consistent with our expectation. At the same time, because the two tumor cells have different migration abilities, the results showed that USP44 overexpression slowed down the migration ability of 786-O cells in the early stage (2h, 3h), and slowed down the migration ability of Caki-1 cells in the late stage (10h, 24h). Then, we performed the wound healing experiment to confirm the migration effect of USP44. In order to avoid the effect of cell proliferation on cell migration, mitomycin pretreatment was performed prior the wound healing experiment. Through wound healing experiments, it can be
demonstrated that the overexpression of USP44 significantly slows down the migration ability of 786-O cells and Caki-1 cells [Fig.3C and 3D]. MMP2 and MMP9 are members of the matrix metalloproteinase (MMP) gene family, zinc-dependent enzymes that clew components of the extracellular matrix and molecules involved in signal transduction. MMP2 and MMP9 are closely related to the tumor blood vessels formation, growth and metastasis [20]. MMP2 and MMP9 are often recognized as the markers of ccRCC cell migration and metastasis [21]. At the molecular level, we also found that USP44 overexpression down-regulated the mRNA and protein expression of MMP2 and MMP9 in two cell lines (Fig.3E-H). Collectively, these results confirmed that USP44 inhibits the cell migration in ccRCC.

**UPS44 knockdown promotes the ccRCC cell proliferation and migration**

In order to further confirm whether knockdown USP44 have an opposite effect on proliferation and migration of ccRCC cells, we tried to verify USP44 role in tumor cells by silencing USP44 expression with shRNA. The two shRNA was constructed successfully to silence the USP44 expression in Caki-1 cell line (Fig.4A). Consistent with our expectation, it was found that USP44 knockdown significantly promoted cell proliferation by CCK-8 assay and Brdu experiment (Fig.4B and C). We also found that USP44 knockdown inhibited P21 expression and upregulated Cyclin D1 expression (Fig.4D and E). Then, cell migration assay was performed and the result showed USP44 deficiency promoted the ccRCC cell migration (Fig.4F), which is associated with MMP2 and MMP9 up regulation (Fig.4G and H). These results confirmed that USP44 knockdown enhance ccRCC cell proliferation and migration.

**USP44 suppresses JNK signaling pathway in ccRCC**

The AKT and MAPK signal pathway plays an important role in the occurrence and development of malignant tumors [22]. To explore the potential underling
mechanism by which USP44 regulated tumor cell proliferation and migration, we detected the activation of AKT, JNK, P38, ERK signal pathway in USP44 OE and Ctrl groups. The results showed that USP44 overexpression decreased the level of JNK but not AKT, p38 and ERK compared with control cells in both cell lines (Fig.4A and 4B). While JNK expression was promoted when USP44 expression was knockdown, but no effect on AKT, P38 and ERK expression (Fig.5C). The above results suggest that JNK signal transduction pathway participated in USP44 function of regulating ccRCC cell proliferation.

Promotional effect of USP44 knockdown on cell proliferation and migration is dependent on JNK pathway.

To further verify whether the USP44’s role in ccRCC progression is dependent on the JNK pathway, we blocked JNK activation via JNK inhibitor and examined cell proliferation and migration (Fig.6A). The results showed that the ability of USP44 knockdown to promote proliferation and migration was significantly reduced after treatment with JNK inhibitor, which proved that USP44 regulated proliferation and migration in ccRCC through JNK signaling pathway (Fig6.B, C).

Discussion

At present, many studies have demonstrated that the molecular mechanism of ccRCC is closely related to apoptosis, autophagy, hypoxia metabolism and immune imbalance [23]. However, the mechanism of pathogenesis and metastasis in ccRCC have not been fully elucidated.

The spindle assembly checkpoint (SAC) is an important mechanism to ensure the procedure of mitosis, while the abnormality of SAC is a key step to lead to the formation of aneuploidy cells and even tumor. The previous research reported that
the important regulatory proteins of the SAC deubiquitinase USP44 was closely associated with tumor [24].

In our study, we explored the tumor marker based on the information from the TCGA data portal and GSE 102010 database. The results showed that USP44 was low expressed in tumor tissues and correlated with pathological stage and degree of tumors. Patients with high USP44 expression showed good survival benefits. These results can conclude that USP44 maybe a good biomarker to predict the ccRCC progression.

