

Antitumor effect of ginsenoside Rg3 on gallbladder cancer by inducing endoplasmic reticulum stress-mediated apoptosis *in vitro* and *in vivo*

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Abstract. Recent studies have highlighted the importance of the endoplasmic reticulum (ER) in apoptotic processes. In the present study, the traditional herbal medicine ginsenoside Rg3 was used to treat gallbladder cancer *in vitro* and *in vivo*. The underlying signaling mechanisms were investigated using various molecular biology techniques, including flow cytometry, western blot analysis, ELISA and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). It was indicated that Rg3 exerted pro-apoptotic activity against the gallbladder cancer cell line GBC-SD through the ER stress-mediated signaling pathway. This was demonstrated by increased expression of phosphorylation of eukaryotic translation-initiation factor 2 α (eIF2 α), activating transcription factor 4 (ATF4), CCAAT/enhancer-binding protein homologous protein and lipocalin 2. In addition, eIF2 α and ATF4 knockdown attenuated the pro-apoptotic effect of Rg3 by inhibiting reactive oxygen species. Furthermore, the results of RT-qPCR analysis indicated that long intergenic non-protein coding RNA-p21 was significantly upregulated following Rg3 treatment. In conclusion, the results of the present study demonstrated that Rg3 inhibited tumor growth in a GBC-SD gallbladder cancer xenograft, by upregulating the ER stress-mediated signaling pathway. Therefore, ER

stress activation is suggested to mediate the antitumor effect of Rg3 in gallbladder cancer activity *in vitro* and *in vivo*.

Introduction

Gallbladder carcinoma (GBC) is the fifth most common neoplasm of the digestive tract and has an overall incidence of 3/100,000 (1). GBC is usually diagnosed at an advanced stage due to a lack of specific symptoms (1). According to epidemiological investigation, it has been reported that the 5-year survival rate for patients with GBC is <10% (1). Despite advances in GBC diagnostic and therapeutic techniques and management for consequent disease remission, prognosis for patients with gallbladder cancer remains poor (2). It has been reported that numerous post-surgery patients are likely to develop recurrence or even metastasis following surgery (2). Therefore, novel therapeutic strategies are required to enhance and improve the response and survival rate of patients with GBC.

Ginseng is a long-living perennial plant that is highly valued in herbal medicine. It has been used for over 2,000 years in various countries of East Asia, such as Korea, China, Japan and Vietnam (3). Ginsenoside Rg3, the most important biologically active compound of ginseng, has been demonstrated to have antitumor effects against several cancer cell lines, including breast cancer, non-small cell lung cancer, melanoma cancer and leukemia (4-7). Zhang *et al* (8) demonstrated that 20(S)-Rg3 inhibited proliferation and survival of GBC cells *in vitro* and *in vivo*. However, the underlying molecular mechanisms of Rg3 in human gallbladder cancer cell lines remain unclear.

The endoplasmic reticulum (ER) serves a major role in the synthesis, folding and the structural maturation of >33% of the total proteins composed in the cell (9). The importance of the endoplasmic reticulum (ER) in apoptotic processes has been recognized previously (10). A range of stressful cellular conditions, including aggregation of unfolded and misfolded proteins in the lumen of the ER, may trigger ER stress. During conditions of prolonged ER stress, pro-adaptive responses have been reported to fail, resulting in apoptosis (10). It has been demonstrated that ER stress triggers different biochemical processes, including leakage of calcium

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into the cytoplasm, leading to the activation of death effectors (10), the unfolded protein response (UPR), designating apoptosis (11) and reactive oxygen species (ROS) production (12). A thorough understanding of these responses may contribute to the development of novel treatments for cancer. Several drugs that activate ER stress have been approved for preclinical and clinical use, including sorafenib, eeyarestatin, tanespimycin, radicicol and MAL3-101 (13). ER stress has been reported to trigger the activation of protein kinase R-like ER kinase (PERK) and the subsequent phosphorylation of eukaryotic translation-initiation factor 2 α (eIF2 α), promoting translation of activating transcription factor 4 (ATF4) (14). In addition, ATF4 has been reported to activate the expression of CCAAT/enhancer-binding protein homologous protein (CHOP), as well as the growth arrest and DNA damage inducible gene (GADD153) (14). Lipocalin 2 (Lcn2), also known as neutrophil gelatinase-associated lipocalin, is a 25 kDa secretory glycoprotein and a member of the lipocalin family. It has been demonstrated that Lcn2 is a novel GADD153 target gene, participating in ER stress-induced apoptosis (15).

In the present study, it was demonstrated that Rg3 induces gallbladder cancer apoptosis by activating the ER stress-mediated apoptotic pathway *in vitro* and *in vivo*.

Materials and methods

Reagents. Rg3 was obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). An annexin V/fluorescein isothiocyanate (FITC)/propidium iodide (PI)-phycoerythrin (PE) apoptosis detection kit was purchased from Nanjing KeyGen Biotech. Co. Ltd. (Nanjing, China). Anti-eIF2 α was purchased from Affinity Biosciences (Cincinnati, OH, USA), and the anti-rabbit secondary antibodies (cat. no. A7016) were from Beyotime Institute of Biotechnology (Haimen, China). ROS, glutathione peroxidase (GSH-PX) and total superoxide dismutase (T-SOD) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). SYBR Green PCR Master mix was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Lipofectamine[®] 2000 transfection reagent was purchased from Thermo Fisher Scientific, Inc.

Cell culture and transfection. The human gallbladder cancer cell line GBC-SD was purchased from Shanghai Xiang Biotech (Shanghai, China). The cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated at 37°C with 5% CO₂. Transfection was performed using Lipofectamine 2000 transfection reagent, according to the manufacturer's protocols. GBC-SD cells were plated at a density of 5x10⁵ cells/well in 1.5 ml medium and cultured for 24 h prior to transfection. Control small interfering RNA (siRNA) (50 nM; siRNA-Scr) or targeted siRNAs (50 nM; eIF2 α -siRNA and ATF4-siRNA) were mixed with 2 μ l Lipofectamine 2000 reagent and incubated at room temperature for 20 min. The mixture was subsequently added to the cells at 250 μ l/well. The cells were cultured for 6 h and the medium was subsequently replaced with fresh RPMI-1640 medium supplemented with 10% fetal bovine serum.

