

Ubiquitin-specific peptidase 17 promotes cisplatin resistance via PI3K/AKT activation in non-small cell lung cancer

SHENGCHAO ZHANG, ZHENGLANG XU, JUN YUAN and HAO CHEN

Department of Thoracic Surgery, Qingpu Branch Zhongshan Hospital, Fudan University, Shanghai 201700, P.R. China

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Abstract. The suppression of ubiquitin-specific peptidase 17 (USP17) has previously been found to result in reduced tumorigenesis and invasion of non-small cell lung cancer (NSCLC) cells. However, the functions and underlying mechanisms of USP17 in NSCLC progression remain unclear. In the present study, cisplatin treatment was found to upregulate USP17 expression in a dose-dependent manner. Furthermore, USP17-overexpressing (USP17-OE) NSCLC A549 and H1299 cells were generated for mechanistic studies. The results from the Cell Counting Kit-8 assay revealed increased cell proliferation in USP17-OE cells compared with that of control cells. Moreover, the viability of USP17-OE cells was significantly higher than that of the control cells, when treated with cisplatin. The results of the biochemical studies demonstrated enhanced PI3K and AKT phosphorylation in USP17-OE NSCLC cells, whereas USP17-knockdown decreased these levels of phosphorylation. By contrast, an AKT inhibitor abolished the USP17-mediated enhancement of proliferation. Moreover, suppression of USP17 or the combination of the AKT inhibitor and cisplatin significantly reduced cell viability. Overall, the results of the present study suggest that PI3K/AKT activation is the underlying mechanism of USP17-mediated cisplatin resistance in NSCLC.

Introduction

Lung cancer is the one of the leading causes of cancer-related death worldwide (1), and non-small cell lung cancer (NSCLC) accounts for almost 80% of these deaths (2). Cisplatin-based chemotherapy is the main treatment used for patients with major NSCLC (2). However, the long-term prognosis for patients with NSCLC still remains poor, with the 5-year survival rate being only ~11% (3). Patients with NSCLC often

show an initial positive response to cisplatin treatment, but in many cases this response is not sustained and cancer cells become resistant to the treatment, this has become a major clinical challenge in the treatment of NSCLC (4). Therefore, the identification of novel therapeutic targets in NSCLC treatment that can complement current therapy is urgently required.

The ubiquitin-proteasome system regulates cellular protein levels with specificity and precision to optimize cellular functions (5,6). Moreover, certain studies have demonstrated that ubiquitination can modify protein functions, including the regulation of subcellular localization and protein-protein interactions (5-7). The association between ubiquitin and tumor biology has long been recognized, and the suppression of the proteasome has proven to be effective in the treatment of various types of cancer, such as myeloma and gastrointestinal cancer (6). Deubiquitinating enzymes (DUBs) can reverse protein ubiquitination by digesting ubiquitin chains (8).

Ubiquitin-specific protease 17 (USP17), a DUB, is involved in the regulation of inflammation (9) and cell motility (10), the development of Th17 cells (11) and oncogenesis (12-17). It has been found that USP17 is highly expressed in several types of tumor, including colon, esophageal and cervical tumors (14). In addition, USP17 regulates the Ras pathway by altering the intracellular localization of Ras and other small GTPases, which are crucial regulators of cellular proliferation and migration (10). USP17 is required for the trafficking and oncogenic signaling of mutant epidermal growth factor receptor (EGFR) in NSCLC cells (15). These studies verified the role of USP17 in promoting tumor growth and metastasis. McFarlane *et al* (16) reported upregulation of USP17 in patients with NSCLC. Moreover, patients with USP17-positive tumors had significantly shorter recurrence-free survival times compared with those with USP17-negative tumors, and USP17 expression was associated with the recurrence of disease at distant sites. In addition, an *in vivo* study, in which human NSCLC cells were inoculated into nude mice, found that the suppression of USP17 in NSCLC cells inhibited tumor growth and invasion (17). However, the biological function of USP17 that directly regulates NSCLC progression has not been studied fully.

The re-emergence of cancer cells is often due to the activation of survival signals, including increased activation of the PI3K/AKT pathway (18-20), which has been associated with NSCLC progression. The PI3K/AKT pathway is an important pathway downstream of EGFR. Deregulation of this pathway,

Correspondence to: Dr Hao Chen, Department of Thoracic Surgery, Qingpu Branch Zhongshan Hospital, Fudan University, 1158 East Gongyuan Road, Qingpu, Shanghai 201700, P.R. China
E-mail: h.chen@fudan.edu.cn

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due to gene amplifications, activating oncogene mutations or the loss of PTEN, has been observed in several types of human cancer, such as colorectal, gastric, lung, ovarian and thyroid cancer (20-23).

