ERp29 downregulation enhances lung adenocarcinoma cell chemosensitivity to gemcitabine by upregulating HSP27 phosphorylation

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Received May 1, 2018; Accepted October 26, 2018

DOI: 10.3892/etm.2018.7040

Abstract. The aim of the current study was to assess the underlying mechanism of endoplasmic reticulum protein 29 (ERp29) in lung adenocarcinoma chemosensitivity to gemcitabine. Western blot analysis was performed to detect ERp29 expression following lung adenocarcinoma cell treatment with gemcitabine. The effects of gemcitabine in combination with ERp29 siRNA on cell apoptosis, cell cycle and heat shock protein 27 (HSP27) expression were assessed. The results demonstrated that ERp29 expression was increased on exposure to gemcitabine. The apoptotic rate of lung adenocarcinoma cells were also increased following gemcitabine treatment and the combined application of gemcitabine and ERp29 siRNA synergistically increased apoptotic rates further. It was also revealed that gemcitabine and ERp29 siRNA synergistically increased the ratio of phosphorylated to total HSP27 protein. In addition, downregulation of HSP27 significantly reduced lung adenocarcinoma chemosensitivity to gemcitabine. These data indicate that ERp29 affects lung adenocarcinoma cell chemosensitivity to gemcitabine by regulating phosphorylated HSP27. ERp29 is a novel target, which may be used to enhance the therapeutic effect of lung adenocarcinoma treatment with gemcitabine.

Introduction

As the most common histologic subtype of lung cancer, lung adenocarcinoma is the leading cause of cancer mortality worldwide (1,2). Molecular targeted drugs have been demonstrated to improve quality of life and therapeutic effects in patients with lung adenocarcinoma whose tumors exhibit driver oncogenes, including epidermal growth factor receptor mutations, anaplastic lymphoma kinase gene rearrangements or ROS proto-oncogene 1 gene fusion (2,3). However, most lung adenocarcinomas lack an identifiable activated oncogene and remain to be treated with conventional chemotherapy (2,3).

Endoplasmic reticulum protein 29 (ERp29), a putative chaperone protein, is located in the endoplasmic reticulum (4). Structurally, ERp29 consists of an N-terminal domain, a flexible loop and a C-terminal domain (4). ERp29 is upregulated on exposure to radiation, homocysteine or dopamine (5-7). ERp29 is abnormally expressed in several types of tumors, including breast cancer, colorectal cancer, basal skin carcinoma and gallbladder adenocarcinoma (8-11). Furthermore, ERp29 expression is associated with the pathological grade, TNM stage, lymph node metastasis, recurrence and prognosis of patients with cancer (8-11). It has also been revealed that ERp29 overexpression enhances breast cancer cell chemoresistance to doxorubicin and nasopharyngeal carcinoma cell radioresistance (9,12).

A previous study has revealed that ERp29 is significantly overexpressed in 75 patients with lung adenocarcinoma and of ERp29 inhibition enhances gemcitabine chemosensitivity (13). Therefore, treatment with gemcitabine in combination with ERp29 expression inhibition may promote therapeutic effects in lung adenocarcinoma. However, underlying mechanisms of this action are yet to be elucidated. It has been revealed that ERp29 is involved in the regulation of heat shock protein 27 (HSP) (5,14). In addition, as a small HSP, HSP27 is associated with gemcitabine chemotherapeutic sensitivity (15,16). Therefore, the current study hypothesized that ERp29 may affect the chemosensitivity of lung cancer cells to gemcitabine by regulating HSP27. In the present study, ERp29 and HSP27 expression was assessed following lung adenocarcinoma cell treatment with gemcitabine. Furthermore, effects of combined treatment with gemcitabine and ERp29 small interfering (si)RNA on cell apoptosis, cell cycle and HSP27 expression were examined in the present study.

Materials and methods

Cell lines and cell culture. A549 and SPC-A1 human lung adenocarcinoma cells (Type Culture Collection of the Chinese Academy of Science, Shanghai, China) were cultured in

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Key words: endoplasmic reticulum protein 29, lung adenocarcinoma, gemcitabine, chemosensitivity, heat shock protein 27

RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and maintained at 37˚C in a 5% CO_2-humidified incubator.

