Abstract. Ubiquitin-specific peptidase (USP)18 belongs to the USP family, and is involved in cleaving and removing ubiquitin or ubiquitin-like molecules from their target molecules. Recently, increasing evidence has suggested that USP18 is constitutively expressed in different types of human tumors, and ectopic expression or downregulation of USP18 expression may contribute to tumorigenesis. However, the role of USP18 in uterine cervical cancer (UCC) remains unclear. Thus, the present study aimed to investigate USP18 expression in a human tissue microarray constructed using UCC and non-cancer cervical tissues, and to determine the potential role and molecular mechanism by which USP18 is implicated in the tumor biology of human UCC HeLa cells. Microarray analysis demonstrated that USP18 protein expression was downregulated in tumor tissues compared with in normal tissues. In addition, in vitro analysis revealed that USP18-knockdown markedly promoted the proliferation, colony formation, migration and aggressiveness of HeLa cells. Mechanistic analysis demonstrated that USP18-knockdown increased the levels of Bcl-2, STAT3 and phosphorylated-ERK in HeLa cells. Notably, USP18 silencing-induced malignant phenotypes were interrupted following exogenous administration of the ERK1/2 inhibitor PD98059. Overall, the results of the present study suggested that USP18 may be a potent inhibitor involved in UCC tumor-associated biological behaviors, which are associated with the ERK signaling pathway.

Introduction

Uterine cervical carcinoma (UCC) is one of the most common types of malignant cancer in women, representing the fourth most frequent malignancy worldwide (1). In 2018, ~570,000 new cases of UCC were identified, and up to 311,000 associated deaths were recorded worldwide (2). Epidemiological data have demonstrated that the incidence and mortality rates of UCC vary across different regions, with more cases in Sub-Saharan Africa and South-Eastern Asia, and less cases in North America, Australia, New Zealand and Western Asia (2). Although it has been reported that UCC incidence and mortality rates have decreased in several regions of the world over the last few decades (3), UCC remains a serious health issue in China, with an estimated 106,430 new cases and 47,739 mortalities in 2018 (3).

Ubiquitin-specific peptidase (USP)18, also known as ubiquitin-specific protease 43 (UBP43), is a member of the USP family and is involved in deubiquitinating activity, thereby resulting in stabilization of substrates (4). It is well known that USPs are the largest sub-family of deubiquitinase enzymes, and exert biological roles through their cysteine
endopeptidase activity (5). The USP family consists of >100 members, which predominantly differ in amino acid sequence and protein size, but are characterized by several highly homologous sequences around the essential domains important for their catalytic activity (6,7). USP18 was originally identified from acute myelogenous leukemia 1- RUNX1 partner transcriptional co-repressor 1 knock-in mice and was characterized by Liu et al (8). Previous studies have demonstrated that USP18 expression is present in multiple types of tissues, including liver, lung, spleen, thymus, bone marrow and adipose tissues (8,9), and is also expressed in different types of cells, such as macrophages, lymphocytes and hematopoietic cells (10,11). The function of USP18 has predominantly been associated with the regulation of cell proliferation, cell differentiation, stress, inflammatory reaction and immune response (9-11). Additionally, it has been suggested that USP18 serves a vital role in regulating T-cell activation and T helper 17 cell differentiation through its ability to remove the transforming growth factor β-activated kinase 1-TAK1-binding protein 1 complex (12).

Several studies have suggested that USP18 may be involved in tumor biology (10,13). USP18 is overexpressed in several types of human cancer including glioblastoma, hepatocellular carcinoma, bladder cancer and breast cancer, and its high expression is associated with a poor prognosis in patients with glioblastoma and bladder cancer (14-17). Furthermore, some studies have demonstrated that USP18 is important for the malignant behaviors of tumor cells, including cellular proliferation, migration, apoptosis and epithelial-to-mesenchymal transition (EMT) (14,17-19). Conversely, it has been demonstrated that USP18 may suppress tumorigenesis due to its involvement in the antitumor immune response (20).

Although the biological functions and clinical presentation of USP18 have been well characterized in several types of human cancer including glioblastoma, breast cancer and melanoma (14,17,19,20), its underlying molecular mechanisms in UCC remain unclear. Thus, the present study aimed to investigate USP18 expression in a cervix tissue microarray, and determine its potential role and molecular mechanism in UCC malignant phenotypes.

