Deubiquitinase OTUD5 Modulates mTOR Signaling Pathways to Promote Bladder Cancer Progression

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Research

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Abstract

Background

The mammalian target of Rapamycin (mTOR) pathway serves as a crucial regulator of various biological processes such as cell growth and cancer progression. In bladder cancer, recent discoveries showing the cancer-promoting role of mTOR complex 1 have attracted wide attention. However, the regulation of mTOR signaling in bladder cancer is complicated and the underlying mechanism remains elusive. Here, we report that the deubiquitinating enzyme, ovarian tumor domain-containing protein 5 (OTUD5), can activate the mTOR signaling pathway, promote cancer progression, and show its oncogenic potential in bladder cancer.

Methods

The expression of OTUD5 in bladder cancer was analyzed using bladder cancer tissue microarrays and Western blotting analysis. Meanwhile, to demonstrate the role of OTUD5-RNF186-Sestrin2-mTOR axis in bladder cancer, we have adopted a series of biochemical and molecular biological methods to verify in vivo and in vitro. The methods used included quantitative real time PCR assay; western blot assay; Immunofluorescence staining assay; MTT assay; colony formation assay; Co-immunoprecipitation assay; In vivo ubiquitination assay; Immunohistochemical assay and Bladder Cancer xenograft animal model.

Results

In our study, we found that OTUD5 deubiquitinated a RING-type E3 ligase, RNF186, and stabilized its function. In addition, the stabilization of RNF186 further led to the degradation of Sestrin2, which is an inhibitor of mTOR signaling pathway.

Conclusion

Together, we first proved that OTUD5 can promote bladder cancer progression through the OTUD5-RNF186-Sestrin2-mTOR axis and provided novel insights into the diagnosis and treatment of bladder cancer.

Background

Bladder cancer places a substantial burden on society with nearly 170,000 deaths per year worldwide[1]. Although the risk factors of its occurrence remain to be elucidated, recent development in sequencing technology has identified several genes and pathways which are key drivers of bladder cancer, including cell cycle-related genes, RAS, and PI-3-kinase/mTOR[2].

The mechanistic Target of Rapamycin (mTOR) signaling pathway plays an important role in homeostasis and coordinates intracellular and extracellular signals to control cell metabolism, growth, and proliferation[3, 4]. Specifically, mTOR complex 1 (mTORC1) activation is triggered by several upstream

signals such as growth factors, energy, and nutrients. Activated mTORC1 can then phosphorylate a variety of downstream targets, including S6K, 4EBP1, TFEB, and ULK1, thereby regulating cell growth, metabolism and autophagy[5]. Not surprisingly, dysregulated mTORC1 signaling is closely related to various diseases including cancers, metabolic diseases and developmental disorders[6].

Recently, post-translational modifications such as ubiquitination and deubiquitination of mTOR signaling components have emerged as a promising research area in mTOR signaling regulation[7]. For example, the tumor suppressor Fbw7 ubiquitin ligase was observed to target mTOR for ubiquitination and degradation[8]. In addition, Deptor as an inhibitor of mTORC1 and mTORC2 can be the substrate of E3 ligase β -TRCP, triggering Deptor ubiquitination and degradation[9]. Recently, some studies have found that deubiquitinating enzymes (DUBs) can remove the ubiquitin chains on mTOR-interacting proteins and regulate mTOR signaling. The above-mentioned Deptor can be deubiquitinated and stabilized by ovarian tumor domain-containing ubiquitin aldehyde-binding protein 1 (OTUB1) belonging to the OTU family DUBs, leading to the inhibition of mTOR signaling[10]. Furthermore, Ubiquitin-specific Peptidase 9, X-linked (USP9X) can remove the ubiquitin chain of Raptor and Rictor to negatively regulate mTOR activities[11].

The OTU family DUBs have been the focus in many studies and shown to function in numerous cellular processes. Recent progress in elucidating the functions of OTUD5 as a member of this family has greatly enhanced our understanding in this area. The first discovered function of OTUD5 is to downregulate the ubiquitination of TRAF3 to negatively regulate IFN- γ expression[12]. In addition, OTUD5 can promote DNA damage repair by stabilizing KU80[13], as well as regulate DNA damage response by regulating FACT-dependent transcription on damaged chromatin[14]. Meanwhile, OTUD5 can enhance anti-tumor immunity by deubiquitinating and stabilizing STING[15]. However, there are few reports addressing the function of OTUD5 in tumorigenesis, especially in bladder cancer.

In this study, we proved that OTUD5 is involved in the progression of bladder cancer and exerts its cancer-promoting effect by activating mTOR signaling. Additionally, our research revealed the OTUD5-RNF186-Sestrin2-mTOR axis and provided novel insights into the diagnosis and treatment of bladder cancer.

Materials And Methods

Cell culture

Human bladder cancer cell lines 253J, UM-UC-14 and T24 were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.). The HEK293T cell line was obtained from Professor Chawnshang Chang (Department of Urology, University of Rochester, Rochester, NY 14642, USA) and maintained in DMEM supplemented with 10% FBS. In addition, the culture medium was supplemented with 100 U/mL penicillin and 0.1 mg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.).