In the present study, the aim was to explore the functions and underlying molecular mechanisms of USP17 in NSCLC cells. Moreover, the effects of inhibiting of USP17 downstream PI3K/AKT pathway in cisplatin sensitivity of NSCLC cells were also investigated.

Materials and methods

Cell lines. The human NSCLC A549 and H1299 cell lines were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. A549 cells were maintained in Dulbecco's modified Eagle's medium, and H1299 cells were maintained in RPMI-1640 medium. All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO₂ at 37°C. All cells were confirmed to be free from mycoplasma contamination.

Plasmids and reagents. USP17 short hairpin (sh)RNA and USP17 overexpression lentiviruses were purchased from Hanyin Biotech Co. Polybrene (cat. no. 107689; Sigma-Aldrich; Merck KGaA) was used as an infection reagent. The target sequence for USP17 shRNA-knockdown (KD) was 5'-CTCTTGAGAATGTGCCGAT-3' (the shRNA was packaged into the lentivirus). The negative control (NC) comprised an empty vector without target sequences. To generate stable cell lines, supernatant containing lentivirus (1x10⁶ TU) was added to A549 and H1299 cells (1x10⁵/well), which were subsequently screened with 1 µg/ml puromycin for 2 weeks. Experiments were performed 72 h after infection.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (100 ng) was used for cDNA synthesis using the Stratagene AffinityScript QPCR cDNA Synthesis kit (Agilent Technologies, Inc.). The temperature protocol for the RT step was 5 min at 65°C, 60 min at 42°C and 15 min at 70°C. The cDNA samples were diluted 10-fold with nuclease-free H₂O, of which 2 µl was combined with Brilliant III Ultra-Fast SYBR® Green qPCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). Human RPL13A was used as an internal reference control. The primer sequences were as follows: Human USP17 forward, 5'-GAGATTCTC CGATGTCACAGGC-3' and reverse, 5'-TCCGTCGTGACA ACTCCACCCA-3'; human RPL13A forward, 5'-CTCAAG GTGTTTGACGGCATCC-3' and reverse, 5'-TACTTCCAG CCAACCTCGTGAG-3'. The relative expression of target genes was determined using the 2^{-ΔΔC_q} method (24). The qPCR cycling conditions comprised an initial denaturation step of 3 min at 95°C, followed by 45 cycles at 95°C (10 sec) and 58°C (45 sec); data were acquired at the end of the annealing/extension phase. Melt curve analysis was performed at the end of each run between 58-95°C and the data were analyzed using Microsoft Excel 2013 (Microsoft Corporation).

Cell Counting Kit-8 assay (CCK-8). Cells (5x10³/well) were treated with cisplatin (1 µM) or MK2206 (1 µM; both purchased from Selleck Chemicals) for 0, 24, 48, 72, 96 and 120 h. The CCK-8 assay was conducted according to the kit instructions (cat. no. CK04; Dojindo Molecular Technologies, Inc.). Cells with or without USP17 overexpression (OE) and cells treated with MK2206 were examined. Briefly, cells in logarithmic growth phase were trypsin-digested and resuspended in RPMI-1640 medium. Cells were plated at equal densities (2,000 cells/100 µl per well) in 96-well plates and incubated at 37°C and 5% CO₂ for continuous detection over a 5-day period. At the beginning of the second day, cell growth was terminated by the addition of 10 µl of CCK-8 solution (5 mg/ml) to the culture medium. After 2 h, the OD490 values were determined using a microplate reader (BioTek Instruments, Inc.).

Transwell assay. USP17-OE cells and those (2x10⁴/well) treated with MK2206 (1 µM) or LY294002 (5 µM; both purchased from Selleck Chemicals) for 48 h were examined. Cells (2x10⁴) were detached and resuspended in serum-free medium and seeded in the upper chamber of Matrigel-coated Transwell (precoating with Matrigel at 4°C for 60 min) inserts with a pore size of 8 µm. Culture medium containing 10% FBS as a chemoattractant was added to the lower chamber. After 24 h of incubation, cells on the upper surface of the insert were gently removed with a cotton swab. Invasive cells (on the lower surface of the insert) were fixed with 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) at room temperature for 15 min, stained with crystal violet (1%) at room temperature for 60 min, and counted under a microscope (Olympus, CKX31); five random microscopic fields were examined for each insert using magnification, x200.

