USP51, an oncogene for pancreatic cancer (PC) cell growth via Wnt/β-catenin pathway

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Abstract

Background: Currently, targeting ubiquitin-specific protease (USP) is popular in cancer treatment. Aim: To study the roles of USP51, a member of USPs, in human pancreatic cancer (PC) and the underlying mechanism.

Method: Expression of USP51 in tumor pancreatic tissue and PC cell lines were assessed. Overall survival rate of PC patients was measured by Kaplan-Meier method. The association between USP51 and β-catenin in PC cells was determined by Co-Immunoprecipitation (Co-IP) and ubiquitination assay. XAV939 was used to inhibit β-catenin. Protein levels of β-catenin and Cyclin D1 were used to assess the mechanism.

Result: Herein, we evidenced high levels of USP51 in tumor tissues of PC patients, which predicted a poor survival. Knockdown of USP51 in MiaPaca2 and SW1990 (two PC cells) inhibited proliferation, arrested MiaPaca2 and SW1990 cells at G0/G1 phases, and impaired tumorigenicity of MiaPaca2 in nude mice, demonstrating the anti-cancer effect of siUSP51 in PC. Moreover, Knockdown of USP51 prevented β-catenin and its downstream Cyclin D1, suggesting the blockade of β-catenin pathway in this process. On the contrary, overexpression of USP51 in ASPC1 (one PC cell) resulted in the opposite effect, facilitating the tumorigenesis of PC and the activation of β-catenin pathway, which were significantly reversed by XAV939. Interestingly, USP51 bound to β-catenin and knockdown of USP51 increased β-catenin ubiquitination.

Conclusion: USP51 is an oncogene that is upregulated in PC. USP51 acted as a deubiquitinase that favored β-catenin accumulation, then activated Wnt/β-catenin pathway and exerted carcinogenesis in PC.

Background

Increased activity of β-catenin, a key transcriptional activator of canonical Wnt pathway, is an essential cause in human pancreatic cancer (PC), and a promising therapeutic target to block this disease [4]. Mechanically, once Wnt signal is stimulated at cell plasma membrane, β-catenin is accumulated in the nucleus to assemble into a complex with lymphoid enhancer binding factor (LEF)/T-cell factor (TCF) to stimulate its downstream oncogenes, such as Cyclin D1, and ultimately contributes to cancer cell pro-proliferation, more aggressive phenotype and chemo-resistance [8]. Usually, level of β-catenin can be determined by the following steps: APC/Axin1 destruction complex-mediated-phosphorylation, E3 ligase (β-TrCP) mediated-ubiquitination and proteasome-dependent degradation [5]. However, the specific regulator in controlling β-catenin levels in PC is not fully understood.

Ubiquitin specific proteases (USPs) functioned as de-ubiquitinating enzymes to favor protein recycle from proteasome degradation, and play a role in protein localization or activation [3]. Recently, UPS appears as an rich source for drug targets in cancer treatment [15]. Evidence suggests that USPs member USP51 is necessary for cell viability, and hindering USP51 expression represses tumor growth [1]. Expression of

USP51 is enhanced and accompanied with a poor survival in human gastric cancer (GC) [17]. However, the roles of USP51 in PC remained largely unknown.

In human cancers, USPs (such as USP4, USP7 and USP47) retarded β-catenin degradation through a deubiquitination process [9, 12, 16]. However, in PC, whether USP51 functioned as a de-ubiquitinating enzyme to control β-catenin expression remained largely unknown. Herein, we found evaluated levels of USP51 in human PC, which correlated with a poor survival. Our data disclosed that knockdown of USP51 inhibited proliferation of PC cells and induced cell cycle arrest. However, overexpression of USP51 resulted in the opposite. We confirmed USP51 as a deubiquitination enzyme to enhanced β-catenin expression and the subsequent accumulation of Cyclin D1 (β-catenin downstream oncogene), which ultimately induced the tumorigenesis of PC cells.

Materials And Methods

Tissue samples

In RT-PCR method, 25 pairs of tumor and adjacent non-tumor tissues in PC patients were acquired from Fudan University Shanghai Cancer Center. In IHC analysis, human PC tissue microarrays (n = 67) and corresponding control healthy (n = 25) were prepared by Shanghai Outdo Biotech Co., Ltd. (Shanghai, China). According to IHC results, USP51 expression in PC tissue were divided into USP51 high (n = 37) and USP51 low (n = 30) group, then the overall survival rate for PC patients were assessed.

Immunohistochemistry (IHC) assay

Expression of USP51 in human PC tissue microarrays, as well as expression of Ki67 in tumor of a Xenograft model was assessed, using IHC method. Paraffinembedded samples (cut into 4 mm thick) from human pancreatic or mice tumor tissue were routinely dewaxing, rehydration, and then blocked using 3% H_2O_2. IHC was conducted using DAB substrate (Long Island Biotech) and hematoxylin (714094, BASO) following manufacturer's instructions. The primary antibody for the IHC was rabbit Ki67 polyclonal antibody (ab16667, abcam) and rabbit USP51 polyclonal antibody (NBP1-86167, NOVUS).

