# USP18 promotes cell proliferation and suppressed apoptosis in cervical cancer cells via activating AKT signaling pathway

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# Abstract

Background: The deubiquitinating (DUB) enzyme ubiquitin-specific protease 18 (USP18), also known as UBP43, is an ubiquitin-specific protease and has been linked to several human malignancies. However, the underlying function of USP18 remains unclear in human cervical cancer. In the current research, we aimed to analyze the role of USP18 and its signaling pathway in cervical cancer.

Methods: Quantitative real time polymerase chain reaction (qRT-PCR) and immunohistochemical staining were performed to analyze USP18 levels in cervical cancer and adjacent-matched tissues. Moreover, RNA interference (RNAi) and lentiviral-mediated vector were performed to silence and overexpression of USP18 in cervical cancer cells. Further, Cell Counting Kit-8 (CCK-8) assay and Annexin V/PI staining were used to assess its biological function in cell proliferation and apoptosis respectively.

Results: Present findings demonstrated that USP18 was overexpressed in cervical cancer specimens and cell lines. Silencing of USP18 in cervical cancer cell lines, SiHa and Caski, inhibited cell proliferation, while induced apoptosis and promoted the expression of cleaved caspase-3. On the contrary, USP18 overexpression showed reversed effects in Hela cells. Gene Set Enrichment Analysis suggested that USP18 was enriched in PI3K/AKT signaling pathway in cervical cancer. Hence, the PI3K/AKT inhibitor LY294002 was used to determine the relationship between USP18 and AKT in cervical cancer cells. Importantly, the PI3K/AKT inhibitor LY294002 deeply abolished the effects of USP18 overexpression in cervical cancer cells.

Conclusions: The current study indicates USP18 was an oncogenic gene in cervical cancer. Our findings not only deepened the understanding the biological function of USP18 in the pathogenesis of cervical cancer but also provided novel insight for cervical cancer therapy. Trial registration: retrospectively registered.


# Background

Cervical cancer is the fourth most frequent malignancy in women worldwide, which is mainly caused by human papillomavirus (HPV) [1]. Although the traditional therapy (radiotherapy or radical surgery) for cervical cancer treatment is widely available, the outcome is far from being fully satisfied. More than one third of patients with cervical cancer will recur, nearly inevitably leading to death. [2] The prognosis of metastatic cervical cancer remains poor and the overall survival has limited within 10 months. [3] Hence, deepened the understanding into the molecule pathogenesis of cervical cancer is a critical step for its novel therapy.

The deubiquitinating (DUB) enzyme ubiquitin-specific protease 18 (USP18), also known as UBP43, is an ubiquitin-specific protease. [4] A previous report has identified that USP18 is increased in certain human tumors. [5]. Silencing of USP18 inhibits the growth of mammary tumor in vivo and promotes the apoptosis of breast cancer cells. [6, 7] Moreover, USP18 silencing significantly increased the apoptosis of glioblastoma cells. [8] Further, Knocking down of USP18 has suppressed the growth and induces

apoptosis of acute promyelocytic leukemia [9]. Furthermore, it has been confirmed that USP18 promotes breast cancer growth via improving the activity of AKT/Skp2 pathway.[10] However, the underlying role and signaling pathway of USP18 is less investigated in cervical cancer cells.

The purpose of present study is to explore the function of USP18 in cervical cancer cells. We induced silencing and overexpression of USP18 in cervical cancer cells using RNA interference (RNAi) and lentiviral-mediated vector. Our analyses not only elucidated the role of USP18 but also demonstrated its potential signaling pathway in cervical cancer cells.

# Methods

Human tissues

A total of 30 pairs of human cervical cancer and adjacent-matched para-cancerous tissues were provided by Shanghai First Maternity and Infant Hospital,Tongji University School of Medicine, Shanghai, China. All patients with cervical cancer were written informed consent.