Western blot analysis. Total protein was extracted from cells using RIPA buffer (Beyotime Institute of Biotechnology). Protein was quantified using a BCA assay. Protein lysates (50 µg/lane) were separated via 8-10% SDS-PAGE and transferred onto nitrocellulose membranes. After blocking with 5% fat-free milk at room temperature for 30 min, the membranes were incubated with primary antibodies (1:500) at 4°C overnight. The primary antibodies used were: Goat anti-human USP17 (cat. no. AP5491b; Abgent, Inc.), mouse anti-actin (cat. no. 3700P), anti-human AKT (cat. no. 4685), anti-human phosphorylated (p)-AKT (cat. no. 4060), anti-human p-PI3K (cat. no. 4228) and anti-human PI3K (cat. no. 4257; all purchased from Cell Signaling Technology, Inc.). Following this, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:3,000; cat. nos. 705-035-147, 705-035-150 and 705-035-152; Jackson ImmunoResearch Laboratories, Inc.) at room temperature for 60 min. Immunoreactive proteins were visualized using an enhanced chemiluminescence reagent (EMD Millipore). Relative expression of p-PI3K and p-Akt was normalized to that of actin, and then relative to the normalized total PI3K or Akt values. Quantity One software version 4.6.9 (Bio-Rad Laboratories, Inc.) was used to quantify the relative band intensities.

Statistical analysis. Quantitative variables were compared using one-way ANOVA to compare differences between two

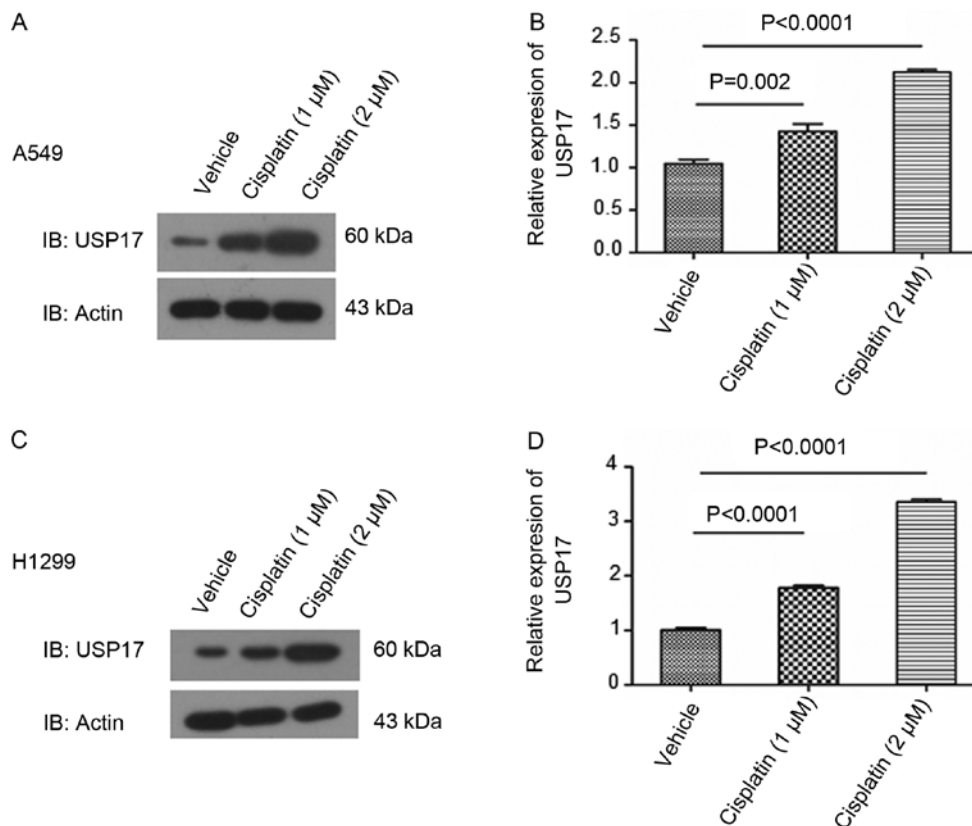


Figure 1. Increased expression of USP17 is observed in non-small cell lung cancer cells treated with cisplatin. Increasing concentrations of cisplatin increased the protein expression level of USP17 in a dose-dependent manner, as shown in (A) western blot images and the (B) corresponding histogram, in A549 cells; and (C) western blot images and the (D) corresponding histogram, in H1299 cells. USP, ubiquitin-specific peptidase.

or more groups, followed by Tukey's test for post-hoc analysis. Statistical analysis was performed using SPSS version 19.0 (IBM Corp.) and GraphPad Prism version 5.0 (GraphPad Software, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

USP17 expression is upregulated in NSCLC cells treated with cisplatin. Western blot analysis revealed increased levels of USP17 in NSCLC cells treated with increasing concentrations of cisplatin, in a dose-dependent manner (vehicle vs. 1 μ M cisplatin in A549, $P = 0.002$, in H1299, $P < 0.0001$; vehicle vs. 2 μ M cisplatin in A549, $P < 0.0001$, in H1299, $P < 0.0001$; Fig. 1).

In order to address the functions of USP17 in the response of NSCLC cells to cisplatin treatment, USP17-OE A549 and H1299 NSCLC cells were generated. Both RT-qPCR and western blot analysis demonstrated high expression levels of USP17 in these cell lines, compared with those in the corresponding NC cells (NC vs. OE, $P < 0.001$; Fig. 2).

