UBR5 inhibits the radiosensitivity of non-small cell lung cancer cells via the activation of the PI3K/AKT pathway

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ABSTRACT

Ubiquitin protein ligase E3 component n-recognin 5 (UBR5) has been identified as an oncogene in diverse cancers; however, whether its expression was associated with radiosensitivities of non-small cell lung cancer (NSCLC) cells remains unclear. Expression levels of UBR5 in NSCLC tissues and cell lines were examined by immunohistochemical staining and western blotting. Colony formation assay, CCK-8 cell viability assay, flow cytometry, and caspase-3 activity assay were performed to evaluate the radiosensitization of UBR5 knockdown in NSCLC cells, and the underlying mechanism in vitro was also investigated. UBR5 was highly expressed in NSCLC tissues, and its high expression was associated with the poor prognosis in 50 patients with NSCLC. After X-ray irradiation, the protein expression levels of UBR5 were also increased in NSCLC cells. UBR5 inhibition enhanced the radiosensitivity of NSCLC cells by inhibiting the cell viability and inducing apoptosis. Further investigation indicated that UBR5 knockdown-mediated radiosensitization involved the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway. Knockdown of UBR5 radiosensitizes NSCLC cells via the inactivation of the PI3K/AKT signal, which provided a novel therapeutic target for NSCLC radiosensitization.

Significance of this study

What is already known about this subject?

- Ubiquitin protein ligase E3 component n-recognin 5 (UBR5) serves as a novel oncogene in many cancers.
- UBR5 is involved in the tumorigenesis of gallbladder cancer by regulating the phosphatase and tensin homolog (PTEN)-mediated phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway.
- Aberrant activation of PI3K/AKT pathway is associated with the radiotherapy resistance in various cancers.

What are the new findings?

- UBR5 is highly expressed in patients with NSCLC and predicts poor prognosis.
- UBR5 is increased in X-ray irradiated NSCLC cells.
- UBR5 inhibition enhances the radiosensitivity of NSCLC cells.
- UBR5 knockdown-mediated radiosensitization involves PI3K/AKT signaling.

How might these results change the focus of research or clinical practice?

- UBR5-mediated PI3K/AKT signaling axis might be a novel therapeutic target for NSCLC radiosensitization.

INTRODUCTION

Lung cancer is one of the leading causes of cancer-related deaths and one of the most common malignancies with high morbidity and mortality worldwide.[1] Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for approximately 80% of all lung cancers, with a 5-year survival rate of 15%.[2] Although radiotherapy is the main non-surgical therapy for NSCLC, the efficacy is limited in recent years, and acquired radioresistance represents the major cause of treatment failure.[3] Therefore, the underlying molecular mechanisms involving radioresistance in NSCLC are urgently required to be investigated.

Ubiquitin protein ligase E3 component n-recognin 5 (UBR5), also known as E3 identified by differential display, is a highly conserved nuclear phosphoprotein localized at chromosome 8q22.3, which is essential for the regulation of gluconeogenesis, DNA damage, cellular proliferation, apoptosis and differentiation.[4–7] Accumulating evidence indicates that UBR5 is overexpressed in diverse types of cancer and serves as a novel oncogene.[8] A previous study suggested that UBR5 might play a crucial role in regulating the proliferation, migration, invasion, cell cycle, and radiosensitivity in laryngeal carcinoma.[9] However, the function of UBR5 in NSCLC and its potential role in radiosensitivity remain unclear. UBR5 overexpression was recently reported to be involved in the tumorigenesis of gallbladder cancer by regulating the PTEN-mediated phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway.[10] A growing body of evidence demonstrates that aberrant activation of the PI3K/AKT pathway is associated with the radiotherapy resistance in various cancers.[11] The present study was designed to

investigate the expression of UBR5 in NSCLC cell lines and to further determine whether UBR5 regulates proliferation, apoptosis, and radiosensitivity in NSCLC cells via PI3K/AKT signaling.

