The Deubiquitinase OTUD3 Stabilizes ACTN4 to Activate NF-κB Signaling Pathway in Hepatocellular Carcinoma

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Research

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Abstract

Background: OTUD3, a deubiquitinating enzyme, has emerged as important role in some cancer. It showed that OTUD3 plays suppressive role in breast cancer whereas oncogenic role in lung cancer. However, the function and mechanism of OTUD3 in hepatocellular carcinoma (HCC) progression remain elusive.

Methods: Gene and protein expression of OTUD3 in HCC tissues were determined by qRT-PCR, western blot and immunohistochemistry. A series of gain- and loss-of-function assays were used to investigated the role of OTUD3 in HCC cells progression. Moreover, massspectroscopic analysis and RNA-seq were used to identify the downstream targets of OTUD3 in HCC cells. The interaction between OTUD3 and ACTN4 was examined through co-IP experiment and in vitro ubiquitination assay.

Results: In our research, OTUD3 was significantly overexpressed in HCC tissues and higher OTUD3 expression was correlated with bigger tumor size, more distant metastasis, and worse TNM stage. Additionally, OTUD3 promoted HCC cells growth and metastasis in vitro and in vivo. Furthermore, ACTN4 was identified as a downstream target of OTUD3 and ACTN4 protein level was significantly related to OTUD3 expression. Rescue experiments indicated that ACTN4 was essential for OTUD3-mediated HCC proliferation and metastasis in vitro and in vivo. Moreover, we identified that NF-κB signaling pathway was activated by OTUD3 through ACTN4 to promote HCC cells progression. Importantly, OTUD3 protein level was correlated with ACTN4 protein level and activity of NF-κB signaling pathway in HCC tissues.

Conclusion: Our findings identify the oncogenic role of OTUD3 in HCC and suggest that OTUD3 can be considered as a pivotal prognostic biomarker and a potential therapeutic target.

Background

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death and it is currently the sixth most common cancer worldwide [1]. As a lethal cancer with limited therapeutics, the 5-year survival rate of HCC is only about 14%–18% and the mortality is mostly due to the high probability of metastasis and rapid growth [2]. Therefore, understanding the molecular mechanisms of HCC progression would provide effective therapeutic strategies to reduce HCC mortality.

Post-translational modifications of proteins can be reversed by some peptidases like deubiquitinase or deubiquitylating enzymes (DUBs), which mediate cleaving and removing of the ubiquitin chains from substrate proteins [3]. The cysteine protease DUBs can be classified into four subfamilies according to their Ub-protease domains: ovarian tumor (OTU), ubiquitin C-terminal hydrolase (UCH), ubiquitin-specific protease (USP) and Machado Joseph disease protease (MJD) [4]. Most members of ovarian tumor (OTU) deubiquitinase act as important modulators of multiple cellular cascades. It has reported that OTUD5 is involved in interferon signaling [5] and OTUD1 regulates DNA damage response [6]. Additionally, OTUD7B and OTULIN play important role in NF-kB signaling pathway [7, 8]. As a member of OUT family, the molecular biological function of OTUD3 (OTU domain-containing protein 3) is seldomly reported.
However, increasing investigations has laid emphasis on the role of OTUD3 in cancer. Zhang et al reported the suppressive function of OTUD3-PTEN axis in breast cancer and OTUD3 can stabilize PTEN through deubiquitylation [9]. Subsequently, their further study showed the pro-oncogenic role of OTUD3 in lung cancer and that OTUD3 drives lung cancer progression through stabilizing GRP78 (glucose-regulated protein 78-kDa) [10]. Additionally, other study revealed that OTUD3 downregulation accelerates growth and motility of colorectal cancer cells [11].

Here, we showed that OTUD3 was significantly upregulated in HCC and the correlation between OTUD3 expression and clinicopathological characteristics of HCC patients was investigated. We also examined the oncogenic role of OTUD3 in promoting HCC cells proliferation, metastasis through in vitro and in vivo experiments. Furthermore, we found the positive correlation between OTUD3 and alpha-actinin 4 (ACTN4) expression and ACTN4 was essential for OTUD3-mediated HCC progression. Mechanistically, OTUD3 can deubiquitinate and stabilize ACTN4 to improve the protein level of ACTN4 in HCC cells. Finally, we identified that OTUD3-ACTN4 axis mediate HCC progression through NF-κB signaling pathway. In clinical, OTUD3 expression was significantly correlated with ACTN4 expression and NF-κB signaling pathway activity in HCC tissues. Thus, our research strongly suggests that OTUD3 might be a novel target for HCC therapy.

Materials And Methods

Patients and Human specimens

Paraffin-embedded and fresh human HCC samples were collected from 115 patients undergoing HCC resection at the Jiangxi Province Tumor Hospital of Nanchang University from June 2013 to July 2020. Fresh specimens obtained after resection were frozen in liquid nitrogen and stored at -80°C for further investigation. Informed consent of the patients was obtained and the investigation was permitted by research ethics committee of the Jiangxi Province Tumor Hospital of Nanchang University. All patients were followed up for 5 years.

Cell culture and treatment

HCC cell lines including Huh7, MHCC97H, HepG2, HCCLM3, Hep3B and human normal hepatocyte cell lines HL-7702 were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences and the Shanghai Institute of Cell Biology in China. The identity of the cell lines was confirmed by short tandem repeat analysis. All cell lines were cultured in Dulbecco's modified Eagle's Medium (Gibco) containing 10% fetal calf serum (FBS, HyClone, USA) at 37 °C in a humidified incubator containing 5% CO2.

quantitative real-time PCR (qRT-PCR)

Total RNA was extracted by the standard Trizol-based protocol (Invitrogen, USA). Complementary DNA (cDNA) was synthesized using the PrimeScript RT Reagent Kit (Invitrogen, USA) and qRT-PCR was
performed using SYBR Premix Ex Taq (TaKaRa Bio, Shiga, Japan), according to the manufacturer's instructions. Information about the gene-specific primers were in Supplementary Table 1.

