

Ghrelin inhibits cisplatin-induced MDA-MB-231 breast cancer cell apoptosis via PI3K/Akt/mTOR signaling

JINYU ZHANG¹ and TIANHAO XIE²

¹Department of Oncology, Medical College of Hebei University; ²Department of General Surgery, Affiliated Hospital of Hebei University, Baoding, Hebei 071000, P.R. China

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Abstract. Ghrelin is a multi-functional peptide, its role on cancer cell apoptosis remains controversial. The present study examined the effects and mechanisms of ghrelin on cisplatin-induced apoptosis in human breast cancer cells. It was identified that ghrelin inhibited apoptosis in MDA-MB-231 cells *in vitro* and reversed the expression of B-cell lymphoma 2 (Bcl2) and Bcl2-associated X, and cleaved caspase-3 induced by cisplatin. Furthermore, ghrelin activated the phosphoinositide 3-kinases/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway after cisplatin treatment. The effects of ghrelin on the cisplatin-induced apoptosis and PI3K/Akt/mTOR signaling were reversed by the growth hormone secretagogue receptor small interfering RNA. The present study suggests that ghrelin may serve as a novel target for cisplatin resistance and a potential indicator of cisplatin sensitivity in breast cancer treatment.

Introduction

Ghrelin is a 28-amino acid peptide produced in the stomach of most organisms, it is the endogenous ligand for the growth hormone secretagogue receptor (GHSR) (1-3). Studies have shown that ghrelin and growth hormone secretagogue receptor are expressed in many tumors and may serve a role in cancer progression (4,5). Ghrelin in particular has been associated with tumor cell proliferation, survival and apoptosis (6-9). Indeed, the biological function of ghrelin in tumor cell proliferation and apoptosis is still controversial.

The effects of ghrelin on cell proliferation and apoptosis mainly depends on the cell type. For example, ghrelin can regulate proliferation of pancreatic adenocarcinoma cells and HT-29 colon cancer cells (10,11). Studies have shown that ghrelin inhibits lipopolysaccharide (LPS)-induced A549 cell apoptosis via phosphoinositide 3-kinases (PI3K)/Akt and

extracellular signal-regulated kinases (ERK) signaling (12). On the other hand, ghrelin appears to inhibit lung cancer cell proliferation (13) and another study reported that ghrelin induces colon carcinoma cell apoptosis (14).

Ghrelin and GHSR have been observed to be expressed in breast cancer and serve a role in breast cancer tumorigenesis (15). Previous studies have suggested ghrelin as a prognostic marker and potential therapeutic target in breast cancer (16). Ghrelin has also been reported to inhibit (17) and promote breast cancer cell proliferation (15). The effects of ghrelin on breast cancer cell apoptosis and its associated molecular mechanisms remain unclear. The present study explores the effects of ghrelin on cisplatin-induced apoptosis in human breast cancer cells and the underlying mechanisms.

Materials and methods

Reagents. Ghrelin was acquired from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratory (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Cell culture reagents, including Dulbecco's modified Eagle's medium (DMEM), glucose, foetal bovine serum (FBS), penicillin and streptomycin, were acquired from Gibco (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA). AnnexinV-FITC was purchased from Pharmingen (San Diego, CA, USA). Propidiumiodide, 4',6-diamidino-2-phenylindole and cisplatin were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Antibodies against PI3K, phospho-PI3K, Akt, phospho-Akt, mammalian target of rapamycin (mTOR), phospho-mTOR, B-cell lymphoma 2 (Bcl2), Bcl2-associated X (Bax) and Caspase-3 were obtained from Cell Signaling Technology, Inc., (Danvers, MA, USA). The *in-situ* apoptosis detection kit was purchased from Roche Diagnostics (Basel, Switzerland). Rapamycin (cat. no. sc-3504), LY 294002 (cat. no. sc-201426), and β -actin (cat. no. sc-47778) were purchased from Santa Cruz Biotechnology, Inc., Dallas, TX, USA.

Cell culture and treatment. MDA-MB-231 cells were purchased from the China Center for Type Culture Collection (Wuhan, China). The cells were cultured in Dulbecco's modified Eagle's medium with glucose (4.5 mg/ml), L-glutamine (Invitrogen; Thermo Fisher Scientific, Inc.) (4 mM), 10% FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml), in

Correspondence to: Dr Tianhao Xie, Department of General Surgery, Affiliated Hospital of Hebei University, 212 East Yuhua Road, Baoding, Hebei 071000, P.R. China
E-mail: 15803329062@163.com

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a humidified atmosphere with 5% CO₂ at 37°C. For *in vitro* studies, MDA-MB-231 cells were pretreated with or without rapamycin (20 nM; Santa Cruz Biotechnology, Inc.) or LY294002 (10 μM; Santa Cruz Biotechnology, Inc.) for 30 min, and then treated with ghrelin (50 nM) and/or cisplatin (25 μM) for 48 h at 37°C and 5% CO₂. MDA-MB-231 cells treated without ghrelin and/or cisplatin were defined as the control group. Cell apoptosis and western blot analyses were performed.