Cells and cell cultures

All the cell lines used in the present study were obtained from the cell bank of the Shanghai Biology Institute (Shanghai, China), including human cervical cancer cell lines Hela, C-33A, Caski, SiHa and normal cervical epithelial cell HcerEpic. Cells were cultured in RPMI-1640 medium (SH30809.01B, Hyclone, USA) containing 10% fetal bovine serum (16000-044, Gibco, USA.) and 1% penicillin-streptomycin (P1400-100, Solarbio, China) and maintained with the conditions at 37℃, 5% CO2. The AKT inhibitor LY294002 (25 μmol/L; S1105, Selleck, USA) was dissolved in DMSO (D2650, Sigma, USA) and used to culture cells.

Overexpression and knockdown of USP18

In brief, the full length of USP18 (NM_017414.4)coding region sequence (CDS) were inserted into the lentiviral-mediator vector (pLVX-Puro). Then, the recombinant vector was transfected into human Hela cells using Lipofectamine 2000 manufacturer's protocols (Cat: 11668027, ThermoFisher, USA) (oeUSP18). The mock vector was transfected as negative control (oeNC).

For silencing, three small interference RNAs that targeting different region of human gene USP18 were synthesized (siUSP18-1 (347–365): CCTGCTGCCTTAACTCCTT; siUSP18-2 (1004–1022): GCCAGATCCTTCC AATGAA; siUSP18-3 (1023–1041): GCGAGAGTCTTGTGATGCT). Then, transfected into Caski and SiHa cells respectively. a nonspecific scrambled siRNA () transfected as negative control (siNC).

Cell proliferation

Cell proliferation profile was examined by using Cell counting kit-8 (CCK-8) (CP002,SAB, USA) following the manufacturer's instruction. The OD450 value was quantified by a microplate reader (DNM-9602，Pulangxin, China). Three replications were needed for each time point.

Flow cytometry

In brief, Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Beyotime, China) were used to stained Hela, Caski and SiHa cells according to the instructions of the manufacturer at 48 h after viral infection. Then, flow cytometer (BD, USA) were used to determine cells.

Real-time PCR

Total RNA from cell samples was extracted using TRIzol Reagent (Invitrogen, USA). Then, the cDNA synthesis kit (Fermentas, Canada) were used for the RNA reverse transcribed into complementary DNA (cDNA) according to the instructions of the manufacturer. The expression of GAPDH was used to normalize the gene expression and counted using the $2^{-\Delta\Delta Ct}$ method. Three replicates are needed for each analysis. The following primers were used to perform PCR: USP18 F 5' TCTGGAG GGCAGTATGAG 3', USP18 R 5' TGGTAGTTAGGATTTCCGTAG 3'; GAPDH F 5' GGATTGTCTGGCAGTAGCC 3', GAPDH R 5'ATTGT GAAAGGCAGGGAG 3'.

Western Blot

Total protein was extracted using RIPA lysis buffer (JRDUN, Shanghai, China). BCA protein assay kit (PICPI23223,Thermo Fisher, USA) was utilized to measure total protein. Proteins that was adjusted to equal content (25 μg) were fractionated in 10% SDS-PAGE and subsequently transferred onto PVDF nitrocellulose membrane (HATF00010，Millipore, USA) for 12 h. Then, the membranes were probed with the primary antibodies at 4 °C overnight followed by the appropriate HRP-conjugated goat anti-rabbit IgG (A0208，Beyotime, China) at 37 °C for 60 min. Protein signals were detected using a chemiluminescence system (5200，Tanon, China). GAPDH served as an endogenous reference. Each analysis was established in triplicate. The primary antibodies as follows: USP18 (AB168478, Abcam, UK), cleaved caspase-3 (AB32042, Abcam, UK), AKT (#4691, CST, Danvers, USA), p-AKT (#4060, CST, Danvers, USA) and GAPDH (#5174, CST, Danvers, USA). Primary antibodies were revealed with HRP-conjugated secondary antibodies anti-rabbit IgG (A0208, Beyotime, Shanghai, China) or anti-mouse IgG (A0216, Beyotime, Shanghai, China).

Immunohistochemistry

The tissue sections were fixed in methanol (4%) for 30 min. Then, eliminated endogenous peroxidase activity through incubating with $H_2O_2$ (3%) for 10 min. After that, the primary antibodies of USP18 (ab115618, Abcam, UK) was used to incubate the tissue sections at room temperature for 1 h, followed by incubation with the HRP-labelled secondary antibody for 30 min. Then, the sections were stained by DAB and re-stained by hematoxylin for 3 min. An upright microscope (ECLIPSE Ni, NIKON, Japan) was

used to take image and analyzed by using the Microscopic image analysis system (DS-Ri2, NIKON, Japan), magnification, 200×.