MATERIALS AND METHODS

Tissue specimens

Paired tumor and adjacent normal tissues were obtained from 50 patients diagnosed with NSCLC who had never undergone preoperative radiotherapy or chemotherapy at Yantai Yantai Shan Hospital. All specimens were collected by surgical dissection with written informed consent and immediately fixed in 4% paraformaldehyde and embedded in paraffin within 30 min after surgery.

Immunohistochemistry

Paraffin-embedded tissues were cut into 4 µm thick sections, followed by deparaffinization and hydration. Immunostaining was then performed after antigen retrieval. The sections were permeabilized with 0.025% Triton X-100 in Tris-buffered saline (TBS) for 10 min at room temperature, blocked in TBS plus 1% bovine serum albumin containing 10% goat serum for 2 hours at room temperature and incubated with rabbit polyclonal primary antibodies to UBR5 (ab70311, 1:1000 dilution; Abcam, Shanghai, China) at 4°C overnight and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (ab205718, 1:20 000 dilution; Abcam) for 1 hour at room temperature. Diaminobenzidine was used as a chromogenic substrate (ab103723, 1:100 dilution; Abcam) and incubated for 10 min at room temperature. Slides were counterstained with haematoxylin and imaged under light microscopy (magnification ×400).

Cell lines

Human NSCLC cell lines (A549, H1229, CALU3 and CALU6) and human bronchial epithelial (HBE) cells were obtained from American Type Culture Collection (Manassas, Virginia, USA). These cells were cultured in Dulbecco's Modified Eagle's Medium (Life Technologies, Carlsbad, California, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin in an incubator with 5% CO_2 at 37°C. The culture medium was replaced every 3 days.

Irradiation treatment

Unless otherwise specified, A549 and H1229 cells were irradiated with X-ray (0 and 4 Gy) at a dose rate of 2 Gy/min using an X-ray irradiator (Elekta Precise, Elekta, Stockholm, Sweden) at room temperature.

Cell transfection

Prior to transfection, A549 and H1229 cells in the logarithmic phase were seeded into six-well plates at a density of 4×10^5 cells/well and cultured overnight at 37°C. Cell transfection with siRNA specifically targeting UBR5 (si-UBR5) or siRNA negative control synthesized by GenePharma Co. (Shanghai, China) was performed using Lipofectamine 2000 (Invitrogen). At 48 hours after transfection, cells were then irradiated with or without 4 Gy X-rays.

Clonogenic survival analysis

Transfected cells were exposed to different doses of irradiation (0, 2, 4 and 6 Gy) and incubated for 14 days at 37 °C to allow for colony formation. Cells were washed three times with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 15 min, and dyed by 0.5% crystal violet for 15 min at room temperature. The colony survival fraction was calculated under a microscope.

Cell viability assay

Cell viability was determined using Cell Counting Kit-8 kit (cat. no. C0037; Beyotime Biotechnology, Shanghai, China) according to the manufacturer's protocol. Briefly, cells in different groups were seeded into a 96-well plate at a density of 2000 cells/well. At 0, 24, 48, and 72 hours, 10 µL CCK-8 reagent was added into each well. After 1-hour incubation at 37°C, the absorbance was measured at 450 nm on a microplate reader (Thermo Scientific).

Cell apoptosis assay

The harvested cells were washed twice with ice-cold PBS and resuspended in the binding buffer at a concentration of 1×10^6 cells/mL. Aliquots (100 µL) of cell solution with 1×10^5 cells were transferred to a 5 mL culture tube and mixed with 5 µL fluorescein isothiocyanate Annexin V (cat. no. 560931; BD Biosciences, San Jose, California, USA) and 10 µL propidium iodide (cat. no. 556463, BD Biosciences). After incubation for 15 min at room temperature in the dark, cells were analyzed by flow cytometry (FACS Calibur; Becton Dickinson, USA) within 1 hour.

Caspase-3 activity assay

Cells were lysed with ice-cold lysis buffer and centrifugated at 20 000 *g* for 10 min to collect the supernatant. After incubation with Caspase 3 Activity Assay Kit (cat. no. C1115, Beyotime Biotechnology) for 2 hours at 37°C, the caspase-3 activity was detected by measuring optical density at 405 nm.