Overexpression of USP17 increases proliferation and sustains viability in NSCLC cells. In order to investigate the biological functions of USP17 in the transformation of NSCLC cells, a CCK-8 assay was conducted to determine the proliferative ability of A549 (Fig. 3A) and H1299 (Fig. 3B) cells (NC vs. OE, $P < 0.001$). Moreover, the viability of USP17-OE cells was significantly higher than that of the control cells when treated with cisplatin (NC vs. OE, $P < 0.001$; Fig. 3C and D). These

results demonstrate that the overexpression of USP17 increases proliferation and viability in NSCLC cells, independent of cisplatin treatment.

Overexpression of USP17 enhances PI3K/AKT pathway activation in NSCLC cells. The importance of the PI3K/AKT pathway as a potent survival mechanism in carcinogenesis and tumor metastasis is well established (18-20). In the present study, the effect of USP17 on the activation of the PI3K-AKT signaling pathway in NSCLC cells was investigated. Western blot analysis demonstrated increased p-AKT and p-PI3K levels in USP17-OE NSCLC cells, compared with those of the control cells (NC vs. OE, $P < 0.0001$; Fig. 4). This finding suggests the activation of the PI3K/AKT pathway by USP17 as the underlying mechanism of the enhanced proliferation and viability in NSCLC cells.

Inhibition of AKT activation abolishes USP17-mediated enhancement of proliferation and viability in USP17-OE cells. MK2206, an allosteric AKT inhibitor, has been demonstrated to decrease cell proliferation in other types of cancer (25-27). Following treatment with 1 μ M MK2206, the viability and proliferation of A549-USP17-OE and H1299-USP17-OE cells were significantly decreased (DMSO vs. MK2206, $P < 0.001$; Fig. 5A and B), which further supports the functional association between USP17 and AKT activation. Moreover, the combination of the AKT inhibitor with cisplatin significantly reduced cell viability and proliferation (DMSO vs. MK2206, $P < 0.001$; Fig. 5C and D).

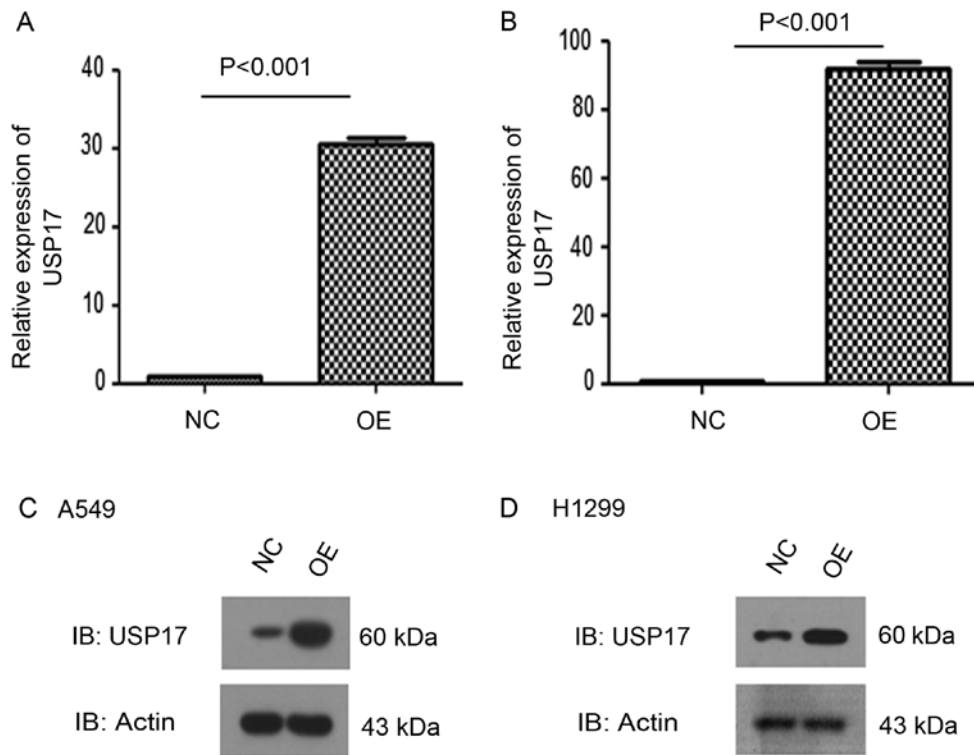


Figure 2. USP17-OE in non-small cell lung cancer cells. Reverse transcription-quantitative PCR analysis of USP17-OE efficiency in (A) A549 and (B) H1299 cells. Western blot analysis of USP17-OE efficiency in (C) A549 and (D) H1299 cells. USP, ubiquitin-specific peptidase; NC, negative control; OE, overexpression.