Gene Set Enrichment Analysis

The data created an ordered list of all genes firstly according to their correlation with USP18 expression, and then a predefined gene set is given an enrichment score (ES) and P value. GSEA was performed using The Cancer Genome Atlas (TCGA) cervical cancer dataset by GSEA version 2.0.

Statistical analysis

Statistical analyses were performed by using GraphPad Prism software Version 7.0 (CA, USA). All data represented as means ± S.E.M from three independent experiments. Statistical significance was assessed by using Student's t-test and one-way analysis of variance (ANOVA). Statistically significant was accepted as the p -value < 0.05.

# Results

USP18 is upregulated in human cervical cancer tissues

To examine the relationship between USP18 and cervical cancer, we collected data from UALCAN (http://ualcan.path.u ab.edu/cgi-bin/TCGA ExResultNe w2.pl? genenam = USP18 &ctype = CESC) database. As presented in Fig. 1A, the level of USP18 is much higher in cervical squamous cell carcinoma (CESC) samples than that in para-cancer samples. Moreover, a total of 30 pairs of cervical cancer and adjacent -matched para-cancer tissues were used to further examine the mRNA level of USP18. Our results also indicated USP18 was overexpressed in human cervical cancer tissues (Fig. 1B). Moreover, results obtained from IHC staining assay suggested that the protein content of USP18 was also increased in cervical cancer tissues than that in para-cancer tissues (Fig. 1C).

Knockdown and overexpression of USP18 in human cervical cancer cells

Next, we compared the relative mRNA and protein levels of USP18 between human normal cervical epithelial (HcerEpic) and cervical cancer cells, including Hela, C-33A, Caski and SiHa. Clearly, both the relative mRNA and protein levels of USP18 were significantly upregulated in cervical cancer cells (except for Hela), especially in Caski and SiHa cells (Fig. 2A & 2B). Therefore, USP18 was induced silencing in Caski and SiHa cells.

For silencing, three siRNAs that targeting different region of human gene USP18 (siUSP18-1, siUSP18-1 and siUSP18-3) and a nonspecific scrambled siRNA (siNC) were synthesized. Then, all of them were transfected into Caski and SiHa cells respectively. The untreated cells were functioned as blank control (Blank). Clearly, both the relative mRNA and protein levels of USP18 were deeply reduced in Caski and SiHa cells that transfected with siUSP18-1, siUSP18-2 and siUSP18-3 (Fig. 2C & 2D). Meanwhile, Hela cells were transfected with a plasmid for overexpressing USP18 (oeUSP18). The mock plasmid was

functioned as negative control (oeNC). As shown in Fig. 2E & 2F, oeUSP18 remarkably promoted the endogenous levels of USP18 in Hela cells respectively.

USP18 silencing inhibited the proliferation and promoted apoptosis in human cervical cancer cells

Then, we examined the proliferation rate of Caski and SiHa cells that transfected with siUSP18-1 or siUSP18-2 respectively using CCK-8 assay. As presented in Fig. 3A & 3B, both siUSP18-1 and siUSP18-2 were significantly abolished the proliferation of Caski and SiHa cells. Moreover, the apoptosis of siUSP18-1 and siUSP18-2 transfected cells were much higher than that in siNC transfected cells in two cell lines (Fig. 3C).

Cleaved caspase-3 is reported as a positive apoptotic factor. [11] Moreover, Gen Set Enrichment Analysis (GSEA) indicated that USP18 was enriched in PI3K/AKT signaling pathway (Figure S1). In the current study, western blotting was performed to quantify the protein contents of USP10, Cleaved caspase-3, p-AKT and AKT in Caski and SiHa that transfected with siUSP18-1 or siUSP18-2. Our results suggested that the protein content of cleaved caspase-3 was significantly increased in siUSP18-1 or siUSP18-2 transfected cells. Interestingly, USP18 siRNAs deeply suppressed the phosphorylation of AKT in Caski and SiHa cells (Fig. 3D & 3E).