Western blot

Total protein was prepared from cultured cells with RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, California, USA). The protein concentration was measured by the Protein BCA Assay Kit (Bio-Rad Laboratories, Hercules, California, USA). The protein extracts were resolved on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The following primary antibodies were used overnight at 4°C: anti-UBR5 (#65344), anti-PI3K (#4249), anti-AKT (#4685), anti-p-AKT (#4060), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#5174) at a dilution of 1000, which were purchased from Cell Signaling Technology (Boston, Massachusetts, USA). HRP-conjugated anti-rabbit IgG secondary antibodies (#7074, 1:2000 dilution; Cell Signaling Technology) were applied at room temperature for 2 hours. The bands were visualized by an enhanced chemiluminescence reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The relative expression of the targets was normalized to GAPDH.

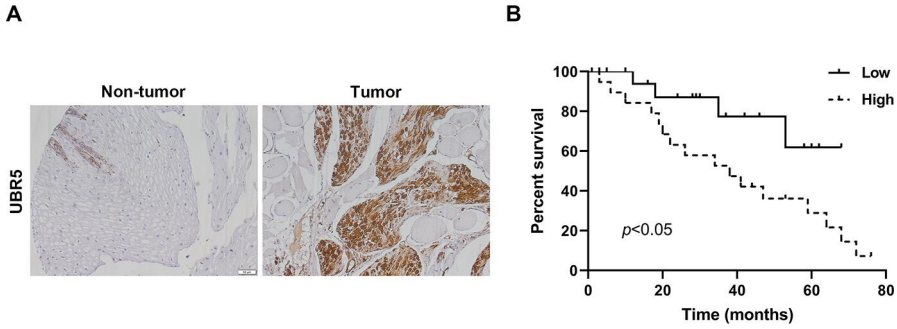

Figure 1 Representative specimens of IHC staining and survival analysis of UBR5 expression in HCC. (A) IHC analysis of the expression of UBR5 protein was detected in 50 cases of NSCLC tissues and corresponding non-tumor tissues. (B) Relationship between UBR5 expression and overall survival of 50 patients with NSCLC was analyzed by Kaplan-Meier analyses (the median expression level of UBR5 was used as the cut-off value). Values are presented as mean±SD, n=50. HCC, hepatocellular carcinoma; IHC, immunohistochemistry; NSCLC, non-small cell lung cancer; UBR5, ubiquitin protein ligase E3 component n-recognin 5.

Statistical analysis

All the experiments were conducted in triplicate. Data are displayed as the mean±SD. Statistical analysis was performed by using SPSS software V.19.0. Kaplan-Meier survival analysis was performed in all 50 cases and compared using the log-rank test. The comparison between two groups and multiple groups was analyzed by Student's t-test and one-way analysis of variance, respectively. A p value of <0.05 was considered statistically significant.

RESULTS

UBR5 is upregulated in NSCLC and associated with poor prognosis

To verify the biological functions of UBR5 in NSCLC development, immunohistochemistry analysis was first performed to determine the expression of UBR5 in 50 pairs of NSCLC tissues and adjacent non-tumor tissues. As shown in figure 1A, UBR5 protein levels were substantially upregulated in NSCLC tissues than those in corresponding non-tumor tissues. In addition, the Kaplan-Meier analysis showed that patients with high expression of UBR5 had a poor prognosis than those with low expression of UBR5 (figure 1B).

