ACTA ABP BIOCHIMICA POLONICA

Vol. 69, No 2/2022
417–422
https://doi.org/10.18388/abp.2020_5871

Regular paper

USP18 contributes to the proliferation and migration of ovarian cancer cells by regulating the AKT/mTOR signaling pathway

Meiqin Liu, Shile Gao✉, Xingjun Xu, Lijie Zhang, Bin Xu and Donghui Lu

Tumor Ward 4, The 901ST Hospital of the Joint Logistics Support Force of PLA, Hefei, Anhui Province, China

Ubiquitin-specific peptidase (USP)18 is elevated in tumor tissues and is associated with tumor malignancy. USP18 functions as an oncogene in different cancers. However, the role of USP18 in ovarian cancer was poorly understood. TCGA database showed that USP18 was elevated in ovarian cancer tissues. Additionally, USP18 mRNA and protein expression was also up-regulated in tumor tissues. The functional assays were then designed *via* siRNA-mediated knockdown of USP18. The results showed that knockdown of USP18 reduced cell viability and ovarian cancer proliferation. Furthermore, cell apoptosis was promoted by USP18 silencing, and interference of USP18 suppressed cell migration and invasion. The expression of phosphorylated AKT (p-AKT) and p-mTOR protein was decreased in ovarian cancer cells by USP18 knockdown. Inhibition of AKT attenuated the decrease in cell apoptosis induced by USP18 overexpression and increased cell viability and migration. In conclusion, USP18 promoted the proliferation and migration of ovarian cancer cells by activating AKT/mTOR signaling.

Keywords: USP18, proliferation, migration, ovarian cancer, AKT/mTOR

Received: 02 September, 2021; revised: 27 November, 2021; accepted: 09 March, 2022; available on-line: 13 June, 2022

✉e-mail: gao_sle@163.com
Acknowledgements of Financial Support: This work was supported by the Wannan Medical College Teaching Hospital Scientific Research Project. (Grant No. JXYY202016).
Abbreviations: USP, Ubiquitin-specific peptidase

INTRODUCTION

Ovarian cancer is one of the three major tumors in obstetrics and gynecology, and its mortality ranks first among gynecological tumors (Juhi Rais 2017). Due to the lack of reliable diagnostic means and typical early symptoms, almost 70% of patients with ovarian cancer are diagnosed as advanced tumor (Marchetti *et al.,* 2019). Despite advances in therapeutic strategies, including targeted therapies and chemotherapy regimens, of ovarian cancer, the mortality rate remains relatively high, with a 5-year survival rate less than 45% (Dinca *et al.,* 2020). Most women with ovarian cancer demonstrate a high recurrence rate and are resistant to chemotherapies (Dinca *et al.,* 2020). Therefore, understanding the pathogenesis in ovarian cancer is urgently needed for the identification of diagnostic, prognostic, and therapeutic biomarkers to improve treatment.

Ubiquitin-specific peptidase (USP) 18 belongs to the USPs family, and it removes ubiquitin from the substrate of the ubiquitinated protein, thus participating in various biological pathways, such as viral infection, antibacterial response, autoimmune diseases and tumor development (Honke *et al.,* 2016). USP18 functions as a deISGylase to cleave the IFN-stimulated gene 15 from conjugated proteins (Pinto-Fernandez *et al.,* 2020), and loss of USP18 promoted ISGylation and destabilization of growth-regulatory protein to induce susceptibility to drug (Potu *et al.,* 2010) and TRAIL (Manini *et al.,* 2013) -induced apoptosis. USP18 deficiency-induced antitumor environment (Burkart *et al.,* 2013), and promoted radiosensitivity and antigenicity of cancer cells (Pinto-Fernandez *et al.,* 2021). Therefore, USP18 was considered an antineoplastic target (Mustachio *et al.,* 2018). Additionally, USP18 also functioned as a deubiquitinating enzyme to stabilize Snail1 and promote the progression of colorectal cancer (Huang *et al.,* 2020). However, in melanoma cells, USP18 enhanced the activities of cytotoxic T lymphocytes to sensitize tumor cells to immunotherapy and immunosurveillance (Hong *et al.,* 2014). However, the role of USP18 in the progression of ovarian cancer has not yet been reported.