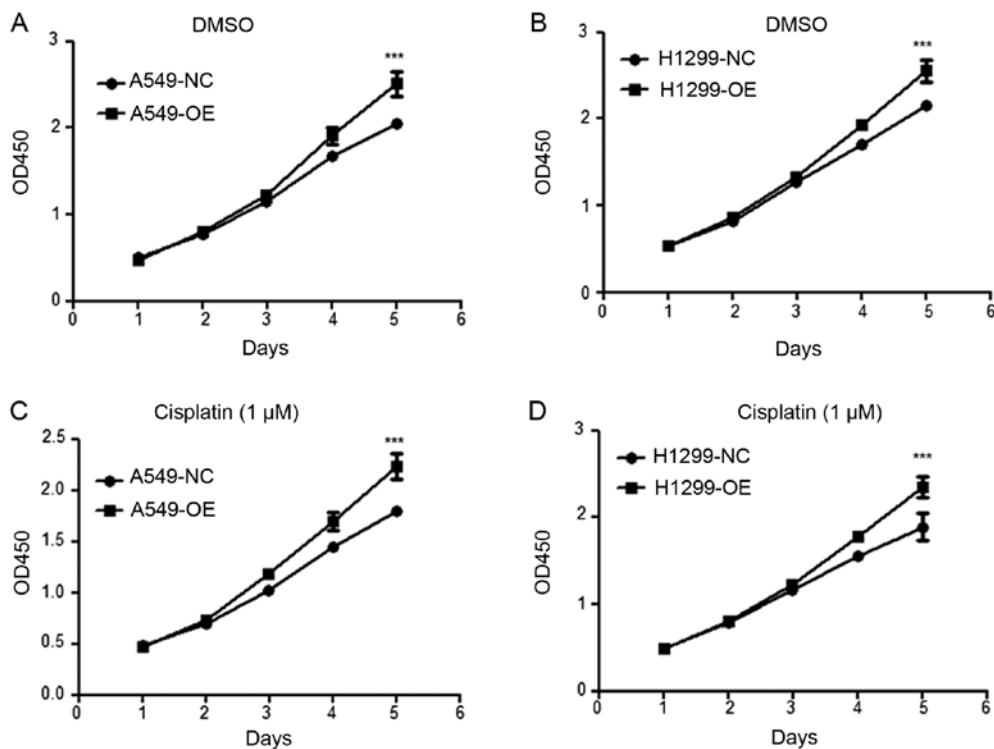


Figure 3. Overexpression of ubiquitin-specific peptidase 17 increases the proliferation and viability of non-small cell lung cancer cells. Cell Counting Kit-8 analysis comparing USP17-OE with the NC in (A) A549 and (B) H1299 cells treated with DMSO; and (C) A549 and (D) H1299 cells treated with cisplatin (1 μ M). $***P < 0.001$ vs. NC. OE, overexpression; NC, negative control; USP, ubiquitin-specific peptidase.

USP17 promotes proliferation and invasion through PI3K/AKT activation in NSCLC. Consistent with the findings from the USP17-OE cells, knock-down (KD) of USP17

decreased the protein expression levels of p-AKT and p-PI3K in NSCLC cells (NC vs. KD, $P < 0.0001$; Fig. 6A-F). In addition, the viability of USP17-KD cells was decreased following