UBR5 is overexpressed under irradiation in NSCLC cells

The expression of UBR5 was further investigated in NSCLC cells using western blotting, and the results showed that the UBR5 protein level was overexpressed in a panel of four human NSCLC cell lines (A549, H1229, CALU3 and CALU6) compared with that in HBE cells, especially in A549 and H1229 cells (figure 2A). To further investigate whether radiation could affect the expression of UBR5 in NSCLC cells, A549 and H1229 cells were treated with or without 4 Gy X-ray, and the protein expression levels of UBR5 were then evaluated. After irradiating A549 and H1229 cells with 4 Gy X-rays, we observed that the expression of UBR5 was significantly increased compared with the untreated cells (figure 2B). Based on the upregulation of UBR5 in response to irradiation, siRNA-mediated UBR5 silencing was performed in A549 and H1229 cells to explore the role of UBR5 in the radiosensitivity of NSCLC cells. Immunoblotting confirmed that the transfection of UBR5 siRNA led to the downregulation of UBR5 in both A549 and H1229 cells (figure 2C).

UBR5 knockdown inhibits the viability and induces apoptosis and radiosensitivity in NSCLC cells

To further explore whether UBR5 knockdown radiosensitizes NSCLC cells, colony formation assay was performed in A549 and H1229 cells using 0–6 Gy doses of radiation after transfection with si-UBR5. Results showed that

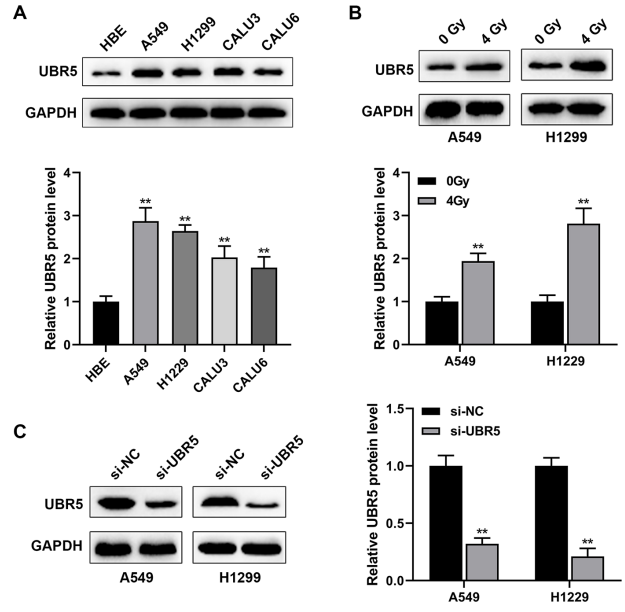

Figure 2 Expression of UBR5 under irradiation condition in NSCLC cells. (A) Western blotting analyses were performed to determine the expression of UBR5 in human NSCLC cell lines (A549, H1229, CALU3 and CALU6) and HBE cells. (B) The expression of UBR5 in A549 and H1229 cells was detected under 0 or 4 Gy irradiation. (C) The transfection efficiency of si-UBR5 was confirmed by western blotting in A549 and H1229 cells. Values are presented as mean±SD, n=3. **P<0.01. HBE, human bronchial epithelial; NSCLC, non-small cell lung cancer; si-UBR5, siRNA specifically targeting UBR5; UBR5, ubiquitin protein ligase E3 component n-recognin 5.

