Curcumin Sensitizes Prolactinoma to Bromocriptine by Activating the ERK/EGR1 and Inhibiting AKT/GSK3β Signaling Pathway

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Research Article

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Abstract

Although bromocriptine (BRC) as first-line drugs are recommended for treating patients with prolactinoma, a minority of patients with prolactinoma resistance to BRC. Moreover, our previous study showed that the difference in drug sensitivity in BRC- treated rat prolactinoma cells, MMQ cells are more resistant to BRC, and GH3 cells are more sensitive to BRC. Curcumin (Cur) has been shown to inhibit proliferation of prolactinoma cell lines. The aim of this study is to further investigate whether Cur could enhance the growth-inhibitory effect of BRC resistance on prolactinoma cell lines and its possible mechanism. CCK-8 kit was used to test cell growth. Cell-cycle analysis and apoptosis was performed by flow cytometry. Electron microscopy was used to test autophagosome. The mRNA expression profiles were analysed using the Affymetrix Gene-Chip array. Western blotting was used to test protein expression. Our data showed that Cur enhanced the growth-inhibitory effect of BRC on GH3 and MMQ cell proliferation. BRC and Cur both induced cell apoptosis, and Cur could significantly increase the apoptosis of BRC on pituitary adenoma cells through the ERK/EGR1 signaling pathway. Moreover, Cur could enhance the autophagic cell death (ACD) of BRC on tumor cell by inhibiting the AKT/GSK3β signaling pathway. The same results were confirmed in vivo study. Taken together, Cur sensitizes rat pituitary adenoma cell to BRC by activating the ERK/EGR1 and inhibiting AKT/GSK3β signaling pathway.

Introduction

Dopamine agonists (DAs) are the first choice treatment for the majority of patients with prolactinoma, and effectively suppress prolactin secretion and shrink tumor volume in most patients [1, 2]. BRC as first-line drug was approved for treating prolactinoma of patients since the 1980s in USA, it was usually very effective in the treatment of prolactinomas [3, 4]. Nonetheless, approximately 20–30% of patients does not respond satisfactorily to BRC and this resistance is defined as a failure to achieve normoprolactinaemia and a 50% or more reduction in macroprolactinoma at maximally tolerated doses[5]. Although patients with BRC resistance shift to cabergoline (CAB) has been shown to be the more effective in normalizing prolactin levels, approximately 20% of BRC-resistant patients still cannot achieve a normal prolactin level[6].

The cellular mechanisms that lead to prolactiomas resistance to DAs have been extensively investigated, and may involve several different molecular alterations in drug-resistant prolactinomas[5]. In most prolactinomas, DAs resistance are associated with reduced expression of subtype 2 dopamine receptor (D2R), but the binding affinity is not altered[7, 8]. However, other molecular alterations downstream of D2R that may may be involved in prolactiomas resistance to DAs[9–11]. Our previous study has shown that BRC mainly through activating the ERK/EGR1 signalling pathway induces the apoptosis of prolactinoma cells, whereas CAB mainly through inhibiting the AKT/mTOR signalling pathway induces autophagic cell death (ACD)[12]. The differential mechanisms in BRC- and CAB-treated prolactinoma cells, which provides a theoretical basis for the prolactiomas resistance to DAs.

Curcumin (Cur) as a traditional herbal medicine is extracted from the dietary spice turmeric[13]. Substantial studies have shown that Cur plays an essential role in anti-proliferative, anti-inflammatory, anti-tumorigenic effects through targeting various molecules[14]. Meanwhile, it has been reported that Cur is mainly through pituitary tumor cell proliferation, induces apoptosis[15]. So far, whether Cur can enhance the inhibitory effect of BRC resistant prolactinomas and the specific mechanism remains unclear. In our previous study, we found that MMQ cells are more resistant to BRC, and that GH3 cells are more sensitive to BRC[12]. Therefore, the purpose of present study was to investigate the mechanism through the effects of Cur combined with BRC on pituitary adenoma cells in vitro and in vivo. It may provide theoretical basis in selection of BRC resistance in the medication treatment of prolactinomas.

Materials And Methods

Cell lines and cell culture

Rat pituitary tumor cell lines GH3 and MMQ were obtained from the Chinese Academy of Medical Science (Beijing, China). GH3 and MMQ cells were cultured in Ham's F10 medium and F12 medium respectively containing 15% horse serum, 2.5% fetal calf serum, 1% penicillin and streptomycin and maintained at 37℃ in a 5% CO_2 atmosphere.