Cell Counting Kit-8 (CCK-8) assay. The anti-proliferative effect of Rg3 on GBC-SD cells was detected using a CCK-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). GBC-SD cells (5,000 cells/well) were seeded into 96-well plates and cultured overnight. Subsequently, the cells were treated with 1, 25, 50 and 100 μ M Rg3, and DMSO (0.1%) (Sigma-Aldrich; Merck KGaA) was added to the negative control (NC) group at 37°C for 72 h. The medium was discarded and 10 μ l CCK-8 solution was added to the wells. GBC-SD cells were then incubated for another 4 h at 37°C with 5% CO₂ and cell viability was analyzed at a wavelength of 450 nm. The experiments were performed in triplicate.

Western blotting. The cells or isolated tumor tissues (100 mg) were lysed using radioimmunoprecipitation assay buffer lysis with protease inhibitors (Sigma Aldrich; Merck KGaA). The protein concentrations were determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of total protein (50 μ g) were separated by SDS-PAGE (10% gel), transferred onto a nitrocellulose membrane and blocked with 5% fat-free dried milk in PBS for 2 h at room temperature. The membrane was subsequently incubated at 4°C overnight with the following primary antibodies: Anti-PERK (cat. no. AF5304; 1:1,000), anti-p-PERK (cat. no. DF7576; 1:1,000), anti-eIF2 α (cat. no. AF6087; 1:1,000), anti-p-eIF2 α (cat. no. AF3087; 1:1,000), anti-ATF4 (cat. no. AF5416; 1:1,000), anti-Lcn2 (cat. no. DF6816; 1:1,000), anti- β -actin (cat. no. AF7018; 1:1,000). Following washing three times with Tris-buffered saline, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (cat. no. A7016; 1:5,000) (Beyotime Institute of Biotechnology) for between 4 and 5 h at 4°C. The immunoreactive bands were visualized using an enhanced chemiluminescence system (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and the signal was analyzed using a Tanon-5200 imagine system (Tanon Science and Technology Co., Ltd., Shanghai, China). All values were normalized to those of β -actin.

Detection of the apoptotic rate by flow cytometry. The apoptotic rate was detected using the annexin V-FITC/PI-PE apoptosis detection kit. Following 100 μ M Rg3 or/and 50 nM siRNA treatment for 72 h, GBC-SD cells were washed with ice-cold PBS and dual-stained with 4 μ l annexin V-FITC and 3 μ l PI-PE for 15 min, according to the manufacturer's protocols. Flow cytometric analysis was performed using a FACSCalibur[™] instrument with Mac Pro CellQPro Dongle software (BD Biosciences, Franklin Lakes, NJ, USA).

ROS detection assay. Following siRNA-Scr, Rg3 or/and siRNA treatment for 72 h, GBC-SD cells were incubated with RPMI-1640 medium containing 10 μ M 2',7'-dichlorofluorescein (DCF) diacetate for 1 h at 37°C, following termination of the reaction with 0.25% trypsin and washed twice with PBS. The intensity of DCF fluorescence was quantified using a fluorescence analyzer (FLX800T; BioTek Instruments, Inc., Winooski, VT, USA) at a wavelength of 408 nm. The cells were transfected with siRNA-Scr (50 nM) as negative control. The experiments were performed in triplicate.

ELISA detection of GSH-PX and superoxide dismutase (SOD). Following Rg3 or/and siRNA treatment for 72 h, GBC-SD cells were washed with PBS and harvested prior to GSH-PX and SOD detection. The determination of cellular GSH-PX and SOD was performed using GSH-PX (cat. no. A005) and SOD ELISA (cat. no. A001-3) kits (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's protocols. A microplate reader (Thermo Fisher Scientific, Inc.) was used to determine the optical density at 450 nm.

RNA preparation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The cells were treated with dimethylsulfoxide or 100 μ M Rg3 for 72 h. Total RNA was subsequently extracted using TRIzol[®] reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RNA was reverse-transcribed into first-strand cDNA in the presence of a poly(A) polymerase with an oligo(dT) adaptor. The cDNA template was amplified by RT-qPCR using the SYBR Green PCR Master mix, according to the manufacturer's protocol. Gene expression in each sample was normalized to GAPDH expression. The primer sequences used were as follows: GAPDH, forward, 5'-CGGAGTCAACGGATTTGGTCGTAT-3', reverse, 5'-AGCCTTCTCCATGGTGGTGAA GAC-3'; long non-coding RNA-p21 (LincRNA-p21), forward, 5'-GGGTGGCTCACTCTTCTGGC-3', reverse, 5'-TGGCCTTGCCCCGGGCTTGTC-3'; metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), forward, 5'-TCTGCA GGGACTACAGCAAG-3', reverse, 5'-TCACATTTGGTGA ATCCGTCT-3'; glutathione transferase Alpha 7 pseudogene (GSTA7P), forward, 5'-ATGACCTATTTACACTTAGC-3', reverse, 5'-AGCATATACTTTGGAAAAC-3'; long intergenic non-protein coding RNA 1093 (LINC01093), forward, 5'-AGT GAGCAATGCAATTCTGGGA-3', reverse, 5'-CATCTTAAA ACATGTTTATTTTCCA-3'. RT-qPCRs were performed using the ABI StepOne system with the StepOne[™] software (version 2.0; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. PCR cycling procedures were as follows: 94°C for 5 min, and 40 cycles of 94°C for 30 sec, 57°C for 35 sec and 72°C for 40 sec and 72°C for 10 min. The relative expression fold change of mRNAs was calculated using the 2^{- $\Delta\Delta$ Cq} method (16).

siRNA sequences synthesis. Using the tool from the Ambion website (<http://www.thermofisher.com/cn/zh/home/brands/ambion.html/>), three siRNA sequences (sense strand and antisense strand) were designed for the human eIF2 α and ATF4 gene mRNAs with the following sequences: eIF2 α -siRNA1, sense, 5'-CAGCCU UACACUACUUCUATT-3', antisense, 5'-UAGAAGUAGUGU AAGGCUGTT-3'; eIF2 α -siRNA2, sense, 5'-GGUAAUAGC UAGCAGAUUTT-3', antisense, 5'-AUCUGUGCUAGCUAU UACCTT-3'; eIF2 α -siRNA3, sense, 5'-CGGUUAAUAAU GGAUACATT-3', antisense 5'-UGUAUCCAUUUAACCC GTT-3'; ATF4-siRNA1, sense, 5'-CAAGTCCTTACACU ACTTCG-3', antisense, 5'-TACAAGCGAGTCCTAAAGGCT GTT-3'; ATF4-siRNA2, sense, 5'-AGGAGCAAACAAGA CAGCATTTT-3', antisense, 5'-ATGCTGTCTTGTTTTGGCT CCTTTT-3'; ATF4-siRNA3, sense, 5'-CTGGATCATGGT GGATAGACCTT-3', antisense, 5'-CGTAACCAGCATTAA CGCGTT-3'. The siRNA scrambled sequence (sense, 5'-CAG

CTTACACUACAACtATT-3'; antisense, 5'-CAGTTACGU AATCGTT-3') was used as control. All single-stranded siRNAs were chemically synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). siRNAs, as aforementioned, at a concentration of 50 nM were transfected into GBC-SD cells for 48 h using Lipofectamine 2000. Western blot analysis was used to determine the transfection efficiency.