Western blot analysis. Total protein was extracted from lung adenocarcinoma cells using radio immunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China). Total protein was quantified using a bicinchoninic acid assay and 30-60 μg protein/lane was separated via SDS-PAGE on a 12% gel using a mini-gel apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The separated proteins were subsequently transferred onto polyvinylidene difluoride membranes and for 1.5 h at room temperature with 5% non-fat dry milk in Tris-buffered saline with Tween-20 (TBST). The membranes were incubated with primary antibodies against ERp29 (1:500; cat. no. ab176573; Abcam, Cambridge, UK), HSP27 (1:1,000; cat. no. 2402), phosphorylated (p)-HSP27 (Ser82; 1:1,000; cat. no. 9709) (both from Cell Signaling Technology, Inc., Danvers, MA, USA) and α-tubulin antibodies (1:3,000; cat. no. T5168; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) overnight at 4˚C. Membranes were washed with TBST. Following primary incubation, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:1,000; cat. no. 7074) and anti-mouse IgG (1:1,000; cat. no. 7076; both from Cell Signaling Technology, Inc.) secondary antibodies for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology). Protein expression was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Following treatment with various doses (0.5, 5 and 50 μM) of gemcitabine (Selleck Chemicals, Houston, TX, USA) for 24 h, ERp29 expression in lung adenocarcinoma cells was detected. HSP27 and p-HSP27 expression levels were detected following treatment with 5 μM gemcitabine for 4, 8 and 24 h in A549 and SPC-A1 cells.

siRNA transfection. ERp29 siRNA, HSP27 siRNA and scrambled siRNA were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The following gene-specific siRNA duplexes: ERP29-siRNA sense, 5'-GUG AGUCCCUUGUGGAAUATT-3' and antisense, 5'-UAUUCC ACAAGGGACUCACTT-3' and HSP27-siRNA sense, 5'-ACC UGUGUGUUCUUUUGAUTT-3' and antisense, 5'-AUCAAA AGAACACACAGGUTT-3'. Cells were transfected with 40 nM siRNA using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Thereafter, cells were harvested for western blot analysis following 72-h transfection.

Detection of apoptosis. Cell apoptosis was analyzed using the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA). A total of $1x10^5$ cells were collected by centrifugation at 170 x g for 5 min at room temperature. The cells were suspended in 100 μl of binding buffer and subsequently stained with 5 μl Annexin V-FITC and 5 μl propidium iodide (PI) in the dark for 15 min at room temperature. Apoptotic cells were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (version 7.6.1; TreeStar, Inc., Ashland, OR, USA). Apoptotic cells were defined as Annexin V-positive cells.

Cell cycle assay. A cell cycle kit (Beyotime Institute of Biotechnology) was used to determine the percentage of cells in G_1, S and G_2 phases of the cell cycle. Lung adenocarcinoma cells were divided into 4 groups: The control group (transfected with control siRNA), the ERp29 siRNA group (transfected with ERp29 siRNA), the gemcitabine group (transfected with control siRNA and treated with gemcitabine) and the combination group (transfected with ERp29 siRNA and treated with gemcitabine). Single-cell suspensions were fixed using 70% ethanol for 24 h at 4˚C. Cells were subsequently washed with phosphate buffered saline, stained with 0.5 ml PI for 30 min at room temperature and analyzed using a BD FACS Calibur flow cytometer and Cell Quest software (version 5.1; BD Biosciences).

Cytotoxicity assay. Gemcitabine cytotoxicity was quantified using a Cell Counting kit-8 (CCK-8) assay (7Sea Biotech, Shanghai, China). Cells were seeded in 96-well plates at a density of $3x10^3$ A549 cells/well or $3.5x10^3$ SPC-A1 cells/well. Following 48-h treatment with gemcitabine (0.6 μM for A549 or 6 μM for SPC-A1 cells) at room temperature, cells were incubated with 10 μl CCK-8 solution for 1 h at room temperature. The optical density (OD) was measured at 450 nm using a Multiskan Go Microplate spectrophotometer (Thermo Fisher Scientific, Inc.). The cell growth inhibition rate of gemcitabine was calculated as follows: (1-OD_{450} of treated cells/OD_{450} of untreated cells) x100%.

Statistical analysis. All statistical analyses were performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard error of the mean. Differences between two groups were assessed using Student's t-test. Multiple comparisons between groups were analyzed using one-way analysis of variance followed by Fisher's least significant difference post-hoc tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Exposure of lung adenocarcinoma cells to gemcitabine increases ERp29 expression. As presented in Fig. 1, ERp29 expression was significantly increased on exposure to gemcitabine ($P<0.05$). Erp29 expression was significantly increased at 50 μM in A459 cells and 5 μM in SPC-A1 cells, respectively.