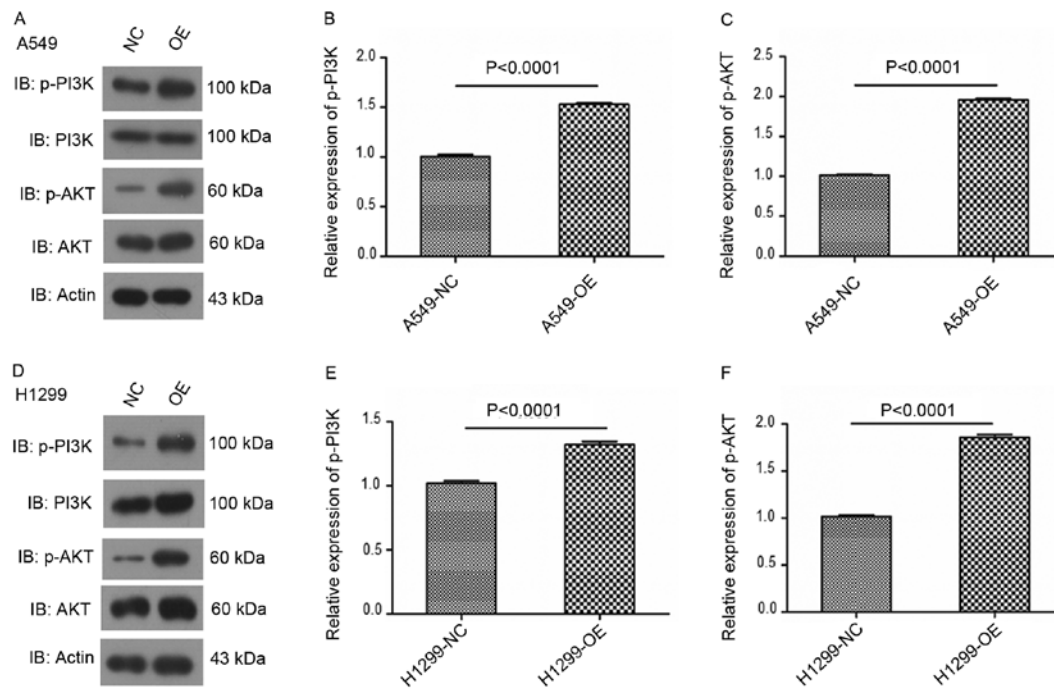


Figure 4. USP17-OE enhances activation of the PI3K/AKT pathway in non-small cell lung cancer cells. Western blot analysis of p-PI3K, PI3K, p-AKT and AKT levels in (A-C) A549 and (D-F) H1299 cells with or without USP17-OE. USP, ubiquitin-specific peptidase; OE, overexpression; NC, negative control; p, phosphorylated.

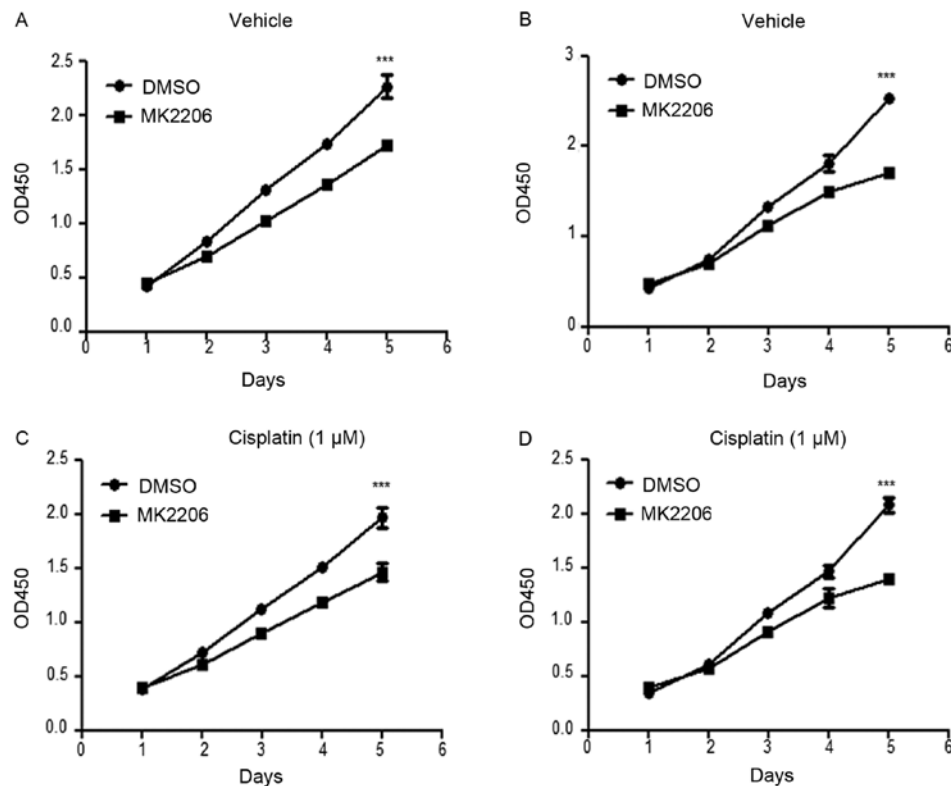


Figure 5. Inhibition of AKT activation abolishes USP17-mediated enhancement of proliferation and viability in USP17-OE cells. Cell Counting Kit-8 assay in USP17-OE A549 (A and C) and H1299 cells (B and D) treated with DMSO control or MK2206, alone or in combination with cisplatin. ***P<0.001 vs. MK2206. DMSO, DMSO negative treatment control; USP, ubiquitin-specific peptidase; OE, overexpression.

cisplatin treatment (NC vs. KD, P<0.001; Fig. 6G-H). Moreover, the invasive capacity of A549-USP17-OE and H1299-USP17-OE cells was significantly decreased in the

presence of MK2206 and the PI3K inhibitor LY294002 (DMSO vs. MK2206, P<0.001; DMSO vs. LY294002, P<0.001; Fig. 7).