Subcutaneous xenograft model. In the present study, 12 5-week-old BALB/c nude male mice (18-20 g) were obtained from Cavens (Changzhou, China). The study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animals were housed at a constant room temperature with a 12-h light/12-h dark cycle and fed on a standard rodent diet and water. GBC-SD cells were harvested, washed and resuspended in PBS. The animal tumor model was generated by subcutaneous injection with 0.2 ml cell suspension containing 5x10⁶ cells into the right flank of nude mice. Tumor sizes were measured every 3 days, and tumor volumes were calculated using the formula: Tumor volume (mm³)=0.5x length (mm) x width² (mm²). When the tumor reached an average volume of 100 mm³, each mouse was administered orally with saline or 20 mg/kg Rg3 once daily for 21 days (n=6). The mice were sacrificed at the end of the treatment period and the tumors were resected for an immunoblotting assay. All animal procedures were approved by the Ethics Committee of Zhejiang Chinese Medical University Animal Center (Hangzhou, China; approval no. 201703345).

Statistical analysis. Data are expressed as the mean \pm standard deviation from ≥ 3 independent experiments. For *in vitro* experiments and *in vivo* mouse experiments, a two-sided Student's t-test was applied for comparison of continuous variables between two groups, and statistical differences among multiple groups were analyzed by one-way ANOVA followed by Dunnett's test (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Rg3 activates the ER stress-mediated PERK pathway in the gallbladder cancer cell line. CCK-8 assay was used to detect the effect of Rg3 on GBC-SD proliferation. As presented in Fig. 1A, Rg3 dose-dependently inhibited GBC-SD proliferation *in vitro*. To further demonstrate the effect of Rg3 on the ER stress-mediated cell apoptotic pathway, GBC-SD cells were stimulated with 1, 25, 50 and 100 μ M Rg3 for 72 h and harvested for western blot analysis. The results indicated that 1 μ M Rg3 did not affect the protein expression of eIF2 α , p-eIF2 α , ATF4 and Lcn2. However, 50 μ M and 100 μ M Rg3 significantly increased the protein expression of p-eIF2 α , ATF4 and Lcn2, but not of eIF2 α (Fig. 1B-F). These results suggested that a higher concentration of Rg3 promoted the UPR-PERK signaling pathway in the gallbladder cancer cell line.

eIF2 α knockdown attenuates Rg3-mediated gallbladder cancer cell apoptosis. To further detect the effect of Rg3