In this study, the expression of USP18 in ovarian cancer was first investigated, and then then the effects of USP18 on cell proliferation, apoptosis, migration,, and invasion were evaluated.evaluated. Furthermore, the potential mechanism involved in the biological role of USP18 in ovarian cancer was also investigated.

MATERIALS AND METHODS

Tissue samples

Patients diagnosed with ovarian cancer (n=50) were recruited at the 901ST Hospital of the Joint Logistics Support Force of PLA. Tumor and para-carcinoma tissues were collected from patients with informed written consents through surgery. The study was approved by the Ethics Committee of the 901ST Hospital of the Joint Logistics Support Force and in accordance with those of the 1964 Helsinki Declaration and its later amendments for ethical research involving human subjects . Ethical principles for medical research involving human subjects.

Cell culture and transfection

Ovarian cancer cells (A2780 and SKOV3) were purchased from Beijing Northland Biotech. Co., Ltd. (Beijing, China). Cells were cultured in a 37°C incubator in RPMI-1640 medium containing streptomycin-penicillin and 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). The pcDNA vector and pcDNA-BDH2 were acquired from Genepharma (Suzhou, China). A549 cells were transfected with the vectors using Lipofectamine 3000 (Invitrogen). A2780 and SKOV3 in the absence or

presence of 20 μM LY294002 (Sigma-Aldrich Corp., St. Louis, MO, USA) were transfected with siRNA-targeting USP18 (siUSP18) or the negative control (siNC), pcDNA-USP18 (USP18) or the negative control (NC) using Lipofectamine 3000 (Invitrogen).

Cell proliferation assays

A2780 and SKOV3 were seeded in 96-well plates and performed with the indicated transfections and treatment for 24 hours. The cells were then incubated in plates for 24, 48 or 72 hours. After incubation with CCK8 solution (Beyotime, Beijing, China) for 4 hours, absorbance at 450 nm of each well was measured *via* microplate reader (Sigma-Aldrich). Furthermore, A2780 and SKOV3 with the indicated transfections and treatment were seeded in 6-well plates and cultured in RPMI-1640 medium for 10 days. After fixation and crystal violet staining, cells were observed under a light microscope (Olympus, Tokyo, Japan).

Flow cytometry

A2780 and SKOV3 with the indicated transfections and treatment were harvested by trypsin treatment and then resuspended in binding buffer from Annexin V-FITC/PI apoptosis kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Following labeling with PI and annexin V-FITC, cells were analyzed by the FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). For cell cycle analysis, cells were incubated with propidium iodide and RnaseA and then analyzed by FACSCalibur flow cytometer.

Cell Migration and Invasion Assays

A2780 and SKOV3 with the indicated transfections and treatment were seeded in 6-well plates. A pipette tip was used to generate a scratch in the plates. The cells were then cultured in plates for 24 hours before observation under a light microscope. For cell invasion, the lower chamber of the matrigel coated well (BD Biosciences)-coated well (Corning, Tewksbury, MA, USA) was filled with RPMI-1640 medium containing 15% fetal bovine serum. The upper chamber was filled with A2780 and SKOV3 in serum-free medium. After incubation at 37°C for 24 hours, the invasive cells in the lower chamber were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The cells were then photographed and counted under the light microscope.

qRT-PCR

The TRIzol method (Invitrogen) was used to extract total RNA from tissues and cells from ovarian cancer. The cDNAs were then synthesized using the reverse transcription system (Applied Biosystems, Carlsbad, CA, USA). SYBR Green Master (Roche, Mannheim, Germany) was used for USP18 qRT-PCR analysis. The mRNA expression of USP18 was normalized to GAPDH. Primers: USP18 (Forward: 5'-TTGGGCTCCTGAGGAAACC-3' and Rerverse: 5'-CGATGTTGTGTAAACCAACCAGA-3') and GAPDH (Forward: 5'-GAAGGTGAAGGTCGGAGTC-3' and Rerverse: 5'-GAAGATGGTGATGGGATTTC-3') were used in this study.