Cell viability assay. To determine a suitable concentration for cisplatin intervention, a CCK-8 assay was used to monitor cell viability. Briefly, MDA-MB-231 cells were seeded in 96-well culture plates with 5x10³ cells in 100 μl of culture medium per well. MDA-MB-231 cells were treated with cisplatin at the concentrations (0, 1, 10, 25 and 50 μM) described in the results for 48 h at 37°C. Cells that did not receive cisplatin treatment were considered the control group. Cell viability was measured using the CCK-8 and the optical density was detected with a microculture plate reader (BioTek Instruments, Inc., Winooski, VT, USA) at 450 nm. Each assay was performed in triplicate.

Cell transfection. For GHSR silencing, MDA-MB-231 cells were transfected with GHSR small interfering (si)RNA: forward, 5'-CCACAAACAGACAGUGAAGUU-3' and reverse, 5'-CUUCACUGUCUGUUUGUGGUU-3' and scrambled siRNA: forward, 5'-CAACAACGAAGCGACAUAUC-3' and reverse, 5'-UUAUGUCGCUUCGUUGUUGUC-3'; obtained from Ribobio (Guangzhou, China). Cells were plated in 6-well plates and cultured for 24 h in media without antibiotics, after which GHSR siRNA or the scrambled siRNA was transfected at a final oligonucleotide concentration of 100 nM using Lipofectamine[®] 2000 (Invitrogen, Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The experiments were performed 48 h after transfection.

Caspase-3 assay. Caspase-3 activation was detected using the Caspase-3 Activity Assay Kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. MDA-MB-231 cells were exposed to test substances for 48 h at 37°C. Subsequently, the culture medium was removed, and cells were resuspended in lysis buffer after washing with ice-cold PBS. Incubation on ice followed for 15 min. After centrifugation at 14,000 x g for 15 min at 4°C, the supernatant was transferred to a fresh tube. Caspase-3 activity was determined using a colorimetric activity assay, which is based on spectrophotometric detection of p-nitroaniline (pNA) after catalysis from the labeled substrate, Ac-DEVD-pNA (Beyotime Institute of Biotechnology). Free pNA was quantified at 405 nm using an enzyme-linked immunosorbent assay reader (BioTek Instruments, Inc., Winooski, VT, USA).

Apoptosis assay by flow-cytometry. MDA-MB-231 cells were seeded in 6-well plates at a density of 2x10⁵ cells/well. Tumor cells were harvested and incubated with AnnexinV-Fluorescein isothiocyanate and propidiumiodide for 15 min in the dark at 25°C. Cell apoptosis was analyzed using a FACScan flow cytometry device with BD CellQuest Pro software 5.1 (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling (TUNEL) assay. Apoptotic cells were detected *in situ* by the TUNEL assay using an *In Situ* Cell Death Detection kit (Roche Applied Science, Penzberg, Germany). Cells were fixed in 4% paraformaldehyde for 30 min at room temperature and washed with PBS. Following this, the cells were resuspended in permeabilisation solution for 2 min on ice. Cells were washed by PBS three times, resuspended in TUNEL reaction buffer mixture and incubated in the dark at 37°C for 1 h. Subsequently, cells were washed with PBS three times and were observed under a fluorescence microscope (magnification, x200). A total of 10 fields-of-view were randomly selected for analysis.

Western blot analysis. Cells were lysed in the lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 140 mM NaCl, 1% (w/v) Nonidet P-40, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF and 10 mg/ml aprotinin. The proteins were separated by SDS-PAGE in an 8-12% gel and electrotransferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 5% skimmed milk for 1 h at room temperature and incubated overnight at 4°C with the primary antibodies against PI3K (cat. no. 4249; 1:1,000 dilution), p-PI3K (cat. no. 4228; 1:1,000 dilution), Akt (cat. no. 4691; 1:1,000 dilution), p-Akt (cat. no. 4070; 1:1,000 dilution), mTOR (cat. no. 2983; 1:1,000 dilution), p-mTOR (cat. no. 5536; 1:1,000 dilution), Bcl-2 (cat. no. 4223; 1:1,000 dilution), Bax (cat. no. 5023; 1:1,000 dilution) and cleaved caspase-3 (cat. no. 9661; 1:1,000 dilution) and β-actin (cat. no. sc-47778; 1:1,000 dilution; all Cell Signaling Technology, Inc.) overnight at 4°C. After washing with TBS with Tween, membranes were incubated with appropriate Horse radish peroxidase-conjugated secondary antibodies (anti-rabbit IgG; cat. no. sc-2357; 1:10,000 dilution; Santa Cruz Biotechnology) at room temperature for 1 h. The bands were visualized using a ChemicDoc XRS system (Bio-Rad Laboratories, Inc.).