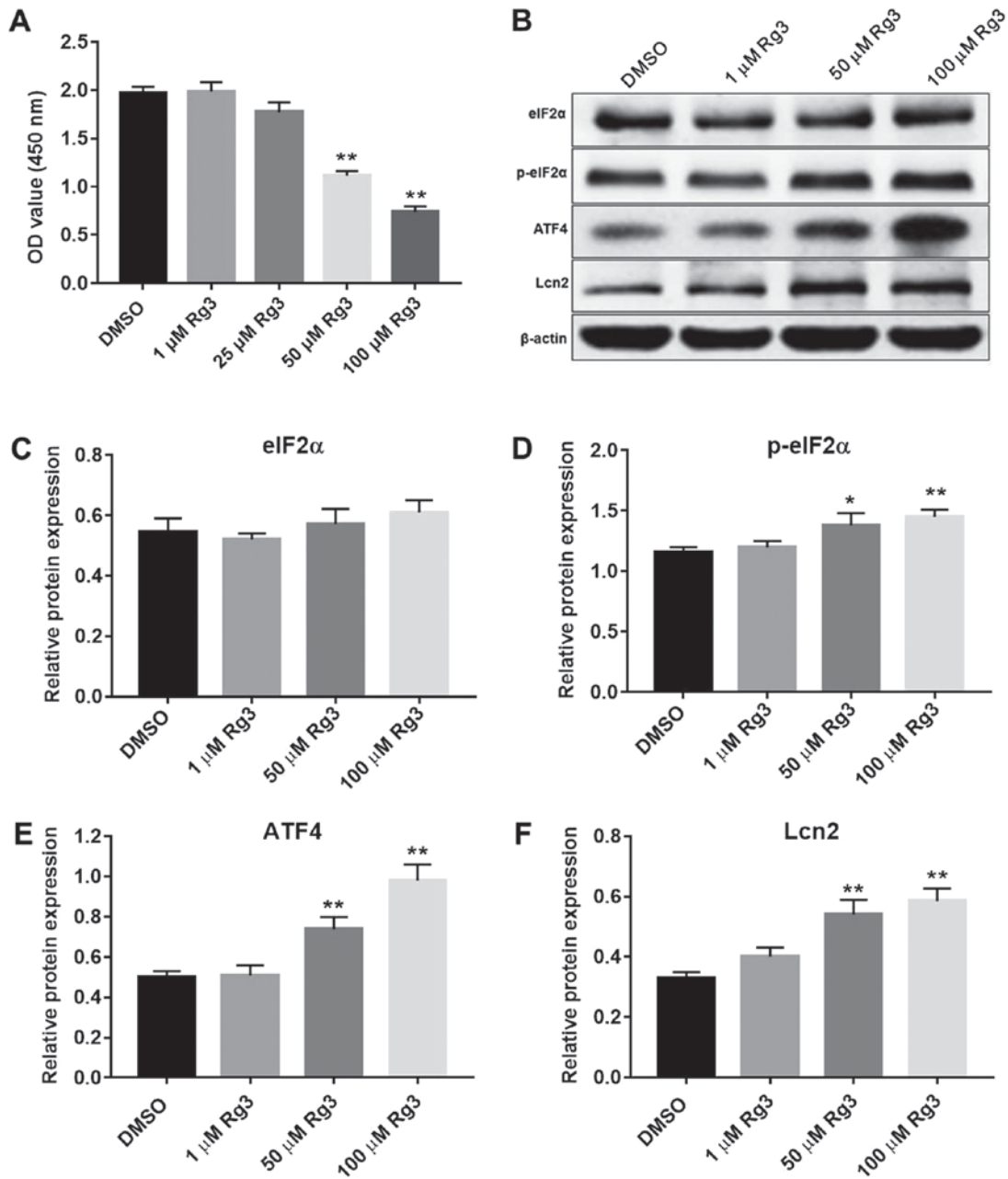


Figure 1. Rg3 activates ER stress in GBC-SD cells. (A) GBC-SD cells were treated with 1, 25, 50 and 100 μ M Rg3 for 72 h, and proliferation was determined using a CCK-8 kit. (B) Rg3 induced p-eIF2 α , ATF4 and Lcn2 expression in GBC-SD cells. Relative protein expression level of (C) eIF2 α , (D) p-eIF2 α , (E) ATF4 and (F) Lcn2 compared with the DMSO group. Relative protein expression was quantified by normalizing to the internal control β -actin (n=3). *P<0.05, **P<0.01 vs. DMSO group. OD, optical density; ER, endoplasmic reticulum; eIF2 α , eukaryotic translation-initiation factor 2 α ; ATF4, activating transcription factor 4; p-, phospho-; DMSO, dimethylsulfoxide; Lcn2, lipocalin 2.

on the UPR-PERK signaling pathway, an RNA interference-mediated method was used to silence eIF2 α expression to determine the biological effects in GBC-SD cells. Western blot analysis revealed a decrease in eIF2 α protein expression levels in siRNA-transfected GBC-SD cells. Compared with eIF2 α -siRNA1 and eIF2 α -siRNA2, eIF2 α -siRNA3 resulted in the most marked suppression of eIF2 α protein expression, whereas control siRNA vectors had no effect on eIF2 α protein expression (Fig. 2A and B). Therefore, eIF2 α -siRNA3 was selected to knock down eIF2 α in subsequent studies. As indicated in Fig. 2C and D, eIF2 α -siRNA3 significantly decreased the protein expression levels of ATF4, Lcn2 and CHOP in the cells. In addition, Rg3-induced ATF4, Lcn2

and CHOP upregulation was reversed by eIF2 α knockdown. These results indicated that Rg3 increased the expression of ATF4, Lcn2 and CHOP, whereas eIF2 α knockdown attenuated the expression of these proteins in gallbladder cancer cells.

Subsequently, the induction of GBC-SD cell apoptosis by Rg3 was assessed. As indicated in Fig. 2E and F, 100 μ M Rg3 significantly induced apoptosis (23.9%) compared with the control (5.71%). However, the apoptotic rate induced by Rg3 was decreased from 23.95 to 11.27% in the presence of eIF2 α -siRNA (P<0.01). The present data suggest that Rg3 may induce gallbladder cancer cell apoptosis, which was significantly reversed by eIF2 α knockdown.