Materials and methods

Cell line, cell culture and cell transfection. Human UCC HeLa cells were purchased from the American Type Culture Collection and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (all Gibco; Thermo Fisher Scientific, Inc) at 37°C with 5% CO2 in a humidified incubator.

To generate USP18-deficient HeLa cells, an RNA interference silencing strategy was used to design and construct a lentivirus vector carrying small interfering (si)RNA sequences targeting USP18 by GeneCopoeia, Inc. The lentiviruses vector carrying small interfering (si)RNA sequences (magnification, x200). Western blot analysis was subsequently performed to detect USP18 protein expression.

Immunohistochemistry (IHC). A commercial cervix tissue microarray containing 15 squamous cell carcinoma tissues, five adenocarcinoma carcinoma tissues, 20 adenocarcinoma tissues, 20 cervical intraepithelial lesions tissues, 14 cervicitis tissues, four unpaired para-cancerous tissues and two cervical canal tissues (cat. no. F801301; Bioaitech Co., Ltd.) was used to detect USP18 expression. The characteristics of the tissues are listed in Table I.

The microarray was processed routinely via deparaffinization, rehydration, endogenous peroxidase quenching and antigen retrieval, and subsequently blocked with 20% normal goat serum (Wuhan Boster Biological Technology, Ltd.) for 30 min at 37°C to remove the background from staining. The microarray was incubated with a mouse antibody against human USP18 (1:50; cat. no. sc-374064; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Following the primary antibody incubation, the microarray was incubated for 35 min at 37°C using the SABC kit (cat. no. SA1021; Wuhan Boster Biological Technology, Ltd.). DAB and hematoxylin were used for visualization and nuclear counterstaining at room temperature for 30 and 5 sec, respectively. The USP18 protein expression profiles were estimated based on the staining intensity and the percentage of positive cells using a fluorescence microscope (magnification, x200). The staining intensity was ranked as follows: 1, weak; 2, moderate; 3, intensive; and 4, super intensive. The percentage of positive cells was scored as follows: 1, <25%; 2, 26-50%; 3, 51-75%; and 4, >75%. The total score for each sample was the sum of the two parameters, as previously described (21).

Cell proliferation assay. The Cell Counting Kit-8 (CCK-8; Wuhan Boster Biological Technology, Ltd.) assay was performed to assess the proliferation of HeLa cells following USP18-knockdown. Briefly, mock- and USP18-siRNA-transfected HeLa cells were seeded into 96-well plates at a density of 1.5x10³ cells/well in a final volume of 100 µl RPMI-1640 medium supplemented with or without 80 µM of the ERK1/2 blocker PD98059 (Sigma-Aldrich; Merck KGaA). Following incubation at 37°C for 24, 48 or 72 h, 10 µl CCK-8 reagent was added to each well and incubated at 37°C for 1 h. Cell proliferation was subsequently analyzed at a wavelength of 450 nm, using a microplate reader (Omega Bio-Tek, Inc.). The inhibitory role of PD98059 on the proliferation of HeLa cells was estimated as follows: Inhibition rate (%)=(treatment with 0 µM PD98059-treatment with 80 µM PD98059)/treatment with 0 µM PD98059 x100.

Clonogenicibility assay. Mock-and USP18-siRNA-transfected HeLa cells were seeded into 24-well plates at a density of 5x10² cells/well in 500 µl RPMI-1640 medium supplemented with 10% fetal bovine serum. Cells were cultured at 37°C and the medium was replaced every 2-3 days. Following incubation for 10 days, the medium was removed and cells were stained with 0.01% crystal violet for 15 min at room temperature.
A minimum of 3 mm diameter or more was considered as a colony. Images of cell colonies were captured using an imaging system (Tanon Science & Technology Co., Ltd.) and counted under a fluorescence microscope (magnification, x50).

Cell migration assay. The migratory ability of HeLa cells was assessed using 24-well Transwell chambers with polycarbonate filter of 8-µm pore size. A total of 2x10⁴ mock- and USP18-siRNA-transfected HeLa cells were plated in the upper chambers of Transwell plates in 100 µl serum-free RPMI-1640 medium. A total of 600 µl RPMI-1640 medium containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) supplemented with or without 80 µM of the ERK1/2 blocker PD98059 was plated in the lower chambers. Following incubation for 24 h at 37°C, cells in the upper chambers were removed using a cotton swab, while the migratory cells were fixed and stained using a solution of crystal violet in ethanol for 15 min at room temperature. Stained cells were counted using a fluorescence microscope (magnification, x200). The inhibitory role of PD98059 on the migration of HeLa cells was estimated as follows: Inhibition rate (%)=(treatment with 0 µM PD98059-treatment with 80 µM PD98059)/treatment with 0 µM PD98059 x100.