**Western blot**

Western blot was performed as previous study [12]. Extraction of total cellular proteins was extracted by RIPA buffer (Beyotime, Shanghai, China) containing protease and inhibitor mixes (Thermo Fisher Scientific, New York, USA) on ice. BCA Protein Assay kit (Thermo Scientific, Waltham, MA, USA) was performed to evaluate protein concentration. Equal amounts of proteins were separated by sodium dodecylsulfonate (SDS) polyacrylamide gel electrophoresis and transferred onto apolyvinylidene fluoride (PVDF) membrane by electroblotting (Millipore, Bedford, MA, USA). Primary antibodies were added and incubated throughout a night at 4°C. Primary antibodies including anti-OTUD3 monoclonal antibody (1:500; HPA028543, Sigma), anti-ACTN4 monoclonal antibody (1:1000, 15145, CST), anti-p65 monoclonal antibody (1:1000, 8242, CST), anti-p-p65 monoclonal antibody (Ser536)(1:1000, 3033, CST), anti-IκBα monoclonal antibody (1:1000, 4812, CST), anti-p-IκBα monoclonal antibody (Ser32) (1:1000, 2859, CST) and anti-Tubulin monoclonal antibody (code ab7291, 1:2000 dilution, Abcam). After being incubated with the second antibody (CST, MA, USA) for 1h at room temperature, the intensity of protein bands was analyzed by Quantity One software (Bio-Rad, Hercules, CA, USA).

**Immunohistochemistry (IHC) staining**

Paraffin-embedded sections (4 mm thick) of human HCC tissues and normal adjacent tissues were deparaffinized. Sections were subjected to antigen retrieval in microwave-heated citrate buffer (pH6.0) for 30 mins. After incubation for 30 mins in goat serum (Solarbio, Beijing, China), tissue sections were incubated by primary antibodies overnight at 4°C. Next, HRP-conjugated secondary antibody (Boster) was used to incubate sections for 2h at room temperature. DAB Detection Kit (Maxim) was adopted for immunostaining for 2 mins. The proportion of positive areas were scored semi-quantitatively by 3 pathologists who were blind to the clinical parameters. In brief, 100 cells were counted randomly at 200X microscopic fields and were classified into five groups according to the percentage of positive staining cells in HCC tissues as follows: 0 = negative; 1 – 3 = 1 – 25%; 4 – 6 = 26 – 50%; 7 – 9 = 51 – 75%; 10 – 12 = ≥76%. The score ranging from 0 to 6 was considered as a low-expression group, whereas the score ranging from 7 to 12 was considered as a high-expression group.

**Stable cell lines and plasmids**

HCC cell lines with stable OTUD3 overexpression (OE) or knockdown were established by transfection of lentivirus containing OTUD3 overexpression plasmid (GV640 vector, Genechem, Shanghai, China) or short hairpin RNA (shRNA) (pGLVH1 vector, GenePharma, Shanghai, China). Cells infected by lentivirus were selected using puromycin (Invitrogen, Carlsbad, CA, USA) for one month. Transient OTUD3 overexpression or knockdown was performed by transfections of OTUD3 OE or knockdown plasmids using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommended protocol [13].
**Immunofluorescence staining**

Paraffin-embedded sections of xenografted tumor tissues were deparaffinized, followed by antigen retrieval in microwave-heated EDTA buffer (pH 8.0) for 30 mins. After incubation for 15 mins in 0.1% TritonX-100 (Solarbio, Beijing, China) and 1% goat serum (Solarbio, Beijing, China) for 30 mins, tissue sections were incubated in dark by primary antibodies overnight at 4°C, followed by secondary incubation for 1h with Alexa Fluor 594 goat anti-rabbit IgG (1:500, Life Technologies). Nuclear were stained with Hoechst 33342 (Life Technologies) for 1 min. Tissues were visualized using a confocal laser scanning microscope (SP-II; Leica Microsystems, Wetzlar, Germany).

**EdU assay**

Cells were seeded in 96-well plates at an initial concentration of 5x10^4 cells per well. After culturing for 24 hours, 5-ethynyl-20-deoxyuridine (EdU; Ribobio) was used to culture cells for 2 hours, followed by three washes with PBS. After cells being incubated with 1xApollo reaction cocktail for 30 minutes, Hoechst 33342 (5 mg/mL) was used to stain the DNA contents of the cells in each well for 25 minutes and was visualized through a confocal laser scanning microscope (SP-II; Leica Microsystems, Wetzlar, Germany).

**Cell Counting Kit-8 assay**

CCK8 assay was used to evaluate cell viability after 24, 48, 72, 96, 120h. Transfected cells were seeded into 96-well plates at an initial concentration of 3x10^3 cells per well. 10μl of CCK-8 solutions (Dojindo Laboratories, Kumamoto, Japan) was added to each well according to the manufacturer's instructions. After incubation in cell incubator for 1h, the absorbance at wavelength of 450 nm was recorded.

**The wound-healing assay**

Transfected cells were incubated into 6-well plates until growing to 80% to 90% confluence. Then 200μl pipette tip was used to scratch across the cells surface followed by three washed with PBS. Subsequently, the cells were incubated at 37 °C and the wound range was imaged by phase-contrast photography at 0h, 24h and 48h. Three randomly selected wound areas were analyzed.

**In vitro migration and invasion assays**

Cell migration assay and invasion assays were performed using a transwell system (Corning, NY, USA) with or without Matrigel matrix (BD bioscience) coated above the membrane. Stably transfected cells were suspended in pure DMEM at a concentration of 1x10^5 /ml. 500μl cell suspension was added in the upper chamber. Fresh medium containing 10% FBS was added in the lower chamber as a chemoattractant. After incubation for 48h, the non-migrated cells on the upper surface of the membrane were removed, and the cells on the lower surface were fixed with methanol and stained by 0.1% crystal violet. The cells in five random microscopic fields were counted and imaged using a light microscope with a DP70 CCD system (Olympus Corp).
**Co-immunoprecipitation experiment**

Cell lysis was incubated with 50µl protein A+G Agarose (Thermo Scientific) and 1 µg of the indicated antibody overnight at 4°C. The protein A/G-agarose were collected by centrifugation. Loading buffer was added to the tube and heated for 15 mins at 100°C. Then the immunoprecipitated proteins were examined by SDS-PAGE and immunoblotting analysis. The intensity of protein bands was analyzed by Quantity One software (Bio-Rad, Hercules, CA, USA).