Western blot

Protein samples isolated from A2780 and SKOV3 via RIPA lysis buffer (Beyotime) were separated by 10% SDS-PAGE and then transferred to a nitrocellulose membrane. The membranes were blocked and probed with specific antibodies: anti-Cyclin D1 (ab226977), anti-CDK4 (ab137675) and anti-p21 (ab227443) (1:1500, Abcam, Cambridge, UK), anti-USP18 (ab168478) and anti-GAPDH (ab8245) (1:2000, Abcam), anti-Bcl-2 (ab59348), anti-Bax (ab216494), and anti-cleaved caspase-3 (ab2302) (1:2500, Abcam), anti-AKT (ab8805) and anti-p-AKT (ab38449) (1:3000, Abcam), anti-E-cadherin (ab231303), anti-N-cadherin (ab76057) and anti-MMP2 (ab97779) (1:3500, Abcam), anti-mTOR (ab2732) and anti-p-mTOR (ab1093) (1:4000, Abcam). After incubation with horseradish peroxidase conjugated secondary antibody (ab205718 and ab97040) (1:5000, Abcam) and peroxidase substrate (tetramethylbenzidine), protein bands were visualized using chemiluminescence (Sigma-Aldrich).

Statistical analysis

All data with at least triple replicates were expressed as mean ±S.E.M. and analyzed using the Student's *t*-test or one-way analysis of variance in SPSS software. A *p*-value of <0.05 was considered statistically significant.

RESULTS

Elevated USP18 in ovarian cancer

Data from the TCGA database showed that USP18 was upregulated in ovarian cancer tissues compared to paracarcinoma tissues (Fig. 1A). The expression of both mRNA (Fig. 1B) and protein (Fig. 1C) expression of USP18 was also elevated in tumor tissues, suggesting the potential relationship between USP18 and ovarian cancer.

USP18 contributed to the proliferation of ovarian cancer cells

Loss of function assays were designed to investigate the role of USP18 in the progression of ovarian cancer. A2780 and SKOV3 were transfected with siUSP18 with reduced expression of USP18 mRNA (Fig. 2A) and protein (Fig. 2B) expression of USP18. The downregulation induced by siUSP18 of USP18 reduced the cell viability of A2780 and SKOV3 (Fig. 2C) and suppressed cell proliferation (Fig. 2D). Transfection of siUSP18 induced cell cycle arrest of A2780 and SKOV3 in the G0/G1 phase (Fig. 2E). However, A2780 and SKOV3 cell apoptosis (Fig. 2F) was promoted by siUSP18 transfection. These results suggested the antiproliferative role of USP18 silencing in ovarian cancer.

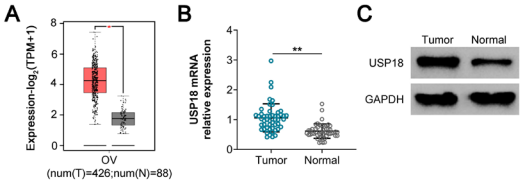

Figure 1. Elevated USP18 in ovarian cancer
USP18 was up-regulated in ovarian cancer tissues (n=426) compared to the para-carcinoma tissues (n=88) based on TCGA database. showed that (Fig. 1A). mRNA expression of USP18 was up-regulated in ovarian cancer tissues compared to the para-carcinoma tissues. Protein expression of USP18 was up-regulated in ovarian cancer tissues compared to the para-carcinoma tissues. **tumor *vs*. normal, $p<0.01$.