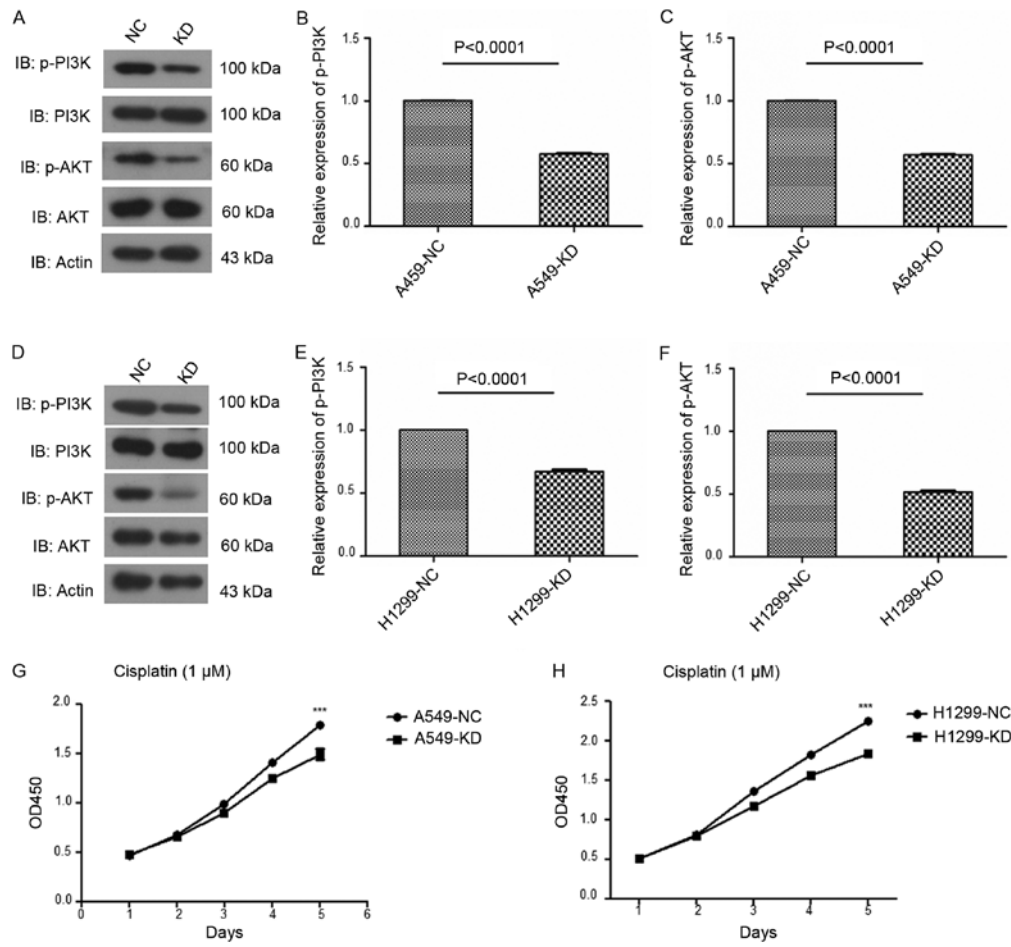


Figure 6. USP17 suppression decreases PI3K/AKT pathway activation and the viability of non-small cell lung cancer cells. Western blot analysis of p-PI3K, PI3K, p-AKT and AKT protein levels in (A-C) A549 and (D-F) H1299 cells with or without USP17 KD. (G) Cell Counting Kit-8 assay in USP17 KD and NC (G) A549 and (H) H1299 cells treated with cisplatin. *** $P < 0.001$ vs. KD. USP, ubiquitin-specific peptidase; KD, knockdown; NC, negative control; immunoblot; p-, phosphorylated.