**Xenografts mouse model**

HCCLM3 cells (2 x10^6 cells per mouse) expressing a luciferase reporter stably transfected with LV-shNC or LV-shOTUD3 lentivirus were subcutaneously injected into the flanks of 8-week-old female BALB/c-nude mice (n=6 per group). The in vivo imaging system (IVIS, PerkinElmer, USA) was employed to monitor and image the growth of tumours regularly. Tumours were measured by caliper every five days to examine tumour volume using the formula: \( V = \frac{\text{length}}{2} \times \text{width}^2 \). Finally, all of the tumour xenografts were harvested and weighted at the 30th day. All animals were randomly divided into different groups by a technician under blinding condition. Animal experiments were approved by the Ethics Committee for Animal Experiments of the Second Affiliated Hospital of Nanchang University.

**Metastasis model**

HCCLM3 cells (2 x10^6 cells per mouse) expressing a luciferase reporter stably transfected with LV-shNC or LV-shOTUD3 lentivirus were injected into the tail vein of 8-week-old female BALB/c-nude mice (n=6 per group). IVIS was employed to monitor and photograph the tumour progression in mice. Organs of mice were harvested after 5 weeks and metastatic nodes in lung sections were evaluated after HE staining. All animals were randomly divided into different groups by a technician under blinding condition. Animal experiments were approved by the Ethics Committee for Animal Experiments of the Second Affiliated Hospital of Nanchang University.

**Statistical analysis**

All results are shown as mean ± SD from at least three independent experiments. Log-rank test was employed to analyze survival of patients. Student’s t-test was used in statistical analyses between two groups. One-way ANOVA was employed for multiple comparisons. GraphPad Prism (version 5) was used in all statistical analyses and \( P< 0.05 \) was considered significant.

**Results**

**OTUD3 is aberrantly upregulated in HCC tissues and is significantly correlated with prognosis in HCC patients**
To examine the expression level of OTUD3 in HCC tissues, we performed IHC staining and examined 115 pairs (including 50 pairs fresh tissues) paraffin-embedded archived HCC and para-cancerous tissues. We found that OTUD3 was highly expressed in HCC tissues (Fig. 1A and B). Consistently, we performed qRT-PCR and western blot in 50 pairs fresh HCC tissues. qRT-PCR results showed that mRNA level of OTUD3 was significantly upregulated in 37 of 50 HCC specimens compared with corresponding adjacent tissues (Fig. 1C). Western blot also indicated that OTUD3 protein was markedly overexpressed in HCC tissues (Fig. 1D and E). Furthermore, we examined the relationship of OTUD3 levels with different clinicopathological characteristics and found that higher OTUD3 expression was correlated with bigger tumor size, more vascular invasion, intrahepatic metastasis and worse TNM stage (Table 1). And univariate survival analysis demonstrated that patients with high OTUD3 expression had worse overall survival (Fig. 1F). Additionally, univariate and multivariate logistic regression analyses indicated that OTUD3 was an independent predictor of poor prognosis for patients with HCC (Table 2). To further verify whether OTUD3 expression was correlated with a poor prognosis, we employed Kaplan–Meier analysis through Kaplan–Meier plotter website. The results demonstrated that liver cancer patients with high OTUD3 expression had markedly poorer overall survival probability than those with low OTUD3 expression. Taken together, OTUD3 was dramatically overexpressed in HCC tissues and upregulated OTUD3 level was associated with advanced diseases and poorer patient outcomes.

**OTUD3 accelerates HCC cells growth in vitro and in vivo**

To obtain insight to the role of OTUD3 in facilitating HCC cells growth in vitro, we investigated the impact of OTUD3 up-/down-regulation on tumor biology behaviors of proliferation. Initially, we examined OTUD3 expression in normal live cell HL7702 and HCC cell lines and compared the expression levels of OTUD3 in different cell lines. We found that OTUD3 mRNA and protein expression was significantly upregulated in HCC cell lines (Fig. 2A-C). Additionally, the knockdown efficiency was verified by western blot and qRT-PCR (Supplementary Fig. S1A and B). Through EdU proliferation assay and CCK8 assay, we observed that OTUD3 stable interference effectively suppressed HCCLM3 cells proliferation ability (Fig. 2D-E). In contrast, EdU and CCK8 assay showed that OTUD3 overexpression significantly facilitated growth ability of Huh7 (Supplementary Fig. S2A and B).

To further determine the biological function of OTUD3 on HCC cells proliferation, we used xenograft mice model to assess for OTUD3 function in HCC growth. Nude mice were injected subcutaneously with luciferase-labeled control or OTUD3 stable knockdown HCCLM3 cells to monitor HCC growth. Image analysis by IVIS showed that tumour sizes of mice in shOTUD3 group was much smaller than that in control group on the 30th day (Fig. 2F). Furthermore, our data showed that OTUD3 stable interference HCCLM3 cells contributed to smaller tumour volume and weight (Fig. 2G and H). Additionally, we performed immunofluorescence staining to detect the cell proliferation biomarker Ki67 expression using subcutaneous xenografts tissue sections of the nude mice. Our results demonstrated that Ki67 expression was markedly lower in shOTUD3 group (Fig. 2I). Moreover, OTUD3 stable overexpression and the control Huh7 cells were subcutaneously injected into the nude mice. Our results showed that tumours of nude mice in OTUD3 overexpression group grew much quicker and finally got higher weights.
compared with the control group (Supplementary Fig. S3A and B). Collectively, our data revealed the crucial function of OTUD3 in facilitating HCC cells growth.