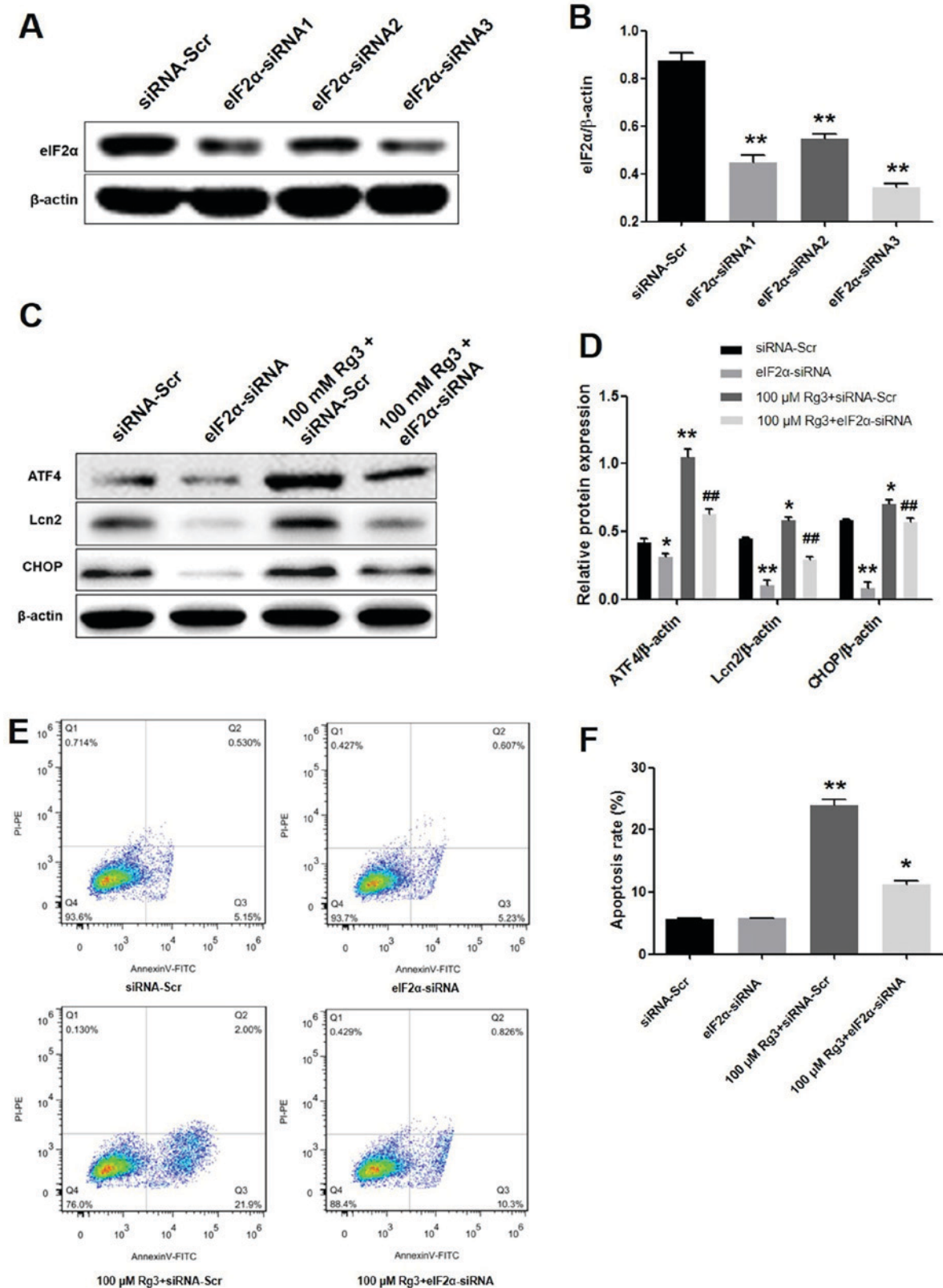


Figure 2. eIF2α knockdown attenuates Rg3-mediated gallbladder cancer cell apoptosis. (A) GBC-SD cells were transfected with siRNA-Scr, eIF2α-siRNA1, eIF2α-siRNA2 and eIF2α-siRNA3. eIF2α-siRNA3 resulted in the highest level of suppression of eIF2α protein expression compared with eIF2α-siRNA1 and eIF2α-siRNA2, whereas siRNA-Scr vectors had no effect on the levels of eIF2α protein expression. (B) Suppression of eIF2α protein expression was significantly increased in the eIF2α-siRNA3 group. **P<0.01 vs. siRNA-Scr control group. Relative protein expression levels were quantified by normalizing to the internal control β-actin (n=3). (C) Effects of eIF2α-siRNA on the protein levels of ATF4, Lcn2 and CHOP induced by Rg3. (D) Quantification of the effects of eIF2α-siRNA induced by Rg3 on the relative protein expression levels of ATF4, Lcn2 and CHOP by normalizing to the internal control β-actin (n=3). *P<0.05, **P<0.01 vs. siRNA-Scr group; ##P<0.01 vs. 100 μM Rg3 + siRNA-Scr group. (E) The cells were treated with eIF2α-siRNA or/and 100 μM Rg3 for 72 h and subsequently stained with annexin V and PI. (F) The apoptotic rate of eIF2α-siRNA induced by Rg3, compared with siRNA-Scr control group. *P<0.05, **P<0.01 vs. siRNA-Scr. eIF2α, eukaryotic translation-initiation factor 2α; siRNA, small interfering RNA; siRNA-Scr, scrambled siRNA; ATF4, activating transcription factor 4; CHOP, CCAAT/enhancer-binding protein homologous protein; DMSO, dimethylsulfoxide; Lcn2, lipocalin 2; PI, propidium iodide; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