Cell culture and treatment

Five human PC cell lines (ASPC1, BXPC3, MiaPaca2, PANC1 and SW1990) and one human normal pancreatic cell line (HPC-Y5) were acquired from ATCC (Manassas, VA, USA). MiaPaca2, SW1990 and ASPC1 cells were allowed a monolayer culture at 37 ^{o}C under 5 % CO_2 in a medium containing 90 % (v/v) of DMEM (Hyclone, Logan, UT), 100 U/ml of penicillin (Solarbio, Beijing, China) and 10 % (v/v) of heat-inactivated fetal bovine serum (FBS) (Gibco Laboratories).

To confirm that USP51 acted through β-catenin to exert carcinogenesis in PC *in vitro*, ASPC1 cells transfected with USP51 overexpression vector (oeUSP51) were subjected to 10 μmol/l of XAV939 (S1180, selleck).

Cell Counting Kit -8 (CCK-8) assay

After treatment, proliferation of MiaPaca2, SW1990 and ASPC1 was assessed using Cell Proliferation and Cytotoxicity Assay Kit (CP002, SAB, Beijing, China), according to a Manufacturer's instruction.

Cell cycle detection

After treatment, MiaPaca2, SW1990 and ASPC1 were dyed at 25 ^{o}C in darkness using 50 μg/ml of propidium iodide solution (C001-200, 7sea Biotech, Shanghai, China) for 10 min. Red fluorescence (488 nm) was recorded on the FL1 channel of flow cytometry (Accuri C6, BD Biosciences, San Jose, CA, USA). Cell cycle of MiaPaca2, SW1990 and ASPC1 cells were assessed using FLOWJO software (Version 6 TreeStar).

Lentivirus

The primers of human USP51 gene (NM_201286.3) were: 5'-CGGAATTCATGGCCCAGGTTCGAGAAAC-3' (forward) and 5'-CGGGATCCAAGAAGTTTCTCGAACCTGGGC-3' (reverse), which were cloned into pLVX-Puro vector (Clontech). pLVX-Puro-USP51 was packaged into lentiviruses in 293T cells according to a provided instructions, and the lentiviruses expressing USP51 were transfected into ASPC1 cells using Invitrogen Lipofectamine® 2000 Reagent following the a manufacturer's proposal. Meanwhile, lentiviruses without USP51 coding sequence were considered as a control Vector.

Three designed shRNA targeting human USP51 mRNA (NM_AB449916.1) were used for siRNA-mediate USP51 depletion (siRNA-USP51), which were cloned into PLKO.1 vector (purchased from Suzhou Synbio Technologies (Suzhou, China)). pLKO.1-shUSP51 was packaged into lentiviruses in 293T cells, and then transfected into ASPC1 cells as mentioned above. Meanwhile, lentiviruses expressing non-specific siRNA were used as a negative control (siNC).

Real-time (RT)-PCR

Total RNA from pancreatic tissue, PC cell lines (ASPC1, BXPC3, MiaPaca2, PANC1 and SW1990), or human normal pancreatic HPC-Y5 cells was extracted by Trizol Reagent (1596-026, Invitrogen) and reverse transcribed using cDNA synthesis kit (Fermentas). The primer targeted USP51 (NCBI NM_201286.3) were: 5'-CCTCAGACACGGAGAAGC-3' (Forward, F) and 5'-GGACCCTGACCAAACTCG-3' (Reverse, R); Pos: 478-575; Size: 98 bps; The primer targeted β-catenin (NM_001904.3) were: 5'-CGACACCAAGAAGCAGAGATG-3' (F) and 5'- GGGACAAAGGGCAAGATTTCG-3' (R); Pos: 1688-1831; Size: 144 bps; The primer targeted cyclin D1 (NM_053056.2) were: 5'-GCGAGGAACAGAAGTGCG-3' (F) and 5'-TGGAGTTGTCGGTGTAGATGC-3' (R); Pos: 203-394; Size: 192 bps; Primers targeted glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NCBI NM_001256799.2) were: 5'-AATCCCATCACCATCTTC-3' (F) and 5'-AGGCTGTTGTCATACTTC-3' (R), Pos: 436-653; Size: 218 bps. mRNA levels of USP51, β-catenin and cyclin D1 were quantified through SYBR Green PCR Kit (Thermo Fisher) on an ABI7300 system (Applied Biosystem), and normalized by GAPDH using the $2^{-\triangle\triangle}$Ct method [7].

Western blot analysis

25 µg of total protein in lysis of pancreatic tissue (20 mg) or PC cells sample, determined by BCA protein assay kit (PICPI23223, Thermo, MA, USA), was separated on 10 % SDS-PAGE. Electrophoretically pure including USP51, β-catenin, cyclin D1 and GAPDH were sent onto PVDF membranes (Millipore, USA) and incubated with anti-USP51 antibody (PA5-35274, Invitrogen), anti-β-catenin antibody (#8480, CST), antibody against cyclin D1 (#2922, CST) and antibody against GAPDH (#5174, CST) at 4 ^{o}C overnight followed by a second antibody (A0208, Beyotime, Shanghai, China) for 1 hour at 25 ^{o}C. Expression of USP51, β-catenin and Cyclin D1 was normalized by GAPDH and quantified using ECL system (GE Healthcare/Amersham Biosciences).