**OTUD3 drives HCC cells invasion and migration in vitro and in vivo**

To further verify the impact of OTUD3 on HCC cells metastasis, we performed transwell migration and invasion assays and wound-healing assay. Through transwell migration and invasion assay, we observed that OTUD3 stable knockdown significantly inhibited HCCLM3 cells migration and invasion (Fig. 3A and B). Meanwhile, wound-healing assay also indicated that OTUD3 knockdown abated HCC cells migration capability (Fig. 3C and D). Conversely, we found that OTUD3 upregulation markedly promoted cells metastatic ability through transwell migration and invasion assays and wound-healing assay (Supplementary Fig. S2C-F).

Consistently in tail-vein injection mice model, luciferase-labeled shNC-HCCLM3 or shOTUD3-HCCLM3 cells were injected into the caudal veins of nude mice. Using IVIS to monitor and image tumor progression in mice, we observed that OTUD3 stable interference markedly inhibited lung metastasis (Fig. 3E). Meanwhile, H&E-stained serial lung sections in the shOTUD3 group indicated less lung metastatic nodules compared with the negative control (Fig. 3F and G). Furthermore, we injected OTUD3 overexpression or the control Huh7 cells into the tail veins of nude mice. H&E-staining results showed that OTUD3 overexpression contributed to more lung metastatic nodules (Supplementary Fig. S3C and D). Thus, our findings disclose the oncogenic role of OTUD3 in promoting HCC cells metastasis.

**OTUD3 is correlated with ACTN4 protein expression in HCC**

To further investigate the molecular mechanism by which OTUD3 regulates HCC cells progression, we analyzed Tandem Mass Tags (TMT)-Mass Spectrometry Proteomics data to determine expression patterns of regulated proteins with OTUD3 downregulation. We found that ACTN4 protein level was significantly decreased (Fig. 4A). To confirm our findings, we further determine the relationship between OTUD3 and ACTN4 mRNA and protein expression in HCC cells. Interestingly, qRT-PCR results showed that neither OTUD3 downregulation nor upregulation had significant effect on ACTN4 mRNA level in HCC cells (Fig. 4B and C). In comparation, western blot results demonstrated that OTUD3 knockdown could downregulate the protein level of ACTN4 whereas OTUD3 overexpression has the opposite effect in HCC cells (Fig. 4D and E). Furthermore, we examined the correlation between OTUD3 and ACTN4 expression in HCC tissues. As expected, the scatter plots analysis showed no significant correlation between OTUD3 and ACTN4 mRNA level whereas a positive correlation between OTUD3 and ACTN4 protein level was found (Fig. 4F and G). Moreover, we performed immunofluorescence staining to detect OTUD3 and ACTN4 expression in mice serial xenografts tissue sections. Our results showed that OTUD3 and ACTN4 were downregulated simultaneously in shOTUD3 group compared with the control group (Fig. 4H).

**ACTN4 is indispensable for HCC cells progression in OTUD3-dependent manner**
To address the potential role of ACTN4 in OTUD3-mediated HCC carcinogenesis, we performed rescue experiments and investigated whether ACTN4 is a critical downstream target of OTUD3 in HCC cells. Through western blot, we found that OTUD3 downregulation dramatically abated the increased protein level of ACTN4 in HCCLM3 cells (Fig. 5A). Furthermore, EdU proliferation assay showed that OTUD3 inhibition markedly suppressed HCC cells growth activity enhanced by ACTN4 upregulation (Fig. 5B, C). Simultaneously, transwell migration and invasion assays demonstrated that ACTN4 upregulation enhanced the HCC cells metastatic ability whereas OTUD3 knockdown reversed this trend effectively (Fig. 5D). To further confirm our findings, we transfected ACTN4 silencing plasmids into OTUD3 overexpression HCC cells and found that decreased protein level of ACTN4 was rescued by OTUD3 overexpression (Fig. 5E). Consistently, EdU assay and transwell migration and invasion assays showed that HCC cells growth and metastatic capabilities suppressed by ACTN4 interference were reversed by OTUD3 upregulation (Fig. 5F-H). These results indicate that ACTN4 is crucial for OTUD3-driven HCC cells progression in vitro.

To further verify whether ACTN4 played the same role in vivo, we constructed tumorigenicity and tail-vein injection mice model. Strikingly, we found that ACTN4 upregulation significantly promoted tumor growth while OTUD3 knockdown effectively inhibited this trend (Fig. 5 I and J). Similarly, OTUD3 stable knockdown HCCLM3 cell dramatically lost the enhanced-metastatic ability induced by ACTN4 overexpression. Herein, our findings reveal that ACTN4 is critical for OTUD3-mediated HCC cells carcinogenesis in vivo.

**OTUD3 deubiquitinates ACTN4 and maintains stabilization of ACTN4**

Having confirmed the correlation between OTUD3 and ACTN4, we aimed to explore the interaction of them. ACTN4 was shown to be degraded through the ubiquitin-proteasome pathway and could be stabilized in human glioblastoma [17]. Given the role of OTUD3 as a deubiquitinase and its function of stabilizing GRP78 through deubiquitylation in lung cancer cells [10], we hypothesized that OTUD3 might deubiquitinate ACTN4 and stabilize it. As expected, co-IP experiments indicated that OTUD3 can bind ACTN4 directly in HCCLM3 and Huh7 cells (Fig. 6A and B). In addition, we performed co-IP experiments to confirm the combination between ACTN4 and ubiquitin in HCC cells. Our results showed that ACTN4 can bind ubiquitin directly (Supplementary Fig. S4A and B). Consistently, confocal microscopic analysis confirmed the co-localization of ACTN4 and ubiquitin in HCCLM3 and Huh7 cells (Fig. 6C). We further demonstrated that ACTN4 could be degraded through the ubiquitin-proteasome pathway through using proteasome inhibitor MG132 (Fig. 6D).