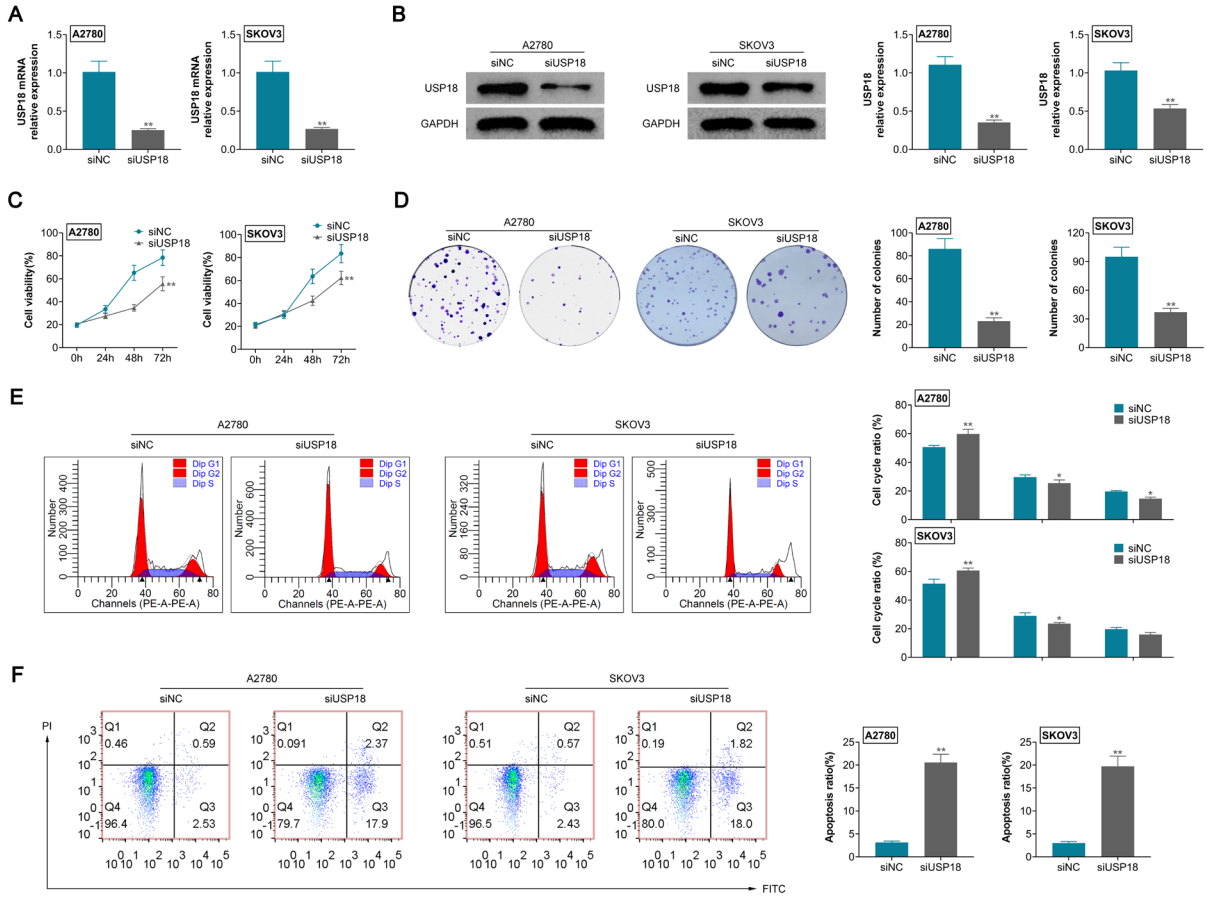

Figure 2. USP18 contributed to ovarian cancer cell proliferation mRNA expression of USP18 was downregulated in A2780 and SKOV3 transfected with siUSP18.
Protein expression of USP18 was downregulated in A2780 and SKOV3 transfected with siUSP18. siUSP18-induced down-regulation of USP18 reduced cell viability of A2780 and SKOV3. siUSP18-induced down-regulation of USP18 suppressed the cell proliferation of A2780 and SKOV3. siUSP18-induced down-regulation of USP18 induced cell cycle arrest of A2780 and SKOV3 at G0/G1 phase. siUSP18-induced down-regulation of USP18 promoted cell apoptosis of A2780 and SKOV3. *, ** *vs*. siNC, $p<0.05$, $p<0.01$.