Co-Immunoprecipitation (Co-IP) and ubiquitination assay

The association between USP51 and β-catenin in MiaPaca2 cells was assessed using Co-IP. Immune complexes of total protein (100 µg) in MiaPaca2 lysis supernatant was obtained using Protein A/G PLUS-Agarose (sc-2003, Santa Cruz). Antibodies used in IP were: IgG (sc-2027, Santa Cruz Biotechnology), anti-USP51 antibody (Orb181545, biorbyt) and antibody against β-catenin (ab227499, Abcam). Antibodies used in Western-blot were anti-USP51 antibody (PA5-35274, Invitrogen) and anti-β-catenin antibody (#8480, CST). Same amount of total protein was reserved for input control.

To study the effect of USP51 on the levels of β-catenin ubiquitination (Ub-β-catenin), Ub-β-catenin, precipitated in the immune-complexes, was determined using Anti-ubiquitin antibody (ab7780), according the western blot.

Xenograft model

Nude mice with 4-6-week-old were provided by Shanghai Laboratory Animal Company. MiaPaca2 cells transfected with siNC/siUSP51 (7×10^6 cells, 100 µl) were subcutaneously injected into nude mice (n = 6 in each group), and then fed separately. Tumor formation in siNC and siUSP51 groups was monitored from 12 th day to 33 th day. On 33 th day, all mice were killed. Tumor weight (g) was recorded. Histological changes in pancreatic tumor tissue were recorded using Hematoxylin-eosin (H&E) staining. IHC was conducted to evaluate the expression of Ki67, using antibody against Rb Ki67 (ab16667, abcam).

2.8 Statistics and data analysis

Data was showed as mean ± standard error of the mean (SEM), calculated from at least 3 parallels. Comparison between two groups was evaluated using Student's test with $P < 0.05$ being statistical significance. Overall survival of PC patients was analyzed by Kaplan-Meier method using medcalc sofeware.

Results

Expression of USP51 was enhanced in PC and predicted a poor survival

Expression of USP51 in PC tissues was detected. **Figure 1A** showed that expression of USP51, assessed by RT-PCR, was significantly enhanced in tumor tissues (n = 25) when compared with adjacent non-tumor tissue (n = 25), suggesting the involvement of USP51 in human PC, which was further confirmed using an IHC method (**Figure 1B**). Besides, similar change tread was also observed in human PC cell lines (ASPC1, BXPC3, MiaPaca2, PANC1 and SW1990) when compared with normal pancreatic HPC-Y5 cells, confirming the up-regulation of USP51 in human PC *in vitro* (**Figure 1C**). We then investigated whether enhanced USP51 levels were correlated with the survival of PC patients. Kaplan-Meier analysis showed that PC patients with lower USP51 levels were more likely to survive than that with the higher USP51 expression (**Figure 1D**, P = 0.0083).

Inhibitory effect of siUSP51 on PC cell proliferation, cycle progression and the activation of β-catenin pathway

Among PC cell lines, USP51 was highly expressed in MiaPaca2 and SW1990 cells while lowly expressed in ASPC1 cells (**Figure 1C**), therefore, we selected MiaPaca2, SW1990 and ASPC1 cells in the subsequent study.

MiaPaca2 and SW1990 cells were transfected with siRNAs (siUSP51-1, siUSP51-2 and siUSP51-3) to knock down USP51 expression. **Figure 2A**, **B** showed that siUSP51 (siUSP51-1, siUSP51-2 and siUSP51-3) dramatically reduced USP51 expression in comparison to siNC, demonstrating a successful construction of USP51 deletion. Among three siRNAs, the most obvious effect was seen in siUSP51-1 and siUSP51-2, thus, we chose siUSP51-1 and siUSP51-2 for the following study.

Proliferation and cell cycle of PC cells transfected with siUSP51 was examined. Our data noted that siUSP51-1 and siUSP51-2 time-dependently reduced PC cell proliferation (**Figure 2C**, **D**), and arrested MiaPaca2 and SW1990 in G0/G1 phase (**Figure 2E**, **F**).

During cycle progression, the transition from G0/G1 to S phase is modulated by Cyclin D1, which is target gene of β-catenin [2]. siUSP51-1 and siUSP51-2 exerted inhibitory effect on protein levels of β-catenin and Cyclin D1 (**Figure 2G**, **H**), as well as mRNA expression of Cyclin D1 (data not shown) in MiaPaca2 and SW1990 cells, suggesting that knockdown of USP51 inhibited PC cell growth likely via blocking β-catenin/Cyclin D1 pathway.

USP51 acted on β-catenin to influence PC cell growth

ASPC1 cells were transfected with oeUSP51 to overexpress USP51. oeUSP51 enhanced USP51 mRNA and protein expression, in comparison to vector (all P < 0.01), indicating the successful construction of USP51 overexpression in ASPC1 cells (**Figure 3A**).