Transfection and establishment of stable clone cells

T24 cells were stably transfected with OTUD5 (T24-OTUD5) and 253J, UM-UC-14 cells were stably transfected with sh-OTUD5 (253J-shOTUD5, UM-UC-14-shOTUD5), and empty vector control sublines (T24-Vec, 253J-shNC, UM-UC-14-shNC) were established. Cells were transfected with various plasmids using Lipofectamine 2000 transfection reagent (Life Technologies, 11668-027) followed the manufacturer's protocol. Various cell lines were infected with lentiviral and retroviral cDNA expressing viruses, which were packaged in HEK293T cells. Then, use hygromycin B (200 µg/ml) or puromycin to select infected cells (1 µg/ml). All established cell lines were cultured for less than 6 months and tested for mycoplasma every month.

Reagents, Antibodies and Plasmids

Everolimus (SML2282) was purchased from Sigma-Aldrich; Merck KGaA and dissolved in DMSO. Dynabeads Protein G (10004D) and TRIzol reagent were purchased from Invitrogen. Antibodies against OTUD5 (PA5-20611) and RNF186 (PA5-42330, PA5-57315) were purchased from Thermo Fisher Scientific. Antibodies mTOR (2983), Phospho-mTOR (Ser2448) (5536,2976), Phospho-p70 S6 Kinase (Thr421/Ser424) (9204), Phospho-4E-BP1 (Thr37/46) (2855), Flag-tag (14793), HA-tag (3724), GST-tag (2624) and β -actin (3700) were purchased from Cell Signaling Technology. Antibody against SESN2 (10795-1-AP) was purchased from Proteintech Group. pcDNA3-Flag-OTUD5, pcDNA3-HA-Sestrin1, pcDNA3-HA-Sestrin2, pcDNA3-HA-Sestrin3, pcDNA3-HA-RNF186 and pGEX-4T-1-GST-OTUD5 plasmid was constructed according to standard protocols by ourselves. His-ubiquitin was purchased from Addgene. pLKO-shOTUD5 (TRCN0000233196, TRCN0000143838) was purchased from Sigma-Aldrich.

Total RNA extraction and Quantitative Real time PCR

Total RNA of bladder cancer cells were extracted with TRIzol. 2µl of extracted RNA for RNA quantification, and reversed by using a Reverse Transcription Reaction Kit (TaKaRa PrimeScript™ RT Master Mix). Then cDNA was amplified using specific primers. Primer sequences were listed as follows:

OTUD5 (forward primer, 5'-GGTTGTGCGAAAGCATTGCAT-3'; reverse primer, 5'-ACCTCCACAGGACGGTTGT-3') and β -actin (forward primer, 5'-TAATCTTCGCCTTAATACTT-3'; reverse primer, 5'-TAATCTTCGCCTTAATACTT-3'). Using β -actin as an internal reference, relative changes in gene expression were normalized against β -actin

Western blot analysis

Cells were washed with ice-cold PBS and lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate; pH 7.4) containing protease inhibitors (Sigma-Aldrich; Merck KGaA) and phosphatase inhibitors (Sigma-Aldrich; Merck KGaA). For immunoblotting analysis, 20-40µg samples of protein (total lysate and mitochondriallysosomal, cytoplasmic and nuclear fractions) are subjected to SDS-PAGE on a 10% or 15% Tris-glycine gel. The separated proteins were then transferred to polyvinylidene fluoride (PVDF) membranes by Western

blotting. The membrane was blocked with 5% milk at room temperature for 1 hour, and then incubated with a primary antibody at 4°C overnight, and then incubated with a peroxidase-conjugated secondary antibody at room temperature (25°C) for 1 hour. Finally, the immune response signal of the protein was tested by the ECL detection system (Thermo Fisher Scientific, Rochester, NY).

Confocal fluorescence microscopy and Immunofluorescence staining

The cells were planted on glass slides and momentarily transfected with Flag-OTUD5, HA-Sestrin2 and HA-RNF186 plasmids for 48 hours. After washing 3 times with pre-cold phosphate buffered saline (PBS), the cells were treated with 4% paraformaldehyde for 15 minutes. The cells were then permeabilized with 0.1% Triton X-100 and incubated with specific primary antibodies OTUD5, Sestrin2 and RNF186 (diluted at 1:200) overnight at 4°C. At room temperature, the cells were stained with fluorescein isothiocyanate (FITC) and TRITC secondary antibody for 1 hour. And then, the cells were stained with DAPI and blocked with glycerol. Use a fluorescence microscope to detect the fluorescence of the cells.

MTT assay

Bladder cancer cells were plated into 96-wells culture plates at the cell density of 5.0×10^4 /ml. And then after 6h, 24h, 48h, 72h, 96h, the supernatant was changed with fresh medium containing 10% MTT (5 mg/ml) for each well and continue incubating for 4 h. Then removing the supernatant and adding another 150 μ L DMSO into each well, using a 96-well microplate reader (Bio-Rad, Hercules, USA) to detect the absorbance at the wavelength of 490 nm.