Effects of gemcitabine and ERp29 siRNA on lung adenocarcinoma cell apoptosis. Following treatment with 20 μM gemcitabine, the apoptotic rate of A549 cells increased from 6.43±0.55 in the control group to 20.90±2.83% in the control + 20 μM gemcitabine group (Fig. 2). Treatment with gemcitabine in combination with ERp29 siRNA significantly increased the apoptotic rate to 30.80±1.41% compared with gemcitabine treatment alone (Fig. 2). Following treatment with 50 μM gemcitabine, the SPC-A1 cell apoptotic rate was increased from 6.10±1.12 in the control group to 19.82±1.76% in the control + 50 μM gemcitabine group. Treatment with

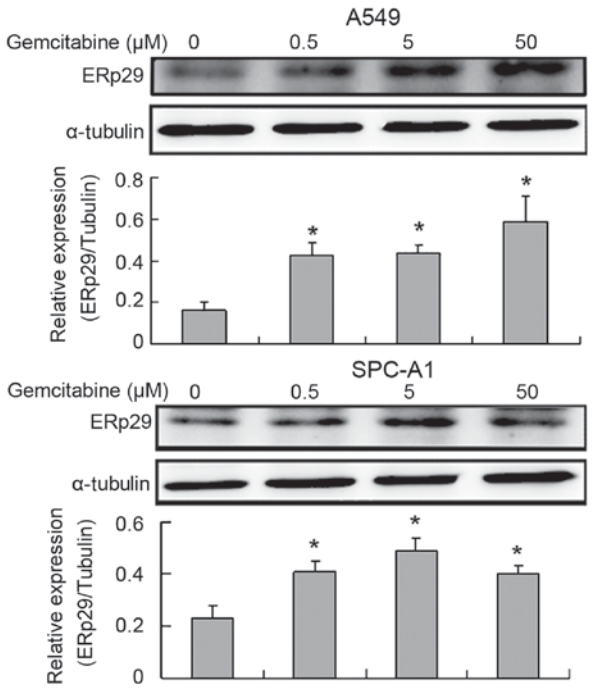

Figure 1. ERp29 expression increases in A549 and SPC-A1 cells exposed to gemcitabine. ERp29 protein expression was assessed by western blot and normalized to α-tubulin. ERp29 expression increased when exposed to gemcitabine. *P<0.05 vs. the 0 μM gemcitabine group. ERp29, endoplasmic reticulum protein 29.

gemcitabine in combination with ERp29 siRNA significantly increased the apoptotic rate to 27.53±1.11% compared with gemcitabine treatment alone (Fig. 2). The apoptotic rate of A549 and SPC-A1 cells in the Si-control and ERp29 siRNA group were not significantly different.

Effects of gemcitabine and ERp29 siRNA on the cell cycle of lung adenocarcinoma cells. Lung adenocarcinoma cells in the gemcitabine and combination groups were treated with 20 μM gemcitabine. As presented in Fig. 3, the percentage of gemcitabine-treated cells in the S phase was significantly increased compared with untreated cells in the S phase in the A549 Si-control group (31.80±2.81 vs. 19.12±1.00%; P<0.05). Similar results were obtained in SPC-A1 cells (29.12±1.84 vs. 19.78±0.39%; P<0.05). The percentage of gemcitabine-treated cells in the S phase was increased compared with untreated cells in the S phase in the A549 Si-ERp29 group and similar results were obtained in SPC-A1 cells. There were no significant differences in the level of G_1 and G_2 cell cycle arrest observed in the ERp29 siRNA or Si-control groups.

Effects of ERp29 siRNA on HSP27 expression in lung adenocarcinoma cells. Lung adenocarcinoma cell lines were transfected with ERp29 or control siRNA. At 72 h following transfection, ERp29, HSP27 and p-HSP27 levels were detected