Chemicals and antibodies

Cur and BRC were obtained from Abcam (Cambridge, MA, USA). The drugs were dissolved in dimethyl sulfoxide (DMSO), and diluted to 25, 50,100μmol/l using serum-free medium (DMSO with final concentration 0.2%). 3-Methyladenine (3-MA) was purchased from Sigma-Aldrich (St.Louis, MO, USA). Fetal bovine serum was purchased from Wisent Biotechnology (Nanjing, China). Antibody were purchased as follows: anti-β-actin, anti-Cdc25A, anti-Cyclin D1, anti-CDK4, anti-phospho-mTOR, anti-phospho-AKT, anti-phospho-GSK3β, anti-LC3B, anti-cleaved-Caspase-3, anti-EGR1 and anti-phospho-ERK1/2 were from Cell Signaling Technology (Danvers, MA); Anti-β-actin were from Bioworld (MN, USA). All antibodies were used according to manufacturers' instructions.

CCK8 assay

Cell viability was measured using the cell counting kit-8 (CCK-8) from Dojindo Laboratories (Kumamoto, Japan) according to the manufacturer's instructions. Cells were transplanted into a 96-well plate with density of 7000 cells/well and then was incubated different concentrations of the drugs for different times. Upon addition of CCK8 solution, 100μLfresh serum-free medium contained 10μLCCK-8 solution was added into each wellthe reaction plate was incubated at 37°C for 2 h, and the optical density value (absorbance) was recorded at 450 nm using an enzyme-linked immunosorbent assay plate reader (Bio-Rad Laboratories, Inc., Berkeley, CA, USA).

Cell cycle analysis

Cell cycle distribution was performed to detect the effect of drug on cell cycle arrest of pituitary tumour cell lines by Fluorescence-activated cell sorting (FACS) analysis. GH3 and MMQ cells were plated in six-well plates at a density of 1 × 107 cells/well. Cells were treated for 48 h, harvested by trypsinization and fixed in 70% ethanol at −20 °C for 1 h. Cells were resuspended and stained in PBS containing 20 mg/ml PI and 10 mg/ml RNAse A, and incubated at 37 °C for 30 min. Following this, cell cycle analysis of DNA content was analysed on a flow cytomete.

Flow cytometry analysis

The two cell types were treated with CAB and BRC for 48 h. Cells were collected, centrifuged (1000 rpm for 5 min), and washed twice with PBS, the supernatant was discarded. The pellet was incubated away from light for 10 min at room temperature with 5μl Annexin V-fluorescein and 5μl propidium iodide (PI) before analysis with a FACSAria III flow cytometer (BD Biosciences, San Jose, CA, USA) according to the standard protocol.

Transmission electron microscopy (TEM)

Samples were processed in the department of pathology at Jinling hospital. Cell pellets were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer. The cells were washed with 0.1 M sodium cacodylate buffer and postfixed with 1% osmium tetroxide. The pellets were then dehydrated in graded ethanol series, infiltrated, and embedded in Spurr’s resin. Samples were then polymerized for 48 h at 60°C, cut into 60-nm-thick sections on LKB-I microtome, positioned on 200 mesh grids, and stained with uranyl acetate and lead citrate. The ultrathin sections were mounted on JEOL JEM-1011 TEM at an accelerating voltage of 120 Kv. Images were acquired with Gatan type UltraScan 4000SP CCD Camera connected to the TEM. The numbers of autophagosome were calculated manually for a minimum of 50 cells for each sample and the photos were taken.

Gene chip analysis

Differential gene expression between MMQ cells treated with Cur (50μM) combined with BRC (50μM) and untreated cells was analyzed. Total RNA of cells was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s instructions. The Affymetrix Rat Genome 230 2.0 Array was used to detect gene expression profiles following the manufacturer's instructions. Preliminary data was analyzed by the software of the Affymetrix microarray suite. The cutoff criterion for differential gene calls was at least a 2-fold between two compared profiles. The microarray data set was submitted to the GEO repository (GSE101012).

Quantitative RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen) from GH3 and MMQ cells pre-treated with Cur and BRC for 48h according to the manufacturer’s protocols. The quality and quantity of the RNA purity were assessed using spectrophotometry and agarose gel electrophoresis. cDNA was synthesized from 1000 ng RNA by reverse transcribed using the Prime ScriptTM RT reagent Kit (Takara, Dalian, China). The

expression levels of mRNA were quantified d in a StepOne Plus thermocycler (Applied Biosystems) using Power SYBR Green (Bio-Rad) according to the manufacturer's protocol. The threshold cycle (Ct) value was assessed as the mRNA expression levels. The relative expression levels of mRNA were defined using the comparative delta-delta Ct method (TaqMan Relative Quantification Assay software), adjusted to β-actin expression level.

Transfection

GH3 and MMQ cells stably expressing control or GSK3β-specific shRNA (GeneChem, Shanghai, China). GH3 and MMQ cells were transduced at ~50% confluence with shRNAs using Lipofectamine^{TM}2000. mRNA expression was detected by QRT-PCR. Protein expression was visualized by western blotting. The rat GSK-3β-shRNA target sequences of the successful shRNA as follows: GSK-3β-shRNA sense: GCUAGGACAACCAAUAUUUTT, anti-sense: AAAUAUUGGUUGUCCUAGCTT. The sequences of negative control as follows: sh-Ctr sense: UUCUCCGAACGUGUCACGUTT, anti-sense: ACGUGACACGUUCGGAGAATT.