To determine whether OTUD3 can regulate ACTN4 degradation through the ubiquitin-proteasome pathway, we used OTUD3 stable knockdown HCCLM3 cells and OTUD3 overexpression Huh7 cells with a 20μM dose of the translation inhibitor cycloheximide (CHX) treatment. At the indicated time, we detected ACTN4 protein level and found that OTUD3 downregulation increased the ACTN4 degradation rate whereas OTUD3 upregulation showed the opposite effect compared with the negative control (Fig. 6E). Meanwhile, our results indicated that neither knockdown nor upregulation of OTUD3 had a significant
effect on the ACTN4 protein level in HCCLM3 cells treated with MG132 compared with those not treated with MG132 (Fig. 6F). Finally, our data revealed that OTUD3 inhibition dramatically upregulated the ubiquitination level of ACTN4, while OTUD3 overexpression showed the opposite impact on ACTN4 (Fig. 6G and H). Thus, our findings demonstrate that OTUD3 can act as a deubiquitinase of ACTN4 and stabilize it.

**OTUD3 activates NF-κB signaling pathway through ACTN4 in HCC cells**

Most members of OUT family deubiquitinases modulate cell signaling pathways and act as complex roles in cancer [7, 15]. To obtain a deeper understanding about the way that OTUD3 drives HCC carcinogenesis, we performed RNA-seq with HCCLM3-shNC and HCCLM3-shOTUD3 cells. Pathway enrichment analysis revealed that NF-κB signaling pathway had the highest correlation with downstream of OTUD3 (Fig. 7A and B). Interestingly, it reported that ACTN4 could act as a selective transcriptional co-activator of p65, a subunit of NF-κB signaling pathway [16]. Additionally, another study also suggested that upregulation of ACTN4 activated NF-κB signaling pathway in glioblastoma [17]. We thus speculated that OTUD3 might regulate NF-κB signaling pathway through ACTN4. To confirm our hypothesis, we performed western blot and found that in OTUD3 knockdown HCCLM3 cells, protein level of ACTN4, p-p65, p-IkBα was decreased (Fig. 7C). In contrast, protein level of ACTN4, p-p65, p-IκBα was increased in OTUD3 overexpression Huh7 cells (Fig. 7C). To determine whether NF-κB signaling pathway was essential for HCC progression in an ACTN4-dependent manner, we employed the phosphorylated IkBα inhibitor BAY11-7082 to inhibit NF-κB signaling pathway. Through western blot, our results showed that ACTN4 overexpression in HCCLM3 cells promoted the phosphorylation of p65 and IkBα whereas BAY11-7082 employment reversed this trend effectively (Fig. 7D). Meanwhile, we performed EdU proliferation assay, transwell invasion assay and wound healing experiment. EdU assay results showed that BAY11-7082 significantly abated the enhanced HCC cells growth ability driven by ACTN4 upregulation (Fig. 7E). Additionally, transwell invasion assay and wound healing experiment demonstrated that BAY11-7082 reversed the strengthened HCCLM3 metastatic capability caused by ACTN4 overexpression (Fig. 7F). Our findings suggest that NF-κB signaling pathway is key for OTUD3/ACTN4 axis-mediated HCC progression.

**OTUD3 expression is markedly correlated with ACTN4 protein level and NF-κB signaling pathway activity in HCC tissues**

To extend our findings in clinic, we examined the relationship among OTUD3, ACTN4 and NF-κB signaling pathway. Through IHC staining, we detected the expression of OTUD3, ACTN4, p-p65 and p-IκBα expression in 50 pairs HCC fresh tissues compared with corresponding normal tissues. Interestingly, our results revealed that protein levels of OTUD3, ACTN4, p-p65 and p-IκBα were markedly upregulated simultaneously (Fig. 8A). Furthermore, we evaluated the protein expression of OTUD3, ACTN4, p-p65 and p-IκBα in the 50 fresh HCC tissues. Scatter plots indicated that OTUD3 protein expression was significantly correlated with p-p65 and p-IκBα protein level (Fig. 8B and C). Meanwhile, ACTN4 protein expression was also dramatically correlated with p-p65 and p-IκBα protein level (Fig. 8D and E). Taken
together, our results suggest that OTUD3 expression in HCC tissues was correlated with ACTN4 protein level and NF-κB signaling pathway activity.

**Discussion**

Deubiquitinating enzymes play important roles in multiple cancers. Understanding the mechanisms about how these enzymes regulate cancer carcinogenesis is critical to develop therapeutic strategies. In this study, we carried out a comprehensive investigation of the function and mechanism of OTUD3 in regulating HCC progression.

Compared with other DUBs, such as BAP1 [18] and USP7 [12, 19], OTUD3 is rarely studied in cancer. Although OTUD3 has been identified as an important oncogenic driver in lung cancer carcinogenesis [10], our study is the first one disclosing the function of OTUD3 in HCC. We find that OTUD3 is highly expressed in HCC tissues and that its expression is markedly correlated with tumor size, distant metastasis and TNM stage of HCC patients. Importantly, we disclose for the first time that ACTN4 can be stabilized by OTUD3 and thus activates NF-κB signaling pathway to drive HCC carcinogenesis.

Alpha-actinin 4 (ACTN4), known as an actinin-binding protein, belonging to the spectrin superfamily, is important for the regulation of cytoskeletal integrity and cell movement [20, 21]. The novel role of ACTN4 in promoting cell motility and cancer invasion was first reported by Honda K et al [22]. In recent years, accumulating evidence showed that ACTN4 enhances migration and lymph node metastasis in colorectal cancer and promotes epithelial-to-mesenchymal transition and carcinogenesis of cervical cancer [23, 24]. In our study, we identify ACTN4 as an essential downstream target of OTUD3 through performing mass spectroscopic analysis and reveal that OTUD3 drives HCC proliferation and metastasis in an ACTN4-dependent manner. Notably, we also found that OTUD3 expression had a significant impact on ACTN4 expression only at the protein level. In addition, ACTN4 can be degraded through the ubiquitin-proteosome pathway and be stabilized in human glioblastoma cells [17]. Considering the deubiquitylation function of OTUD3, we speculated that OTUD3 might interact with ACTN4 to deubiquitinate it. As expected, we suggest that ACTN4 can interact with OTUD3 and it can also be degraded through the ubiquitin-proteosome pathway in HCC cells. Furthermore, our data indicates that OTUD3 upregulation inhibits ACTN4 ubiquitination whereas OTUD3 knockdown shows the opposite effect. In other words, OTUD3 can deubiquitinate ACTN4 to inhibit its degradation in HCC cells.