A previous study has demonstrated that USP44 overexpression is closely associated with the colorectal neoplasia, gastric cancer and so on. However, there is a lot of controversy. Some studies suggest that USP44 overexpression promotes tumor development, while other studies suggest that USP44 inhibits tumor cell proliferation [10, 11, 25, 26]. Thus, we examined the USP44 effect in the proliferation of ccRCC. Therefore, two types of renal clear cell carcinoma cell lines were used. One is the classic renal clear cell carcinoma named 786-O, the other is the human renal clear cell carcinoma skin metastatic cell line named Caki-1. The results showed that the USP44 overexpression inhibited renal clear cell cancer proliferation in various methods. The genes associated with cell proliferation are also regulated by USP44 overexpression.

The metastatic potential of ccRCC is the main factor leading to the decrease in survival rate of recipients [27]. The treatment of metastatic ccRCC has changed greatly over the past years [28]. The USA FDA had approved agents to treat the metastatic ccRCC, which include immunotherapeutic drugs, antiangiogenic agents, and mTOR inhibitors [1, 29]. Nevertheless, even with these treatments, many patients with metastatic ccRCC have a very short survival term. We also
demonstrated that USP44 overexpression inhibited migration of tumor cells through wound healing test and Transwell chamber experiments.

MMP family are involved in the breakdown of extracellular matrix (ECM) in normal physiological processes, as well as in disease processes, such as metastasis [20]. MMP2 and MM9 are two typical representatives of MMP family, which are closely related to the invasion and metastasis of many tumors [30]. The result showed that USP44 overexpression in both cell lines also reminded that the ccRCC metastasis is related with MMP2 and MMP9 expression. Based on the result from Caki–1 cell with USP44 silence by shRNA, we also demonstrated that USP44 inhibits ccRCC progression in reverse.

At present, there is no evidence that the deubiquitination enzyme is directly involved in cell proliferation, apoptosis, autophagy and cell cycle, etc. Whether the deubiquitination enzyme play a role in promoting or inhibiting cancer is closely related to the function of its substrate protein [31]. The substrate molecules regulate many tumor-associated signaling pathways, including p53, NF-κB, Wnt, TGF-β, and histone epigenetic modifications, and these signaling pathways interact with each other. Up-regulation of a USP molecule in tumor cells often suggests that its substrate protein can promote the malignant progression of cancer cells [32]. The down-regulated expression of USP molecule is the opposite, and its substrate is usually tumor suppressor. Each USP has multiple substrates, and the same substrates may be regulated by multiple USPs [33]. Therefore, the regulatory network of USP on tumor cell signaling pathway is extremely complex.

PI3K/AKT is a serine/threonine protein kinase, which is involved with the tumorigenesis in many tumor including ccRCC[34]. When cells are stimulated by extracellular signals, PI3K activates AKT activity and AKT further activates its
downstream factor mTOR. The MAPK signal transduction pathway plays a crucial role in the occurrence, development, treatment and prognosis of malignant tumors[35]. The downstream signaling pathway includes the JNK, ERK and P38, which are associated with the tumor cell growth and proliferation[36]. AKT-JNK/P38/ERK has been proved with tumor progress in lung cancer and pancreatic cancer[34, 37]. In this study, we detected the protein activity of JNK, AKT, ERK and p38, and the results showed that USP44 inhibits JNK pathway but not AKT, ERK and p38. The rescue experiment showed silencing USP44 expression to promote tumor cell proliferation and migration can be blocked by JNK inhibitor. Of course, we can’t ignore that JNK activation in USP44 knock down may be a result of stress response activation due to chromosome mis-segregation as previously reported in the literature[38]. The ubiquitin proteasome system regulates oncogenic factors post-transcriptionally at the epigenetic level. Existing experiments have shown that important tumor-related factors such as EGFR, sarbox-2, c-myc, and McL-1 are regulated by USP. However, little is known about the catalytic substrates of USP44. In current study, overexpression of USP44 promoted the malignancy of glioma by stabilizing tumor-promoter securing[12]. USP44 induced the tumorigenesis of prostate cancer cells partly by stabilizing EZH2[39]. Therefore, further studies are needed to determine whether USP44 regulates the promoter or tumor suppressor in ccRCC cells.