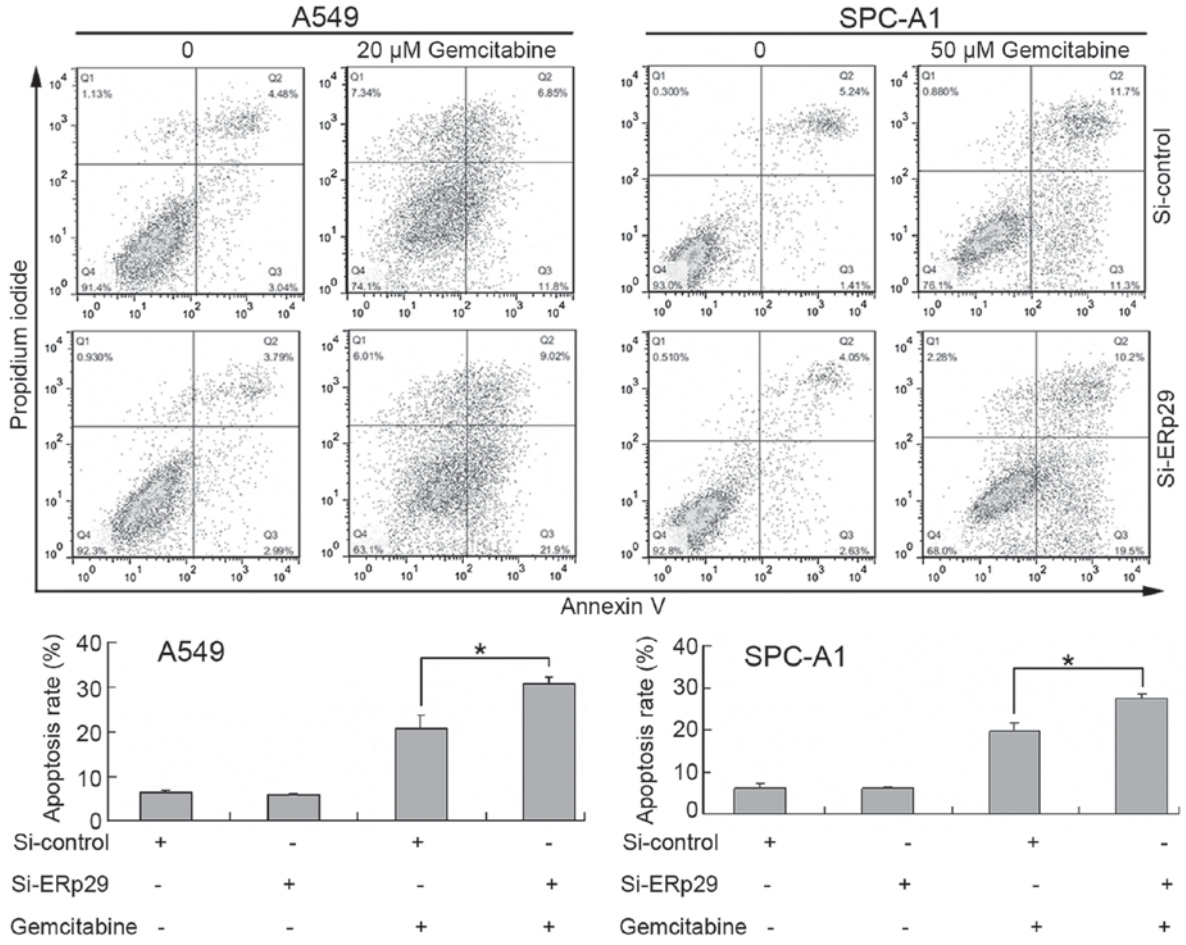

Figure 2. Combined gemcitabine and ERp29 siRNA treatment increases apoptosis in lung adenocarcinoma cells. Flow cytometry was performed to analyze apoptosis rates. *P<0.05. Si, small interfering RNA; ERp29, endoplasmic reticulum protein 29.

