OTUB1* Promotes Progression and Proliferation of Prostate Cancer via Deubiquitinating and Stabbling Cyclin *E1

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Research

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Abstract

Background: Prostate cancer (PCa) is currently the most common and highest incidence of cancer in men all over the world. OTUB1 has been reported to be a critical role in various kinds of cancers, closely related with proliferation, migration and clinical prognosis. The aim of this research was to investigate the influence of OTUB1 in PCa, and to identify the mechanism underlying function.

Methods: Using TCGA database, we identified OTUB1 higher expression in PCa. We observed the changes of functional behavior in PC3 and C4-2 cells through overexpression or knock down of OTUB1. Subcutaneous ectopic tumors were conducted and IHC of tumors tissue in nude mice also carried out. Western blotting and co-immunoprecipitations assays were conducted to identify that OTUB1 interacted with cyclin E1.

Results: We found that the expression of OTUB1 was significant higher in PCa than in Benign prostatic hyperplasia (BPH). The overexpression of OTUB1 promoted obviously proliferation and migration in PC3 and C4-2 cells via regulating Cyclin E1 protein stability, and contrary observation in OTUB1 knock down. The nude mice experiment further explained our conclusions. We finally identified that OTUB1 promoted the progression of PCa by deubiquitinating and stabilizing cyclin E1.

Conclusions: Our findings reveal the important role of OTUB1 in PCa, OTUB1 promoted cyclin E1 protein stability in PCa. Knock down of OTUB1 can significantly repressed development of cancer. OTUB1 might serve a newly and potential therapy target for PCa.

Background

Prostate cancer (PCa) is the most common malignant tumor in America. The prevalence of PCa approximately accounted for 20% in American men and ranks the first among all kinds of cancers, about 174650 new cases and 31620 deaths from PCa in 2019(1). Early detection of PCa usually accounts for a 5-year survival rate of nearly 100%. However, many patients of PCa were diagnosed at a very late stage and their survival rate reduced drastically, due to no obvious symptoms apart from those of urinary tract infection(2). Currently the main and standard treatment is androgen deprivation therapy (ADT) because of the irreplaceable role of androgen receptor (AR) in progression of PCa(3, 4). ADT mainly include medical castration and surgical castration, but most patients finally progressed to castration-resistant prostate cancer (CRPC), even metastasis castration resistant prostate cancer (mCRPC) with no effective treatment(5, 6). CRPC and mCRPC still were the trickiest trouble during diagnosis and treatment of prostate cancer. With the development of urology in recent years, many different treatments occurred, including chemotherapy, radiation therapy, targeted therapy and immunotherapy, the overall survival rate had rase markedly(7-9). It still lacks an effective and sensitive treatment drugs for prostate cancer, especially the acute demand for novel drugs in treating CRPC(10).

Ubiquitination was a critical pathway for protein degradation, and served a crucial moderator in many cellular signal pathway(11). As a member of the ovarian tumor domain (OTU) deubiquitylating cysteine

proteases subfamily(12). OTUB1 could stable the expression level of target protein and maintain its function by inhibiting its ubiquitination (13). Many researchers had proved that OTUB1 regulated lots of important cellular processes, such as DNA-repair, cell signaling, growth and apoptosis(14, 15). Furthermore, the status of OTUB1 in the domain of cancer is becoming more and more important and irreplaceable, for example, OTUB1 was found to up-regulated in colorectal cancer(16), gastric adenocarcinoma(17), esophageal cancer(18), ovarian cancer(19), human glioma(20) and hepatocellular carcinoma(21), and promoted tumor invasion and predicted a poor prognosis. OTUB1 promoted tumor progression mainly via two patterns, the one was to stable the expression of tumorigenesis genes by inhibiting ubiquitination of target protein depending on its deubiquitination function, and another mode didn't depend on its deubiquitination manner(22), by direct interacting with E2 ubiquitin ligase. These results implied us that OTUB1 might serve a tumor associated diagnosis biomarker and candidate target for cancer treatment. Currently, the relationship of OTUB1 and PCa had been researched preliminary, Our group verified that OTUB1 promoted prostate cancer invasion in vitro and aggravate tumorigenesis in vivo via regulating RhoA activity and p53 levels(23). Cell cycle related specific cyclin/ Cdk complexes were the primary regulator during the various stages of mitosis, they could influence the conversion within different cell phases via phosphorylation of cell phase-specific substrate proteins(24, 25). Cyclin E1 was known to an conserved protein whose essential function was to promote G1/S transition, and Cyclin E1/Cdk2 axis has been identified to involve proliferation in various cancers in previous studies(26, 27). The anticancer role of Cyclin E1/Cdk2 complex was been extensive noticed by lots of researchers, including ovarian cancer(28), liver cancer(29, 30) and so on(31).

In this research, we focused on further characterize the involvement of OTUB1 in cell cycle progression and investigated the specific mechanism of OTUB1 promote tumor progress. Further exploring the possibility of OTUB1 serves a candidate treatment target and diagnosis biomarker for PCa.

Methods

Clinical samples

Clinical tissue samples were acquired from patients who underwent transurethral resection of the prostate at the Tianjin Medical University second affiliated Hospital (Tianjin, China) and inspected by a professional pathologist in order to score Gleason grade. This research was approved by the Ethics Committee of the Tianjin Medical University, and the Helsinki Declaration of Human Rights was strictly complied with.

Prostate cancer cell lines

The human prostate cancer cell lines (PC3 and C4-2) were obtained from American ATCC cell bank. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at the humidified atmosphere environment containing 5% CO₂ at 37 °C.

Cell transfection and Inhibitor

6×10^5 PC3 and C42 cells were seeded in 6-well plates, Twenty-four hours later, the cells were transfected with 2 μ g of plasmid or 100 nM siRNA using LipofectamineTM 2000 (Invitrogen) according to the manufacturer's protocol. And the amplified OTUB1 expression was generated by transfecting with the FLAG-OTUB1 plasmid and the expression of otub1 c91s was generated via transfecting pcDNA3.1-OTUB1^{C91S} plasmid in PC3 cell and C42 cell. The knockdown expression of OTUB1 was generated for transient OTUB1 siRNA transfections. After 48 h, the cells were collected for western blotting, MTT, transwell and migration assays. Relative si RNA primers were listed in supplementary data table 1. RO-3306, a cyclin inhibitor, was purchased from MCE, and different dose RO-3306 were added into six well plate respectively.