Statistical analyses. All data are presented as the mean ± standard error of the mean. Various treatment groups were compared with analysis of variance, followed by the Least Significant Difference test for differences. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Ghrelin inhibits cisplatin-induced apoptosis of MDA-MB-231 cells. To determine the working concentrations of ghrelin, MDA-MB-231 cells were treated with 1, 10, 25, 50 and 100 nM of ghrelin. The CCK-8 assay showed that none of the concentrations tested had marked effects on cell viability (Fig. 1A). In addition, dose-dependent assays were performed to assess the cytotoxicity of cisplatin on MDA-MB-231 cells. Cells were treated with 1, 10, 25, 50 and 100 μM of cisplatin and cell viability was determined at 48 h. It was observed that cisplatin treatment induced a dose-dependent reduction of cell viability (Fig. 1B), which was inhibited by ghrelin (Fig. 1C). A previous study reported that 10-100 nM ghrelin did not affect MDA-MB-231 cells viability (15). Based on these results, 25 μM cisplatin and 50 nM ghrelin was chosen for subsequent

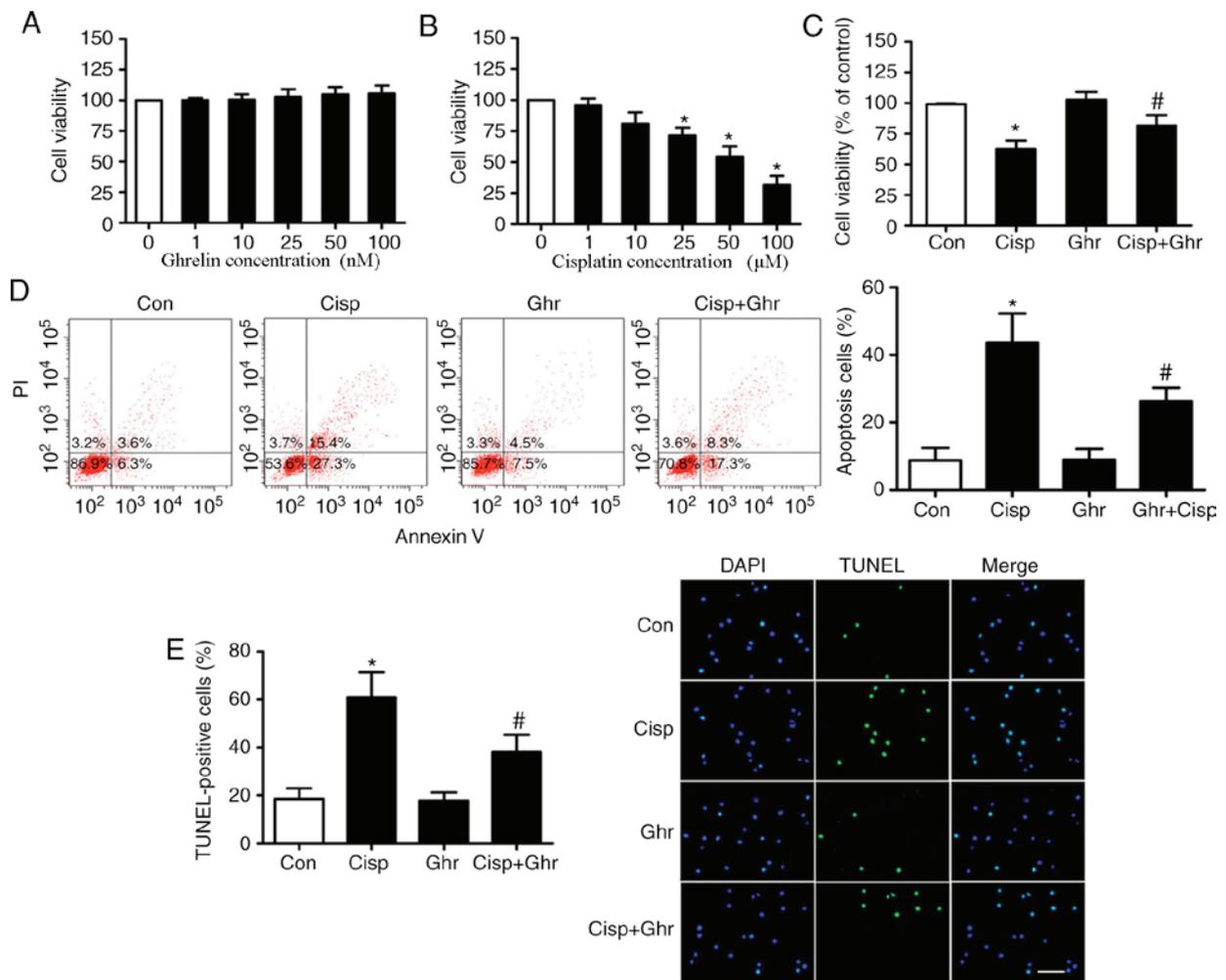


Figure 1. Effects of ghrelin and cisplatin on MDA-MB-231 cell viability. MDA-MB-231 cells were treated with (A) ghrelin, (B) cisplatin at the concentrations indicated on the x-axis respectively and (C) 25 μM cisplatin and/or 50 nM ghrelin. Control indicated cells were treated without cisplatin or ghrelin. (D) Apoptosis of MDA-MB-231 cell on treatment with 50 nM ghrelin and/or 25 μM cisplatin was analysed by flow cytometry and (E) by Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling staining. Scale bar, 100 μm. *P<0.05 vs. control; #P<0.05 vs. cisplatin treated alone. Cisp, cisplatin; Con, control; Ghr, ghrelin; PI, Propidiumiodide.

experiments. Subsequently, whether ghrelin affected apoptosis of MDA-MB-231 cells after cisplatin treatment was examined. Results showed ghrelin significantly inhibited apoptosis in MDA-MB-231 cells in comparison with cells treated with cisplatin only (Fig. 1D and E).