The PI3/AKT inhibitor LY294002 disrupted the function of oeUSP18 in human Hela cells

To assess the relationship between USP18 and AKT in cervical cancer cells, the PI3/AKT inhibitor LY294002 was used to silence the endogenous activity of AKT. As shown in Fig. 4A, USP18 overexpression significantly improved the proliferation of Hela cells, while this function was deeply disrupted by the inhibitor LY294002. Moreover, oeUSP18 deeply reduced the apoptosis of Hela cells, whereas this suppression was significantly released by the inhibitor LY294002 (Fig. 4B).

Further, the protein level of cleaved caspase-3 was deeply suppressed in Hela cells transfected with oeUSP18. However, the inhibitor LY294002 remarkably increased the level of cleaved caspase-3 in oeUSP18 transfected cells. Importantly, the phosphorylation of AKT was much higher in oeUSP18 transfected cells, whereas deeply suppressed by the inhibitor LY294002 (Fig. 4C).

# Discussion

Cervical cancer is a common malignant tumor in women with high mortality rate worldwide. Currently, the main treatments for cervical cancer include surgery, postoperative radiotherapy and chemotherapy, which are related to damaging side effects and cause great pain for patients.[12] Therefore, the novel therapy methods are urgently desired.

The ubiquitin proteasome system (UPS) is critical in the regulation of the biological processes of tumor cells [13]. Dysregulated ubiquitination and de-ubiquitination are closely associated the progresses of cervical cancer [14, 15]. In the present study, USP18 was suggested as an oncogene in human cervical cancer. Therefore, targeting USP18 presented novel insight in the treatment for cervical cancer.

Previous report has demonstrated that silencing of USP18 suppressed the proliferation and promoted apoptosis in hepatocellular cancer cells. [16] In the current study, downregulation of USP18 also significantly suppressed the proliferation of cervical cancer cells, whereas induced its apoptosis. Moreover, USP18 overexpression presented the opposite function. Therefore, these results demonstrated that USP18 was a pro-proliferation an anti-apoptosis factor in cervical cancer cells.

Growing evidences have indicated that PI3K/AKT pathway plays a key function in tumor progression and metastasis [17, 18]. Targeting PI3K/AKT is confirmed as a potential therapeutic strategy for multiple human cancers, including pancreatic cancer, breast cancer and bladder cancer.[19–21] Moreover, it has been confirmed that activated PI3K/AKT is correlated with the progression and metastasis of cervical cancer cells.[22] In this study, knockdown of USP18 deeply suppressed the phosphorylation of AKT in cervical cancer cells. The PI3K/AKT inhibitor LY294002 deeply suppressed the function of oeUSP18 in cervical cancer cells. Hence, USP18 was involved in the regulation of PI3K/AKT pathway in cervical cancer cells. USP18 might promote the proliferation and inhibited apoptosis of cervical cancer cells through regulating PI3K/AKT pathway.

## Conclusions

In the present study, we explored the function of USP18 in human cervical cancer cells. Our findings firstly indicated that USP18 was increased in human cervical cancer tissues and promoted the progression of human cervical cancer cells. Moreover, our results not only elucidated the possible signaling pathway of USP18 in human cervical cancer cells but also disclosed its potential value as a target in cervical cancer treatment.

## Abbreviations

DUB: deubiquitinating; USP18: ubiquitin-specific protease 18; qRT-PCR: Quantitative real time polymerase chain reaction; RNAi: RNA interference ; CCK-8: Cell Counting Kit-8; HPV: human papillomavirus cDNA: complementary DNA; TCGA: The Cancer Genome Atlas; ES: enrichment score; ANOVA: analysis of variance; CESC: cervical squamous cell carcinoma

## Declarations

# Ethics approval and consent to participate

Our research was approved by the independent ethics committee of Shanghai First Maternity and Infant Hospital，Tongji University School of Medicine, Shanghai, China and was in accordance with the Declaration of Helsinki.

# Consent for publication

Not applicable

# Availability of data and materials

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

# Competing interests

The authors declare that they have no competing interests.