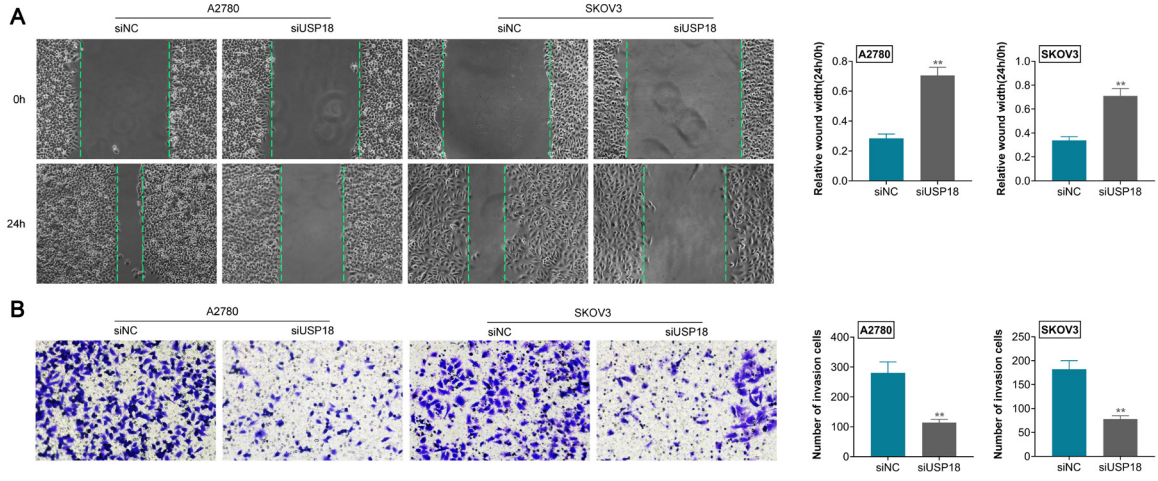

Figure 3. USP18 contributed to ovarian cancer cell invasion siUSP18-induced down-regulation of USP18 repressed cell migration of A2780 and SKOV3.
siUSP18-induced down-regulation of USP18 repressed cell invasion of A2780 and SKOV3. ** *vs*. siNC, $p<0.01$.