The subfamily of OTU DUBs have emerged as important modulators of cell signaling pathways. OTU DUBs members like OTUD1, OTUD7B, OTUD2 and OTUD5 are involved in multiple signaling cascades [6, 7, 25, 26]. In our study, we identified NF-κB signaling pathway as an important downstream signaling pathway correlated with OTUD3 in HCCLM3 cell through RNA-seq and pathway enrichment analysis. The NF-κB signaling pathway plays important roles in driving HCC carcinogenesis [27, 28]. There exist over 150 different stimuli which can phosphorylate and activate NF-κB signaling pathway [29]. Notably, some OTU DUBs members like OTUD7B and OTULIN can regulate NF-κB signaling pathway [7, 8]. In this research, we find that OTUD3 can markedly regulate protein level of ACTN4, phosphorylated-p65 (p-p65)
and phosphorylated-IκBα (p-IκBα) simultaneously. Moreover, ACTN4 has been shown to act as a selective transcriptional co-activator of p65 in NF-κB signaling pathway [16]. Another study also demonstrated that ACTN4 promotes human glioblastoma progression through activating NF-κB signaling pathway [17]. Through employing the phosphorylated IκBα inhibitor BAY11-7082 to suppress NF-κB signaling pathway, we revealed that NF-κB signaling pathway was key for ACTN4-mediated HCC cell proliferation, migration and invasion. Collectively, NF-κB signaling pathway is key for HCC cells progression mediated by OTUD3/ACTN4 axis.

Some study has reported the relationship between ACTN4 and NF-κB signaling pathway in cancer [16, 17]. However, the correlation among OTUD3, ACTN4 and NF-κB signaling pathway in HCC has not been investigated. In this research, we initially disclosed the positive correlation between protein levels of OTUD3 and ACTN4 in HCC tissues. After identifying the key role of NF-κB signaling pathway in HCC progression driven by OTUD3-ACTN4 axis, we further examined the relationship among these three participants in HCC. Intriguingly, the expression of OTUD3, ACTN4, p-p65 and p-IκBα were overexpressed simultaneously in HCC tissues. Moreover, both OTUD3 and ACTN4 protein expression were positively correlated with p-p65 and p-IκBα protein levels in HCC tissues.

**Conclusion**

In conclusion, our findings demonstrate that OTUD3 is aberrantly upregulated in HCC tissues and is markedly correlated with worse prognosis of HCC patients. OTUD3 plays an essential in accelerating HCC cells growth, migration and invasion *in vitro* and *in vivo*. In addition, ACTN4 is key for OTUD3-mediated HCC progression. Mechanistically, OTUD3 deubiquitinates and stabilizes ACTN4 to activate the NF-κB signaling pathway and thus accelerates HCC progression. This study contributes to our ever-increasing understanding of the role of OTUD3 in malignant carcinoma and highlight the potential role of OTUD3 as a prognostic indicator and a therapeutic target in HCC.

**Abbreviations**

DUBs: Deubiquitinases; HCC: Hepatocellular carcinoma; OTUD3: OTU domain-containing protein 3; ACTN4: Alpha-actinin 4; CCK-8: Cell counting kit-8; EdU: 5-Ethynyl-2′-deoxyuridine; qRT-PCR: quantitative real-time PCR; IHC: Immunohistochemistry; Co-IP: Co-immunoprecipitation.

**Declarations**

**Declarations:** The authors declare that they have no conflict of interest.

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None

**Authors’ contributions**
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Tables
Table 1. Relationship between OTUD3 expression and clinicopathological features in 115 HCC patients

| Parameters                  | Total case | OTUD3          | P value |
|-----------------------------|------------|----------------|---------|
|                             | 115        | High expression| Low expression | |
| Age(years)                  |            |                | 0.523   |
| ≥60                         | 71         | 36             | 35      |
| ≥60                         | 44         | 25             | 19      |
| Gender                      |            |                | 0.817   |
| Female                      | 49         | 19             | 30      |
| Male                        | 66         | 27             | 39      |
| Tumor size (cm)             |            |                | 0.023*  |
| ≥5                          | 31         | 13             | 18      |
| ≥5                          | 84         | 55             | 29      |
| Tumor nodule number         |            |                | 0.497   |
| Single                      | 71         | 39             | 32      |
| Multiple                    | 44         | 27             | 17      |
| AFP(ng/ml)                  |            |                | 0.401   |
| <400                        | 69         | 37             | 32      |
| ≥400                        | 46         | 21             | 25      |
| Cirrhosis                   |            |                | 0.701   |
| Absence                     | 33         | 16             | 17      |
| Presence                    | 82         | 43             | 39      |
| Liver function              |            |                | 0.546   |
| Child-Pugh A                | 88         | 21             | 67      |
| Child-pugh B                | 27         | 8              | 19      |
| Lobe                        |            |                | 0.329   |
| Right                       | 81         | 39             | 42      |
| Left                        | 34         | 13             | 21      |
|                          |      |      |      |
|--------------------------|------|------|------|
| **TNM**                  | 0.034* |      |      |
| I/II                     | 53   | 22   | 31   |
| III/IV                   | 62   | 38   | 24   |
| **Vascular invasion**    | 0.046* |      |      |
| Negative                 | 37   | 18   | 19   |
| Positive                 | 78   | 53   | 25   |
| **Intrahepatic metastasis** | 0.034* |      |      |
| Negative                 | 37   | 16   | 21   |
| Positive                 | 78   | 51   | 27   |
Table 2. Univariate and multivariate analysis of OTUD3 in overall survival of 115 HCC patients (Cox proportional hazards regression model)