Ghrelin inhibits cisplatin-induced mitochondria-dependent apoptosis of MDA-MB-231 cells. Caspase-3 activity is a pivotal biomarker of apoptosis. It was found that cisplatin increased caspase-3 activity in MDA-MB-231 cells in comparison with the control, while ghrelin reduced caspase-3 activity (Fig. 2A). As expected, it was observed that the protein levels of cleaved caspase-3 increased after cisplatin treatment (Fig. 2B), while ghrelin significantly inhibited caspase-3 activation induced by cisplatin. Furthermore, ghrelin inhibited the cisplatin-induced inhibition of Bcl-2 expression and upregulation of Bax, which resulted in an increased Bcl-2/Bax ratio (Fig. 2B-D). These results indicate ghrelin inhibits cisplatin-induced apoptosis in MDA-MB-231 cells through mitochondria-dependent processes.

The inhibitory effect of ghrelin is mediated by PI3K/Akt/mTOR signaling. Activation of PI3K/Akt and mTOR is known

to be associated with cell proliferation, apoptosis and survival (12,18,19). Whether ghrelin regulates the expression of p-PI3K/p-Akt and p-mTOR in MDA-MB-231 cells was investigated. It was observed that the level of p-PI3K/p-Akt and p-mTOR decreased after cisplatin treatment in comparison with control, whereas ghrelin increased levels of p-PI3K/p-Akt and p-mTOR (Fig. 3A). It was estimated that ghrelin could promote MDA-MB-231 cell survival by activating PI3K/Akt/mTOR signaling. To understand this effect, PI3K inhibitor LY294002 and mTOR inhibitor rapamycin to block PI3K/Akt and mTOR signaling in MDA-MB-231 cells were studied. In the presence of LY294002 or rapamycin, the effect of ghrelin on cisplatin-induced Bax activation, and the induction of Bcl-2 expression were reversed (Fig. 3B and C). LY294002 or rapamycin partially reduced the pro-survival effects of ghrelin on MDA-MB-231 cells treated with cisplatin (Fig. 3D). In summary, these results suggest PI3K/Akt/mTOR signaling mediates the inhibitory effects of ghrelin on cisplatin-induced apoptosis in MDA-MB-231 cells.