ASPC1 cells with oeUSP51 were treated with XAV939 (a specific β-catenin inhibitor). Our data showed that oeUSP51 time-dependently enhanced cell proliferation (**Figure 3B**), promoted the transition of ASPC1 from G0/G1 to S phase (**Figure 3C**), and up-regulated β-catenin and Cyclin D1 expression (**Figure 3D**) when compared with vector. However, oeUSP51-induced those changes were reversed by XAV939 ($P < 0.01$).

USP51 regulated β-catenin ubiquitination

Our data showed that siUSP51-1 and siUSP51-2 significantly reduced β-catenin protein expression, yet, had no distinguished effect on β-catenin mRNA expression (data not shown). We further studied whether USP51 (a deubiquitinase) regulates β-catenin ubiquitination (Ub-β-catenin). CO-IP analysis showed that USP51 likely bound β-catenin within PC cells (**Figure 4A**, **B**), and knockdown of USP51 significantly enhanced Ub-β-catenin (**Figure 4C**).

Knockdown of USP51 suppressed tumorigenicity of MiaPaca2 cells in nude mice

Ki67, a nuclear associated antigen, indicates the nuclei of proliferating, and can be used to label the proliferative activity of pancreatic tumors. Evidence suggests that Ki67 is the most reliable factor in the prognosis of PC [6]. In the present study, after tumor formation using MiaPaca2 cells transfected with siUSP51 in nude mice, tumor growth and its pathological observation, as well as the expression levels of Ki67 were measured. Our data showed that siUSP51 remarkably inhibited tumor growth and weight (**Figure 5A, B**), obviously reduced cell infiltration and viability, as evidenced by relatively sparse cytoplasm and dispersed chromatin around cell membrane (**Figure 5C**), and reduced the proportion of Ki67-labeled cells (**Figure 5D**), suggesting the inhibitory effect of siUSP51 on tumor growth in PC *in vivo*.

Discussion

Currently, more than 30 USPs have been implicated in cancer, considering the diversity roles of different USPs in regulating cellular functions, and various substrates involved and modulated by them [10]. Evidence suggests that USP51 is overexpressed and indicate a poor survival in GC [17], which was further confirmed in PC, substantiating the involvement of USP51 in human cancers.

Cancer cells are benefits from USPs for their sustained growth and progression of cell cycle [13]. USP51 is implicated in cell proliferation and cycle progression, and also involved in tumor growth modulation [1]. Presently, we firstly proposed USP51 as an oncogene in PC. Knockdown of USP51 suppressed PC cell proliferation and tumor growth, and overexpression of USP51 resulted in the opposite. Besides, we also evidenced the inhibited effect of USP51 knockdown on tumorigenicity of PC cells in nude mice, further confirming the anti-cancer effect of siUSP51 in PC.

89–90% of intracellular proteins can be degraded by an important process, named deubiquitination [10]. Evidence suggested that USPs, as deubiquitinating enzymes, regulates β-catenin ubiquitin, and is emerging as an activator for β-catenin [9, 12, 16]. In this study, we confirmed an up-regulation of β-catenin

in PC tumorous tissue. Interference experiment using XAV939 suggested that USP51 regulated β-catenin to determin PC cell growth. Besides, our data further substantiated that USP51 deubiquitinates β-catenin, thereby leading to accumulation of β-catenin protein. Furthermore, oncogene Cyclin D1, a key regulator for transition from G1 to S phase and a downstream of β-catenin, is overexpressed in many types of cancers [14]. Cyclin D1 is regulated by USP2 in a deubiquitination process [11]. Our data suggested that down-regulation of cyclin D1 is implicated in the inhibitory effect of siUSP51 on PC cells growth, however, whether USP51 was a specific deubiquitinase for cyclin D1, and whether there was a USP51/β-catenin/cyclin D1 axis that contributes to PC cell growth needed further confirmed.

In Conclusion

USP51 was up-regulated in PC and associated with a poor survival. USP51 enhanced β-catenin accumulation via deubiquitination process, and then favoring the tumor progression of PC both *in vivo* and *in vitro*. Targeting USP51 may represent a therapeutic strategy in PC treatment.

Declarations

Abbreviations

Not applicable

Consent for publication

Not applicable

Acknowledgements

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Conflict of Interest

None

Authors' contributions

Lu-ming Liu designed the study; Yong-qiang Hua, Yun He, Xiao-bo Ding and Ke Zhang performed the experiment and made data entry; Jie Sheng, Li-tao Xu, Zhou-yu Ning, Hao Chen, Zhen Chen, Zhi-qiang

Meng and Qing-xing Zheng made data collection/entry/analysis, Lu-ming Liu drafted the manuscript, and given final approval of the version to be published

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

In this study, the use of PC patients was permitted by the ethics committee of Fudan University Shanghai Cancer Center, and written informed consent was acquired.