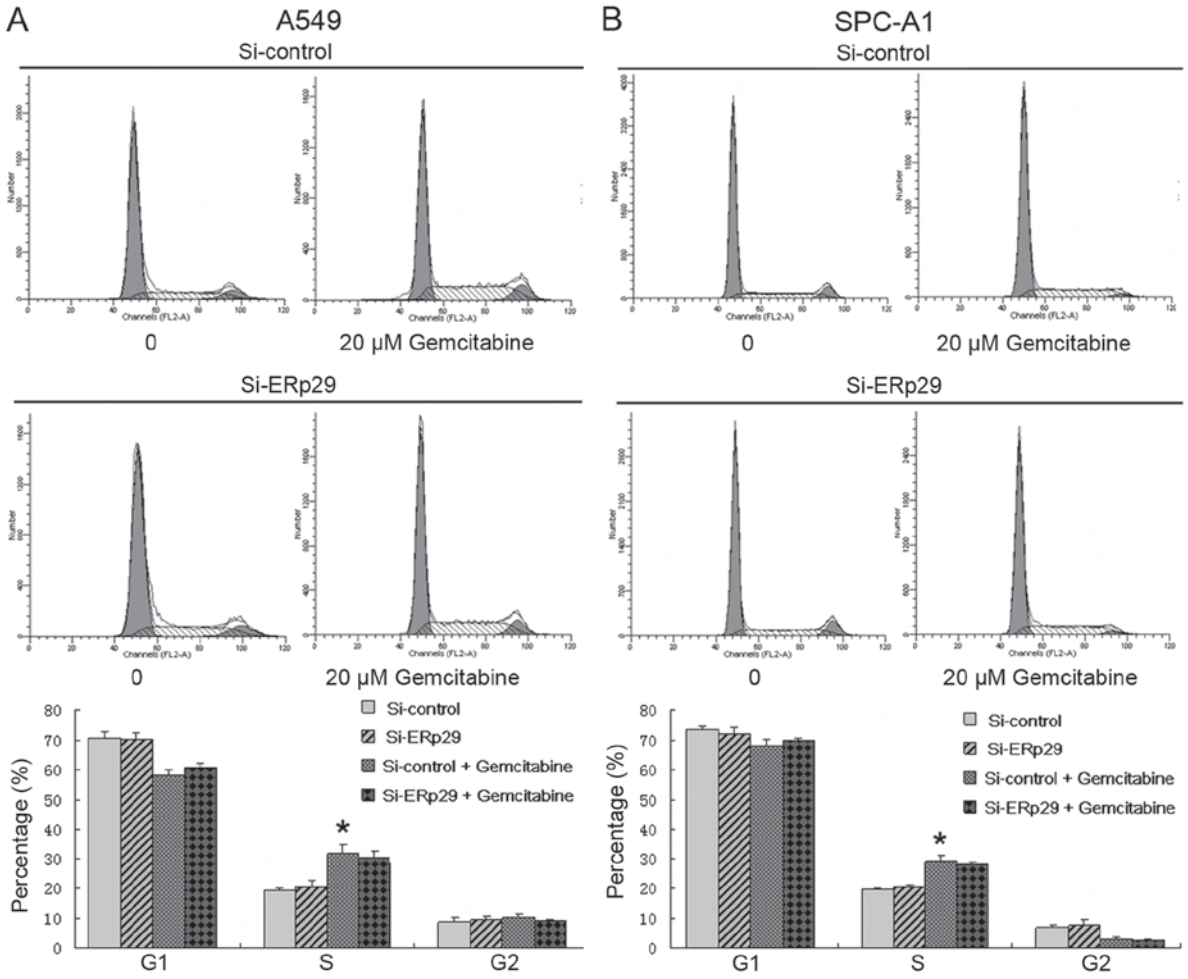

Figure 3. Gemcitabine changes the cell cycle of lung adenocarcinoma cells. The cell cycle of treated (A) A549 and (B) SPC-A1 cells was determined by flow cytometry. The percentage of cells in the S phase of the gemcitabine group was significantly higher than that of the control group. There were no significant differences in the cell cycles of the ERp29 siRNA and Si-control group, and in combination and gemcitabine group. Si, small interfering RNA; ERp29, endoplasmic reticulum protein 29. *P<0.05 vs. 0 μM group.

using western blotting. The downregulation of ERp29 significantly increased the ratio of p-HSP27 to total HSP27 (P<0.05; Fig. 4).

Effects of gemcitabine on HSP27 expression in lung adenocarcinoma cells. Phosphorylation of HSP27 was significantly increased in gemcitabine-treated cells compared with the 0 h group (P<0.05; Fig. 5). However, gemcitabine-induced HSP27 phosphorylation was not found to be time-dependent.

Effects of gemcitabine in combination with ERp29 siRNA on HSP27 expression. Lung adenocarcinoma cells in the gemcitabine and combination groups were treated with 5 μM gemcitabine. Phosphorylation of HSP27 in the combination group was significantly higher compared with the other three groups (P<0.05; Fig. 6).

Effects of HSP27 siRNA on lung adenocarcinoma cell chemosensitivity to gemcitabine. Lung adenocarcinoma cells were transfected with HSP27 siRNA or control siRNA. As presented in Fig. 7, HSP27 expression was effectively reduced in A549 and SPC-A1 cells transfected with HSP27 siRNA compared with the control RNA. The downregulation of HSP27 expression in A549 cells significantly decreased the cell growth inhibition rate of gemcitabine compared with the control (37.79±2.99 vs. 55.63±4.28%; P<0.05). Furthermore, the cell growth inhibition rate of gemcitabine in HSP27-inhibiting SPC-A1 cells was significantly lower than that of the control (54.82±3.15 vs. 64.80±0.62%; P<0.05).