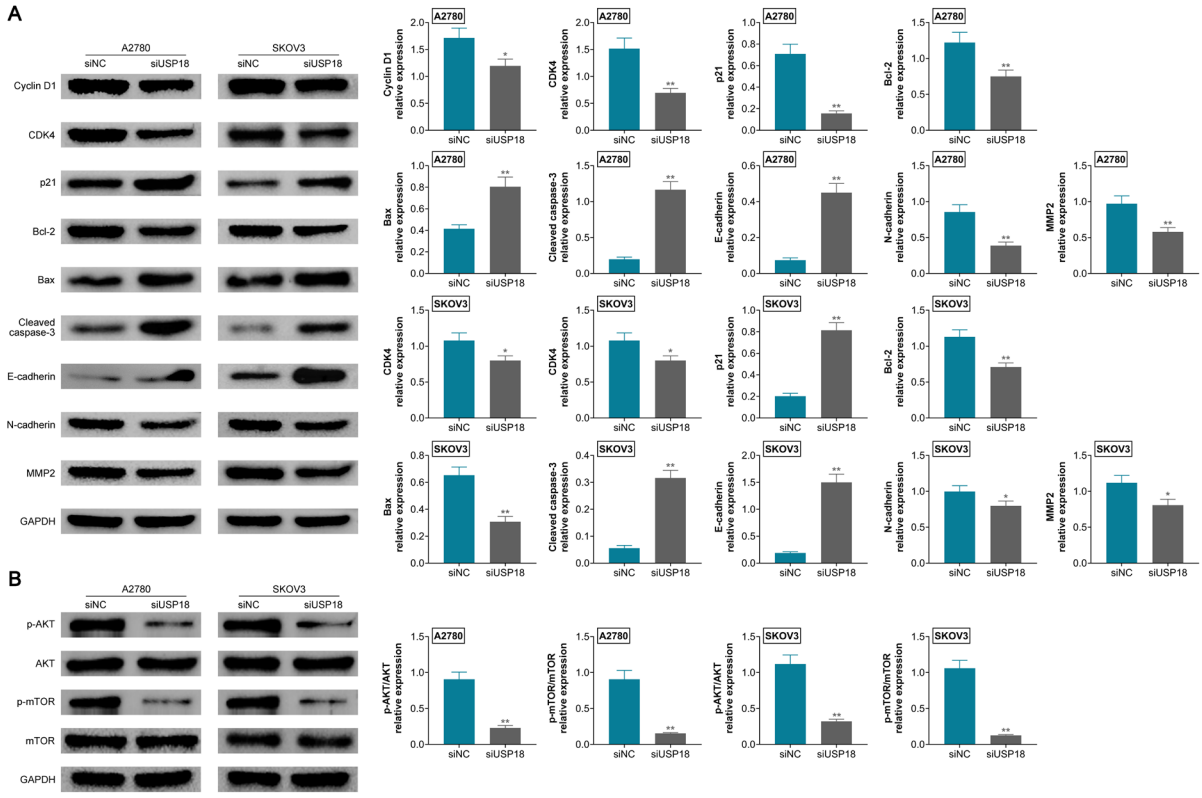

Figure 4. USP18 contributed to activation of AKT/mTOR in ovarian cancer siUSP18-induced down-regulation of USP18 reduced protein expression of Cyclin D1, CDK4, Bcl-2, N-cadherin, MMP2, while enhanced p21, Bax, cleaved caspase-3 and E-caherin in A2780 and SKOV3.
siUSP18-induced down-regulation of USP18 reduced protein expression of p-AKT and p-mTOR in A2780 and SKOV3. ** *vs*. siNC, $p<0.01$.