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Figures

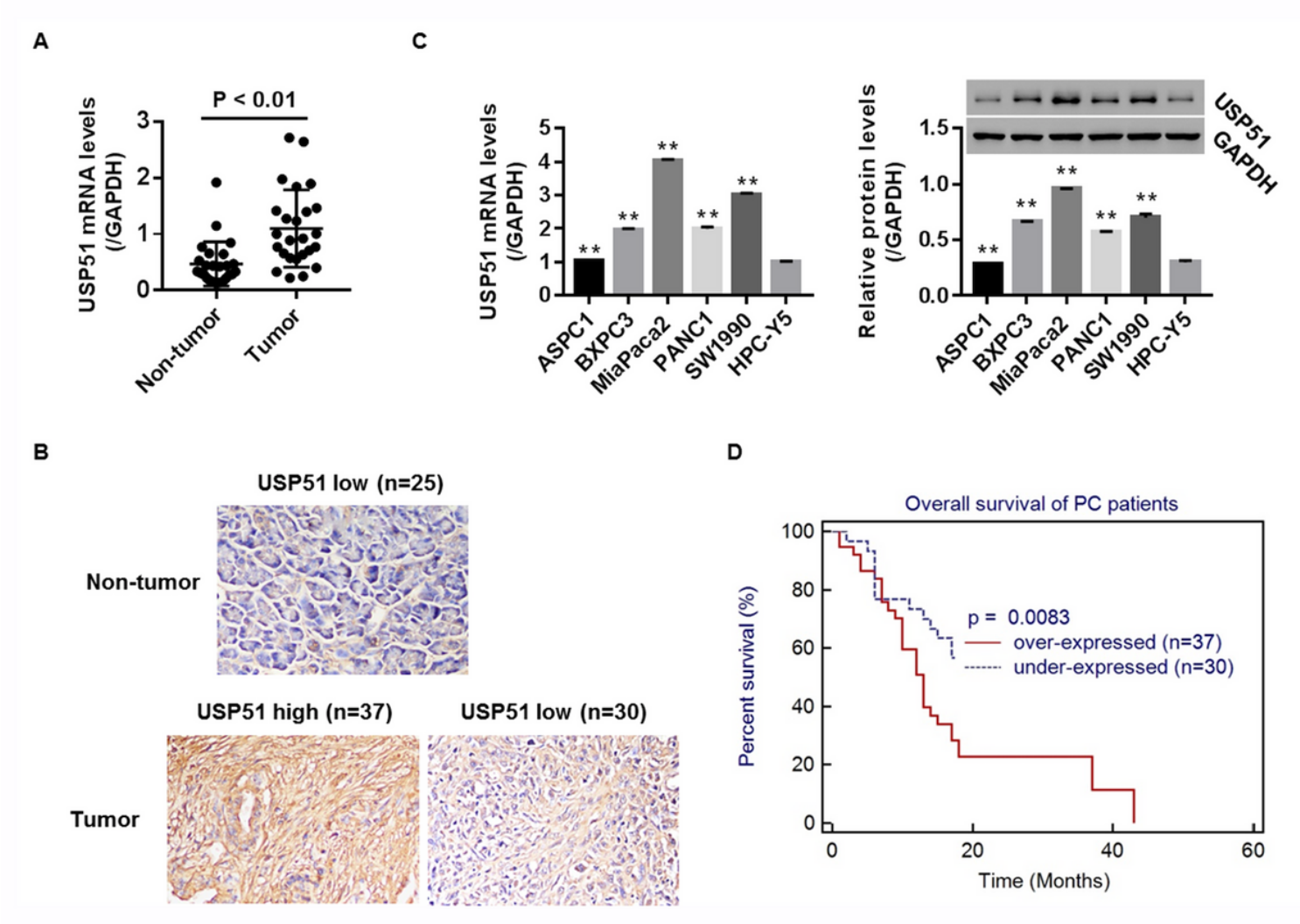

Figure 1

Expression of USP51 in human PC. A. mRNA level of USP51 in 25 pairs of tumor and adjacent non-tumor pancreatic tissue, detected using RT-PCR. B. IHC staining showed that USP51 was enhanced in tumor tissue of PC patients (n = 67) when compared with non-tumor tissue (n = 25). Scale bar: 50 μm. C. mRNA and protein level of USP51 five human PC cell lines (ASPC1, BXPC3, MiaPaca2, PANC1 and SW1990) and one normal pancreatic cell line (HPC-Y5), assessed by RT-PCR and Western blot, respectively. D. Human tissue microarrays from PC patients (n = 67) were divided into USP51 high-expression and USP51 low-expression groups, according to USP51 expression levels, assessed using IHC assay, and then the effect of USP51 on overall survival of PC patients were analyzed by Kaplan-Meier method. #P < 0.01 vs. precancerous tissue; **P < 0.01 vs. HPC-Y5 cells.