Discussion

The present study investigated the role of ERp29 in lung adenocarcinoma cell chemosensitivity to gemcitabine. The results demonstrated that ERp29 expression in A549 and SPC-A1 cells was increased on exposure to gemcitabine. Furthermore, ERp29 downregulation significantly increased apoptosis induced by gemcitabine. It was further demonstrated that gemcitabine and ERp29 siRNA synergistically increased HSP27 phosphorylation and HSP27 downregulation significantly reduced chemosensitivity to gemcitabine.

A previous study revealed that ERp29 was overexpressed in patients with lung adenocarcinoma and ERp29 inhibition significantly enhanced the cytotoxic effects of gemcitabine (13). However, the underlying mechanism of this effect is poorly understood. Zhang *et al* (17) demonstrated that

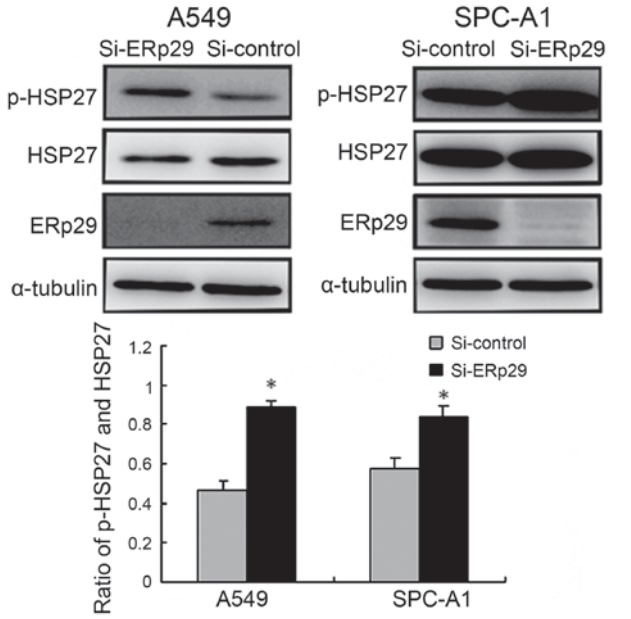

Figure 4. ERp29 downregulation increases HSP27 phosphorylation in lung adenocarcinoma cells. ERp29 siRNA treatment significantly increased HSP27 phosphorylation as determined by western blot analysis. *P<0.05 vs. Si-control group. ERp29, endoplasmic reticulum protein 29; Si, small interfering RNA; HSP27, heat shock protein 27.

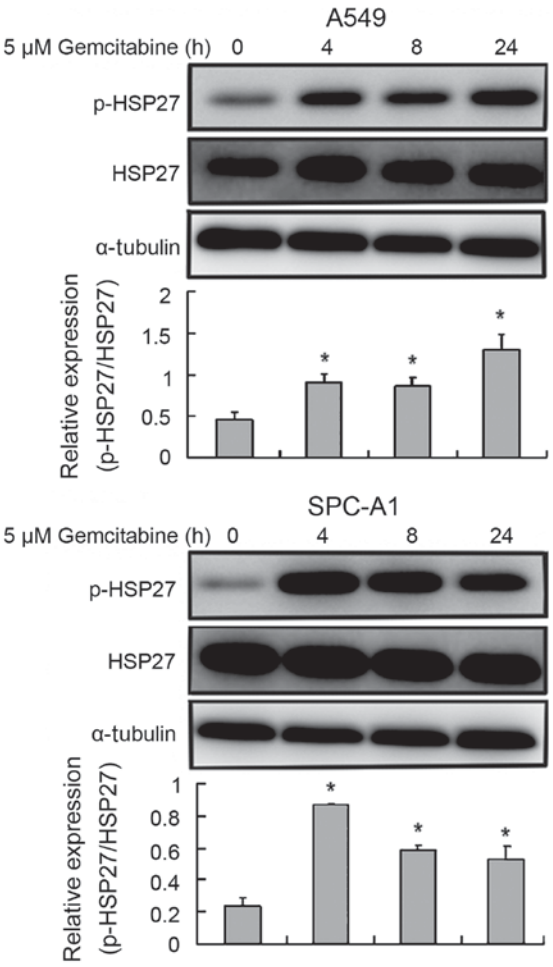

Figure 5. Gemcitabine treatment increases HSP27 phosphorylation in lung adenocarcinoma cells over time. Western blot analysis was performed to assess HSP27 phosphorylation in A549 and SPC-A1 cells over time. *P<0.05 vs. the 0 h group. HSP27, heat shock protein 27; p, phosphorylated.