Colony formation assay

Bladder cancer cells in the logarithmic growth phase were seeded in a 6-well plate at 1,000 cells/well, and cultured with DMEM medium supplemented with 10% FBS in an incubator at 37°C with a volume fraction of 5% CO₂. After 1 week when clonal formation is visible, stop the culture; discard the medium, washed with phosphate-buffered saline (PBS) 3 times. Then fixed with 4% paraformaldehyde for 15 min, stained with crystal violet for 15 min. The staining solution was slowly washed away with running water and air dry, take pictures and count the number of clones

Protein structure prediction and protein-protein docking

The full length structure of OTUD5 and RNF186 was predicted by trRosetta. The visualization of interface was used pymol. The detail analysis of OTUD5/RNF186 complex was used PDBePISA. ΔG indicates the solvation free energy gain upon formation of the interface, in kcal/M. ΔiG P-value indicates the P-value of the observed solvation free energy gain. The P-value measures the probability of getting a lower than observed ΔiG , when the interface atoms are picked randomly from the protein surface, such as to amount to the observed interface area.

Co-immunoprecipitation

After being transfected with particular plasmid, the cells were lysed with IP buffer [50 mM Tris HCl, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100] containing with protease inhibitors (Sigma-Aldrich; Merck KGaA) and phosphatase inhibitors (Sigma-Aldrich; Merck KGaA). The Beckman Coulter DU-800 spectrophotometer is used to detect protein concentration using Bio-Rad protein assay reagents (Bio-Rad Laboratories, CA). The protein was incubated with M2 agarose beads (Sigma-Aldrich) bound with anti-FLAG or anti-HA antibodies for 4 hours at 4°C with gentle shaking. Subsequently, the cell lysate was washed with IP buffer, and protein was extracted from the beads by boiling at 95°C for 5 minutes.

***In Vivo* Ubiquitination Assays**

293T cells were transfected with His-ubiquitin and the constructs were displayed for 42 hours, and then treated with 20mM MG132 for 6 hours. Afterwards, cells were lysed with buffer A (6 M guanidine-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, and 10 mM imidazole [pH 8.0]) and sonicated for 15 seconds. After incubating with nickel-nitrilotriacetic acid (Ni-NTA) beads (QIAGEN) for 3 hours at room temperature, the proteins were washed twice with buffer A, twice with buffer A/TI (1 volume buffer A and 3 volumes buffer TI), and then eluted once with buffer TI (25 mM Tris-HCl and 20 mM imidazole [pH 6.8]). Denature the pulled-down protein by boiling at 95°C for 5 minutes, and then perform immunoblotting by SDS-PAGE separation.

Bladder Cancer xenograft animal model

All animal experiments were approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University and their care was in accordance with institution guidelines. 4-week-old Balb/c male nude mice were purchased from the Experimental Animal Center of Xi'an Jiaotong University. The cultured UM-UC-14/shNC, UM-UC-14/shOTUD5-01 and UM-UC-14/shOTUD5-02 cells were detached by trypsinization, washed, and resuspended in serum-free DMEM medium containing matrigel (Sigma-Aldrich; Merck KGaA). The cells (2×10^6 cells in 100 μ L) were then injected subcutaneously into the right flank of nude mice to initiate tumor growth. Measure the size of the tumor every three days through the caliper 1 week after implantation. The mice were separated to two groups, UM-UC-14/shNC, UM-UC-14/shOTUD5-01 and UM-UC-14/shOTUD5-02 control group (n=6) and UM-UC-14/shNC, UM-UC-14/shOTUD5-01 and UM-UC-14/shOTUD5-02 Everolimus treatment group (2.5 mg/kg, n=6). All mice were operated every 3 days, and the tumor volume was calculated as follows: volume (mm^3) = $1/2 \times (\text{length}) \times (\text{width})^2$. All treatments were administered for 30 days. At the end of the experiment, tumors were excised, weighed, and then fixed in 4% paraformaldehyde or stored in liquid nitrogen for further analyses.

Immunohistochemical assay

The BCa specimens were obtained from the First Affiliated Hospital of Xi'an Jiaotong University in China and the usage of these specimens was approved by the Institute Review Board of the First Affiliated Hospital of Xi'an Jiaotong University. For IHC in brief, the samples were deparaffinized and incubated with primary antibody against OTUD5 or p-mTOR. After incubating with a horseradish peroxidase-

conjugated secondary antibody at room temperature for 1 hour, the specimen was detected by using diaminobenzidine (DAB). Finally, the sample was observed under a microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

Statistical analysis.

All the data were presented as mean $\pm$ standard deviation SD of 3 independent experiments. All statistical analyses were performed using GraphPad Prism 5.2 software (GraphPad Software, Inc.). The contrast between only two groups was analyzed by Student's t test. The differences between the groups are determined by one-way analysis of variance. $P < 0.05$ was served as the standard to represent the significant difference.