| Parameters                                      | Univariate analysis |                       |          |                          | Multivariate analysis |                       |          |
|-------------------------------------------------|---------------------|-----------------------|----------|--------------------------|-----------------------|-----------------------|----------|
|                                                 | HR                  | 95%CI                 | P value  | HR                       | 95%CI                 | P value               |         |
| Age (≥ 60/  < 60)                               | 0.833               | 0.631-1.305           | 0.531    | –                        | –                     | –                     |         |
| Gender (Male/Female)                            | 1.875               | 1.079-2.759           | 0.177    | –                        | –                     | –                     |         |
| Tumor nodule number (Single/Multiple)           | 1.164               | 0.631-2.153           | 0.467    | –                        | –                     | –                     |         |
| AFP(ng/ml) (<400/≥400)                          | 0.653               | 0.322-1.316           | 0.275    | –                        | –                     | –                     |         |
| Cirrhosis (Absence/Presence)                    | 0.756               | 0.431-1.623           | 0.335    | –                        | –                     | –                     |         |
| Liver function (Child-Pugh A/Child-pugh B)      | 1.632               | 0.731-3.115           | 0.312    | –                        | –                     | –                     |         |
| Lobe (Right/Left)                               | 0.693               | 0.413-1.531           | 0.364    | –                        | –                     | –                     |         |
| Tumor size (≥ 5/ < 5)                           | 1.862               | 0.952-2.879           | <0.01**  | 1.503                    | 0.911-2.651           | 0.035*                |         |
| TNM (III, IV/I, II)                             | 2.431               | 1.469-4.021           | <0.01**  | 2.213                    | 1.336-4.536           | 0.012*                |         |
| Vascular invasion (Positive/Negative)            | 2.512               | 1.381-3.513           | <0.01**  | 1.756                    | 1.351-3.125           | 0.031*                |         |
| Intrahepatic metastasis (Positive/Negative)      | 2.058               | 1.617-3.031           | <0.01**  | 1.739                    | 1.412-2.814           | 0.026*                |         |
| OTUD3 expression (High/Low)                     | 2.213               | 1.271-3.731           | <0.01**  | 1.82                     | 1.572-2.681           | 0.016*                |         |

Figures
OTUD3 is significantly upregulated in human HCC tissues a, Representative images for OTUD3 IHC staining in HCC tissues and corresponding normal tissues (N=115; scale bar: 50μm, 100μm). b, Diagram of OTUD3 staining score in IHC staining. c, qRT-PCR analysis of OTUD3 mRNA level in 50 cases of HCC tissues and corresponding normal tissues. Left, a log 2 (T/N) value > 0 indicates that OTUD3 expression is overexpressed in the HCC samples; right, a log 2 (T/N) value < 0 indicates that OTUD3 expression is
downregulated in the HCC samples. The OTUD3 mRNA levels are normalized to the GAPDH mRNA levels.

d. Determination and quantification of OTUD3 protein levels in HCC tissues and paired non-tumour tissues by western blot (N=50). e. Representative images of western blot. f. Kaplan–Meier analysis of the correlation between the OTUD3 level and overall survival of HCC patients with high and low OTUD3 expression in the IHC staining. g. Kaplan–Meier analysis of OTUD3 expression in 364 liver cancer tissues using the Kaplan–Meier plotter website (www.kmplot.com). Data were mean ± S.D. of three independent determinations. Student’s t-test was used for P value assessment. *P< 0.05, **P<0.01.

Figure 2
Figure 2

OTUD3 knockdown suppresses HCC cell growth in vitro and in vivo. a, qRT-PCR analysis of OTUD3 mRNA level in normal liver cell HL7702 and HCC cell lines. b and c, Western blot showing the protein expression of OTUD3 in normal liver cell HL7702 and HCC cell lines. d, EdU assay evaluating the proliferation ability for HCCLM3 cells transfected with control shRNA or shRNA targeting OTUD3. Right panel is quantification of the results of the EdU assay. e, CCK8 examining the effect of OTUD3 knockdown on the proliferation of HCCLM3 cell. f, Luciferase intensity of the nude mice (n=6 per group) injected with luciferase-expressing HCCLM3 cells stably transfected with shNC or shRNA targeting OTUD3 were detected by IVIS, Representative images obtained are shown. g and h, Tumour sizes and tumour weights of HCCLM3-shNC or HCCLM3-shOTUD3 group of nude mice were measured and corresponding tumour growth curves were obtained. i, Immunofluorescence staining of Ki67 in subcutaneously tumour of nude mice injected with HCCLM3 cells stably transfected shOTUD3 or shNC (Scale bar: 100 μm; Magnification: 200X). *P< 0.05, **P<0.01 by t-tests.
Figure 3