ERp29 overexpression in cortical neurons following axotomy prevents the reduction of neurite length and neuron number, and protects from apoptosis. Furthermore, a previous study revealed that ERp29 is upregulated on exposure to radiation and ERp29 expression protects cells from damage caused by ionizing radiation (5). In the present study, lung adenocarcinoma cells were exposed to gemcitabine and ERp29 expression was significantly increased. This may be due to certain stimuli in the tumor microenvironment causing ERp29 upregulation, which may serve a cytoprotective role (9,17). In the current study, lung cancer cells were treated with gemcitabine for 24 h and the analysis of time-dependent effects of gemcitabine on ERp29 expression requires further examination.

Cell apoptosis is one of the main mechanisms of antineoplastic activity employed by chemotherapeutic agents (18). Gemcitabine exerts cytotoxic effects primarily by inducing tumor cell apoptosis via blockage of DNA synthesis and repair (19,20). In the present study, A549 and SPC-A1 apoptotic rates were increased following treatment with gemcitabine and the combined application of gemcitabine and ERp29 siRNA synergistically increased apoptotic rates further. Zhang and Putti (9) demonstrated that ERp29 downregulation significantly increased doxorubicin-induced apoptosis and ERp29 overexpression decreased cell apoptosis. Additionally, ERp29 inhibition enhances cell apoptosis induced by ionizing radiation and cigarette smoke extract via caspase-3 and -7 expression (5,12,21,22).

Gemcitabine is a cell cycle-specific drug that acts primarily in the S phase (23,24). In the present study, A549 cells were treated with 20 μM gemcitabine for 48 h. The percentage of cells in the S phase of the gemcitabine group was significantly higher than that of the control group. However, the combined application of gemcitabine and ERp29 siRNA did not alter the effect of gemcitabine. Few studies have assessed the role of ERp29 in cell cycle control and the exact mechanism requires further investigation (5,9).

HSPs can be divided into 5 families: HSP110, HSP90, HSP70, HSP60 and small HSP (25). HSP27, a small HSP with a molecular weight of 27 kDa, consists of 205 amino acid residues (26). The C-terminus of HSP27 contains a highly conserved α-crystallin domain and the N-terminus comprises a WDPF domain and a PSRLFDQXFGEXLL sequence (26). In the present study, ERp29 downregulation and treatment with gemcitabine combined with ERp29 siRNA were revealed to significantly increase HSP27 phosphorylation. Nakashima *et al* (27) have revealed that HSP27 phosphorylation is increased following pancreatic cancer cell treatment with gemcitabine. Similar results were observed in the current study. The phosphorylation of HSP27 is primarily catalyzed by mitogen-activated protein kinase-activated protein kinase (MAPKAPK)-2, MAPKAPK-3 and MAPKAPK-5, protein kinase (PK) A, PKB and PKC (28,29). Furthermore, MAPKAPK2 activation enhances damaging effects of gemcitabine on DNA and inhibits DNA repair (30). However, the association between ERp29 and HSP27 requires to be further elucidated.

In the current study, HSP27 downregulation significantly reduced chemosensitivity to gemcitabine in A549