Ghrelin inhibits cisplatin-induced apoptosis through GHSR. The effects of ghrelin appear to be mainly mediated by

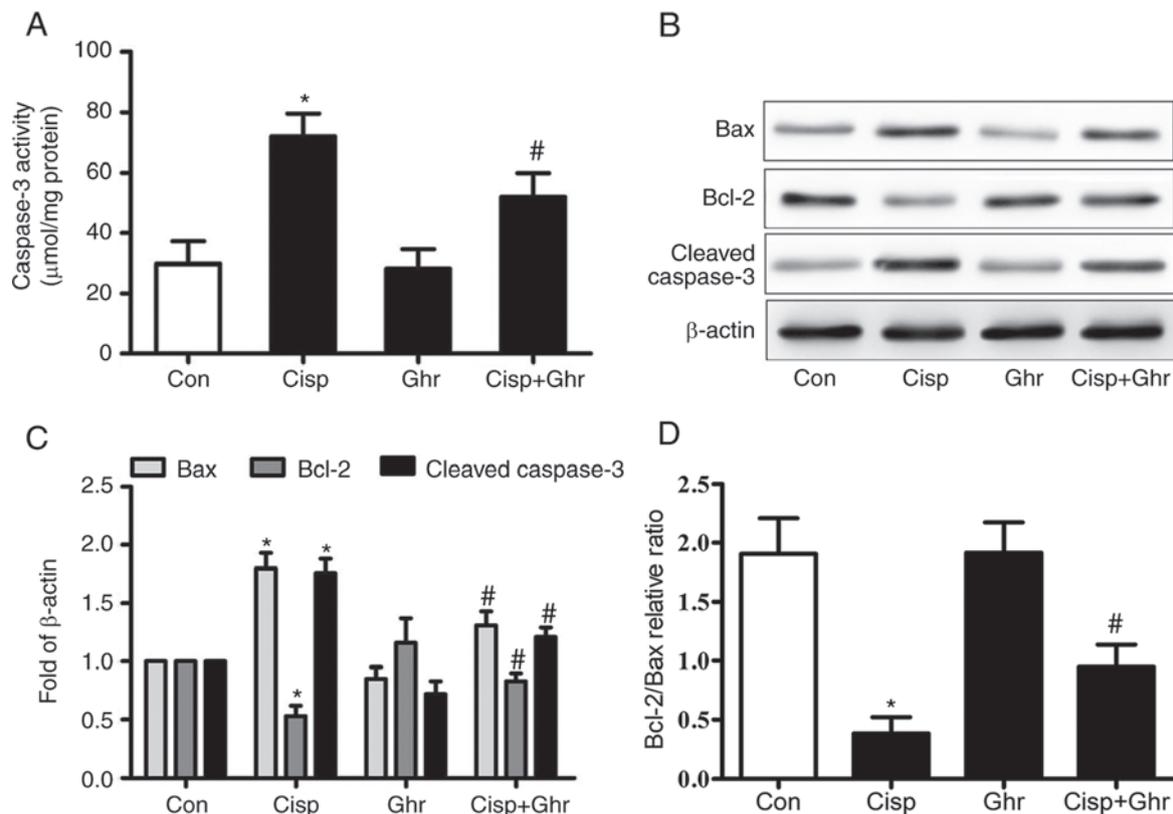


Figure 2. Effects of cisplatin and ghrelin on the expression of Bax, Bcl-2 and cleaved caspase-3. MDA-MB-231 cells were treated with 50 nM ghrelin and/or 25 μ M cisplatin for 48 h. (A) Caspase-3 activity was measured by caspase-3 colorimetric activity assay. (B) The expression of Bax, Bcl-2 and cleaved caspase-3 in treated MDA-MB-231 cells was detected by western blotting. (C) The relative quantities of Bax, Bcl-2, cleaved caspase-3 protein normalised to β -actin. (D) Bcl-2/Bax relative ratio. * $P < 0.05$ vs. control; # $P < 0.05$ vs. cisplatin treated alone. Bax; B-cell lymphoma 2-associated X; Bcl2, B-cell lymphoma 2; Cisp, cisplatin; Con, control; Ghr, ghrelin.

GHSR (20). To test this, MDA-MB-231 cells were transfected with GHSR siRNA, and the expression of GHSR was validated by western blotting (Fig. 4A). As expected, knockdown of GHSR inhibited the effects of ghrelin on cisplatin-induced apoptosis in MDA-MB-231 cells (Fig. 4B and C). In addition, GHSR siRNA reduced ghrelin-induced phosphorylation of PI3K/Akt/mTOR (Fig. 4D). These results suggest that GHSR mediates the effects of ghrelin on cisplatin-induced apoptosis in MDA-MB-231 cells.

Discussion

The effects of ghrelin on tumor cell apoptosis are controversial. The present study investigated the potential effects of ghrelin on cisplatin-induced apoptosis in MDA-MB-231 cells and the underlying mechanisms involved. It was observed that ghrelin inhibited the cisplatin-induced apoptosis *in vitro* and reduced MDA-MB-231 cell sensitivity to cisplatin *in vivo*. Furthermore, ghrelin-mediated effects were accompanied by activation of PI3K/Akt/mTOR signaling, which was inhibited by cisplatin. Moreover, the effect of ghrelin was significantly attenuated by GHSR siRNA, indicating ghrelin inhibits cisplatin-induced apoptosis in MDA-MB-231 cells through GHSR and activation of PI3K/Akt/mTOR signaling.