Immunoprecipitation and western blot analysis

Total protein was extracted from PC3, C42 cell lines and tumor tissues using RIPA(Biosharp) and PMSF, and the BCA kit was used for protein concentration measure. An equal amount of protein sample(40 μ g per lane) was separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 10% acrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membrane(Millipore, Billerica, MA). The membrane was blocked in 5% fat-free milk and incubated at 4°C overnight with the following primary antibodies: rabbit anti-OTUB1(1:1000 dilution; affinity), rabbit anti-GAPDH (1:1000dilution; Abcam), mouse anti- β -actin (1:1000 dilution; CST), rabbit anti-Cyclin E1 (1:1000 dilution; CST), and rabbit anti-FLAG(1:1000 dilution; SIGMA). Next day, the blots were washed and incubated with anti rabbit or anti mouse IgG for 1h. Bands were analyzed using an enhanced chemiluminescence system (ECL; Beyotime Institute of Biotechnology). Data and results were quantified by densitometry using software Image J.

Immunohistochemistry staining

Clinical tissue samples were collected from prostate surgery and the tumor of null mice were collected and stored in formalin. The specimens are frozen, embedded in paraffin, and cut into 5 micron thick sections. Tissue sections were griddled at 65°C for 45min, next de-waxed in xylene and rehydrated in graded alcohol. Antigen retrieval was performed with citrate buffer (pH adjusted to 6.0) under high fire for 5min and middle-low fire for 10min in turn. Endogenous peroxidase was blocked in 0.3% hydrogen peroxide for 10 min and blocked using 1.5% horse serum. Incubation with primary antibody was done in humidified chamber overnight at 4°C (anti-OTUB1, 1:100 from affinity ; anti-Cyclin E1 1:100 from affinity; Ki67 1:100 from Abcam). After applying Poly-HRP anti-rabbit IgG (30min), secondary antibody detection was performed using the Ultraview DAB detection kit (Zhongshan Co, China). In the case of OTUB1, ki-67 and Cyclin E1, only nuclear staining was considered as positive and was scored. All immunostained sections were evaluated under Zeiss microscope ($\times 200$).

Wound healing assay

PC3 and C42 cells were seeded on six-well plates and grown to paved overnight. 24 hours after transfection, a sterile 10 μ L micropipette tip was used to scratch a single channel on the monolayer cell. Then PC3 and C42 cells were washed twice with PBS, and cultured in 10% FBS 1640 at 5% CO₂, 37°C for

an additional 24 h. Photographs were taken by an inverted Leica phase contrast microscope at 0h and 24h time points.

Clone formation assay

PC3 and C42 cells were digested and 2.0×10^3 cells of each group were seeded into a well of 6-well plates. After 24 h, cells were transfected with OTUB1 siRNA, negative control siRNA, OTUB1-overexpression and OTUB1^{c91s} respectively. Cells culture was performed for 1 week. After washing twice with phosphate-buffered saline (PBS) buffer, 4% paraformaldehyde was used for 20 min to fixate. Then, an appropriate amount of crystal violet solution was added and staining was performed for 30 min. After washing again with PBS and air drying, software Image J was used for clones counting.

MTT Assay

48h after of transfection, 2.0×10^3 cells each well were seeded into 96-well plates and cultured at 37°C for 24 h, 48h, 72h, 96h. Then 30uL MTT solution was added into every well at indicated times every time point and cultured for another 2 h at 37°C. Subsequently, MTT solution was removed and 150uL dimethyl sulfoxide (DMSO) each well was added to dissolve formazan crystals. The absorbance value was measured at 490 nm using a microplate reader.

Transwell migration assay

PC3 and C42 cells were transfected respectively with OTUB1-siRNA or negative-control siRNA and pcDNA3.1-OTUB1 plasmid were suspended in 1640 containing 10% FBS, and PC3 and C42 cells transfected pcDNA3.1 plasmid were cultured in the same medium as the mock groups. 2×10^4 cells were added to the top chambers of 24-well transwell plates (Corning, 8 μ m pore size), and 1640 with 10% FBS was added to the bottom chambers. After incubating at 37°C for 48h, the chambers were washed with PBS twice, and cells which migrated to the bottom chambers were fixed with paraformaldehyde and stained with crystal violet. Then we counted the number of migrating cells in five fields under $\times 200$ magnifications for all the chambers. Experiment data were obtained from triplicate chambers and the experiments were repeated three times.

Animal studies

five-week-old male Babl/c mice (HFK Bio-Technology Co. Ltd, Beijing) were injected subcutaneously with 2×10^6 PC3 cells with control, otub1 and otub1-c91s groups suspended in 0.1mL of Matrigel (BD Biosciences) and 1640, and were implanted subcutaneously into the dorsal flank on both sides of the mice. Once the size of tumors reached a stage about 50 mm³, the volume of tumor were measured daily for 12 days. Tumor volume was recorded by digital caliper and estimated using the formula $LW^2/2$ (L=length of tumor and W=width). At 12 day point of the mice were killed and tumors extracted and measured. The tumors were fixated with paraformaldehyde, then immunohistochemistry staining were performed for OTUB1, Ki 67 and Cyclin E1. All procedures involving mice were approved by the University

Committee on Use and Care of Animals at the Tianjin Medical University and conform to all regulatory standards.

Results

OTUB1 was upregulated in prostate cancer

We filtered cell cycle progression and in prostate cancer from TCGA database, identified 3480 genes up-regulated and 2592 genes down-regulated, in up-regulated genes, we observed the expression of OTUB1 in PCa is higher than para-carcinoma tissue (Fig 1A). To identify the deubiquitinating enzymes driving the prostate cancer progression, we conducted subsequent experiments and assays.