Wound healing assay. Mock- and USP18-siRNA-transfected HeLa cells were seeded into 6-well plates at a density of 2x10⁶ cells/well in 1 ml RPMI-1640 medium supplemented with 10% fetal bovine serum. After incubation until confluent, the culture medium was replaced with serum-free medium, and a cell-free wound zone was created by scraping the monolayer with a sterile pipette tip. The images of the wounds were captured and the numbers of migrating cells were counted at 0 and 24 h after wounding using a fluorescence microscope by eye (magnification, x100).

Western blotting. Untransfected parental, mock- and USP18-siRNA-transfected HeLa cells were harvested and lysed using a commercial RIPA buffer kit (cat. no. P0013C; Beyotime Institute of Biotechnology) supplemented with phenylmethylsulfonyl fluoride. Protein concentrations were determined using the bicinchoninic acid protein assay kit (cat. no. P0010; Beyotime Institute of Biotechnology) and 40 µg protein/lane was subjected to 12% SDS-PAGE. The separated proteins were subsequently transferred onto polyvinylidene difluoride membranes and blocked with 5% skimmed milk in PBS for 60 min at 37°C. The membranes were incubated with primary antibodies against USP18 (1:1,000; cat. no. DF7968; Affinity Biosciences), Bcl-2 (1:1,000; cat. no. AF6139; Affinity Biosciences), STAT3 (1:1,000; cat. no. CY5165; Shanghai Abways Biotechnology Co., Ltd.), ERK (1:1,000; cat. no. CY5487; Shanghai Abways Biotechnology Co., Ltd.), phosphorylated (p)-ERK (1:1,000; cat. no. CY5277; Shanghai Abways Biotechnology Co., Ltd.) and β-actin (1:5,000; cat. no. AB0011; Shanghai Abways Biotechnology Co., Ltd.) overnight at 4°C. Following the primary antibody incubation, membranes were incubated with HRP-coupled goat anti-rabbit and anti-mouse secondary antibodies (1:10,000; cat. nos. BA1054 and BA1050, respectively; Wuhan Boster Biological Technology, Ltd.). Protein blots were visualized using ECL reagent (Thermo Fisher Scientific, Inc.). The protein expression levels of USP18, Bcl-2 and STAT3 were normalized to β-actin, whereas p-ERK expression was normalized to total ERK expression using ImageJ software (version 1.45 s; National Institutes of Health).

Statistical analysis. Statistical analysis was performed using SPSS 22.0 software (IBM Corp.). All experiments were performed in triplicate and data are presented as the mean ± SD. Comparisons between two groups were analyzed using unpaired Student’s t-test (if the variance was homogeneous) or Cochran and Cox separate variance estimation t-test (if the variance was not homogeneous). Comparisons among multiple groups were analyzed using one-way ANOVA followed by Student-Newman-Keuls post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

USP18 expression is downregulated in UCC tissues. To evaluate the clinical significance of USP18, USP18 expression was determined via IHC analysis in a commercial cervix tissue microarray (Fig. I). The final score demonstrated that USP18 was expressed at significantly lower levels in UCC tissues compared with in normal tissues (Table II). However, there was no significant difference in USP18 expression between different stages, grades, age and lymph node metastasis (Table II).

USP18 is expressed in HeLa cells. To assess the potential involvement of USP18 in the malignant phenotypes of HeLa cells, USP18 protein expression was detected. Western blot analysis confirmed that USP18 protein was expressed in untransfected parental HeLa cells (Fig. 2A). Following transfection with lentiviruses, fluorescent imaging demonstrated that siRNA- and mock-transfected cells expressed high-intensity screening fluorescence, suggesting that the cells were effectively infected by lentiviruses (Fig. 2B). Subsequent western blotting showed that USP18 protein expression was significantly decreased in siRNA-transfected HeLa cells.
cells compared with untransfected parental and mock-transfected HeLa cells (Fig. 2A and C), which indicated that USP18-deficient HeLa cells were successfully established.