Results

OTUD5 is overexpressed in bladder cancer

In order to explore the role of OTUD5 in bladder cancer, we first performed immunohistochemical analysis of tissue samples from patients with bladder cancer (Figure 1A and B). The result showed significantly higher OTUD5 expression in bladder cancer tissues compared with the adjacent tissues. Furthermore, Western blot (Figure 1C) and RT-PCR (Figure 1D) analyses showed increased OTUD5 expression in bladder cancer cell lines (RT4, 5637, UM-UC-14 and 253J, not including T24) compared with the urothelial cell line SV-HUC. At the same time, we analyzed the expression of OTUD5 in paired samples of bladder cancer tissues and normal bladder tissues from 12 patients through Western blot. The result once again showed increased OTUD5 expression in bladder cancer tissues (Figure 1E and F). These results indicate that OTUD5 may play a cancer-promoting role in bladder cancer.

OTUD5 is an oncogene in bladder cancer

Next, we investigated the role of OTUD5 in bladder cancer by constructing cell lines with stable OTUD5 knockdown and overexpression (Figure 2A and B). Plate cloning experiment using 253J and UM-UC-14 cells showed reduced cell proliferation in the OTUD5 knockdown group when compared with the control group (Figure 2C). In contrast, plate cloning experiment using T24 cells showed enhanced cell proliferation in the OTUD5 overexpression group when compared with the control group (Figure 2D). Further MTT assays yielded results similar to those shown in the plate cloning experiment (Figure 2E-G). As shown in Figure 2H-J, OTUD5 knockdown can inhibit xenograft growth *in vivo*. Taken together, these results indicate that OTUD5 can promote tumor proliferation in bladder cancer, and it may be involved in the occurrence and development of bladder cancer as an oncogene.

OTUD5 positively regulates the mTOR signaling pathway

The results of our previous studies have shown that OTUD5 can promote the proliferation of bladder cancer cells. However, the underlying mechanism remained unclear. In order to explore how OTUD5 affects tumor progression, we used mass spectrometry to detect the proteins that OTUD5 may bind

(Figure 3A). Through enrichment analysis of the mass spectrometry results, it was found that OTUD5 and mTOR signaling pathway were obviously related (Figure 3B). Next, we used Western blot to verify the relationship between OTUD5 and the mTOR pathway. In 253J and UM-UC-14 cells, compared with the control group, the expression levels of key mTOR pathway genes such as p-mTOR, p-S6K and p-4EBP were reduced in the OTUD5 knockdown group (Figure 3C). In T24 cells, compared with the control group, the expression levels of p-mTOR, p-S6K and p-4EBP1 were all increased in the OTUD5 overexpression group (Figure 3D). Meanwhile, we performed amino acid starvation treatment in UM-UC-14 cells for 6 h, and then resumed amino acid culture for 1 h. Through Western blot detection, we found that compared with the control group, the increase of p-S6K and p-4EBP1 expression was less apparent in the OTUD5 knockdown group. This suggested that knocking down OTUD5 can weaken the recovery of mTOR signaling pathway (Figure 3E). Similarly, when amino acid was readded to the amino acid-starved T24 cells, the increase of p-S6K and p-4EBP1 expression was more apparent in the OTUD5 overexpression group compared with the control group. This suggested that overexpression of OTUD5 can enhance the recovery of mTOR signaling pathway (Figure 3F). Thus, these results indicate that OTUD5 plays an important role in mTOR signaling. Though everolimus is an mTOR inhibitor and several studies have confirmed that everolimus negatively regulates mTOR signaling in other diseases, it has been rarely studied in bladder cancer. To confirm everolimus is regulating this intended target in bladder cancer, we examined p-S6K and p-4EBP1 levels in the bladder cancer cell lines after everolimus treatment. In UM-UC-14 cells, everolimus group showed inhibited mTOR signaling and this inhibition become even more pronounced when there was a simultaneous OTUD5 knockdown (Figure 3G). In T24 cells, everolimus group also showed the inhibition of mTOR signaling, and this inhibition could reverse the mTOR signaling activation caused by OTUD5 overexpression (Figure 3H). Thus, these results indicate that everolimus could negatively regulate mTOR signaling in bladder cancer cells. Moreover, the various inhibition effects in bladder cancer cells with different levels of OTUD5 expression may provide new insights into the treatment of bladder cancer in the future. Next, we investigated the relationship between p-mTOR and OTUD5 in bladder cancer tissues using immunohistochemistry. We found that p-mTOR was highly expressed in tissues with high OTUD5 expression; and p-mTOR expressed at low levels in tissues with low OTUD5 expression (Figure 3I and J). These results indicate that the protein levels of OTUD5 and p-mTOR are positively correlated in bladder cancer tissues.