In order to identify whether clinical data was consistent with the database, we collected clinical prostate cancer tissue and immunohistochemical staining was performed with OTUB1 and ki-67 antibody (Fig 1C, D). The expression of OTUB1 and Ki67 in ADPC and CRPC groups were higher than that in BPH group. The mean optical density values of OTUB1 and Ki67 in ADPC group were higher than BPH, the difference was statistically significant ($P \leq 0.05$). The concise statistical results were showed that the positive ratios of OTUB1 and Ki67 in ADPC and CRPC groups were higher than BPH group (Fig 1E).

OTUB1 promoted proliferation and invasion of PCa cell.

Wildtype OTUB1 vector was transfected into prostate cancer PC3 and C42 cells, and the expression level of OTUB1 was detected by Western blotting. The cells with significantly increased OTUB1 expression level were obtained for cell functional experiments. The results of Western blotting showed that, compared with the cells in the control group, the expression level of OTUB1 in the cells in the otub1-c91s group (cells introduced by the mutated OTUB1 fragment) was not significantly changed, but the expression level of OTUB1 protein in the otub1 cells was significantly increased (Fig 2A). Image analysis software Image J was used to detect the grayscale value of OTUB1 expression in OTUB1 cells with stable and high expression (Fig 2A), and it was found that the expression level of OTUB1 was more than three times that of OTUB1 C91S cells or control group. For further explanation, we transfected PC3 and C4-2 cells with OTUB1 siRNA, and the expression level of OTUB1 was decreased distinctly compared with si control group (Fig 2B).

Recent studies have shown that OTUB1 is highly expressed in invasive tumor cells and plays an important role in their proliferation and invasion(16-18, 32-34). The migration experiment results of this project showed that in PC3 and C4-2 cells of prostate cancer, the migration and invasion ability of OTUB1 overexpression cells was significantly enhanced compared with control cells (Fig 2C,2E). And the opposite result was found in the PC3 and C4-2 cells transfected with OTUB1 siRNA, the migration and invasion ability of PC3 and C4-2 cells with decreased OTUB1 expression attenuated dramatically (Fig 2D,2F).

Cell scratch method is one of the methods to determine the motion characteristics of tumor cells. Using the experimental model of in vitro cell injury and healing, the tumor cell migration ability was observed by applying scratch injury to monolayer cells cultured in vitro and adding low concentration serum medium to eliminate the interference caused by cell proliferation. An empty scar appeared on the cell layer. After the culture with low concentration serum, the effect of OTUB1 expression level on the migration ability of PC3 and C4-2 cells in prostate cancer was observed. The results showed that, compared with the control group, the migration ability of otub1 overexpression cells was significantly improved (Fig 2G), and the otub1-c91s group has no significant difference with control group. and the difference was statistically significant ($P < 0.05$). The similar conclusions also were found in the PC3 and C4-2 cells transfected with OTUB1 siRNA, the migration ability of PC3 and C4-2 cells with decreased OTUB1 expression attenuated dramatically (Fig 2H). The results showed that OTUB1 expression could significantly influence the migration ability of the cells (Fig 2G, H).

Effects of increased OTUB1 expression on PC3 and C4-2 cells cycle distribution

Cell growth curves were drawn to detect the proliferation of control, otub1 and otub1 c91s cells at 24h, 48h, 72h and 96h, respectively. After the increase of OTUB1 expression level, the changes of PCa cell growth levels at 24h, 48h, 72h and 96h were observed. The results showed that the growth ability of the cell lines with increased OTUB1 expression was significantly higher than that of control group, while the proliferation ability of otub1 c91s cells with OTUB1 mutation have no significant change with control group (Fig 3A, B). At the same way, we transfected PC3 and C4-2 cells with si otub1, we found the opposite results (Fig 3C, D). Our results showed that OTUB1 expression significantly influenced the proliferation of PC3 and C42 cells ($P < 0.05$) (Fig 3A-D).

The cells are made into a dispersed suspension and inoculated at a low density (2×10^3 cells /chamber). The rate at which a single cell grows, proliferate and forms small groups of cells (clones) is called the clonogenesis rate. All the cells formed obvious colonies after 10 days of culture, and the clonogenesis ability of PC3 and C4-2 cells was significantly enhanced after the increase of OTUB1 expression level, and the difference was statistically significant ($P < 0.01$) (Fig 3E, F). At the same way, we decrease the expression of OTUB1 via transfecting PC3 and C4-2 cells with si otub1, the clonogenesis ability of PC3 and C4-2 cells also decreased significantly (Fig 3G, H). From above results, we could easily find that the expression level of OTUB1 can influence the ability of forming colonies.

Cell cycle refers to the whole process of continuously dividing cells from the end of the last mitosis to the completion of the next mitosis, including the quiescent phase (G0), early DNA synthesis phase (G1), DNA synthesis phase (S), late DNA synthesis phase (G2), and division phase (M). After the increase of OTUB1 expression level, the proliferation ability of prostate cancer cells was significantly enhanced ($P < 0.05$) (Fig 3A-D). Cell cycle test results of PC3 and C42 showed that the ratio of G0/G1 phase decreased after OTUB1 expression level increased, while the ratio of G2/M+S phase increased, and the difference was statistically significant ($P < 0.05$) (Fig 3I, J). Overall, these experiments suggested that OTUB1 may influence PCa cells proliferation through altering cell cycle distribution (Fig 3I, J).

OTUB1 rescue Cyclin E1 from proteasomal degradation

We next investigated how OTUB1 promoted the G1 cell cycle progression, a series of OTUB1-relative proteins were analyzed via geneMANIA online database. Cyclin E1, a cell cycle-relative regulative key protein, was found to interact with OTUB1 closely (Fig 4A). To further explore the relationship between OTUB1 and Cyclin E1, we observed that the expression level of Cyclin E1 augment on the increased OTUB1 group (Fig 4B). We also observed that the protein expression level of OTUB1 and Cyclin E1 after PC3 cell was transfected with OTUB1 siRNA (Fig 4C). From above results, we can speculate that up-regulated OTUB1 could promote the expression of Cyclin E1, we had enough reasons to predict that Cyclin E1 may be regulated by OTUB1.