**Downregulation of USP18 promotes the proliferation, colony formation, migration and aggressiveness of HeLa cells.** The CCK-8 assay was performed to assess the effect of USP18
on the malignant behavior of HeLa cells. Following incubation for 24, 48 and 72 h, the results demonstrated that the proliferative ability of siRNA-transfected HeLa cells was significantly increased compared with mock-transfected HeLa cells (Fig. 3A). The results of the colony formation, migration and wound healing assays demonstrated that silencing of USP18 in HeLa cells significantly increased their colony forming (Fig. 3B and C), migratory (Fig. 3D and E) and aggressive abilities (Fig. 3F and G).

**USP18-knockdown alters ERK-signaling pathway-specific protein expression.** The potential molecular mechanism by which USP18 regulates the malignant process of HeLa cells was assessed via western blotting. The results demonstrated that the protein expression levels of Bcl-2, STAT3 (Fig. 4A and B) and p-ERK (Fig. 4C and D) were significantly increased following USP18-knockdown in HeLa cells, suggesting the involvement of ERK signaling in the malignant phenotypes of UCC cells.

**USP18 silencing-induced malignant behaviors of HeLa cells depend on ERK signaling.** To further clarify whether the roles of USP18 silencing on HeLa cells were mediated by ERK signaling, the ERK1/2 blocker PD98059 was used. The results demonstrated that there were no significant differences in cell proliferation at 24 or 48 h (Fig. 5A) and cell migration at 24 h (Fig. 5C and D) between siRNA- and mock-transfected cells following treatment with PD98059. Although the proliferative rate of siRNA-transfected cells was significantly higher

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Figure 3. Roles of USP18-deficiency in HeLa cell malignant behaviors *in vitro*. (A) Proliferation analysis from mock- and USP18-siRNA-transfected HeLa cells using Cell Counting Kit-8 reagent. (B) Representative images of clonogenic analysis from mock- and USP18-siRNA-transfected HeLa cells. (C) Statistical analysis of colony formation ability. (D) Representative images of Transwell analysis from mock- and USP18-siRNA-transfected HeLa cells. Magnification, x200. (E) Statistical analysis of cell migratory capacity. (F) Representative images of wound healing assay from mock- and USP18-siRNA-transfected HeLa cells. Magnification, x100. (G) Statistical analysis of cell aggressiveness capacity. *P<0.01 vs. mock. USP18, ubiquitin-specific peptidase 18; siRNA, small interfering RNA.
compared with that of mock-transfected cells after 72 h of exposure to PD98059 (P<0.05; Fig. 5A), the difference between the two groups was slightly lower compared with that between the two groups treated without PD98059 (Fig. 3A). Following
treatment with PD98059, the inhibition rates exerted by PD98059 on the proliferation (Fig. 5B) and migration (Fig. 5E) of siRNA-transfected HeLa cells were significantly higher compared with those in mock-transfected HeLa cells. Overall, the present results suggested that USP18 silencing-induced malignant responses in HeLa cells depend on the activation of the ERK signaling pathway.

**Discussion**

USP18 is a major isopeptidase, which was initially identified based on its role to efficiently deconjugate interferon-stimulated gene 15 (ISG15), a two-domain ubiquitin-like protein, from ISGylation (9). In addition to ISG15, USP18 is highly induced by type I and III interferons, and it has been proposed that USP18 is a vital blocker of the type I interferons signaling pathway (22). Furthermore, a study demonstrated that type III interferons may induce USP18 production (23). In the presence of USP18, type III interferons acquire higher properties to weaken type I interferons-mediated actions by repressing JAK-STAT signaling (23).