OTUD5 regulates the protein stability of Sestrin2, a feedback inhibitor of mTOR

Previous findings have shown the positive correlation between OTUD5 and mTOR. However, the reason for this correlation was not clear. By analyzing mass spectrometry results, we found that Sestrin2 may be a potential target molecule of OTUD5 in mTOR signaling pathway (Figure 4A). As a feedback inhibitor in mTOR signaling pathway, Sestrin2 can inhibit the function of mTOR[16, 17]. Then we found a colocalization of OTUD5 and Sestrin2 by immunofluorescence (Figure 4B) and detected a binding between OTUD5 and Sestrin2 by immunoprecipitation (Figure 4C). These results indicate that OTUD5 may bind to Sestrin2 and function through deubiquitinating Sestrin2. Surprisingly, when we detected the expression of Sestrin2 in cell lines with stable OTUD5 knockdown and overexpression, it turned out that the expression level of Sestrin2 was increased when OTUD5 was knocked down, while the expression

level of Sestrin2 was decreased when OTUD5 was overexpressed (Figure 4D). These results were contradictory to our expectation that OTUD5 can stabilize Sestrin2 by deubiquitination. Therefore, we speculated an indirect interaction between OTUD5 and Sestrin2 instead of the direct deubiquitination. To test our speculation, we constructed the GST-OTUD5 protein and ran the GST pull down experiment. The result showed that there was no direct binding between OTUD5 and Sestrin2 (Figure 4E). Next, we investigated the possibility that OTUD5 could deubiquitinate Sestrin2 through its deubiquitinating activity. Thus, we produced the wild type OTUD5 (OTUD5/WT) and the catalytically inactive OTUD5 mutant (OTUD5/C224S). In vitro ubiquitination experiment showed that OTUD5/WT could increase Sestrin2 ubiquitination. While OTUD5/C224S theoretically cannot play the function of deubiquitination, the ubiquitination level of Sestrin2 was reduced (Figure 4F). Considering the role of OTUD5 as a deubiquitinase, we studied how OTUD5 affected the stability of Sestrin2 by using cycloheximide (CHX). The CHX analysis showed that OTUD5 knockdown prolonged the half-life of Sestrin2 (Figure 4G and H), while OTUD5 overexpression reduced the half-life of Sestrin2 (Figure 4I and J). All the above results indicate that there is no direct binding between OTUD5 and Sestrin2. Since OTUD5 could not directly deubiquitinate Sestrin2, we speculate that OTUD5 may regulate the expression of Sestrin2 through some unknown intermediate proteins.

OTUD5 stabilizes RNF186 by deubiquitination, leading to Sestrin2 degradation

In order to identify the interactions connecting OTUD5 and Sestrin2, we used protein binding prediction and found that OTUD5 may bind to RNF186. The docking results showed that OTUD5 and RNF 186 had high potential and affinity to form a complex. According to the ΔG value of OTUD5/RNF186 complex, we predicted the binding affinity between OTUD5 and RNF 186 to be less than 0.1 nM (Figure 5A). As an E3 ubiquitin ligase, RNF186 can affect the stability of the substrate protein. Moreover, it has been reported that it could ubiquitinate Sestrin2 to control nutrient sensing[18]. Therefore, we speculated that RNF186 is the key molecule connecting OTUD5 and Sestrin2. Then, we explored the potential binding between OTUD5 and RNF186 by immunofluorescence assay (Figure 5B) and immunoprecipitation assay (Figure 5C-E). Meanwhile, we detected the expression of RNF186 in stable OTUD5 knockdown and overexpression cell lines. The result showed the RNF186 expression decreased when OTUD5 was knocked down and increased when OTUD5 was overexpressed (Figure 5F). This indicate that OTUD5 may directly deubiquitinate RNF186. Thus, the GST pull down experiment was performed, and the result revealed the direct binding between OTUD5 and RNF186 (Figure 5G). In addition, the in vitro ubiquitination experiment showed that OTUD5/WT could significantly deubiquitinate RNF186, while OTUD5/c224s could not (Figure 5H). Next, we found that OTUD5 knockdown reduced the stability of RNF186 (Figure 5I and J), while OTUD5 overexpression prolonged the half-life of RNF186 (Figure 5K and L). Taken together, the results we have presented so far indicate that OTUD5 stabilizes RNF186 by deubiquitination, Then, through ubiquitination, RNF186 promotes the degradation of Sestrin2, which is a feedback inhibitor of mTOR signaling pathway. Therefore, OTUD5 regulates mTOR through the OTUD5-RNF186-Sestrin2-mTOR axis.

OTUD5 knockdown combined with everolimus inhibits bladder cancer growth in vitro and in vivo

First, we investigated the in vitro inhibitory effect of OTUD5 knockdown combined with everolimus on the proliferation of bladder cancer cells. Through the plate cloning experiment (Figure 6A) and MTT experiment (Figure 6B), we found that compared with the NC group, OTUD5 knockdown could inhibit the proliferation of UM-UC-14 cells. Interestingly, the combination of OTUD5 knockdown and everolimus showed a more significant inhibitory effect on bladder cancer cell proliferation than the everolimus treatment alone. Then, we evaluated the in vivo inhibitory effect in a mouse model which was established by transplanting UM-UC-14 WT/shOTUD5 cells. The results showed that OTUD5 knockdown can inhibit xenograft growth, and OTUD5 knockdown combined with everolimus showed a more significant growth inhibition of the xenografts (Figure 6C). Consistent with the tumor volume data (Figure 6D), the same trend was observed in the tumor weight data (Figure 6E). Furthermore, we detected the tumor tissues by Western blot (Figure 6F) and immunohistochemistry (Figure 6G). The results showed that compared with the control group, the expression of p-mTOR and RNF186 were downregulated while the expression of Sestrin2 was upregulated in the OTUD5 knockdown group. These results are consistent with the proposed OTUD5-RNF186-Sestrin2-mTOR axis and suggest OTUD5's role in the regulation of mTOR signaling pathway.