Further experiments are needed to verify this conclusion. Next, we explored how OTUB1 influence the expression level of Cyclin E1, we treated PC3 cell transfected with negative control siRNA and OTUB1 siRNA with DMSO and 10uM MG132 for 8h respectively. The results demonstrated the stability of Cyclin E1 could be decreased in OTUB1 siRNA transfected group, adding MG132 could preserved the stability partly, showing OTUB1 influenced the expression of Cyclin E1 via a proteasome dependent manner (Fig 4D). To further explained above results, we treated PC3 cell transfected with negative control and OTUB1 siRNA with 10mg/ml cycloheximide (CHX), a protein synthesis inhibitor in eukaryotic cells, in 0h, 4h, 8h and 24h. The results showed that CHX could promote the decease of Cyclin E1 protein, and the decrease rate of Cyclin E1 increased in PC3 cell transfected with OTUB1 siRNA. The detailed decrease rates were showed (Fig 4E, F). Above results implied us that Cyclin E1 might be regulated by OTUB1 via a deubiquitination degradation manner, to identify this hypothesis, we applied 10uM MG132 and 200uM chloroquine to PC3 cell transfected with OTUB1 siRNA for 8h. We found that MG132 could retain part stability of Cyclin E1 in PC3 cell transfected with OTUB1 siRNA, and chloroquine not (Fig 4G). The results demonstrated that Cyclin E1 might be degraded in a ubiquitination depend manner instead of lysosomal degradation pathway. To identify Cyclin E1 did be degraded in a ubiquitination depend manner, we conducted an immunoprecipitation assay to determined that OTUB1 could interact with Cyclin E1 (Fig 4H,I). Next, we transfected si OTUB1 in PC3 cell, then another immunoprecipitation assay was conducted to confirm that knockdown OTUB1 could strengthen the intensity of Cyclin E1 ubiquitination (Fig 4J). The results demonstrated that OTUB1 could promote tumor process and proliferation in prostate cancer via stabling the function of Cyclin E1 and increasing its expression level. We analyzed the correlation between OTUB1 and CCNE1 via GEPIA online database, the result presented that OTUB1 was positively related to CCNE1 ($R=0.19$; $P<0.01$), the result was displayed in the Figure 4J.

The OTUB1/Cyclin E1 axis promotes prostate cancer cell proliferation

To further explore the influence of OTUB1/ Cyclin E1 upon PCa, we transfected PC3 cell with si Cyclin E1 and otub1 wt. 48h after transfection, the protein expression level of Cyclin E1 was detected, the results demonstrated that otub1 promoted the expression of Cyclin E1 and Cyclin E1 expression was decreased obviously after transfecting si Cyclin E1, interesting that knockdown Cyclin E1 attenuated the effect of otub1 wt increasing the expression of Cyclin E1 (Fig 5A). The proliferation ability was tested by MTT

assay, the results demonstrated that knockdown Cyclin E1 obviously postponed proliferation comparing with control transfected group, and the effect of OTUB1 overexpression promoting proliferation was restrained significantly accompany with si Cyclin E1 (Fig 5B). This result was reinforced by healing assay and clone formation experiments, these results were consistent with previous results (Fig 5C, D). These results demonstrated that OTUB1/Cyclin E1 axis could promote proliferation and migration ability in prostate cancer. Disturbing the contact between OTUB1 and Cyclin E1 might serve a potential therapy for prostate cancer.

To identify the possibility of targeting OTUB1/ Cyclin E1 axis treatment in prostate cancer, we treated PC3 cell with RO-3306, a Cyclin E1/CDK2 related inhibitor, in 0, 340nM, 1uM, 2uM, 3uM and 5uM. 24h after treating with RO-3306, we extracted protein and conducted western blotting. We found that the expression of Cyclin E1 was decreased depended on RO-3306 dose. Interesting that RO-3306 also attenuated the effect of otub1 overexpression promoting Cyclin E1 (Fig 5E). More experiments were performed to further explore the influence, the results of MTT and clone formation experiments demonstrated that the proliferation ability would gradually decline with the RO-3306 concentration increasing (Fig 5F, H). And the results of healing assay were consistent with the abovementioned results (Fig 5G). RO-3306 treatment postponed the migration and proliferation ability, the application of RO-3306 to clinical treatment for prostate cancer might be a potential and effective treatment measure, play a role with decreasing the mortality rate of prostate cancer patients.

Increased expression level of OTUB1 promote PC3 cell tumor growth in vivo.

The animal model was male nude mice at 4-6 weeks. We planted 2×10^6 PC3 cells control and its overexpressed OTUB1 cloned cells otub1 in the groin of mice respectively. When the tumor grew to a diameter of 0.5 cm, we began to measure the tumor size daily with a vernier caliper (Fig 6C). After 12 days, the animals were sacrificed for cervical dislocation, and solid tumor was removed under sterile conditions. The volume of the tumor blocks of otub1 mice overexpressed OTUB1 and control group have distinct difference, and the OTUB1 c91s group has no obvious change with control group (Fig 6A, B). We found that the tumorigenesis rate of OTUB1 overexpressed cells in mice was significantly higher than that of control cells, especially after two weeks of tumorigenesis, which demonstrated the promoting effect of OTUB1 on tumor formation. We observed that the tumor of OTUB1 overexpression group also have high expression of OTUB1, ki-67 and Cyclin E1 with cells experiments compared with control group (Fig 6D).

Discussion

In our research, we found the expression of OTUB1 was up regulated in PCa, and OTUB1 could promote proliferation and progression in PCa via deubiquitinating and stabilizing Cyclin E1 protein expression.