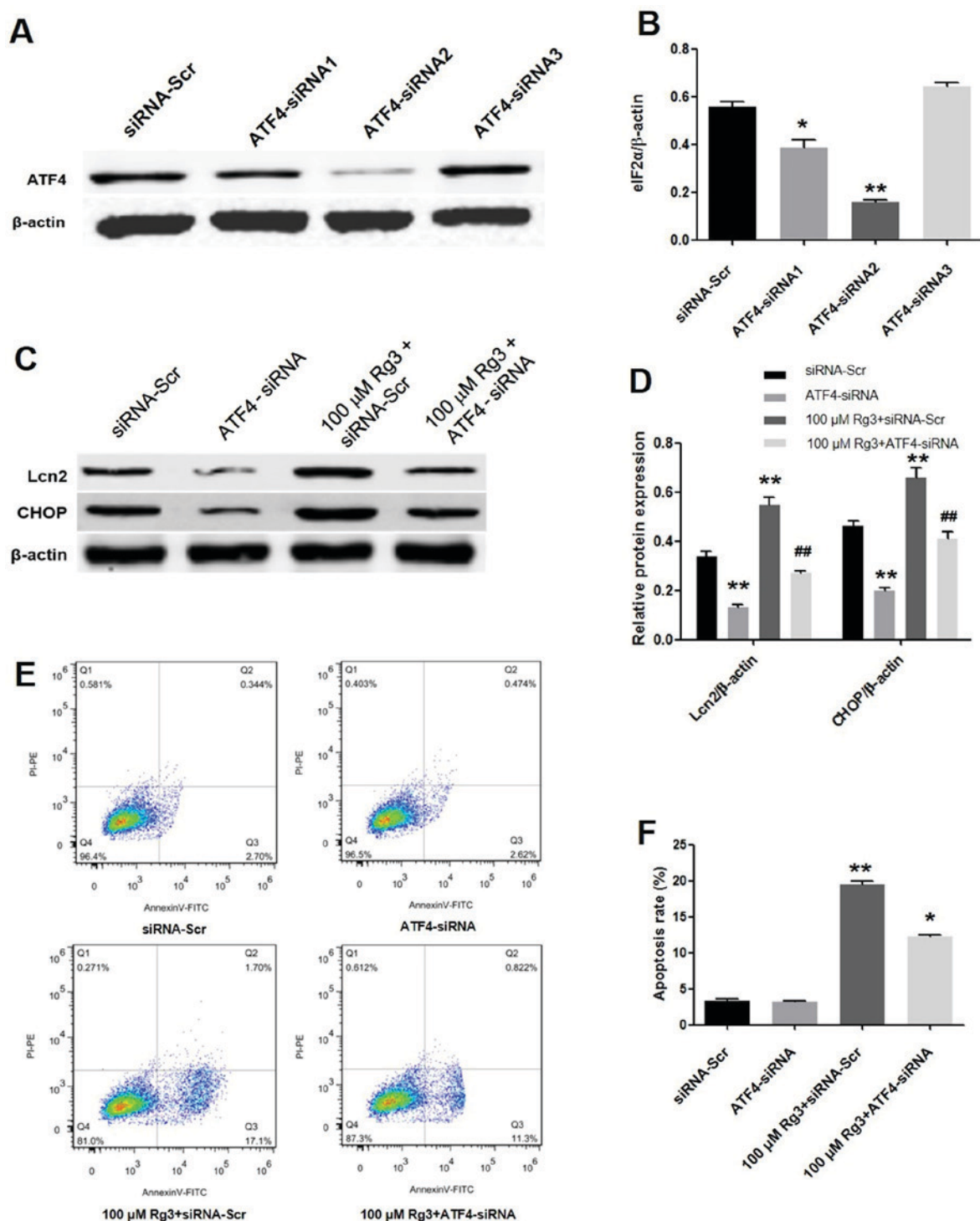


Figure 3. ATF4 knockdown attenuates Rg3-mediated gallbladder cancer cell apoptosis. (A) Western blot analysis of GBC-SD cells transfected with siRNA-Scr control, ATF4-siRNA1, ATF4-siRNA2 and ATF4-siRNA3. (B) Quantification of relative protein expression levels by normalizing to the internal control β -actin ($n=3$). * $P<0.05$, ** $P<0.01$ vs. siRNA-Scr group. (C) Western blot analysis of protein levels of cells treated with ATF4-siRNA2 or/and Rg3. (D) Quantification of the effects of ATF4-siRNA induced by Rg3 on the relative protein expression levels of ATF4, Lcn2 and CHOP by normalizing to the internal control β -actin ($n=3$). ** $P<0.01$ vs. siRNA-Scr group; ## $P<0.01$ vs. 100 μ M Rg3 + siRNA-Scr group. (E) Cells were treated with eIF2 α -siRNA or/and 100 μ M Rg3 for 72 h and subsequently stained with annexin V and PI. (F) The apoptotic rate of ATF4-siRNA induced by Rg3, compared with siRNA-Scr group ($n=3$). * $P<0.05$, ** $P<0.01$ vs. siRNA-Scr. eIF2 α , eukaryotic translation-initiation factor 2 α ; siRNA, small interfering RNA; siRNA-Scr, scrambled siRNA; ATF4, activating transcription factor 4; CHOP, CCAAT/enhancer-binding protein homologous protein; DMSO, dimethylsulfoxide; Lcn2, lipocalin 2; PI, propidium iodide; PE, phycoerythrin.

ATF4 knockdown attenuates Rg3-mediated gallbladder cancer cell apoptosis. A total of three ATF4-siRNAs were constructed to interfere with the expression of ATF4. Western blot analysis revealed that ATF4-siRNA2 exhibited

the highest inhibition of ATF4 expression compared with other vectors (Fig. 3A and B). Subsequently, GBC-SD cells were transfected with ATF4-siRNA2 and the levels of PERK pathway proteins were analyzed. As presented in

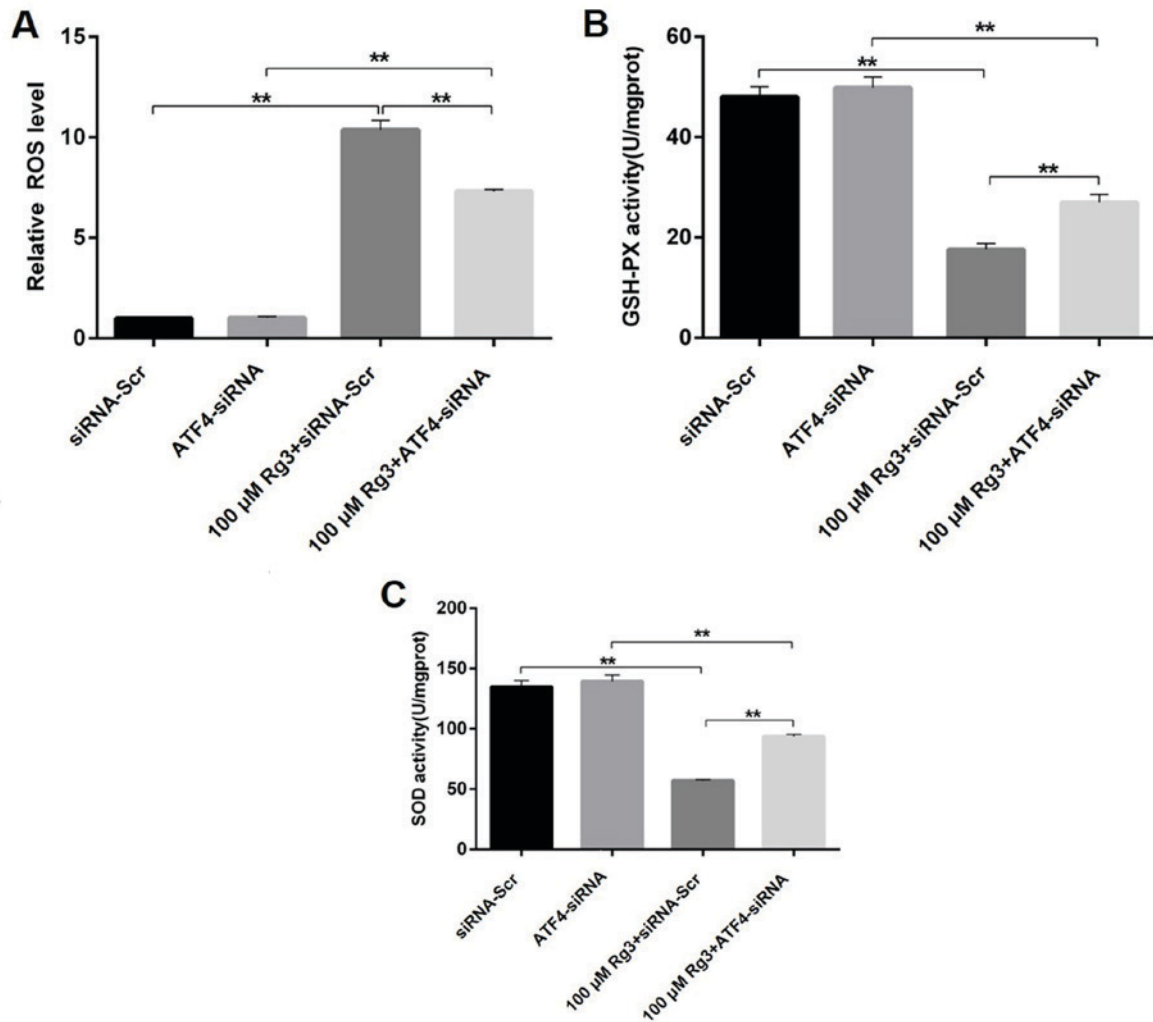


Figure 4. ATF4 knockdown attenuates Rg3-mediated ROS generation in gallbladder cancer cells. (A) GBC-SD cells were treated with ATF4-siRNA2 or/and Rg3 and the ROS generation was detected using a 2',7'-dichlorofluorescein diacetate assay after 72 h of culture. ** $P < 0.01$; $n = 3$. (B) GSH-PX, and (C) SOD activities were detected by ELISA. ** $P < 0.01$; $n = 3$. siRNA, small interfering RNA; ROS, reactive oxygen species; siRNA-Scr, scrambled siRNA; GSH-PX, glutathione peroxidase; SOD, superoxide dismutase; ATF4, activating transcription factor 4.