Discussion

In this study, we first discovered that OTUD5 as an oncogene promotes the progression of bladder cancer. Compared with that in normal bladder epithelial cells, OTUD5 mRNA and protein showed a trend of overexpression in bladder cancer cell lines. In addition, Western blot and immunohistochemistry found overexpressed OTUD5 in cancer tissues obtained from bladder cancer patients. Furthermore, we demonstrated that knocking down or overexpressing the OTUD5 gene could respectively decrease or increase the growth of bladder cancer cells in clone formation and MTT experiments, indicating the cancer-promoting role of OTUD5 in bladder cancer, and suggesting a potential target for early diagnosis and treatment of bladder cancer.

Consistent with the recent published data that OTUD5 can positively regulate mTOR signaling[19], we found that OTUD5 might regulate mTOR signaling pathway through enrichment analysis of mass spectrometry results and further confirmed the OTUD5-induced mTOR signaling activation in bladder cancer, unveiling the mechanism underlying the cancer-promoting role of OTUD5.

Since the abnormal mTOR signaling pathway is related to many diseases, especially cancer[20]. The regulation of mTOR signaling has attracted wide attention in developing treatment for these diseases. For example, everolimus as a derivative of rapamycin can significantly inhibit the activity of mTOR. It has been reported that everolimus inhibits tumor cell proliferation and induces apoptosis and autophagy[21, 22]. In addition, everolimus inhibits the malignant progression of various tumors, including breast cancer[23], ovarian cancer[24], colorectal cancer[25], pancreatic cancer[26], bladder cancer[27] and renal cell carcinoma[28]. In this study, we examined the inhibitory effect of everolimus in bladder cancer. Interestingly, our in vitro and in vivo data suggested that everolimus treatment could inhibit mTOR signaling and this inhibition becomes more significant when there is a simultaneous OTUD5 knockdown.

This phenomenon indicates that OTUD5 expression levels may be used to predict everolimus sensitivity when treating bladder cancer patients in the future. Though the everolimus treatment with a simultaneous OTUD5 knockdown seems to be an ideal strategy, there is currently no specific inhibitor for OTUD5, and future discoveries of OTUD5 inhibitors may lead to some more effective bladder cancer treatments.

Further mechanistic studies of the OTUD5-induced cancer-promoting effects revealed that OTUD5 regulates mTOR signaling through the OTUD5-RNF186-Sestrin2-mTOR axis in bladder cancer. First, the mass spectrometry results indicated an interaction between OTUD5 and Sestrin2 which was further confirmed by IF and IP. It has been reported that Sestrin2 as an inhibitor of mTOR can negatively regulate mTOR signaling[16, 29]. But interestingly, when OTUD5 was knocked down, the expression of Sestrin2 increased instead. This made us wonder whether there are other proteins that play the role of a bridge between OTUD5 and Sestrin2. Ring finger protein 186 (RNF186) as an E3 ubiquitin ligase can ubiquitinate the substrate protein to promote its degradation. It has been reported that RNF186 regulates ER stress-mediated apoptosis through its interaction with BNip1[30]. Another research team has found that RNF186 can negatively regulate NF-kappaB in colorectal cancer[31]. And a recent study has shown that RNF186 can ubiquitinate and degrade Sestrin2, thereby activating mTOR[18]. Our research found that OTUD5 deubiquitinates and stabilizes RNF186. Then, the stabilized RNF186 further ubiquitinates and degrades Sestrin2, thereby activating mTOR signaling in bladder cancer.

Conclusion

In summary, our research first discovered that OTUD5 plays an oncogenic role in bladder cancer. Thus, it may become a potential target for the diagnosis and treatment of bladder cancer in the future. Mechanistically, we found that OTUD5 exerts its cancer-promoting effects through the OTUD5-RNF186-Sestrin2-mTOR axis. In addition, bladder cancer cells and transplanted tumors with OTUD5 knockdown are more sensitive to the mTOR inhibitor everolimus, which provides new ideas for personalized treatment of bladder cancer patients in the future.

Abbreviations

BCa: Bladder cancer; CoIP: Co-immunoprecipitation; RT-PCR: Quantitative reverse transcription polymerase chain reaction; RNF186: ring finger protein 186; mTOR: The mammalian target of Rapamycin; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OTUD5: ovarian tumor domain-containing protein 5; IHC: Immunohistochemistry; FBS: Fetal bovine serum; shRNA: Short hairpin RNA.