Many previous researches had reported that OTUB1 were frequently up regulated in many cancer metastasis, such as esophageal squamous cell carcinoma and colon cancer(35). Based on these conclusions from previous researches, OTUB1 promoted esophageal squamous cell carcinoma metastasis by modulating snail stability(36), and colorectal cancer migration and progression were influenced with different level of OTUB1 via ERR α or miR-542-3p regulating(33, 34). OTUB1 regulated not only cancer metastasis but also chemotherapy resistance, U Karunarathna et al had found that OTUB1 inhibited the ubiquitination and degradation of FOXM1 in breast cancer and mediated epirubicin resistance(37). Recently, some researchers had proved OTUB1 could weaken interferon response to hepatitis B virus infection(38). The effect of OTUB1 in varies tumors reminded us that OTUB1 might play important role during cancer evolution process and some physiological activities.

In our research, we first found the expression of OTUB1 was up regulated in PCa compared with normal prostate tissue from TCGA database. And next, we conducted immunohistochemistry staining to identify above conclusion with PCa tissues and BPH. The results were consistent with the database conclusions (Fig 1). To further explored the specific role of OTUB1 in the progression of prostate cancer, we performed a series of experiments to monitor the changes of migration ability by altering the expression of OTUB, and the results demonstrated that high expression level of OTUB1 could significantly promote migration and invasion in both PC3 cell and C4-2 cell. When down regulated expression of OTUB1, the ability of influencing migration and invasion obviously decreased compared with untreated PC3 cell and C4-2 cell (Fig 2). Previous study about the influence of OTUB1 in prostate cancer, their results presented that different expression level of OTUB1 had no significant bias in cancer proliferation(23). In this research, we found that high expression of OTUB1 could promote proliferation of PC3 cell and C4-2 cell. We also found that cell cycle G1 phase shortened when the expression of OTUB1 was elevated (Fig 3). To explore how OTUB1 influence proliferation, we found that high expression level of OTUB1 could increase the expression of Cyclin E1. Further experiments presented that Cyclin E1 interacted with OTUB1. OTUB1 could mediate deubiquitination of Cyclin E1 and stable the expression level and function of Cyclin E1 and the ability of proliferation would decline when PC3 cell co-transfected with overexpression OTUB1 and knock down Cyclin E1 compared with simply transfected with overexpression OTUB1 (Fig 4-5). Above results reminded us that OTUB1 might promote the proliferation in prostate cancer via mediating Cyclin E1. The results of vivo experiments in animals were consistent with the results of above cell experiments (Fig 6). So far, we had much evidence to identify the hypothesis that OTUB1 promoted the progression and proliferation in prostate cancer via mediating and stabling Cyclin E1. Previous researches had proved that Cyclin E1 belonged to the highly conserved Cyclin family, whose members are characterized by a dramatic periodicity in protein abundance through the cell cycle(26). Cyclins function as regulators of CDK kinases(39, 40). This cyclin forms a complex with and functions as a regulatory subunit of CDK2, whose activity is required for cell cycle G1/S transition(41). Many researches had presented that Cyclin E1 could mediate progression of many tumors, such hepatocellular carcinoma(41, 42), ovarian cancer(43) and breast cancer(44). Previous researches had proved that Cyclin E1 had serval functional domains, mainly including a central cyclin homology district (interacting with CDK2), a unique N-terminal region and a C-terminal PEST sequence, which was often detected in protein degraded through the

ubiquitin system(45-48). As an unstable protein, Cyclin E1 was degraded by two distinct pathways involving the ubiquitin-proteasome system mediated by Cul1 or Cul3, which both belonged to the cullin-RING family of ubiquitin ligases(49, 50). And Cul1 mediated-degradation requires phosphorylation of Cyclin E at T77 and T395 in order to ubiquitylation of cyclin E to arise(51). While previous studies had shown that Cul3 degrades Cyclin E that was not bound to Cdk2 and regulation of Cyclin E by Cul3(49, 52). Although our research found that the deubiquitinase OTUB1 promoted Cyclin E1 stability and prostate cancer progression, the specific mechanism with OTUB1 mediated function of Cyclin E1 needed to research in further. The interaction among OTUB1, Cul1 and Cul3 might become the internal reasons for stable Cyclin E1 and its subsequent functions. The next aims of our research focus on the relationship between OTUB1 and ubiquitin ligases.

Not only prostate cancer and above mentioned cancers, recently, Kai Zhou et al had found that OTUB1 could promote the progression of renal cell carcinoma via mediating deubiquitination of FOXM1 up-regulated the expression of ECT-2(32). Other researchers had proved that SP1 regulated the progression of non-small-cell lung cancer by recruiting OTUB1(53).

Based on above results, we found that the high expression level of OTUB1 promoted the PCa cell migration and invasion ability, and knockdown the expression of OTUB1 decreased migration and invasion of PCa cell. Previous studies on the biological function of OTUB1 were contradictory. The original study found OTUB1 attributed to the stability of P53 protein, and resulting in inhibiting cell proliferation. Recent research indicated that OTUB1 involved in the invasion and migration of malignant tumors. But in our study, we discovered that high level of OTUB1 promoted proliferation, and low expression on the contrary. And we further found that OTUB1 promoted the progression via mediating the stability and interacting with Cyclin E1. Cyclin E1 was mainly degraded in a ubiquitination manner, yet OTUB1 could inhibited the ubiquitination of Cyclin E1 and stabled the function to promote the proliferation. This mechanism and relationship reminded us that OTUB1-Cyclin E1 pathway might serve a potential treat target during the treatment of PCa, when we interfere or interrupt the connection between OTUB1 and Cyclin E1, the proliferation and progression might slow down or cease. The results of RO-3306 treatment was the directed evidence for targeting OTUB1/ Cyclin E1 axis treatment in prostate cancer. This newly target treatment maybe become an effective therapy for lots of patients with PCa. The specific mechanism of OTUB1/Cyclin E1 axis needs to further experiments to investigate and reinforce.

Conclusions

Here, our researches have presented that OTUB1 interacted and mediated with Cyclin E1 to promote progression, migration and proliferation in prostate cancer. The OTUB1/Cyclin E1 axis may serve a potential treatment target for patients with prostate cancer. The prognosis of patients with prostate cancer maybe improve when the connection between OTUB1 and Cyclin E1 are interrupted or disturbed.