# Fundings

Not applicable

# Author contributions

MD and HC designed this project and wrote the manuscript; WD performed the experiments; QG, CZ, YS, HF and YC analyzed the data and edited diagrams.

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# Figures

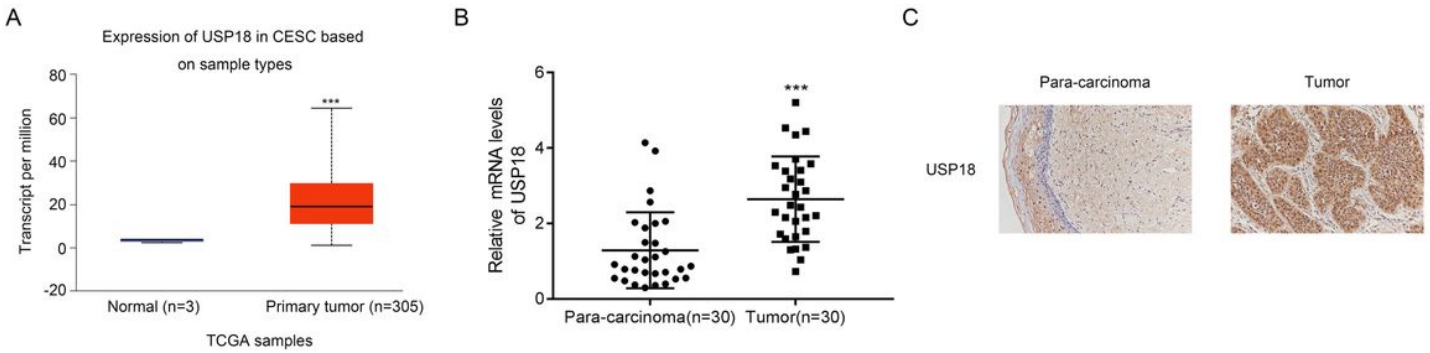
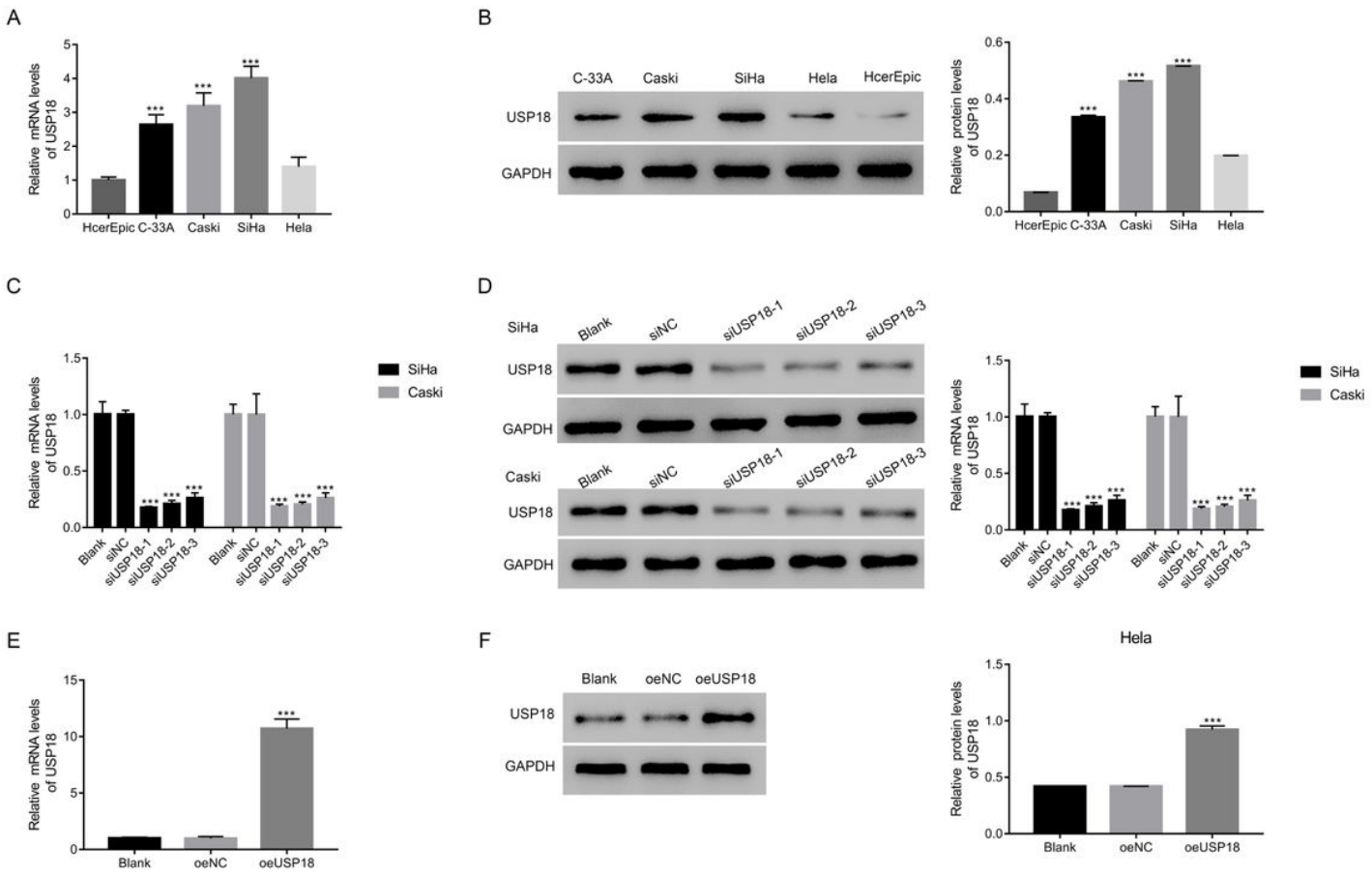