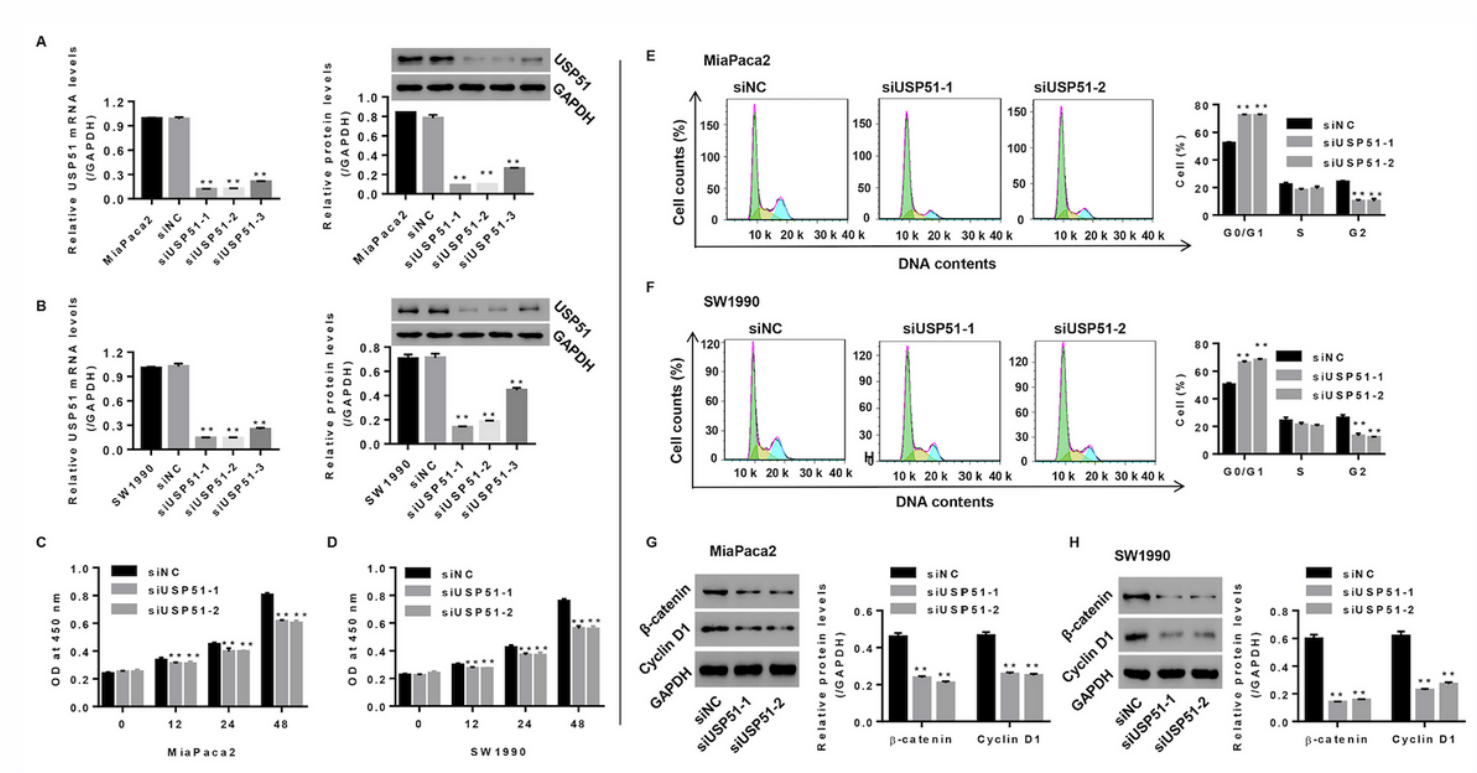

Figure 2

Knockdown of USP51 inhibited PC cell growth and prevented the activation of Wnt/β-catenin pathway. siUSP51 (siUSP51-1, siUSP51-2 and siUSP51-3) significantly reduced mRNA and protein levels of USP51 in A. MiaPaca2 and B SW1990 cells, suggesting a successful establishment of knockdown of USP51 within those two PC cell lines. After transfection with siUSP51-1 and siUSP51-2, proliferation of C. MiaPaca2 and D. SW1990, was determined using CCK-8 method. Cell cycle of E. MiaPaca2 and F. SW1990, was determined using flow cytometry. Protein levels of β-catenin and Cyclin D1 within G. MiaPaca2 and H. SW1990 were assessed using western blot analysis. **P < 0.01 vs. siNC.