Abbreviations

OTUB1: ovarian tumor domain deubiquitination 1; PCa: prostate cancer; ccne1: Cyclin E1; AR: Androgen receptor; BPH: Benign prostate hyperplasia; IP: Immunoprecipitation; UB: Ubiquitination; DUB: Deubiquitination; ADT: androgen deprivation therapy; CRPC: castration-resistant prostate cancer; mCRPC: metastasis castration resistant prostate cancer.

Declarations

Disclosures

None

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Research Ethics Committee of The Second Hospital of Tianjin Medical University and with the 1964 Helsinki declaration and its later amendments.

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

None of the authors have any relevant conflicts of interest pertaining to the studies and data in this manuscript.

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Authors' contributions

Yihao Liao: Project development, Perform experiment, Data analysis, Manuscript writing

Ning Wu: Project development

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Miaomiao Wang: Data analysis

Youzhi Wang: Perform experiment

Jie Gao: Data collection

Boqiang Zhong: Data collection

Fuling Ma: Project development

Yudong Wu: Data analysis

Ning Jiang: Project development

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References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA: a cancer journal for clinicians*. 2019;69(1):7-34.
2. Nguyen-Nielsen M, Borre M. Diagnostic and Therapeutic Strategies for Prostate Cancer. *Seminars in nuclear medicine*. 2016;46(6):484-90.
3. Bastos DA, Antonarakis ES. CTC-derived AR-V7 detection as a prognostic and predictive biomarker in advanced prostate cancer. *Expert review of molecular diagnostics*. 2018;18(2):155-63.
4. Murillo-Garzón V, Kypta R. WNT signalling in prostate cancer. *Nature reviews Urology*. 2017;14(11):683-96.
5. Gasnier A, Parvizi N. Updates on the diagnosis and treatment of prostate cancer. *The British journal of radiology*. 2017;90(1075):20170180.
6. Hossain MK, Nahar K, Donkor O, Apostolopoulos V. Immune-based therapies for metastatic prostate cancer: an update. *Immunotherapy*. 2018;10(4):283-98.
7. Komura K, Sweeney CJ, Inamoto T, Ibuki N, Azuma H, Kantoff PW. Current treatment strategies for advanced prostate cancer. *International journal of urology : official journal of the Japanese Urological Association*. 2018;25(3):220-31.
8. Altwaijry N, Somani S, Dufès C. Targeted nonviral gene therapy in prostate cancer. *International journal of nanomedicine*. 2018;13:5753-67.
9. Sebesta EM, Anderson CB. The Surgical Management of Prostate Cancer. *Seminars in oncology*. 2017;44(5):347-57.
10. Smolle MA, Bauernhofer T, Pummer K, Calin GA, Pichler M. Current Insights into Long Non-Coding RNAs (LncRNAs) in Prostate Cancer. *International journal of molecular sciences*. 2017;18(2).
11. Popovic D, Vucic D, Dikic I. Ubiquitination in disease pathogenesis and treatment. *Nature medicine*. 2014;20(11):1242-53.

12. Sivakumar D, Kumar V, Naumann M, Stein M. Activation and selectivity of OTUB-1 and OTUB-2 deubiquitinylases. *The Journal of biological chemistry*. 2020;295(20):6972-82.
13. Wiener R, Zhang X, Wang T, Wolberger C. The mechanism of OTUB1-mediated inhibition of ubiquitination. *Nature*. 2012;483(7391):618-22.
14. Nakada S, Tai I, Panier S, Al-Hakim A, Iemura S, Juang YC, et al. Non-canonical inhibition of DNA damage-dependent ubiquitination by OTUB1. *Nature*. 2010;466(7309):941-6.
15. Liu T, Jiang L, Tavana O, Gu W. The Deubiquitylase OTUB1 Mediates Ferroptosis via Stabilization of SLC7A11. *Cancer research*. 2019;79(8):1913-24.
16. Zhou Y, Wu J, Fu X, Du W, Zhou L, Meng X, et al. OTUB1 promotes metastasis and serves as a marker of poor prognosis in colorectal cancer. *Molecular cancer*. 2014;13:258.
17. Weng W, Zhang Q, Xu M, Wu Y, Zhang M, Shen C, et al. OTUB1 promotes tumor invasion and predicts a poor prognosis in gastric adenocarcinoma. *American journal of translational research*. 2016;8(5):2234-44.
18. Sun J, Deng Y, Shi J, Yang W. MicroRNA-542-3p represses OTUB1 expression to inhibit migration and invasion of esophageal cancer cells. *Molecular medicine reports*. 2020;21(1):35-42.
19. Wang Y, Zhou X, Xu M, Weng W, Zhang Q, Yang Y, et al. OTUB1-catalyzed deubiquitination of FOXM1 facilitates tumor progression and predicts a poor prognosis in ovarian cancer. *Oncotarget*. 2016;7(24):36681-97.
20. Xu L, Li J, Bao Z, Xu P, Chang H, Wu J, et al. Silencing of OTUB1 inhibits migration of human glioma cells in vitro. *Neuropathology : official journal of the Japanese Society of Neuropathology*. 2017;37(3):217-26.
21. Ni Q, Chen J, Li X, Xu X, Zhang N, Zhou A, et al. Expression of OTUB1 in hepatocellular carcinoma and its effects on HCC cell migration and invasion. *Acta biochimica et biophysica Sinica*. 2017;49(8):680-8.
22. Saldana M, VanderVorst K, Berg AL, Lee H, Carraway KL. Otubain 1: a non-canonical deubiquitinase with an emerging role in cancer. *Endocrine-related cancer*. 2019;26(1):R1-r14.
23. Iglesias-Gato D, Chuan YC, Jiang N, Svensson C, Bao J, Paul I, et al. OTUB1 de-ubiquitinating enzyme promotes prostate cancer cell invasion in vitro and tumorigenesis in vivo. *Molecular cancer*. 2015;14(1):8.
24. Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. *Nature reviews Cancer*. 2009;9(3):153-66.
25. Wei R, Thanindratarn P, Dean DC, Hornicek FJ, Guo W, Duan Z. Cyclin E1 is a prognostic biomarker and potential therapeutic target in osteosarcoma. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society*. 2020;38(9):1952-64.
26. Masaki T, Shiratori Y, Rengifo W, Igarashi K, Yamagata M, Kurokohchi K, et al. Cyclins and cyclin-dependent kinases: comparative study of hepatocellular carcinoma versus cirrhosis. *Hepatology (Baltimore, Md)*. 2003;37(3):534-43.