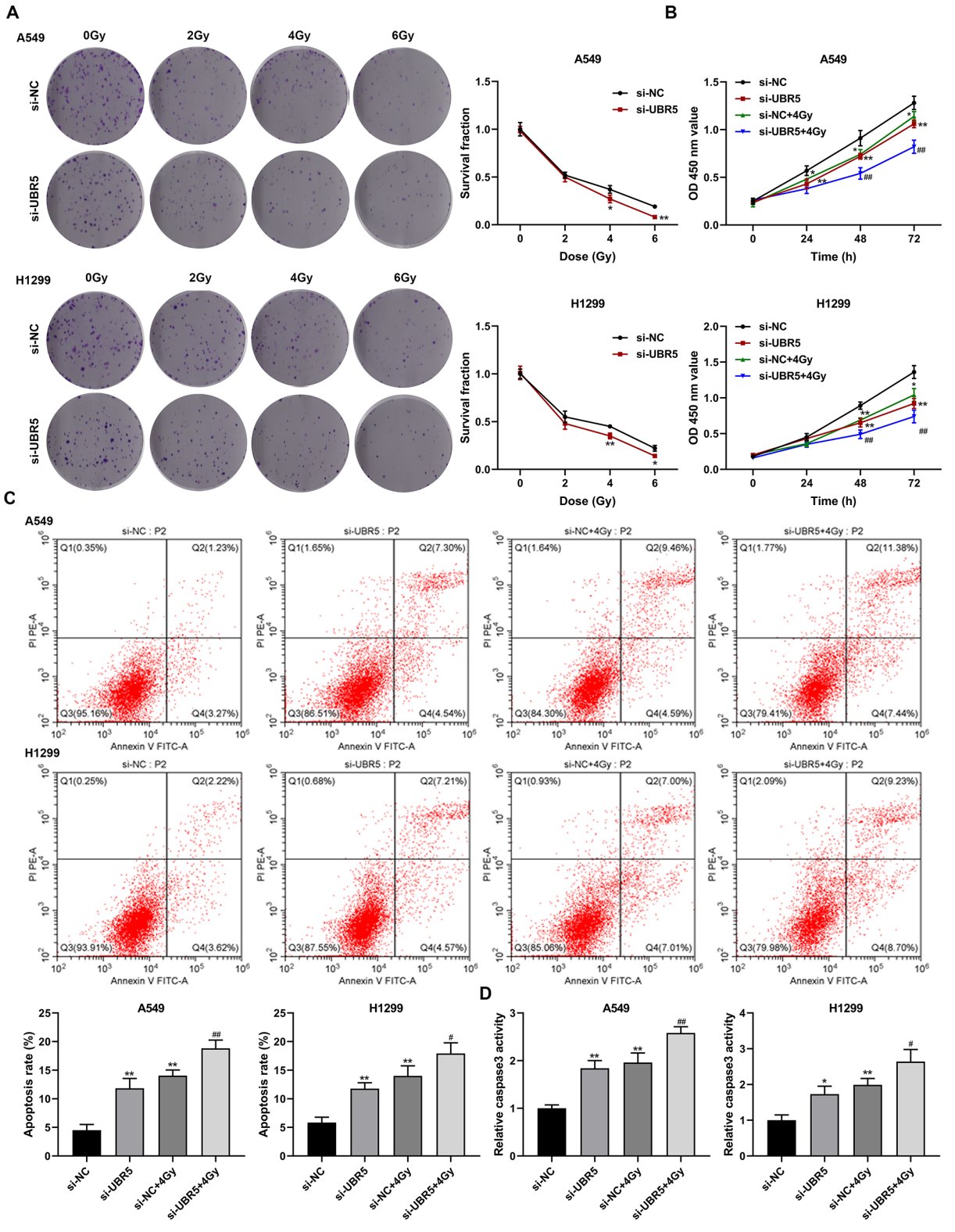

Figure 3 UBR5 knockdown inhibits the viability and induces apoptosis and radiosensitivity in NSCLC cells. (A) Colony formation assay was performed in A549 and H1229 cells treated with 0–6 Gy radiation after transfection with si-UBR5 or si-NC. (B) CCK-8 assay was performed to evaluate cell viability at 0, 24, 48, and 72 hours in A549 and H1229 cells exposed to 4 Gy irradiation after transfection with si-UBR5 or si-NC. (C) Flow cytometry analyses were carried out to determine the apoptosis rate of A549 and H1229 cells transfected with si-UBR5 or si-NC under 4 Gy irradiation. (D) Caspase-3 activity assay was used to detect the caspase-3 activity in A549 and H1229 cells transfected with si-UBR5 or si-NC under 4 Gy irradiation. Values are presented as mean±SD, n=3. * #P<0.05; ** ##P<0.01. FITC-A, fluorescein isothiocyanate A; NSCLC, non-small cell lung cancer; si-NC, siRNA negative control; si-UBR5, siRNA specifically targeting UBR5; UBR5, Ubiquitin protein ligase E3 component n-recognin 5.