Conclusion

In conclusion, our results demonstrated that USP44 was under expressed in ccRCC, and USP44 overexpression significantly inhibited cell proliferation and migration. JNK pathway is involved in the function of USP44 in the regulation of ccRCC cell.
proliferation and migration.

Abbreviations

AKT, protein kinase B; ECM, extracellular matrix; ccRCC, clear cell Renal Cell Cancer; OE, over expression; MMP, matrix metalloproteinase; SAC, spindle assembly checkpoint; USP44, Ubiquitin-specific protease 44.

Declarations

• Ethics approval and consent to participate
All the animal experiments were proved by ethic committee of Wuhan University Renmin Hospital.

• Consent for publication
All the authors express agreement for publication.

• Availability of data and material
All the data and materials are availability.

• Competing interests
The authors have no conflicts of interest.

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• Authors’ contributions
TQ and JQZ designed the research study and performed the research; ZBC and XXM contributed essential reagents or tools; LZ and JLZ analyzed the data; TQ and TYW wrote the paper.
TYW and TQ contributed equally as the first co-authors in this manuscript.
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Tables

Table 1. Relationship between the expression of USP44 and Clinicopathological characteristics

| Category     | Subcategory | Cases | USP44 expression mean rank | P value |
|--------------|-------------|-------|----------------------------|---------|
| Age          | ≤60         | 249   | 31.02941                   | 0.05706 |
|              | >60         | 288   | 23.13299                   |         |
| Gender       | Male        | 352   | 27.92778                   | 0.57303 |
|              | Female      | 186   | 24.54313                   |         |
| Grade        | I-II        | 248   | 33.035699                  | 0.00504 |
|              | III-IV      | 282   | 21.315288                  |         |
| Stage        | T1-T2       | 348   | 29.613193                  | 0.01157 |
|              | T3-T4       | 190   | 21.52742                   |         |
| Nodes        | N0          | 240   | 23.622062                  | 0.0041  |
|              | N1          | 16    | 15.18371                   |         |
| Metastasis   | Yes         | 427   | 27.59757                   | 0.00019 |
|              | No          | 78    | 19.761654                  |         |
### Table 2. Primers for qPCR detection

| Gene name | Forward primer (Human) | Reverse primer (Human) |
|-----------|------------------------|------------------------|
| USP44     | AAACGATTCAGGTGGTCAGG   | AGTGTACCAGAACCCTCCCT  |
| P21       | TGGAGACTCTCAGGGTCGAAA  | TTCCTCTTGGAGAAGATCAGC |
| CyclinD1  | CAGATCATCCGAAACACGC    | AGGCGGTAGTAGGACAGGA   |
| MMP2      | CCGTCGCCCATCATCAAGTT   | CCGCATGGTCTCGATGGTAT  |
| MMP9      | TTTGAGTCCGGTGGACGATG   | TTGTCCGCGATAAGGAAGGC  |
| β-ACTIN   | CATGTACGTTGCTATCCAGGC  | CTCCTTAATGTCACGCAGCA  |

**Figures**
USP44 is involved with the occurrence and development of ccRCC. (A) The USP44 mRNA expression of normal vs tumor samples.

Figure 1
USP44 overexpression inhibits ccRCC cells proliferation. (A, C) mRNA expression of USP44 in 786-O and Caki-1 cells as well as Caki-1 cell line (J). *P < 0.05, **P < 0.01 vs. the ctrl group. All data shown as the mean ± SD.
USP44 overexpression inhibits ccRCC cell migration. (A, B) The image of Transwell
USP44 knockdown promotes ccRCC cell proliferation and migration. (A) mRNA expression level of USP44 in shctrl, sh#1, and sh#2 group of Caki-1 cell. **P < 0.01 vs. the ctrl group. All data shown as the mean ± SD.
The JNK signaling pathway is related to the regulation of USP44 function in the development of ccRCC.

**Figure 5**
USP44 knockdown promotes ccRCC cell proliferation and migration through JNK activity.