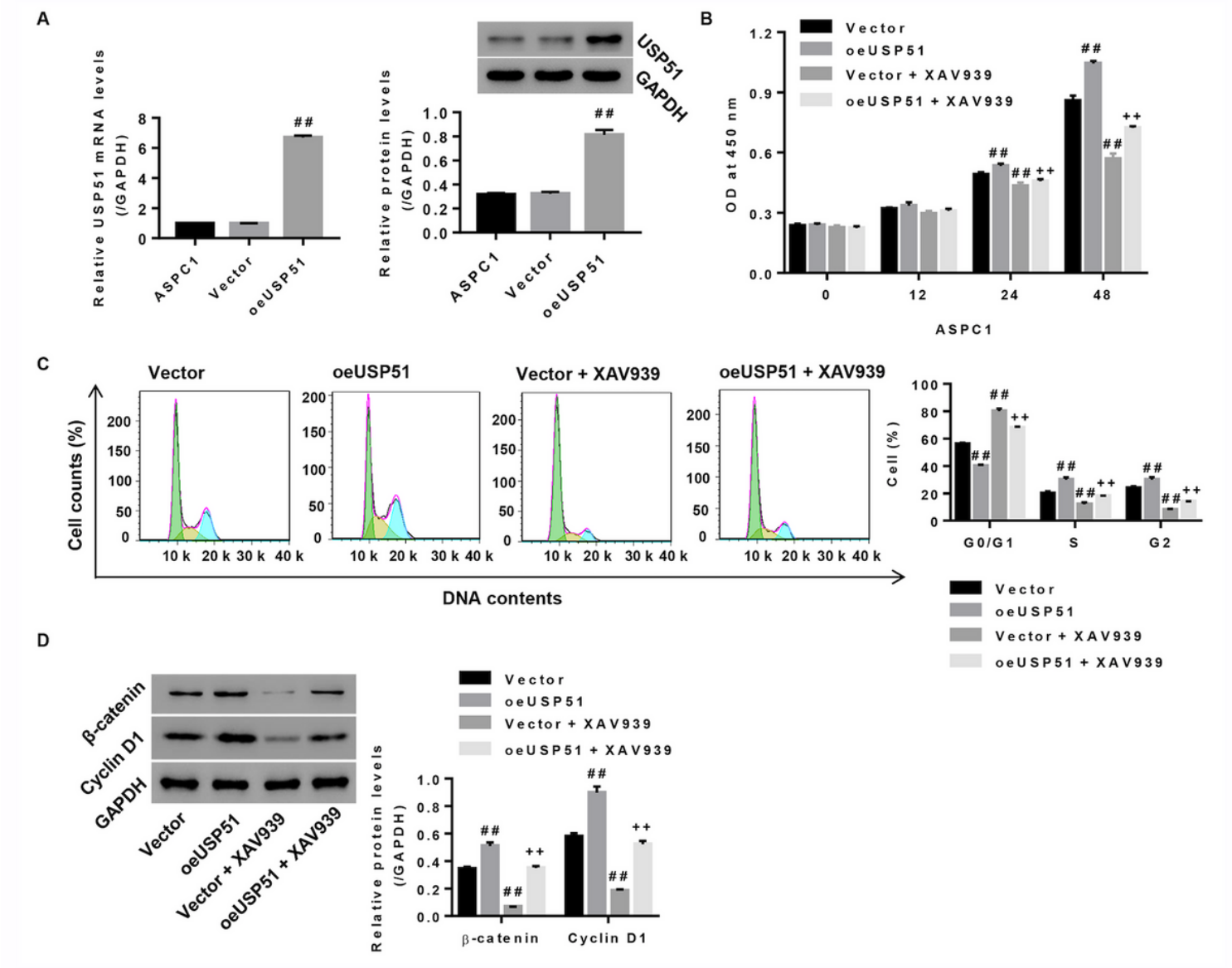

Figure 3

Overexpression of USP51 promoted PC cell growth via activating Wnt/β-catenin pathway. A. Successful establishment of lentiviruses-mediated USP51 overexpression within ASPC1 cells. ASPC1 cells transfected with USP51 overexpression were treated with 10 μmol/L XAV939, and then B cell proliferation, C proportion of ASPC1 cell in cell cycle phases, and D. protein expression of β-catenin and Cyclin D1 were assessed. ##P < 0.01 vs. Vector; ++P < 0.01 vs. Vector + XAV939.