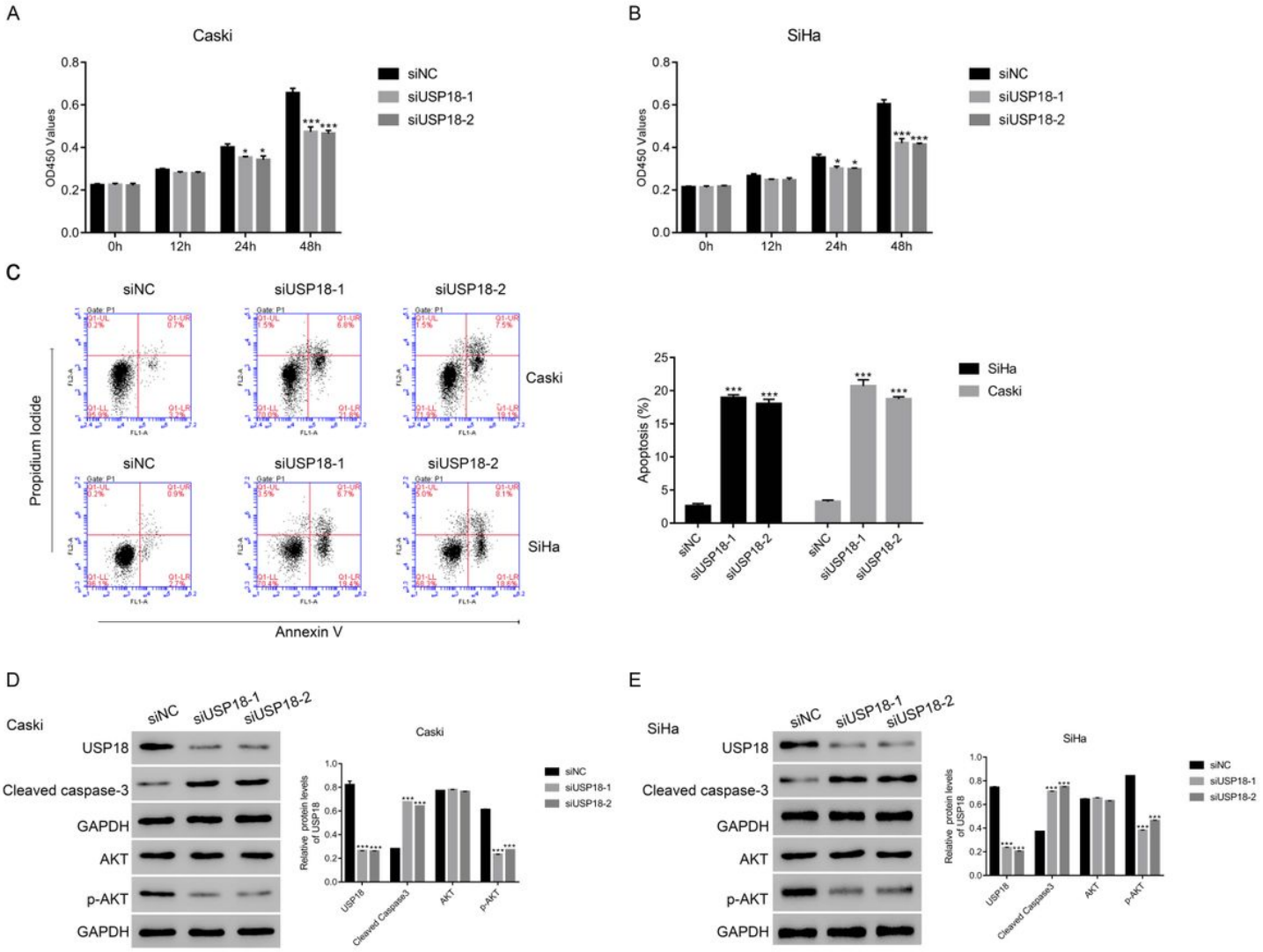

**Figure 1**

USP18 is upregulated in human cervical cancer tissues. A. USP18 is upregulated in primary cervical cancer samples. Data collected from TCGA database CESC dataset. *** $p < 0.001$ vs Normal. B & C. The relative mRNA and protein levels of USP18 were much higher in human cervical cancer tissues than that in para-cancer tissues. *** $p < 0.001$ vs Normal.




## Figure 2

Knockdown and overexpression of USP18 in human cervical cancer cells. A & B. The relative mRNA and protein levels of USP18 were upregulated in cervical cancer cells, including Hela, C-33A, Caski and SiHa. *** $p < 0.001$ vs HcerEpic C & D. USP18 siRNAs (siUSP18-1, siUSP18-2 and siUSP18-3) deeply suppressed the endogenous mRNA and protein levels of USP18 in SiHa and Caski cells. *** $p < 0.001$ vs siNC. E & F. oeUSP18 significantly improved the endogenous mRNA and protein levels of USP18 in Hela cells. *** $p < 0.001$ vs oeNC.




## Figure 3

USP18 silencing suppressed the proliferation and induced apoptosis in human cervical cancer cells. A & B. siUSP18-1 or siUSP18-2 deeply suppressed the proliferation of Caski and siHa cells. * $p < 0.05$ vs siNC; *** $p < 0.001$ vs siNC. C. The apoptosis of Caski and siHa cells were significantly upregulated after transfecting with siUSP18-1 or siUSP18-2. *** $p < 0.001$ vs siNC. D & E. western blot was used to examine the protein contents of USP18, Cleaved caspase-3, AKT and p-AKT in Caski and siHa cells that transfected with siUSP18-1 or siUSP18-2 respectively. *** $p < 0.001$ vs siNC.