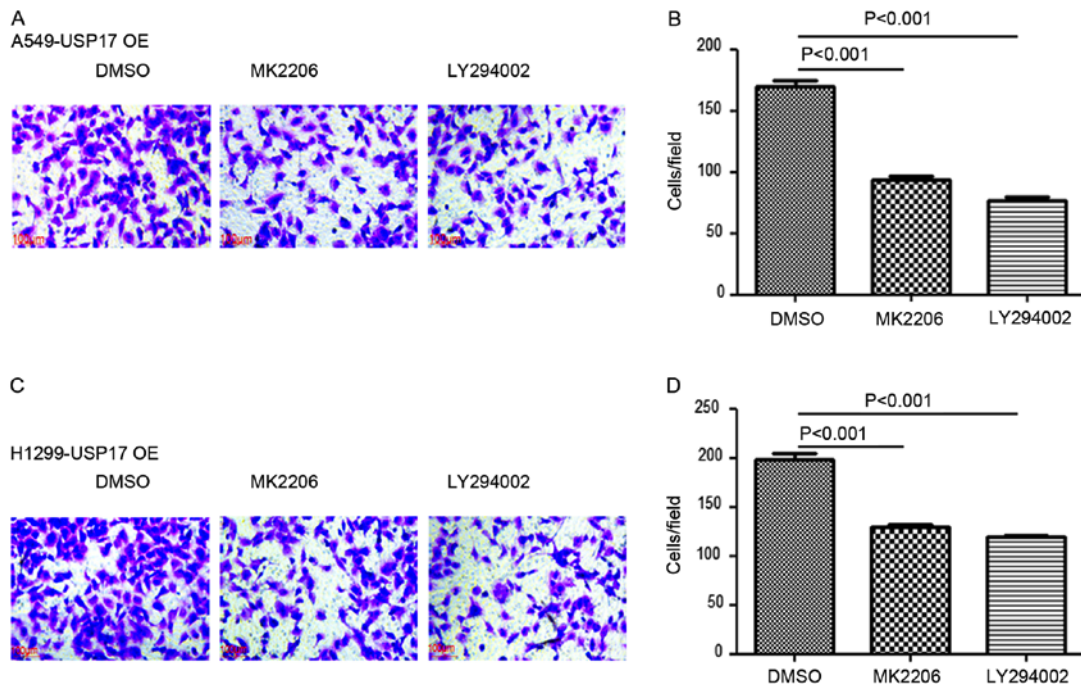


Figure 7. USP17-induced non-small cell lung cancer cell invasion is mediated by the PI3K/AKT pathway. Transwell assay using USP17-OE (A and B) A549 and (C and D) H1299 cells treated with the DMSO control, MK2206 or LY294002. Magnification, x200; scale bar, 100 μ m. USP, ubiquitin-specific peptidase; OE, overexpression; DMSO, DMSO negative treatment control.

Overall, the findings of the present study demonstrate the promotion of NSCLC cell proliferation and viability by USP17, via the activation of the PI3K/AKT pathway.

Discussion

The USP family is one of the five subfamilies of DUB enzymes, which cleave polyubiquitin chains from proteins, a number of studies have reported that USP17 has oncogenic characteristics. Firstly, it has been shown that high levels of USP17 in lung, colon, esophageal and cervical tumor samples promoted G1/S transition and cellular proliferation (14-17). Additionally, it was demonstrated that USP17 was expressed highly in NSCLC tissues, and patients with high levels of USP17 exhibited lower survival rates (16,17). Moreover, USP17 can be induced by cytokines such as interleukin (IL)-4 and IL-6 (28). In the present study, it was found that cisplatin treatment upregulated USP17 expression in a dose-dependent manner. Moreover, increased cell proliferation was found in USP17-OE cells compared with that of control cells, which is consistent with previous studies (14-17). Furthermore, it was demonstrated that the viability of USP17-OE cells was significantly higher than that of the control cells, when treated with cisplatin. In the present study USP17 regulated cisplatin sensitivity in NSCLC. USP17 was initially identified as a regulator of cell viability via signaling pathways associated with cell death in cervical cancer (29).

It was previously reported that USP17 expression could regulate Ras cellular localization and activation, via the deubiquitination of Ras-converting enzyme 1, thereby inhibiting phosphorylation of the downstream kinases, dual specificity mitogen-activated protein kinase kinase and ERK (30). In osteosarcoma, USP17 facilitated cell migration and invasion by deubiquitinating and stabilizing SMAD4 (31). USP17- and Skp1-cullin-1-F-box protein β F-box/WD repeat-containing protein 1A-regulated degradation of differentially expressed in chondrocytes protein 1 has been shown to control the DNA-damage response (32,33), thus indicating that the expression of USP17 may be associated with sensitivity to chemotherapeutics, including cisplatin.

In the present study, USP17 was found to promote the growth of NSCLC cells via the activation of the PI3K/AKT pathway. Aberrant activation of the PI3K/AKT pathway is often detected in numerous types of human cancer; hence, targeting this pathway may have therapeutic potential for the management of these tumors (34-37). Emerging evidence indicates that the activation of PI3K/AKT signaling by hypoxia may be a contributing factor to drug resistance in certain types of human cancer, including NSCLC and prostate cancer (38-40). PI3K activation is regulated by various molecules in NSCLC, such as DIX domain-containing protein 1, GRB2-associated-binding protein 2 and microRNAs (41-43).

There are limitations of the present study. Firstly, the functions of USP17 in the response of NSCLC cells to cisplatin treatment require further investigation, specifically using *in vivo* animal models (44,45). Moreover, as the underlying mechanisms of USP17 in the cisplatin response have not been investigated in this study, it should be examined in the future.

Considerable effort has been made to identify agents that target the activity of DUBs. However, no effective drugs have yet entered into clinical trials. The findings from the present study

demonstrate the therapeutic potential of targeting PI3K/AKT pathways downstream of USP17 to prevent NSCLC progression.

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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

SZ and HC contributed to designing research studies, conducting experiments, acquiring data, analyzing data, providing reagents and writing the manuscript. ZX and JY contributed to conducting experiments and acquiring data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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