Declarations

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Author contributions

T.H. and W.C.D. contributed equally to this work. J.Z., Y.Z.F. and L.L. designed and supervised all the experiments and contributed to manuscript preparation. T.H., W.C.D., T.J.L., B.L., Y.W., T.T.Q., Y.Z.S.L. and Z.X.W. performed the experiments, analyzed the data and contributed to the manuscript preparation. T.H. and W.C.D. collected animal samples and participated in the animal experiments. T.H., C.Y.Y., Y.Z.F. and L.L. wrote the manuscript.

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Availability of data and material

The datasets used and analyzed in this study can be obtained from corresponding authors upon reasonable request.

Ethics approval and consent to participate

All experiments involving human subjects were carried out in accordance with the code of ethics of the World Medical Association (Helsinki Declaration). All animal experiments were approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University and their care was in accordance with institution guidelines.

Consent for publication

The manuscript has been approved for publication by all authors.

Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

Figure 1

OTUD5 is overexpressed in bladder cancer A. Representative images of OTUD5 immunohistochemistry (IHC) staining in bladder cancer tissue and adjacent normal bladder tissue samples. Scale bars, upper 500 μm, lower 100 μm. B. Statistical analysis of OTUD5 expression in adjacent normal bladder tissues and bladder cancer tissues. ***P < 0.001. C and D. Western blot analysis and quantitative real-time RT-PCR of OTUD5 expression in the normal human bladder cell line and bladder cancer cell lines. E. Western blot analysis of OTUD5 protein expression in adjacent normal bladder tissues and cancer tissues. β-actin was used as the loading control. F. The quantitative analysis of the relative expression of OTUD5 protein.

Figure 2

OTUD5 is an oncogene in bladder cancer A and B. Quantitative real-time RT-PCR and western blotting analysis of OTUD5 mRNA expression in 253J or UM-UC-14 cell lines transfected with OTUD5 shRNAs and shNC, and T24 cell line infected with OTUD5 lentivirus and negative control. 18S was applied as the endogenous control for quantitative real-time RT-PCR, and β -actin was used as a loading control for western blotting assay. C. Colony formation assays and quantification results of 253J and UM-UC-14

cells with OTUD5 knockdown. ***P < 0.001. D. Colony formation assays and quantification results of T24 cells with OTUD5 overexpression. Vec, vector. ***P < 0.001. E, F and G. MTT assay was performed to detect viability in 253J and UM-UC-14 cells with OTUD5 knockdown and T24 cells with OTUD5 overexpression. **P < 0.01. H. The growth curves of xenografts in different groups. Tumor volumes were measured every three days from day 12 to day 30. ***P < 0.001. I. Illustration of tumors excised from male nude mice in each group. J. After 30 days, the nude mice were sacrificed, and UM-UC-14 cell xenografts were weighed. ***P < 0.001.

Figure 3

OTUD5 positively regulates the mTOR signaling pathway A. Flag-OTUD5 was immunoprecipitated (IP) from 293T cells transfected with Flag-OTUD5 plasmid and the lysates were subjected to SDS-PAGE and Coomassie Blue staining. B. Enrichment analysis of mass spectrometry results showed that OTUD5 is involved in the mTOR signaling pathway. C. IB analysis of whole-cell lysates (WCL) derived from 253J and UM-UC-14 cells stably expressing shOTUD5 and shNC. shNC was used as the negative control. p-mTOR, t- mTOR, p-S6K, p-4EBP1 and OTUD5 were detected. β -actin was used as the loading control. D. IB analysis of WCL derived from T24 cells stably overexpressing OTUD5 and vector. Vector was used as the negative control. E and F. UM-UC-14 shNC/shOTUD5 cells (E) and T24 Vec/oeOTUD5 cells(F) were subjected to amino acid starvation for 6 hours and resupplementation for 1 hour. p-S6K, p-4EBP1 and OTUD5 were detected. G and H. UM-UC-14 shNC/shOTUD5 cells (G) and T24 Vec/oeOTUD5 cells (H) were treated with or without Everolimus. I. Representative images of OTUD5 and p-mTOR IHC staining in patient-derived bladder cancer tissues. Scale bars, left 500 μ m, right 100 μ m. J. Correlation analysis of OTUD5 and p-mTOR expression in patient-derived bladder cancer tissues.

Figure 4

OTUD5 regulates the protein stability of Sestrin2, a feedback inhibitor of mTOR A. The interaction diagram between OTUD5 and specific proteins in the mTOR signaling pathway was obtained by mass spectrometry analysis. B. Confocal immunofluorescence microscopic analysis of OTUD5 and Sestrin2 in 253J and UM-UC-14 cells. Scale bars represent 10 μ m. C. IB analysis of WCL and anti-HA immunoprecipitates (IPs) derived from 293T cells transfected with Flag-OTUD5 and HA-tagged Sestrin 1-3. pcDNA3.1 was used as the control. D. IB analysis of WCL derived from 253J and UM-UC-14 cells stably