27. Geng Y, Yu Q, Sicinska E, Das M, Schneider JE, Bhattacharya S, et al. Cyclin E ablation in the mouse. *Cell*. 2003;114(4):431-43.
28. Kanska J, Zakhour M, Taylor-Harding B, Karlan BY, Wiedemeyer WR. Cyclin E as a potential therapeutic target in high grade serous ovarian cancer. *Gynecologic oncology*. 2016;143(1):152-8.
29. Bisteau X, Caldez MJ, Kaldis P. The Complex Relationship between Liver Cancer and the Cell Cycle: A Story of Multiple Regulations. *Cancers*. 2014;6(1):79-111.
30. Ehedego H, Mohs A, Jansen B, Hiththetiya K, Sicinski P, Liedtke C, et al. Loss of Cyclin E1 attenuates hepatitis and hepatocarcinogenesis in a mouse model of chronic liver injury. *Oncogene*. 2018;37(25):3329-39.
31. Fang D, Huang S, Su SB. Cyclin E1-CDK 2, a potential anticancer target. *Aging*. 2016;8(4):571-2.
32. Zhou K, Mai H, Zheng S, Cai W, Yang X, Chen Z, et al. OTUB1-mediated deubiquitination of FOXM1 up-regulates ECT-2 to promote tumor progression in renal cell carcinoma. *Cell & bioscience*. 2020;10:50.
33. Yuan L, Yuan P, Yuan H, Wang Z, Run Z, Chen G, et al. miR-542-3p inhibits colorectal cancer cell proliferation, migration and invasion by targeting OTUB1. *American journal of cancer research*. 2017;7(1):159-72.
34. Zhou Y, Jia Q, Meng X, Chen D, Zhu B. ERR α Regulates OTUB1 Expression to Promote Colorectal Cancer Cell Migration. *Journal of Cancer*. 2019;10(23):5812-9.
35. Liu X, Jiang WN, Wang JG, Chen H. Colon cancer bears overexpression of OTUB1. *Pathology, research and practice*. 2014;210(11):770-3.
36. Zhou H, Liu Y, Zhu R, Ding F, Cao X, Lin D, et al. OTUB1 promotes esophageal squamous cell carcinoma metastasis through modulating Snail stability. *Oncogene*. 2018;37(25):3356-68.
37. Karunarathna U, Kongsema M, Zona S, Gong C, Cabrera E, Gomes AR, et al. OTUB1 inhibits the ubiquitination and degradation of FOXM1 in breast cancer and epirubicin resistance. *Oncogene*. 2016;35(11):1433-44.
38. Xie M, Yin Y, Chen L, Yin A, Liu Y, Liu Y, et al. Scavenger receptor A impairs interferon response to HBV infection by limiting TRAF3 ubiquitination through recruiting OTUB1. *The FEBS journal*. 2020;287(2):310-24.
39. Asghar U, Witkiewicz AK, Turner NC, Knudsen ES. The history and future of targeting cyclin-dependent kinases in cancer therapy. *Nature reviews Drug discovery*. 2015;14(2):130-46.
40. Hu W, Nevzorova YA, Haas U, Moro N, Sicinski P, Geng Y, et al. Concurrent deletion of cyclin E1 and cyclin-dependent kinase 2 in hepatocytes inhibits DNA replication and liver regeneration in mice. *Hepatology (Baltimore, Md)*. 2014;59(2):651-60.
41. Xu J, Huang F, Yao Z, Jia C, Xiong Z, Liang H, et al. Inhibition of cyclin E1 sensitizes hepatocellular carcinoma cells to regorafenib by mcl-1 suppression. *Cell communication and signaling : CCS*. 2019;17(1):85.

42. Sonntag R, Giebler N, Nevzorova YA, Bangen JM, Fahrenkamp D, Lambertz D, et al. Cyclin E1 and cyclin-dependent kinase 2 are critical for initiation, but not for progression of hepatocellular carcinoma. *Proceedings of the National Academy of Sciences of the United States of America*. 2018;115(37):9282-7.
43. Au-Yeung G, Lang F, Azar WJ, Mitchell C, Jarman KE, Lackovic K, et al. Selective Targeting of Cyclin E1-Amplified High-Grade Serous Ovarian Cancer by Cyclin-Dependent Kinase 2 and AKT Inhibition. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2017;23(7):1862-74.
44. Turner NC, Liu Y, Zhu Z, Loi S, Colleoni M, Loibl S, et al. Cyclin E1 Expression and Palbociclib Efficacy in Previously Treated Hormone Receptor-Positive Metastatic Breast Cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2019;37(14):1169-78.
45. Lew DJ, Dulić V, Reed SI. Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. *Cell*. 1991;66(6):1197-206.
46. Richardson HE, O'Keefe LV, Reed SI, Saint R. A *Drosophila* G1-specific cyclin E homolog exhibits different modes of expression during embryogenesis. *Development (Cambridge, England)*. 1993;119(3):673-90.
47. Honda R, Lowe ED, Dubinina E, Skamnaki V, Cook A, Brown NR, et al. The structure of cyclin E1/CDK2: implications for CDK2 activation and CDK2-independent roles. *The EMBO journal*. 2005;24(3):452-63.
48. Rath SL, Senapati S. Why are the truncated cyclin Es more effective CDK2 activators than the full-length isoforms? *Biochemistry*. 2014;53(28):4612-24.
49. Davidge B, Rebola KGO, Agbor LN, Sigmund CD, Singer JD. Cul3 regulates cyclin E1 protein abundance via a degron located within the N-terminal region of cyclin E. *Journal of cell science*. 2019;132(21).
50. Welcker M, Singer J, Loeb KR, Grim J, Bloecher A, Gurien-West M, et al. Multisite phosphorylation by Cdk2 and GSK3 controls cyclin E degradation. *Molecular cell*. 2003;12(2):381-92.
51. Minella AC, Loeb KR, Knecht A, Welcker M, Varnum-Finney BJ, Bernstein ID, et al. Cyclin E phosphorylation regulates cell proliferation in hematopoietic and epithelial lineages in vivo. *Genes & development*. 2008;22(12):1677-89.
52. McEvoy JD, Kossatz U, Malek N, Singer JD. Constitutive turnover of cyclin E by Cul3 maintains quiescence. *Molecular and cellular biology*. 2007;27(10):3651-66.
53. Xie JJ, Guo QY, Jin JY, Jin D. SP1-mediated overexpression of lncRNA LINC01234 as a ceRNA facilitates non-small-cell lung cancer progression via regulating OTUB1. *Journal of cellular physiology*. 2019;234(12):22845-56.