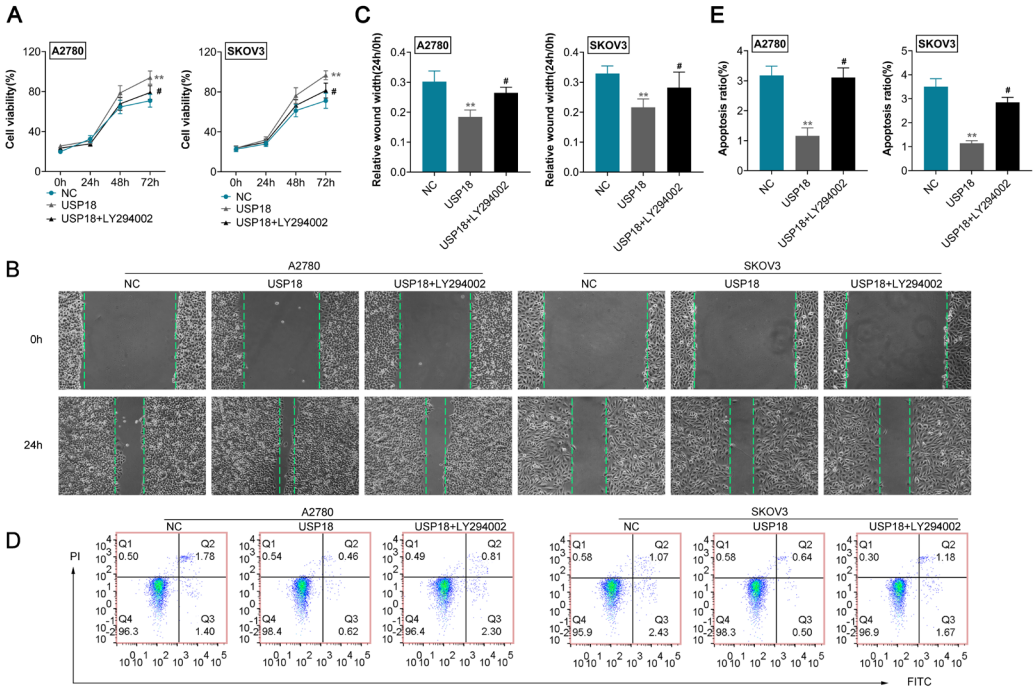

Figure 5. USP18 contributed to ovarian cancer cell proliferation and migration through AKT/mTOR LY294002 incubation attenuated USP18 over-expression-induced increase of cell viability in A2780 and SKOV3.
LY294002 incubation attenuated USP18 over-expression-induced increase of cell migration in A2780 and SKOV3. Relative wound width in A2780 and SKOV3 transfected with pcDNA-USP18 in the absence or presence of LY294002. LY294002 incubation attenuated USP18 over-expression-induced decrease of cell apoptosis in A2780 and SKOV3. Relative apoptosis ratio in A2780 and SKOV3 transfected with pcDNA-USP18 in the absence or presence of LY294002. ** *vs*. NC, $p<0.01$. # *vs*. USP18, $p<0.05$.

USP18 contributed to the invasion of ovarian cancer cells

Knockdown of USP18 repressed the migration of A2780 and SKOV3 cells (Fig. 3A). Furthermore, A2780 and SKOV3 cell invasion (Fig. 3B) was also delayed by siUSP18 transfection, demonstrating the anti-invasive effect of USP18 silencing on ovarian cancer.

USP18 contributed to the activation of AKT/mTOR in ovarian cancer

Cyclin D1 and CDK4 protein expression levels were reduced in A2780 and SKOV3 with siUSP18 transfection (Fig. 4A), while p21 expression was enhanced (Fig. 4A). Furthermore, USP18 knockdown decreased Bcl-2 expression, while increasing Bax expression and cleaved caspase-3 in A2780 and SKOV3 (Fig. 4A). E-cadherin was upregulated, while N-cadherin and MMP2 were downregulated by silencing of USP18 (Fig. 4A). The elimination of USP18 in A2780 and SKOV3 (Fig. 4B) had no significant effect on the expression of AKT and mTOR protein. However, the expression of p-AKT and p-mTOR in A2780 and SKOV3 was reduced by silencing of USP18 (Fig. 4B), indicating that silencing of USP18 suppressed AKT/mTOR activation in ovarian cancer.

USP18 contributed to ovarian cancer cell proliferation and migration through AKT/mTOR

To investigate the effects of the USP18/AKT/mTOR axis on ovarian cancer progression, A2780 and SKOV3 were transfected with pcDNA-USP18 in treatment with LY294002, AKT inhibitor. USP18 overexpression improved the cell viability of A2780 and SKOV3 (Fig. 5A) and promoted cell migration (Fig. 5B and 5C). However, incubation with LY294002 reduced cell viability (Fig. 5A) and repressed migration (Fig. 5B and 5C). Incubation with LY294002 incubation also attenuated the decrease induced by USP18 overexpression in cell apoptosis in A2780 and SKOV3 (Fig. 5D and 5E), revealing that USP18 contributed to ovarian cancer cell proliferation and migration through activation of AKT/mTOR.