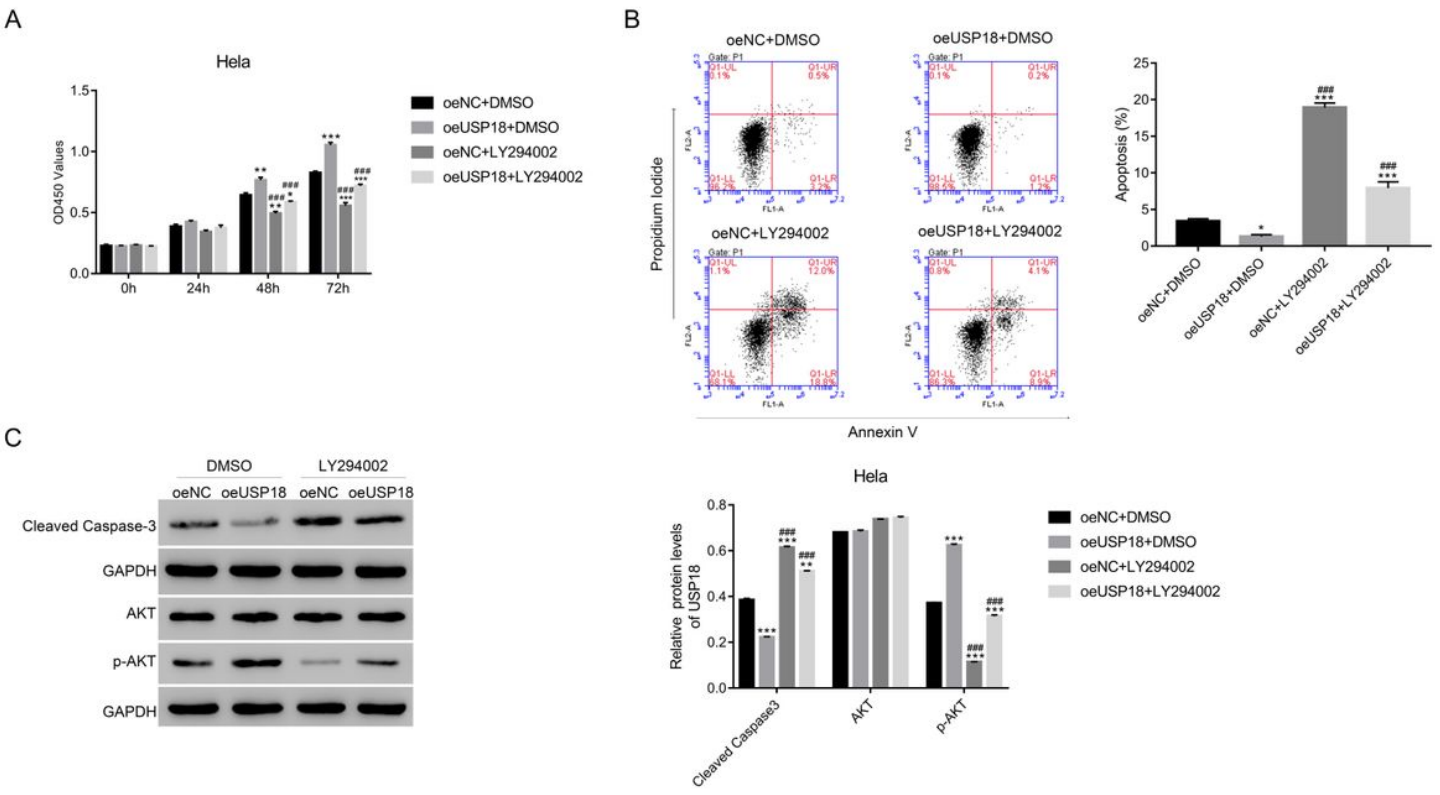



**Figure 4**

The PI3/AKT inhibitor LY294002 disrupted the function of oeUSP18 in human Hela cells. A. The proliferation of oeUSP18 transfected cells was deeply suppressed in the presence of the PI3/AKT inhibitor LY294002. * $p < 0.05$ vs oeNC + DMSO, ** $p < 0.01$ vs oeNC + DMSO, *** $p < 0.001$ vs oeNC + DMSO; ### $p < 0.001$ vs oeUSP18+DMSO. B. The PI3/AKT inhibitor LY294002 promoted the apoptosis of oeUSP14 transfected cells. * $p < 0.05$ vs oeNC + DMSO, *** $p < 0.001$ vs oeNC + DMSO; ### $p < 0.001$ vs oeUSP18+DMSO. C. Western blot was used to examine the protein contents of cleaved caspase-3, AKT and p-AKT in oeNC or oeUSP18 transfected cells with or without the treatment of the PI3/AKT inhibitor LY294002. *** $p < 0.001$ vs oeNC + DMSO; ### $p < 0.001$ vs oeUSP18+DMSO.

# Supplementary Files

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- 3SiHa.tif
- 3Caski.tif
- 4.tif
- 2SiHa.tif
- 2Hela.tif

- FigureS1.jpg
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