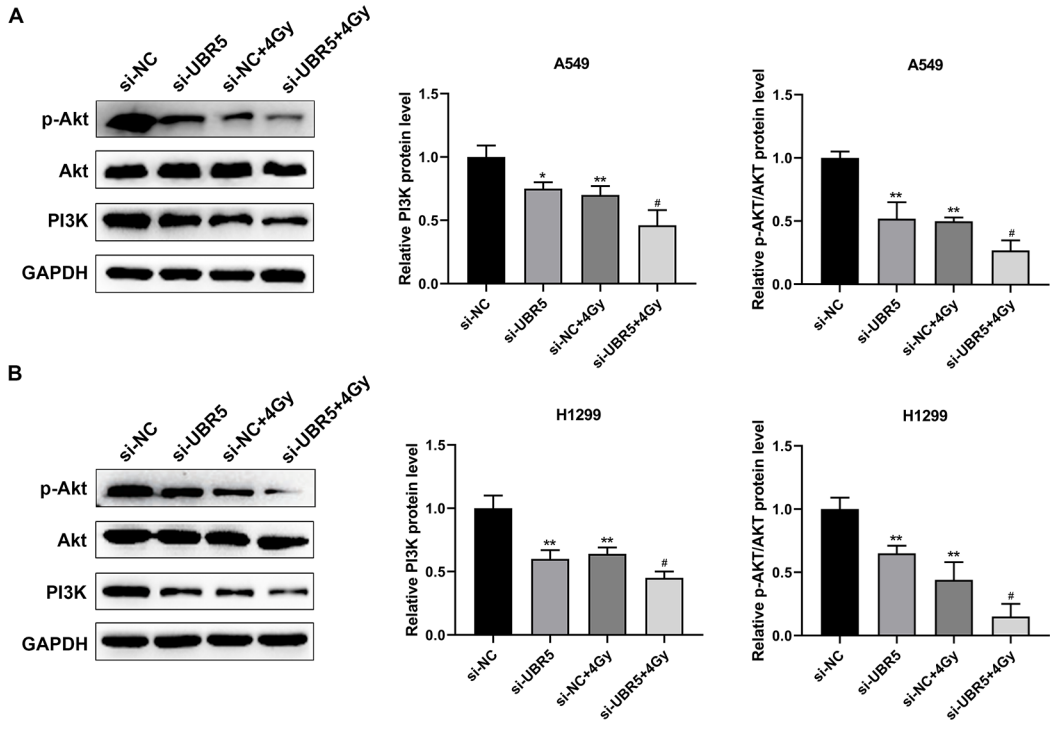

Figure 4 UBR5 mediates the radiosensitivity of NSCLC cells by regulating the PI3K/AKT pathway. Representative western blots and determination of the total protein levels of PI3K and the ratio of the p-AKT/AKT expression were shown in A549 (A) and H1229 (B) cells transfected with si-UBR5 or si-NC under 4 Gy irradiation. Values are presented as mean±SD, n=3. * #P<0.05, **P<0.01. NSCLC, non-small cell lung cancer; PI3, phosphatidylinositol 3-kinase; si-NC, siRNA negative control; si-UBR5, siRNA specifically targeting UBR5; UBR5, ubiquitin protein ligase E3 component n-recognin 5.

UBR5 inhibition markedly decreased the survival fraction of both A549 and H1229 cells in a radiation dose-dependent manner (figure 3A). Cell viability detection by CCK-8 assay showed that UBR5 downregulation decreased the cell viability of irradiated A549 and H1229 cells in a time-dependent manner (figure 3B). Meanwhile, apoptosis assessment by flow cytometry showed that UBR5 silencing observably enhanced the apoptotic rate of A549 and H1229 cells exposed to radiation (figure 3C). Apoptosis was also determined by caspase-3 activity assay. As expected, siRNA-mediated UBR5 suppression significantly resulted in a decrease in caspase-3 activity in A549 and H1229 cells following irradiation treatment (figure 3D).