Ghrelin has been reported to regulate tumor cell proliferation, apoptosis and survival (21-23). In cancer studies, ghrelin has shown different proliferative and anti-proliferative effects in various colon cancer cell lines (14,24). Ghrelin has been reported to induce HT-29 cell proliferation via GHSR and the

Ras/PI3K/AKT/mTOR pathway (24). Studies have also reported ghrelin induces apoptosis in human colorectal carcinoma HCT116 cells (14). Previous findings have also indicated ghrelin inhibits LPS-induced apoptosis in A549 cells (12). In addition, ghrelin appears to induce proliferation of A549 cells via PI3K/Akt/mTOR/P70S6K and ERK signaling. Indeed, ghrelin seems to serve an important role in cancer cell proliferation and apoptosis. The effects of ghrelin on human breast cancer cell apoptosis and its associated mechanisms remain unclear. The present study identified that cisplatin reduced tumor cell viability in a dose-dependent manner, with ghrelin markedly reducing the death rate of MDA-MB-231 cells. In addition, flow cytometry and TUNEL analysis indicated ghrelin inhibited cisplatin-induced apoptosis in MDA-MB-231 cells.

It has been reported that PI3K/Akt and mTOR signaling pathways are activated by ghrelin, serving important roles in cell growth, apoptosis and survival (25-27). The present study assessed whether the PI3K/Akt and mTOR pathways were activated by ghrelin in MDA-MB-231 cells. The results demonstrated that ghrelin reversed cisplatin-induced inhibition of PI3K/Akt and mTOR activity in MDA-MB-231 cells. The results suggested PI3K/Akt/mTOR signaling participates in the effects of ghrelin on cisplatin-induced apoptosis in MDA-MB-231 cells. Nevertheless, other signaling pathways may be also activated by ghrelin in MDA-MB-231 cells, and further studies are required to examine whether other pathways intervene in the effects of ghrelin on breast cancer cell apoptosis.