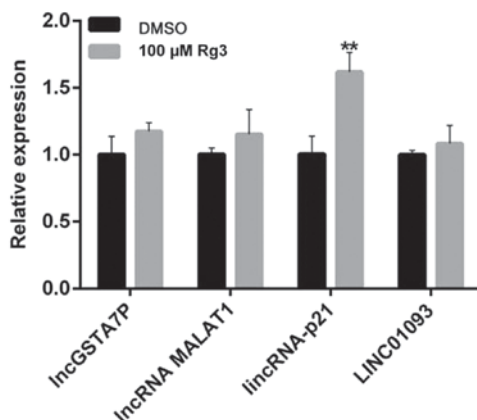


Figure 5. Effects of Rg3 on the expression of lncRNAs in gallbladder cancer cells. GBC-SD cells were treated with 100 μM Rg3 for 72 h, and the reverse transcription-quantitative polymerase chain reaction was performed to determine the expression of lincRNA-p21, lncMALAT1, lncGSTA7P and LINC01093. The relative expression levels were normalized to the DMSO control ($n = 3$). ** $P < 0.01$ vs. DMSO group. DMSO, dimethylsulfoxide; lincRNA-p21, long intergenic non-coding RNA-cyclin-dependent kinase inhibitor 1A; lncRNA, long non-coding RNA; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; GSTA7P glutathione transferase Alpha 7, pseudogene; LINC01093, long intergenic non-protein-coding RNA 1093.

Fig. 3C and D, Rg3-induced Lcn2 and CHOP upregulation were reversed by ATF4 knockdown. Flow cytometric analysis of the apoptosis assay indicated that ATF4 inhibition significantly decreased apoptosis induced by Rg3 ($P < 0.01$; Fig. 3E and F).

ATF4 knockdown attenuates Rg3-mediated ROS generation in gallbladder cancer cells. ROS are small molecules that are highly reactive as a result of the presence of unpaired electrons. An increase in the protein-folding load in the ER can lead to the accumulation of ROS. GSH-PX and SOD are the primary enzymes, which work synergistically to neutralize ROS (17). In order to further confirm the role of ER stress during Rg3-induced GBC-SD apoptosis, the generation of ROS, GSH-PX and SOD were determined. As presented in Fig. 4A, 100 μM Rg3 significantly increased the ROS level compared with the scrambled control group, whereas ATF4 knockdown diminished this effect. The ELISA data also indicated that the enzyme activities of GSH-PX and SOD were significantly decreased by Rg3 treatment, which were partly recovered in the presence of ATF4-siRNA ($P < 0.01$; Fig. 4B and C). These results suggest that Rg3 induced ER stress-mediated cell death

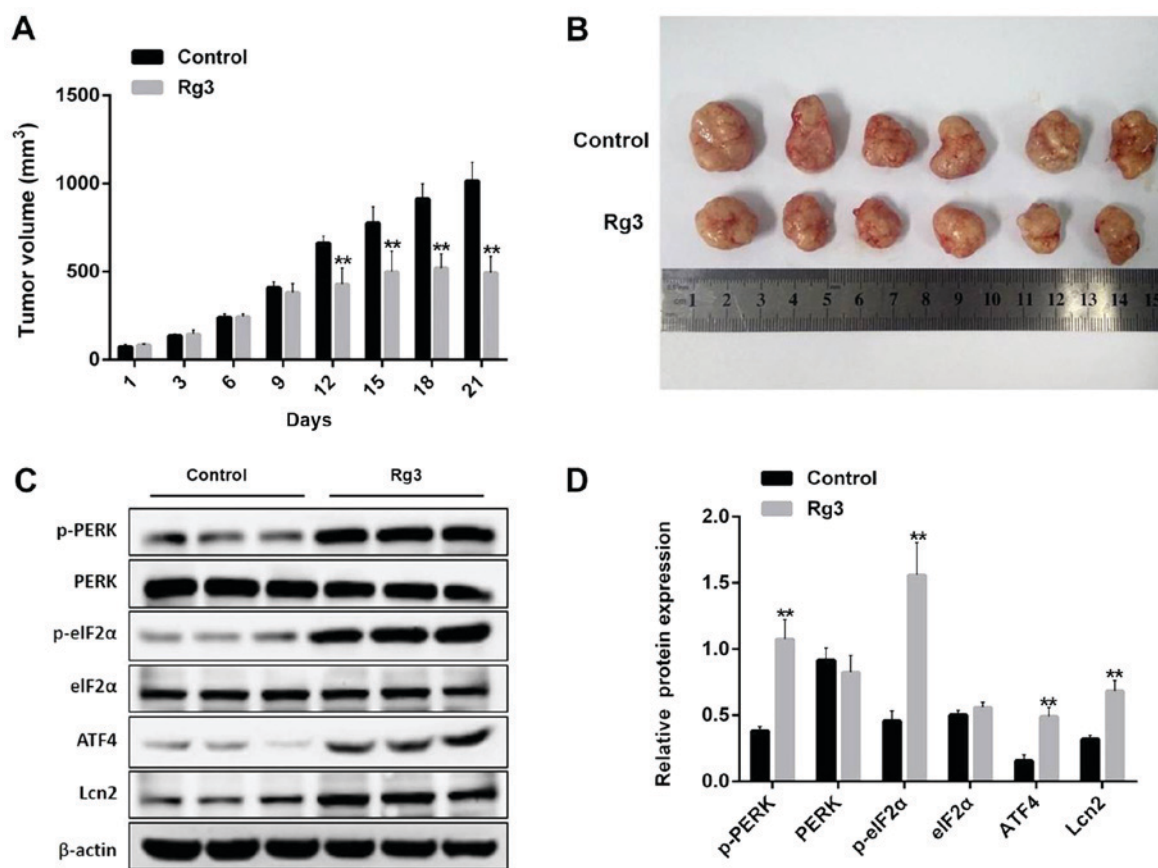


Figure 6. Rg3 inhibits tumor growth through the ER stress-mediated pathway in a xenograft model. (A) The GBC-SD xenograft mice were treated once a day with 0.2 ml saline or 0.2 ml Rg3 (20 mg/kg) over a period of 21 days and tumor volumes were determined. ** $P < 0.01$ vs. control group. (B) The mice were sacrificed on day 21 and the tumors were isolated and measured with a caliper ($n = 6$). (C) Tumor tissues from each group were processed for the proteins p-PERK, PERK, p-eIF2 α , eIF2 α , ATF4 and Lcn2 detection. (D) Relative protein expression levels of p-PERK, PERK, p-eIF2 α , eIF2 α , ATF4 and Lcn2 were quantified by normalizing to internal control β -actin ($n = 3$). ** $P < 0.01$ vs. control group. ATF4, activating transcription factor 4; ER, endoplasmic reticulum; PERK, eukaryotic translation initiation factor 2 α kinase 3; p-, phospho-; Lcn2, lipocalin 2; eIF2 α , eukaryotic translation-initiation factor 2 α .

due to increased ROS generation, which was partly reversed by ATF4 knockdown.