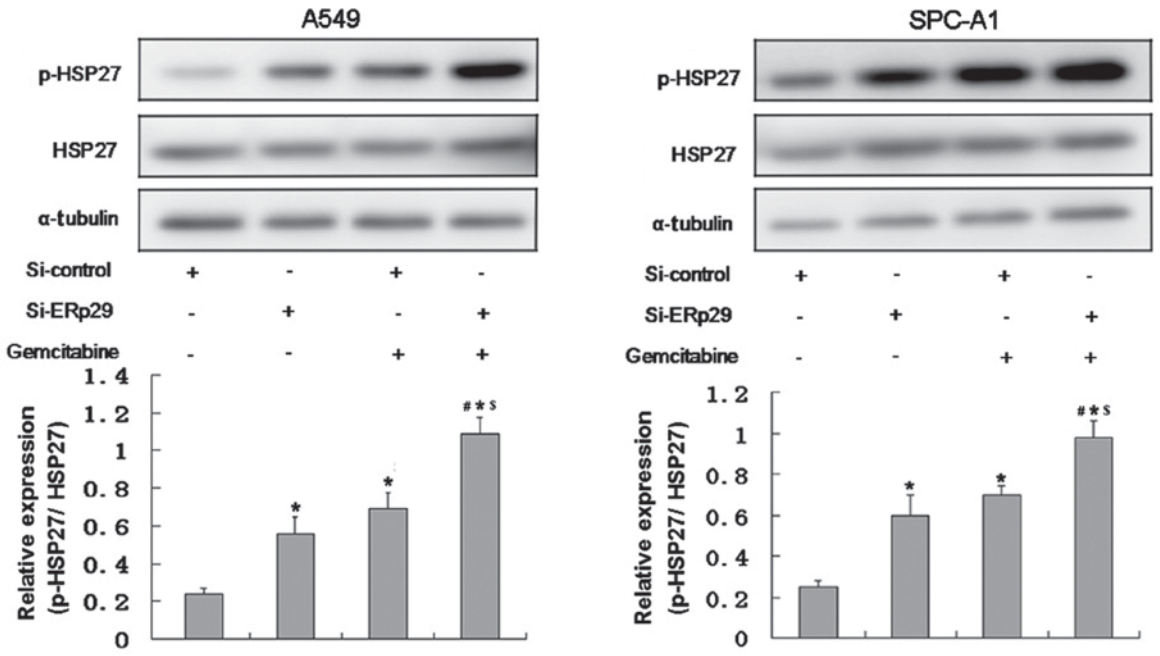

Figure 6. Combined gemcitabine and ERp29 siRNA treatment increases HSP27 phosphorylation. Gemcitabine and ERp29 siRNA synergistically increased HSP27 phosphorylation as determined by western blot analysis. *P<0.05 vs. Si-control; #P<0.05 vs. Si-ERp29; and $P<0.05 vs. Si-control + Gemcitabine. ERp29,endoplasmic reticulum protein 29; Si, small interfering RNA; HSP27, heat shock protein 27; p, phosphorylated.

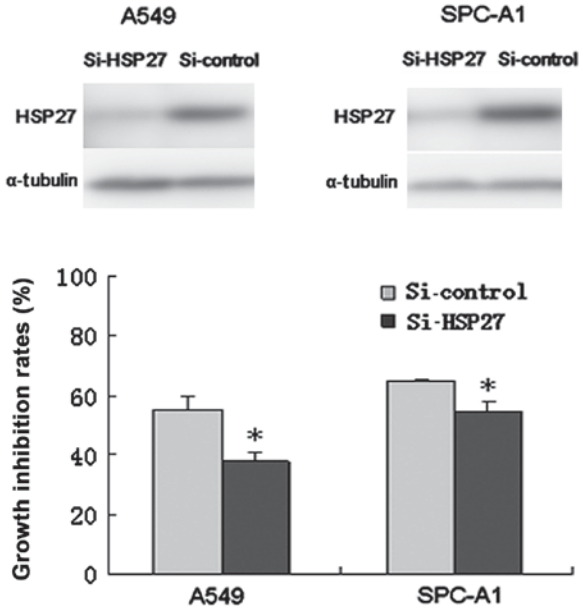

Figure 7. HSP27 downregulation reduces lung adenocarcinoma cell chemosensitivity to gemcitabine. HSP27 siRNA treatment significantly reduced HSP27 expression as determined by western blot and further decreased cell growth inhibition rates determined using cell counting kit-8 assays. *P<0.05 vs. the Si-control group. HSP27, heat shock protein 27; Si, small interfering RNA.

and SPC-A1 cells. This finding was consistent with a report by Schäfer *et al* (15), who revealed that HSP27 expression inhibition in AsPC-1 pancreatic cancer cells attenuated gemcitabine cytotoxicity. HSP27 downregulation has been demonstrated to inhibit apoptosis by regulating caspase-3, B-cell lymphoma/leukemia-2 (Bcl-2), Bcl-2 associated X protein and poly-ADP-ribose polymerase (PARP) (31,32). Guo *et al* (16) revealed that the combination of gemcitabine and HSP27 overexpression synergistically increased apoptosis in pancreatic cancer cells and increased PARP expression and caspase-3, -8 and -9 cleavage.

In summary, ERp29 expression was upregulated on exposure to gemcitabine and increased ERp29 expression protected lung adenocarcinoma cells from cytotoxic effects of gemcitabine. It was further revealed that ERp29 inhibition increases apoptotic rates induced by gemcitabine, which is one of the main mechanisms of its antitumor effect (18,33). ERp29 may therefore affect lung cancer cell chemosensitivity to gemcitabine by regulating HSP27 phosphorylation. However, further studies, including *in vivo* research, are required to verify these results.

ERp29 is a novel target, inhibition of ERp29 expression may be used to enhance the therapeutic effect of lung adenocarcinoma treatment with gemcitabine.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All datasets used and/or generated during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WY and GQ designed the study. WY, ZL, TT and JD performed the experiment. XZ, HW and XL analyzed the data

and prepared the manuscript. 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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