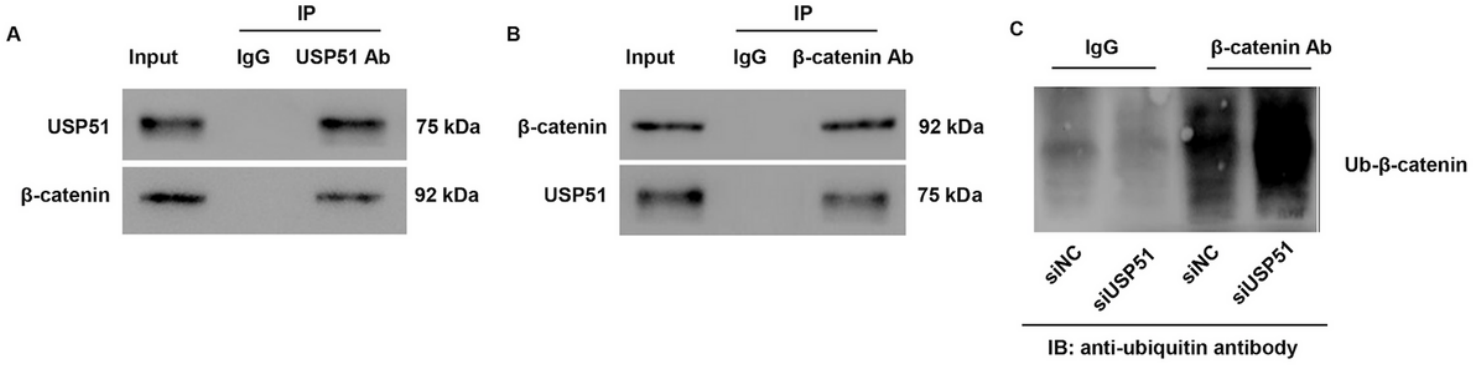

Figure 4

USP51 regulated Ub-β-catenin in human PC cells. Following the Co-IP with A. anti-USP51 antibody and B. anti-β-catenin antibody, the presence of USP51 and β-catenin in MiaPaca2 cells was measured by Western blot. C. Following the Co-IP with anti-β-catenin antibody, the presence of Ub-β-catenin in siUSP51 transfected MiaPaca2 cells was immunoblotted with anti-ubiquitin antibodies.

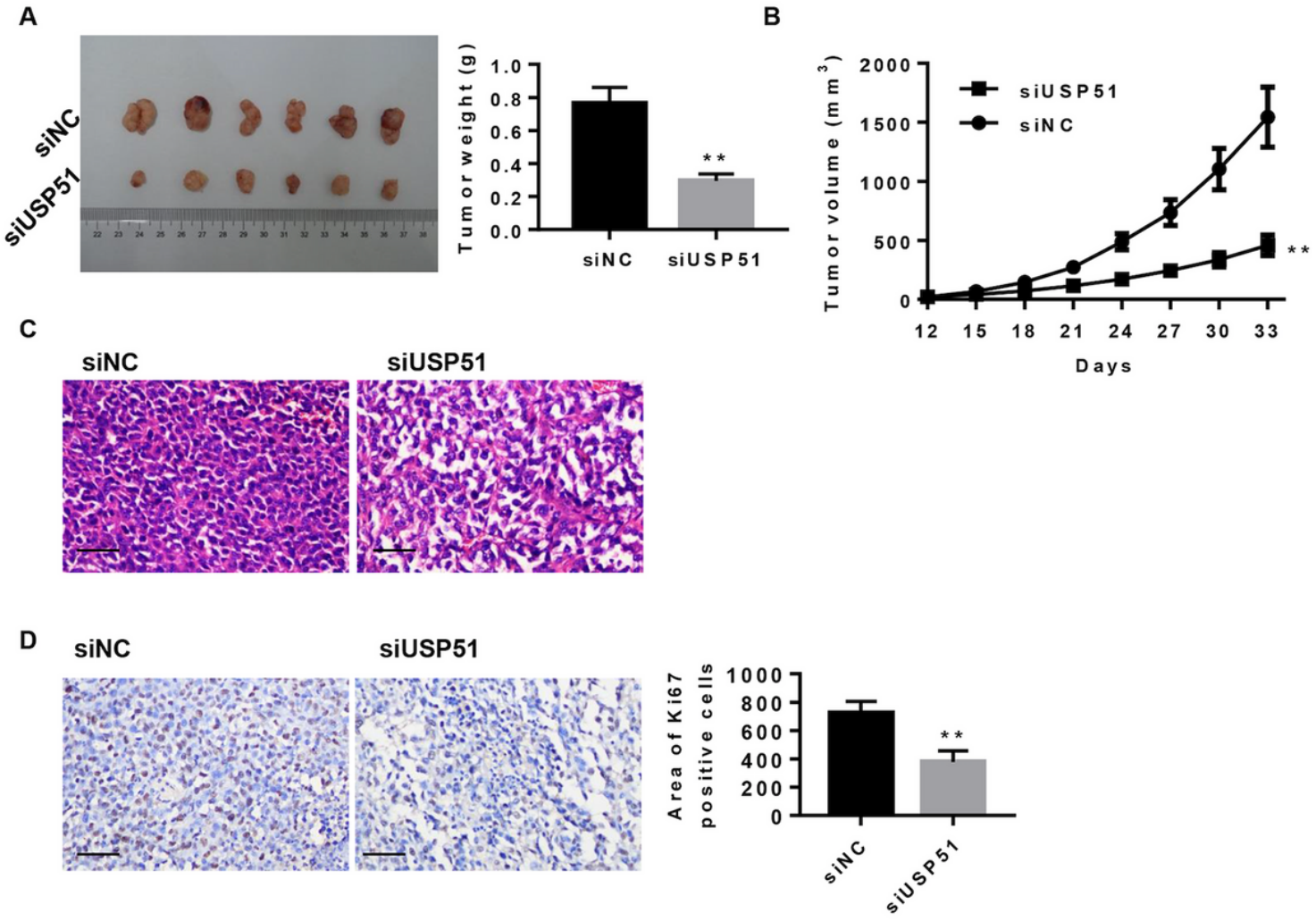

Figure 5

Knockdown of USP51 inhibited tumorigenicity of MiaPaca2 cells in a Xenograft model. A. Tumor weight on 33 th day; B. Tumor volume (mm3) from 12 th day to 33 th day; C. Pathological observation using H&E staining.; D. expression of Ki67 in tumor tissue, measured by IHC. Scale bar: 50 μm. **P < 0.01 vs. siNC.