OTUD3 downregulation inhibits HCC cells migration and invasion in vitro and in vivo. a and b, Invasion and migration assays were employed to evaluate the effect of OTUD3 knockdown on HCCLM3 cells metastatic ability (Magnification 200X). c and d, Scratching assay was performed to detect migration ability of OTUD3 knockdown HCCLM3 cells compared with the control group. e, In vivo tumour metastasis was examined using the nude mice (n=6 per group) injected with luciferase-expressing
HCCLM3 cells stably transfected with shNC or shRNA targeting OTUD3 and was detected by IVIS from day 0 to day 35. Representative images obtained are shown. f, Quantification of metastatic lung nodules with shNC and shOTUD3 HCCLM3 cells by tail-vein injection. g, Images of H&E staining of paraffin-embedded lung tissues from shNC and shOTUD3 nude mice group (Magnification: 100X, 200X, 400X). The unpaired two-sided Student’s t test was used for comparing between two groups of equal variances. Error bars represent mean ± SD from three independent experiments. *P<0.05, **P< 0.01.
ACTN4 protein level is significantly correlated with OTUD3 expression in HCC a, Mass spectroscopic analysis listed the top 20 downregulated proteins with OTUD3 knockdown in HCCLM3 cells. b, qRT-PCR analyses were used to detect ACTN4 mRNA levels in OTUD3 knockdown HCCLM3 and MHCC97H cells compared with the control group. c, qRT-PCR analysis evaluating ACTN4 mRNA levels in OTUD3 upregulation Huh7 and HepG2 cells compared with the control group. d, Western blot detecting ACTN4 protein expression in OTUD3 knockdown and the control HCCLM3 and MHCC97H cells. e, Western blot showing ACTN4 protein expression in OTUD3 overexpression and the control Huh7 and HepG2 cells. f, Scatter plots showed no significant correlations between OTUD3 and ACTN4 at the mRNA levels in 50 HCC tissues. g, Scatter plots showed positive correlations between OTUD3 and ACTN4 at the protein levels in 50 HCC tissues. h, Immunofluorescence staining of ACTN4 and OTUD3 in subcutaneously tumour of nude mice injected with HCCLM3 cells stably transfected shOTUD3 or shNC (n=6 per group; scale bar: 100μm; Magnification: 200X). The unpaired two-sided Student’s t test was used for comparing between two groups of equal variances. Error bars represent mean ± SD from three independent experiments. NS: not significant, **P< 0.01.
Figure 5

ACTN4 is critical for OTUD3-mediated HCC cells progression in vitro and in vivo. a, Western blot confirming the downregulation of OTUD3 abated increased ACTN4 expression in HCCLM3 cells. b and c, EdU assay evaluating the effect of OTUD3 knockdown on accelerated HCC cell growth enhanced by ACTN4 upregulation. d, Quantification of HCC cell transwell migration and invasion results. e, Western blot showing the overexpression of OTUD3 rescued the decreased ACTN4 protein level caused by ACTN4.
knockdown. f and g, EdU assay identifying the effect of OTUD3 upregulation on inhibited HCC cell growth caused by ACTN4 downregulation. h, Quantification of HCC cell transwell migration and invasion results. i and j, Tumor sizes and tumor weights of 24 nude mice (6 mice per group) were measured and corresponding tumor growth curves were obtained in the rescue experiments. f, In vivo lung metastasis rescue experiment was examined in 24 nude mice (6 mice per group). Representative H&E staining of lungs are shown (Scale bar: 100μm; magnification: 200X), along with the number of lung metastases in the four groups of nude mice. The unpaired two-sided Student’s t test was used for comparing between two groups of equal variances. Error bars represent mean ± SD from three independent experiments. NS: not significant, *P< 0.05, **P< 0.01.
OTUD3 enhances the stability of ACTN4 through deubiquitylation a and b, co-IP experiments between endogenous OTUD3 and ACTN4 in HCCLM3 and Huh7 cells. ACTN4 was detected in the immunoprecipitate when the anti-OTUD3 antibody was used as bait. c, Colocalization of OTUD3 and ACTN4 in HCCLM3 and Huh7 cells (Scale bar: 14µm). d, HCCLM3 and Huh7 cells were treated with 15μM proteasomal inhibitor MG132 for the indicated time, and the levels of ACTN4 were then detected. e,
HCCLM3 cell transfected with OTUD3 shRNA or shNC together with stably OTUD3 overexpressing Huh7 cells and negative control were treated with 20μM cycloheximide (CHX). Cells were collected at different time points and were detected ACTN4 protein expression. f, HCCLM3 cells with OTUD3 knockdown or OTUD3 overexpression were treated with MG132 (15μM). Cells were collected at 6 h and immunoblotted with the antibodies indicated. g and h, the knockdown or upregulation of OTUD3 altered the ubiquitination of ACTN4 in both HCCLM3 and Huh7 cells. The cells in each group were treated with MG132 (15μM). The levels of ubiquitin-attached ACTN4 were detected by western blot analysis with ubiquitin (Ub) antibody.
NF-κB signaling pathway is essential in OTUD3/ACTN4 axis-mediated HCC cells progression

a, Heatmap representation of genes differentially represented in RNA-seq data from HCCLM3-shOTUD3#2 versus HCCLM3-shNC cell lysis (n= 3).

b, Pathway enrichment analysis of genes differentially represented in RNA-seq data from HCCLM3-shOTUD3#2 versus HCCLM3-shNC cell lysis (n= 3).

c, Western blot analysis evaluating the indicated protein expression in OTUD3-knockdown HCCLM3 cells or OTUD3
overexpressing Huh7 cells compared with the control group. d, Western blot showing indicated protein levels in HCCLM3 cells stably overexpressing ACTN4 or treated with Bay11-7082 (6μM,12h). e, EdU assay was employed to detect growth ability of HCCLM3 cells with ACTN4 upregulation and/or treated with Bay11-7082 (6μM,12h) compared with the negative control. f, Cell migration and invasion activity was measured in HCCLM3 cells with ACTN4 overexpression and/or treated with Bay11-7082 (6μM,12h) compared with control group. Error bars represent mean ± SD from three independent experiments. NS: not significant, *P< 0.05 by t-tests.

Figure 8
Figure 8

The correlation among OTUD3, ACTN4 and NF-κB signaling pathway activity in clinical a, IHC staining of paraffin-embedded serial sections of HCC tissues and adjacent normal tissues. The expression of OTUD3, ACTN4, p-p65 and p-IκBα were evaluated (Scale bar:50μm; magnification: 100X, 200X, 400X). b, Scatter plots showing the relationship between protein expression of OTUD3 and p-p65. r=0.305, **P <0.01. c, Scatter plots evaluating the correlation between protein expression of OTUD3 and p-IκBα. r=0.288, **P <0.01. d, Scatter plots showing the relationship between protein expression of ACTN4 and p-p65. r=0.265, P =0.015. e, Scatter plots evaluating the correlation between protein expression of ACTN4 and p-IκBα. r=0.271, **P <0.01.

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