expressing shOTUD5 and T24 cells stably overexpressing OTUD5. p-mTOR, p-4EBP1 and Sestrin2 were detected, β -actin was used as the loading control. E. GST pull-down assay revealed no direct interaction between Sestrin2 and OTUD5. The upper panel presents the result of IB using the antibody against HA, and the lower panels show Coomassie blue staining of the purified proteins. F. IB analysis of WCL and Ni-NTA pull-down products derived from 293T cells transfected with Flag-OTUD5 WT, Flag-OTUD5 C224S, HA-Sestrin2 and His-Ub. 20 μ M MG132 was added 6 hours before harvesting the cells. G. OTUD5 knockdown cells (shOTUD5) as well as parental UM-UC-14 cells (shNC) were treated with 100 μ g/ml cycloheximide (CHX) for the indicated time period before harvesting. Equal amounts of WCL were immunoblotted with the indicated antibodies. H. Quantification of the band intensities in (G). Sestrin2 levels were normalized to the corresponding β -actin levels, then normalized to the t = 0h Sestrin2 level. I. IB analysis of WCL derived from 293T cells transfected with HA-Sestrin2, Flag-EV and Flag-OTUD5. Cells were treated with 100 μ g/ml CHX for the indicated time period before harvesting. EV, empty vector. J. Quantification of the band intensities in (I). Sestrin2 levels were normalized to the corresponding β -actin levels, then normalized to the t = 0h Sestrin2 level.

Figure 5

OTUD5 stabilizes RNF186 by deubiquitination, leading to Sestrin2 degradation A. Protein binding prediction chart for OTUD5 and RNF186. B. Confocal immunofluorescence microscopic analysis of OTUD5 and RNF186 in 253J and UM-UC-14 cells. Scale bars represent 10 μm . C. IB analysis of WCL and anti-HA immunoprecipitates (IPs) derived from 293T cells transfected with Flag-OTUD5 and HA-tagged RNF186, pcDNA3.1 was used as the control. D and E. OTUD5 interacts with RNF186. Co-

immunoprecipitation (co-IP) of OTUD5 and RNF186 was assayed in 253J and UM-UC-14 cells. Immunoprecipitation (IP) was performed using the antibody against OTUD5, and the endogenous interaction between OTUD5 and RNF186 was determined by Western blotting using the antibody against RNF186. F. IB analysis of WCL derived from 253J and UM-UC-14 cells stably expressing shOTUD5 and T24 cells stably overexpressing OTUD5. RNF186 and Sestrin2 were detected, β -actin was used as the loading control. G. GST pull-down assay revealed the direct interaction between RNF186 and OTUD5. The upper panel presents the result of IB using the antibody against HA, and the lower panels show Coomassie blue staining of the purified proteins.. H. IB analysis of WCL and Ni-NTA pull-down products derived from 293T cells transfected with Flag-OTUD5 WT, Flag-OTUD5 C224S, HA-RNF186 and His-Ub. 20 μ M MG132 was added 6 hours before harvesting the cells. I. OTUD5 knockdown cells (shOTUD5) as well as parental UM-UC-14 cells (shNC) were treated with 100 μ g/ml cycloheximide (CHX) for the indicated time period before harvesting. Equal amounts of WCL were immunoblotted with the indicated antibodies. J. Quantification of the band intensities in (I). RNF186 levels were normalized to the corresponding β -actin levels, then normalized to the t = 0h RNF186 level. K. IB analysis of WCL derived from 293T cells transfected with HA-RNF186, Flag-EV and Flag-OTUD5. Cells were treated with 100 μ g/ml CHX for the indicated time period before harvesting. EV, empty vector. L. Quantification of the band intensities in (I). RNF186 levels were normalized to the corresponding β -actin levels, then normalized to the t = 0h RNF186 level.

Figure 6

OTUD5 knockdown combined with everolimus inhibits bladder cancer growth in vitro and in vivo. A. Colony formation assays and quantification results of UM-UC-14/WT and UM-UC-14/shOTUD5 cells treated with or without Everolimus (10 nM). ** $P < 0.01$, *** $P < 0.001$. B. The growth curves of UM-UC-14/WT and UM-UC-14/shOTUD5 cells treated with or without Everolimus (10 nM). *** $P < 0.001$. C. The growth curves of xenografts in different treatment groups. Tumor volumes were measured every three days from day 12 to day 30. *** $P < 0.001$. D. Illustration of tumors excised from male nude mice in each treatment group. E. After 30 days, the nude mice were sacrificed, and UM-UC-14 cell xenografts were

weighed. **P <0.01, ***P <0.001. F. The expressions of OTUD5, p-mTOR, RNF186 and Sestrin2 in the xenograft tumors were measured by immunohistochemistry. (Scale bar, 20 μ m). G. The expressions of OTUD5, p-mTOR, RNF186 and Sestrin2 in the xenograft tumors were measured by Western blot. β -actin was used as the loading control. n = 6 mice per treatment group, data are presented as mean $\pm$ SD.

Figure 7

Schematic models. A. OTUD5 exerts its functions through the OTUD5-RNF186-SESTRIN2-mTOR axis to promote bladder cancer proliferation. B. Downregulation of OTUD5 and Everolimus show inhibitory effects on bladder cancer growth.