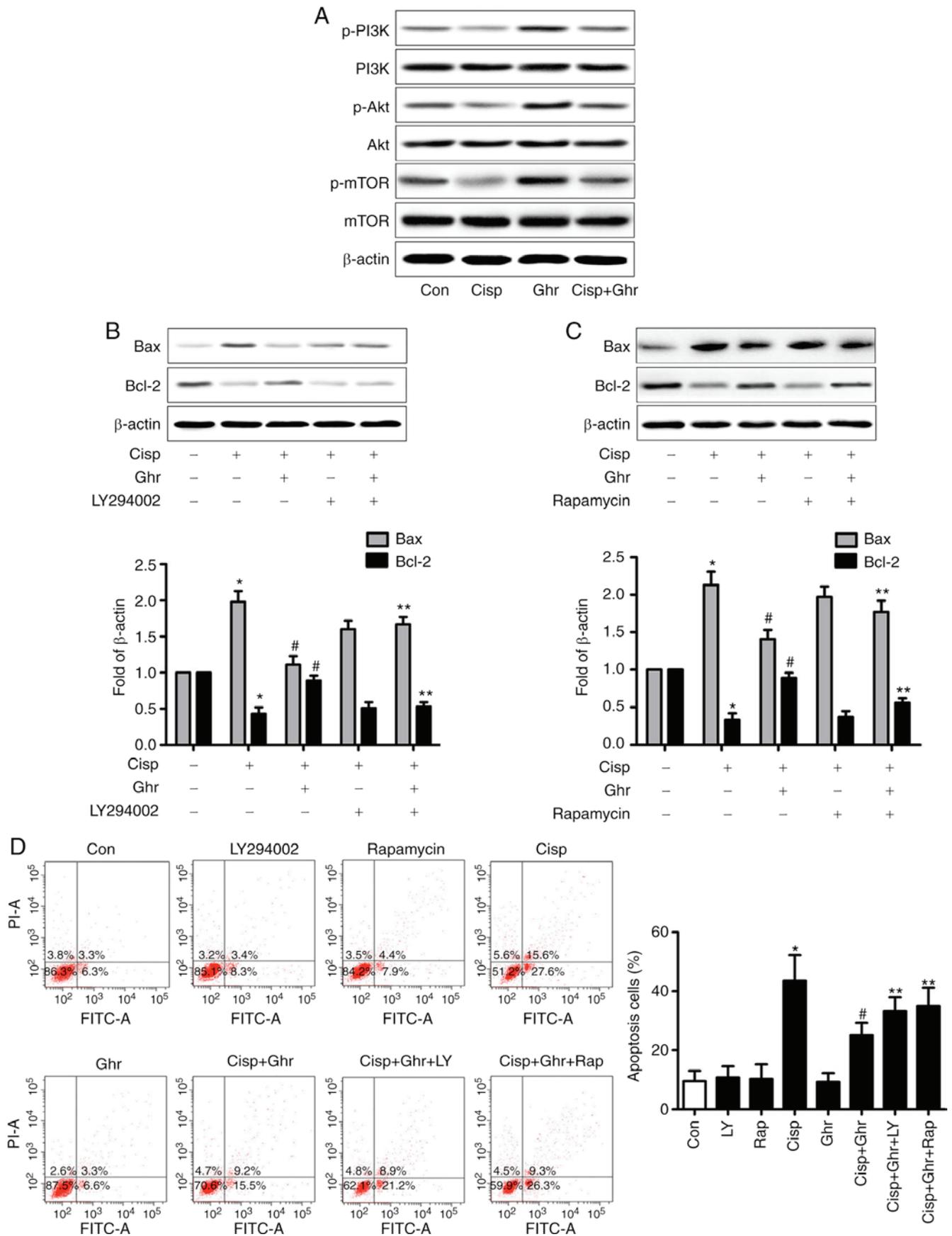


Figure 3. Involvement of p-PI3K/p-Akt and p-mTOR on the effects of cisplatin and ghrelin. (A) Phosphorylation of PI3K/Akt/mTOR was detected by western blotting. (B and C) Expression of Bax and Bcl-2 was detected by western blotting, PI-3K inhibitor, LY294002 (10 μM) or rapamycin (20 nM) was added 30 min before ghrelin and/or cisplatin treatment. (D) Apoptosis of MDA-MB-231 cells was analysed by flow cytometry. *P<0.05 vs. control; #P<0.05 vs. cisplatin treated alone; **P<0.05 vs. cisplatin + Ghrelin. Bax; B-cell lymphoma 2-associated X; Bcl2, B-cell lymphoma 2; Cisp, cisplatin; Con, control; FITC-A, Annexin V-Fluorescein isothiocyanate; Ghr, ghrelin; PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; siRNA, small interfering RNA.