Increasing evidence has suggested that USP18 is implicated in a variety of physiological and pathological processes in different tissues and cells, including cell development, viral infection, viral replication and antibacterial response (10,24,25). However, a vast expansion in the understanding of USP18 expression and its association with tumor biology has occurred. It has been reported that USP18 is frequently overexpressed in different types of cancer, including breast cancer, bladder cancer and hepatocellular carcinoma, and its overexpression is positively associated with several pathological tumor characteristics (15-17). For example, a recent study indicated that USP18 methylation is predominantly downregulated, whereas its expression is upregulated in breast cancer, which is positively associated with increasing TNM stage, worse disease-free survival rate and HER2+ patients, but negatively associated with apoptosis (17). Accordingly, it has been suggested that USP18 may be used as a predictive marker for poor prognosis in muscle invasive bladder cancer, since high USP18 expression is a significant risk factor for cancer-specific death, and decreased USP18 expression is markedly associated with longer cancer-specific survival (16). USP18 has also been the focus of investigations evaluating its functions in tumorigenesis. USP18 silencing in a mouse model for breast cancer exhibited a significant decrease in tumor growth, and USP18-deficiency in breast cancer MCF-7 cells in vitro triggered an increase in the induction of apoptosis (18,19). In addition, downregulation of USP18 expression in glioblastoma cells may protect against tumor cell invasion and migration by repressing EMT (14), an essential event for cancer metastasis, by which tumor cells obtain increased motility and invasiveness. Gain-of-function assays in vitro have demonstrated that overexpression of USP18 has an important role in regulating tumor progression due to its contribution in enhancing breast cancer tumor cell proliferation, colony formation and cell cycle progression (17). However, studies have also revealed that USP18 may exert an opposing role in the control of cancer development. For example, ectopic USP18 expression in B16 melanoma cancer cells may suppress tumorigenesis, restraining cancer cell-mediated inhibition of T-cell proliferation and activation, thus facilitating cancer cells to specific immune responses (20). In human leiomyosarcoma, downregulation of USP18 is associated with a poor clinical outcome, and USP18-deficient mice exhibited an enhanced ability to develop these sarcomas (26).

Although a recent study demonstrated that USP18 is a critical regulator for the tumorigenicity of cervical cancer CaSki and SiHa cells (27), the results of the present study demonstrated that USP18 expression was downregulated in UCC compared with in normal tissues. USP18 expression was knocked down in HeLa cells, and the malignant behaviors in cells, including proliferation, colony formation and migration, were enhanced. Mechanistically, p-ERK expression was significantly upregulated following USP18-knockdown in HeLa cells. Previous studies have reported that several signaling pathways, such as the PTEN/AKT (28), AKT/Skp2 (16) and JAK/STAT (29) signaling pathways, are implicated in USP18 associated-biological roles. To the best of our knowledge, the present study was the first to illustrate an involvement of ERK signaling in the function of USP18 in tumorigenesis. ERKs are a family of protein-serine/threonine kinases, which serve vital roles in the control of diverse cell functions, such as cell differentiation, proliferation and survival, by phosphorylating several substrates including transcription factors, protein kinases and phosphatases (30). Increasing evidence has suggested that amplification or activation of ERK signaling frequently occurs in several malignant tumors, such as gastric adenocarcinoma and lung cancer (31,32), which results in increased cell proliferation, promotion of cell cycle progression and repressed apoptosis of tumor cells (33). Given that the ERK signaling pathway participates in several aspects of tumorigenesis by regulating the expression of its downstream signaling molecules, such as NF-κB (34), Bcl-2 (35) and STAT3 (36), the present study assessed whether the expression levels of these genes were affected following USP18-knockdown. The results demonstrated that Bcl-2 and STAT3 expression was upregulated, whereas NF-κB expression remained unchanged (data not shown) following USP18-knockdown in HeLa cells. Bcl-2 is a novel gene encoding a unique apoptosis inhibitor that efficiently suppresses apoptosis induced by the p53 tumor suppressor protein (37). STAT3 is an important proto-oncogene essential for modulating the transition from the G1 to S phase of the cell cycle (38), and most cancer cases arise due to proliferating cells losing control of cell cycle regulation, in which loss of the G1/S-phase transition checkpoint is a major cause of cancer (39).

In conclusion, the results of the present study demonstrated that USP18 expression was downregulated in UCC tissues, and USP18-knockdown facilitated tumor cell proliferation and migration by affecting the expression levels of genes associated with the ERK signaling pathway. Overall, the current results provide a novel mechanism for USP18-deficiency, which may serve a crucial role in UCC progression in an ERK-dependent manner.

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

Authors' contributions
AP and YL participated in cell experiments and drafted the manuscript. JG and PZ participated in statistical analyses. YH, LS and JRW performed immunohistochemistry assay. CZ, YC, and QR participated in cell transfection and cell experiments. SL, SF and TZ performed western blot analyses. AP, YL and JTW confirm the authenticity of all the raw data. WW and JTW designed the study and performed the revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The medical ethics committee at Jiamusi University (Jiamusi, China) approved all procedures performed in the present study involving animals and human participants, which were in accordance with ethical standards, and all patients provided written consent prior to participation in this study.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that there are no competing interests.

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