Figures

Figure 1

OTUB1 was upregulated in prostate cancer. (B) OTUB1 are upregulated in prostate cancer according to TCGA. (C) The expression of OTUB1 was upregulated in human prostate cancer in IHC staining. (D) The expression of Ki-67 was upregulated in human prostate cancer in IHC staining. (E) The IHC was statistical analysis according to the strength of the expression.

Figure2.

Figure 2

OTUB1 promoted proliferation and invasion of PCa cell. (A,B) C4-2 and PC3 cells were transfected with OTUB1 overexpression (otub1 wt) and otub1 c91s, lysed 48h post transfection. Whole cell extracts were analyzed by western blotting with OTUB1 antibodies. (C) transwell migration and invasion assays in PC3 cells transfected with OTUB1 overexpression and otub1 c91s. (D) transwell migration and invasion assays in PC3 cells transfected with OTUB1 siRNAs. (E) transwell migration and invasion assays in C4-2 cells transfected with OTUB1 overexpression and otub1 c91s. (F) transwell migration and invasion assays in C4-2 cells transfected with si otub1. (G,H) Wound healing assays with OTUB1 overexpression, otub1 c91s and si otub1 in PC3 and C4-2 cells. Migration of cells to the wound was visualized at 0 and 24h with an inverted Leica phase-contrast microscope.

Figure3.

Figure 3

Effects of increased OTUB1 expression on PC3 and C4-2 cells cycle distribution. (A,B)MTT assays in PC3 and C42 cells transfected with otub1 overexpression and otub1 c91s. (C,D)MTT assays in PC3 and C42 cells transfected with si otub1. (E,F) Colony formation assays in PC3 and C42 cells transfected with otub1 overexpression and otub1 c91s. (G,H)Colony formation assays in PC3 and C42 cells transfected with si otub1. (I,J)Effects of increased otub1 expression on the cycle distribution of PC3 and C42 cells.

[illegible]

OTUB1 rescue Cyclin E1 from proteasomal degradation. (A) the relationship between OTUB1 and Cyclin E1 was analyzed via geneMANIA database. (B) PC3 and C4-2 cells were transfected with *otub1* over-expression and *otub1* c91s, lysed 48h post transfection. Whole cell extracts were analyzed by western blotting with the indicated antibodies (OTUB1, Cyclin E1 and GAPDH). (C) PC3 and C4-2 cells were transfected with two different si *otub1* and lysed 48h post transfection. The expression of Cyclin E1 and OTUB1 were analyzed by western blotting. (D) PC3 cell was transfected with an increasing *otub1* over expression, the protein expression of OTUB1 and Cyclin E1 were analyzed. (E) PC3 cell was transfected with OTUB1 siRNA and control siRNA respectively, treated with DMSO and 10uM MG132 for 8h. And the expression levels of proteins were analyzed by western blotting. (F) The protein expression of Cyclin E1 and OTUB1 were analyzed between OTUB1 siRNA transfected group and control group treated with 10mg/ml CHX for 0h, 4h, 8h and 24h. (G) PC3 cell were transfected with OTUB1 siRNA, respectively treated with 10uM MG132 for 8h, 200uM chloroquine for 8h and treated with DMSO as control for 8h. The expression level of Cyclin E1 and OTUB1 were analyzed by western blotting. (H) Cyclin E1 protein was enriched via IP assay, blotting with Cyclin E1 and OTUB1 antibody and monitoring the change of the

protein expression (the Upper). Purified FLAG-OTUB1 protein was enriched via IP assay, blotting with Cyclin E1 and OTUB1 antibody and monitoring the change of the protein expression (the Lower). (I) The effect of si otub1 on Cyclin E1 ubiquitination in PC3 cell. (J) The correlation between OTUB1 and Cyclin E1 was analyzed online ($R=0.19$, $P\leq 0.01$).

Fig 5

Figure 5

The OTUB1/Cyclin E1 axis promotes prostate cancer cell proliferation. (A) The effect of otub1 wt and si cyclin e1 on protein expression levels of Cyclin E1 in PC3 cell. (B, D) The effect of otub1 wt and si cyclin e1 on cell proliferation of PC3 cell. (C) The effect of si cyclin e1 and otub1 wt on cell migration of PC3 cell. (E) The effect of otub1 and different dose RO-3306 on protein expression levels of Cyclin E1 in PC3 cell. (F, H) The effect of different dose RO-3306 on cell proliferation of PC3 cell. (G) The effect of different dose RO-3306 on cell migration of PC3 cell.

Figure 6.

Figure 6

Increased expression level of OTUB1 promote PC3 cell tumor growth in vivo.. (A)PC3 cells transfected with otub1 overexpression and otub1 c91s were implanted subcutaneously in nude mice. The effect of otub1 wt and otub1 c91s on PCa tumor growth. (B)Tumor volumes were measured with calipers. (C)Tumor volumes were measured daily after implanting in nude mice. (D)IHC staining with OTUB1, ki-67 and Cyclin E1 were performed in the tumor from nude mice.

Supplementary Files

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