UBR5 mediates the radiosensitivity of NSCLC cells by regulating the PI3K/AKT pathway

PI3K/AKT signaling-related gene expression was further examined to explore the underlying mechanism by which UBR5 regulated the radiosensitivity in NSCLC cells. As demonstrated in figure 4A,B, the total protein levels of PI3K and the ratio of p-AKT/AKT expression were decreased by UBR5 depletion in radiation-treated A549 and H1229 cells.

DISCUSSION

A large quantity of evidence has revealed that UBR5, a key regulator of the unfolded protein response, is aberrantly expressed in cervical cancer, gastric cancer, colorectal cancer, and breast cancer, and could regulate tumor cell proliferation, cell cycle, apoptosis, and metastasis.[12–15] Although these studies have well characterized the involvements of UBR5 in the etiology of various cancers, its importance in NSCLC has not been conceivably described. UBR5 has recently been demonstrated to promote the proliferation and xenograft tumor growth in lung adenocarcinoma.[16] Targeting UBR5 as a chemotherapeutic strategy in ovarian cancer has attracted interest.[17–19] Accumulating evidence has strongly implied that UBR5 downregulation reduced cell proliferation and reversed the radiosensitivity of NSCLC cells by targeting the p38/MAPK signaling pathway.[9] The results of the present study found that UBR5 protein was overexpressed in NSCLC tissues compared with non-tumor tissues; an increased expression of UBR5 was also observed in NSCLC cell lines. High UBR5 expression positively correlated with poor prognosis in patients with NSCLC according to the survival analysis, indicating UBR5 might be a novel potential independent prognostic factor for patients with NSCLC. Intriguingly, the expression level of UBR5 was significantly upregulated in selected NSCLC cells after exposing to X-ray irradiation in a dose-dependent manner. Furthermore, suppression of UBR5 expression by siRNA decreased cell viability and clonogenic potentials of NSCLC cells and led to a distinct increase of caspase-3-mediated apoptosis under irradiation, suggesting that UBR5 silence improved the radiosensitivity of NSCLC cells.

The PI3K/AKT signaling pathway is implicated in diversified cancers and plays a pivotal role in the regulation of cell survival, growth, and proliferation.[20] Te hPI3K/AKT signaling pathway is reported to be critical for the radiosensitivity in various types of tumors, including NSCLC. For example, Jiang *et al* found that miR-21 overexpression

decreased the sensitivity to radiotherapy and apoptosis of NSCLC cells by targeting PDCD4-mediated PI3K/AKT/mTOR pathway.[21] Kang *et al* showed that delphinidin sensitized radioresistant NSCLC cells through autophagy and apoptosis induction by inhibiting the PI3K/AKT pathway.[22] To further explore the mechanism by which UBR5 regulated the sensitivity of radiotherapy in NSCLC cells, the expressions of PI3K/AKT signaling pathway-related proteins were detected after radiotherapy, since Zhang *et al* reported that UBR5 activated the PI3K/AKT signaling pathway, thereby contributing to the carcinogenesis of gallbladder cancer.[10] Our results suggested that UBR5 silencing blocked the activation of the PI3K/AKT signaling pathway in NSCLC cells following radiation. These results indicated that the effect of UBR5 on radiosensitivity in breast cancer cells was at least partly mediated by the PI3K/AKT signaling pathway. The relationship between UBR5 expression and clinicopathological characteristics requires further investigation. Furthermore, whether UBR5 expression levels were associated with radiotherapeutic efficacy also requires further examination.

In summary, we demonstrated that UBR5 was required for radioresistance in NSCLC cells by modulating the PI3K/AKT signaling pathway. Therefore, UBR5 might be a potential predictor of radiosensitivity in patients with NSCLC.

Contributors Y-FG conducted most of the experiments, designed the study and wrote the manuscript; X-PG conducted the experiments and analyzed the data. All authors have read and approved the manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval The present study was approved by the ethic committee of Yantai Yantai Shan Hospital of Science and Technology.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplemental information. All data in this study can be obtained by proper request from the authors.

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