Effects of Rg3 on the expression of long non-coding RNA (lncRNA) in gallbladder cancer cells. lncRNAs are transcribed RNA molecules of >200 nucleotides and with no significant protein-coding capacity (18). Important research into the role of lncRNAs in the diagnosis and prognosis of various types of cancer, such as gallbladder carcinoma, is being conducted (18). In order to investigate the role of lncRNA in the Rg3-induced ER stress activation, lincRNA-p21, lincRNA MALAT1, lincRNA MALAT1P and LINC01093 were evaluated. As presented in Fig. 5, Rg3 significantly increased the expression of lincRNA-p21 ($P < 0.01$), but had no effect on the other three lncRNAs. LincRNA-p21 is a downstream lncRNA transcript of p53, which participates in diverse biological processes, including apoptosis, cell cycle, metabolism and pluripotency. LincRNA-p21 is implicated in the development and progression of a number of human diseases, particularly cancer (19). The results of the present study revealed that lincRNA-p21 was involved in Rg3-induced ER stress activation.

Rg3 inhibits tumor growth through the ER stress-mediated pathway in a xenograft model. To assess the antitumor effect of Rg3 on gallbladder cancer *in vivo*, a GBC-SD subcutaneous

xenograft model was used. As presented in Fig. 6A and B, tumor growth was significantly inhibited with Rg3 treatment from day 12, compared with the vehicle group. In addition, western blot analysis of tumor samples identified that the expression of p-PERK, p-eIF2 α , ATF4 and Lcn2 was increased by Rg3 treatment compared with the vehicle group (Fig. 6C and D). Furthermore, treatment with Rg3 did not reveal any adverse effect on the body weight of the mice, in comparison with the vehicle group, during the experimental period (data not shown), indicating the safety of Rg3. The aforementioned data were consistent with the *in vitro* study, which further demonstrated Rg3 induced gallbladder cancer apoptosis through the ER stress pathway.

Discussion

Ginsenoside Rg3 is a bioactive ginseng constituent that has been reported to inhibit proliferation of numerous cancer cell lines; however, the underlying mechanism remains unclear (5-7). In the present study, it was demonstrated using flow cytometry analysis with annexin V-FITC/PI staining that Rg3 treatment led to significant GBC-SD human gallbladder cancer cell apoptosis. Zhang *et al* (8) also revealed that Rg3 induced a dose-dependent increase in GBC-SD cell apoptosis, which is consistent with the results of the present

study. Furthermore, it was demonstrated that Rg3 inhibited tumor growth in a GBC-SD xenograft nude mice model, in accordance with the results of Zhang *et al* (8), who used NOZ cells to construct the animal model.

At the molecular level, it was revealed that pathological ER stress activation could be the key signaling mechanism in gallbladder cancer cells. The hallmarks of ER stress, p-eIF2 α and ATF4, were upregulated in Rg3-treated GBC-SD cell lines and xenograft mice models. Wang and colleagues indicated that Rg3 induced anti-gallbladder cancer cell activity *in vitro* and *in vivo* was mediated by ER stress activation (8). The activation of CHOP, PERK and inositol-requiring enzyme 1 (IRE1) were additionally involved. It has been reported that, under ER stress, binding immunoglobulin protein chaperone dissociates from the luminal domain of PERK, which leads to the activation of three sensors, PERK, IRE1 and ATF6 (20). One of the mechanisms of ER stress-induced apoptosis involves sequential steps of PERK-mediated eIF2 α phosphorylation, preferential translation of ATF4/cyclic AMP-response element-binding protein 2 mRNA and induction of CHOP/GADD153. In the present study, Rg3 was identified to inhibit GBC-SD cell apoptosis through the PERK/p-eIF2 α /ATF4/CHOP/Lcn2 signaling pathway *in vitro* and *in vivo*, which was further validated by knockdown of eIF2 α and ATF4 in GBC-SD cells. Increased expression of ATF4 and CHOP results in increased protein synthesis, which produces ROS, a necessary signal to induce apoptosis in response to ER stress. Increased ROS expression was also detected along with decreased GSH-PX and SOD activity. These results indicated that Rg3-induced gallbladder cancer cell apoptosis was mediated through ER stress signal pathway.

An important result of the present study is that lincRNA-p21 was significantly increased by Rg3 treatment in GBC-SD cells. lincRNA-p21 interacts with a number of RNA-binding proteins, microRNAs and mRNA targets, and regulates the expression of the targets (21). lincRNA-p21 has been identified to be a novel regulator of proliferation, apoptosis and DNA damage response (21). The results of the present study revealed that p21 is involved in Rg3-induced ER stress activation in gallbladder cancer cells.

Prognosis, preoperative diagnosis, surgical management and systemic treatment of gallbladder cancer remains a problem. In the present study, the traditional herbal medicine Rg3 was used to treat gallbladder cancer *in vitro* and *in vivo*. The results revealed pathological ER stress activation mediated the antitumor effect of Rg3 on gallbladder cancer *in vitro* and *in vivo*, providing a novel strategy for anticancer drug design and development based on Rg3.

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Availability of data and materials

All datasets used in this study are available from the corresponding author upon reasonable request.

Authors' contributions

KW, JH, NL and TX performed experiments, analyzed data and were the major contributors in writing the manuscript. WC and ZY performed experiments. KW and JH collected tissues, interpreted the patient data and reviewed the final version of the manuscript.

Ethics approval and consent to participate

All animal procedures were approved by the Ethics Committee of The First Affiliated Hospital of Zhejiang Chinese Medical University (Hangzhou, China; approval no. 201703345).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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