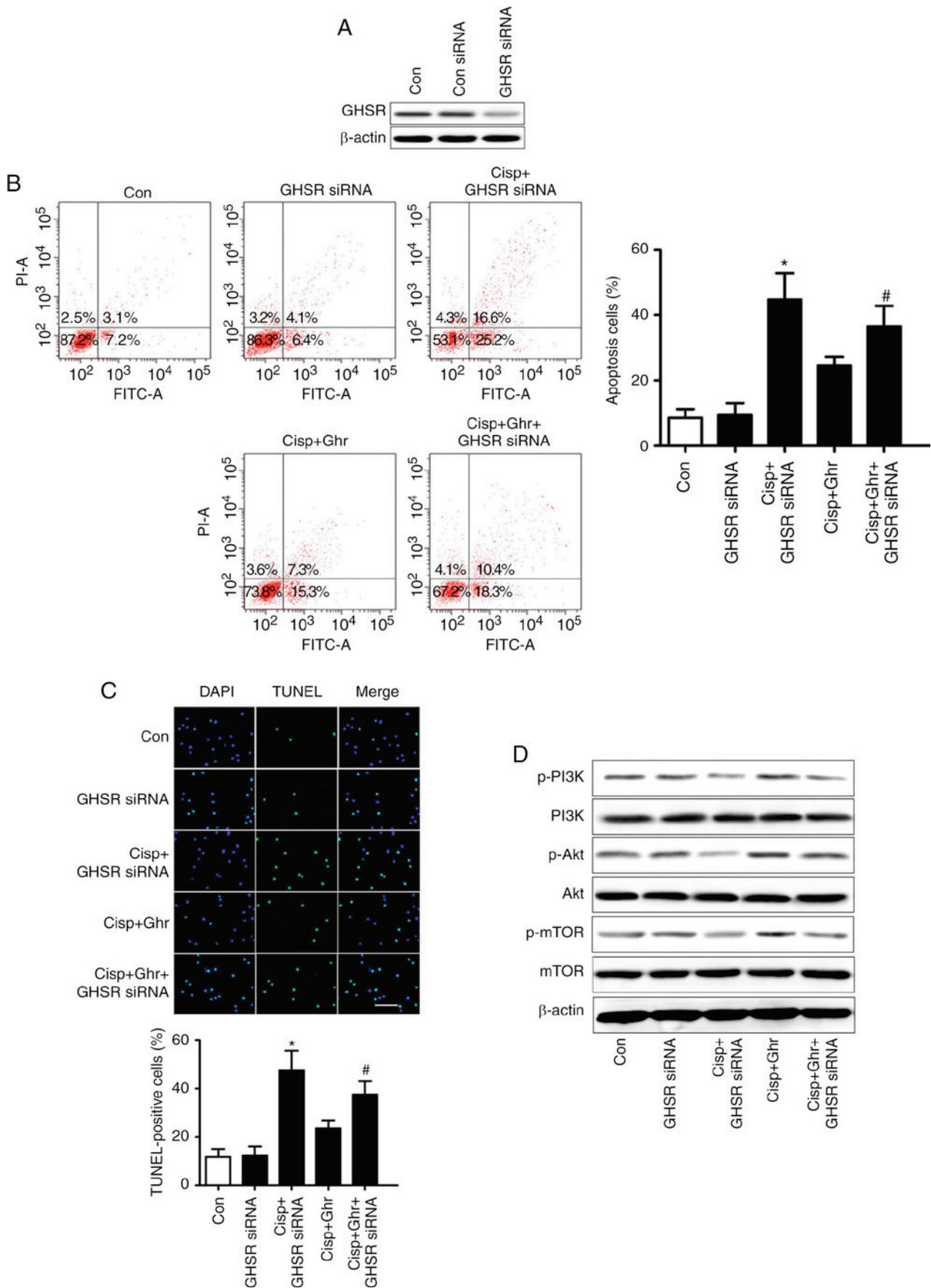


Figure 4. GHSR mediates the effects of ghrelin on apoptosis and PI3K/Akt/mTOR phosphorylation of MDA-MB-231 cells. (A) The protein level of GHSR was measured by western blotting. (B) Apoptosis of MDA-MB-231 cells were analysed by flow cytometry or (C) by Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling staining. Scale bar, 100 μ m. (D) PI3K/Akt/mTOR phosphorylation was detected by western blotting. * $P < 0.05$ vs. GHSR siRNA; # $P < 0.05$ vs. cisplatin + Ghrelin. Cisp, cisplatin; Con, control; FITC-A, Annexin V-Fluorescein isothiocyanate; Ghr, ghrelin; GHSR, growth hormone secretagogue receptor; mTOR, mammalian target of rapamycin; si, small interfering.

The Bcl-2 family includes several important regulators of intracellular apoptotic signal transduction, and the Bcl-2/Bax ratio determines cell survival or apoptosis (26). Bcl-2 protein levels decreased following treatment with cisplatin, whereas ghrelin increased Bcl-2 expression and inverted the Bcl-2/Bax ratio. Inhibition of PI3K and mTOR largely attenuated the ghrelin-mediated changes of the Bcl-2 and Bax expression. This provides strong evidence that PI3K/Akt/mTOR signaling mediates ghrelin-induced anti-apoptosis in MDA-MB-231 cells treated with cisplatin. Furthermore, ghrelin inhibited cisplatin-induced caspase-3 activation, consistent with previous observations in A549 and INS-1 cells (12,28).

Ghrelin exerts its physiological function by binding to GHSR. This receptor is expressed in many cancers, including lung cancer, breast cancer, renal cell carcinoma, colorectal cancer and ovarian cancer (12,17,29-31). Previous studies have reported the use of GHSR siRNA or (D-Lys3)-GHRP-6 to block the biological functions of ghrelin (22,26,27). The present study determined the involvement of GHSR in the ghrelin-mediated anti-apoptotic effects in MDA-MB-231 cells through the knockdown of GHSR with GHSR siRNA. This method reversed the inhibitory effect of ghrelin on cisplatin-induced apoptosis in MDA-MB-231 cells. The results suggested the anti-apoptotic effects of ghrelin to be GHSR-dependent. However, previous studies have implied the effects of ghrelin on intestinal epithelial cell survival and apoptosis may be dependent on an uncharacterized GHSR subtype. These discrepancies may be attributed to different GHSR subtype expression in cancer cells.

In summary, the present study provides evidence that ghrelin inhibits cisplatin-induced apoptosis in MDA-MB-231 cells through mechanisms dependent on GHSR and PI3K/Akt/mTOR signaling, but this needs further investigation.

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Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Authors' contributions

TX designed the experiments and wrote the manuscript. JZ carried out the experiments and analysed the data. All authors read and approved the final manuscript, and each author believes that the manuscript represents honest work.

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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