DISCUSSION

Post-translational modification, such as ubiquitination of target proteins by the ubiquitin-conjugating system, is implicated in the pathogenesis of tumorigenesis (Wang & Wang, 2021). USPs function as main deubiquitinase and are found to be involved in tumor cell cycle progression, apoptosis, metastasis, cancer cell stemming, DNA damage repair, and tumor-associated microenvironment (Young *et al.,* 2019). Therefore, USPs are considered therapeutic targets for cancer prevention (Young *et al.,* 2019). In ovarian cancer, USPs have been shown to be oncogenes through promotion of cell proliferation, metastasis and epithelial-mesenchymal transition (Du *et al.,* 2019; Yan *et al.,* 2019; Nakae *et al.,* 2021). Since USP18 was reported to be involved in the physiology and pathogenesis of cancers (Kang & Jeon 2020), the biological role of USP18 in ovarian cancer was investigated.

USP18 was verified to be up-regulated in tissues of ovarian cancer in this study. Previous studies have shown that the silence of USP18 promoted hepatocellular cancer cell apoptosis and suppressed cell proliferation (Cai *et al.,* 2017). The functional assays in the current study demonstrated that USP18 overexpression repressed ovarian cancer cell apoptosis, while it promoted cell proliferation and migration. However, USP18 knockdown promoted cell apoptosis, while retarding cell proliferation, migration, and invasion. These results confirmed the oncogenic role of USP18 in ovarian cancer.

Increasing evidence has shown that PI3K/Akt/mTOR signaling is crucial for cell survival and growth, thus participating in the pathological condition of cancers (Porta *et al.,* 2014). Inhibition of PI3K/Akt/mTOR signaling is widely investigated in cancer immunotherapies (O'Donnell *et al.,* 2018). PI3K/Akt/mTOR signaling was also reported to be altered in ovarian cancer (Cheaib *et al.,* 2015), and PI3K/Akt/mTOR signaling was considered a therapeutic target for ovarian cancer (Li *et al.,* 2014). USP18 has been shown to activate the AKT pathway to promote cervical (Diao *et al.,* 2020) and breast (Tan *et al.,* 2018) cancer cell proliferation and migration. The results of this study revealed that the protein expression of p-AKT and p-mTOR was reduced in ovarian cancer cells by silencing USP18. Furthermore, incubation with LY294002, an AKT inhibitor, attenuated the increase in cell viability induced by USP18 overexpression and the decrease in cell apoptosis in ovarian cancer. Deficiency of USP18 promoted degradation of interferon gene stimulator through enhancing K48-linked ubiquitination (Zhang *et al.,* 2016), and the interferon gene pathway stimulator was implicated in the pathogenesis of ovarian cancer (Huvila *et al.,* 2021). USP35 has been reported to regulate the interferon gene pathway stimulator and participate in ovarian cancer progression and drug resistance (Zhang *et al.,* 2021). USP18 might also regulate the ubiquitination of the interferon gene stimulator to be involved in ovarian cancer progression.

In summary, USP18, which is upregulated in tissues of ovarian cancer, functioned as an oncogene in ovarian cancer through activation of AKT/mTOR pathway. This study provided evidence that USP18 could be used as a potential target for the treatment of ovarian cancer. However, the *in vivo* suppressive role of USP18 silencing in the growth of ovarian cancer should be investigated in further research.

Declarations

Acknowledgements. Not applicable.

Competing Interests. The authors state that there are no conflicts of interest to disclose.

Availability of data and materials. All the data generated or analyzed during this study are included in this published article.

Ethics approval. Ethical approval was obtained from the Ethics Committee of the 901ST Hospital of the Joint Logistics Support Force of PLA

Statement of Informed Consent. Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

Authors' Contributions. Meiqin Liu and designed the study, supervised the data collection, Xingjun Xu and Lijie Zhang analyzed the data, interpreted the data, Bin Xu, Donghui Lu, and Shile Gao prepare the manuscript for publication, and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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