Western blot analysis

Cell extracts for Western blotting were prepared in RIPA buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenyl methylsulfonyl fluoride, and protease inhibitors). Lysates were separated by SDS-PAGE and were transferred to PVDF membranes (Millipore, MA, USA). The apoptosis and autophagy associated protein was detected by standard techniques with primary antibodies against cleaved-caspase-3, LC3B, EGR1, p-ERK1/2, p-AKT, p-GSK-3β. The HRP-conjugated goat antirabbit IgG was used as a secondary antibody for primary antibodies. The immune complex was detected by chemiluminescence luminol reagents (Millipore, MA, USA). The densitometry of the immunoblotting bands were quantified by public software ImageJ (National Institutes of Health, USA).

Tumor cell xenograft nude mice models

Nude mice experiments protocols were approved by the Institutional Animal Committee of Jinling Hospital. To determine the therapeutic efficacy of Cur combined with BRC against rat pituitary cell tumor xenograft growth, four to six week female athymic BALB/c nude mice (n=15) were obtain from Department of Comparative Medicine (Jinling Hospital, China) and maintained in specific pathogen-free (SPF) conditions at the Jinling Hospital. GH3 cells (approximately1.0×10^{7}) were transplanted subcutaneously to develop a mouse xenograft model of prolactinoma. Tumor volumes were measured every day using vernier caliper, and calculated as (volume = 1/2 ×length × width^{2}). The tumors were allowed to grow to 2-4 mm in size and the mice were assigned randomly to three groups. The mice of control group were treated with 100 μl of 0.9% saline by oral gavage. The mice of BRC-treated group were treated with BRC (1 mg/kg/d) by oral gavage in 100 μl of 0.9% saline and with 100 μl olive oil by intraperitoneal injection every day. Cur was dissolved in (15 mg/kg) and administered (i.p., 100 μl) to the mice of Cur combined with BRC-treated group every day from day 1 until day 14. Fourteen days after

treatment, all tumors were harvested from animals were measured, weighed and submitted for immunohistochemical staining analysis.

Immunohistochemical staining

Immunohistochemical staining was performed on sections from paraffin-embedded tumor tissue to detected the protein expression levels. After Deparaffinization and re-hydration of tissue slide, antigen retrieval with 10 mmol/l sodium citrate buffer (containing 0.05% Tween 20, pH 6.0) was performed by microwave. The sections of tissues were incubated with a primary antibody against overnight at 4°C, they were incubated with HRP-conjugated secondary antibody at room temperature for 2 hour. Slides were counterstained with hematoxylin to detect nuclei, all sections were viewed and photographed with a microscope equipped with a digital camera

Immunofluorescence staining

To determine the protein expression and distribution of LC3 in all the experimental groups, immunofluorescence staining was adopted 48 h after treatment. Cells were grown on coverslips, fixed in 4% paraformaldehyde for 15 min at room temperature, incubated by 5% BSA for 30 min at 37°C, and treated with antibodies specific for LC3B (1:100 dilution) at 4 °C overnight. After being rinsed by PBS, the slides were incubated for 30 min at room temperature with goat anti-rabbit IgG (1:2000 dilution), and the nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). The images were photographed under an immunofluorescence microscope (Olympus IX81, Japan).

TUNEL staining

The TUNEL staining was performed to detecte apoptotic cells according to the manufacturer's instructions. After Deparaffinization of tissue slide, the section was washed 3 times with PBS and permeabilized with 0.2% Triton X-100 in PBS-Tween for 30 minutes. The sections were incubated with 3% H_2O_2 in PBS for 10 minutes to block endogenous peroxidase activity. All sections were incubated with terminal deoxynucleotidyl transferase (TdT) Reaction Buffer for 10 minutes, and then incubated in TdT Reaction Mixture for 1 hours at at room temperature in humidified chamber. After rinse sections, the sections were incubated with 100μl Streptavidin-HRP in PBS for 20 minutes at room temperature. The sections were incubated with 100μl DAB for 2 minute. The sections were counterstained with hematoxylin for 30 seconds and dehydrated through 95% ethanol for 5min, 100% ethanol for 2x3min. The number of TUNEL-positive cell in ten microscopic fields was counted.

Statistical analysis

All experiments were replicated in triplicate at least. The SPSS version 19.0 software (SPSS Inc., Chicago, IL, USA) was applied for statistical analysis. Comparisons between treated groups and vehicle control were performed using independent t test, and expressed as mean ± standard deviation (SD). $P < 0.05$ were considered statistically significant.

Results

Cur strengthens BRC inhibits rat prolactinoma cell proliferation

The CCK-8 assay kit was used to test the cell proliferation. To examine whether Cur strengthens BRC inhibits the cell proliferation. GH3 and MMQ cells were treated with Cur and BRC at concentrations of 25, 50, and 100 μM for 24, 48, and 72 h. As the results shown in (**Fig. 1A, B**). BRC and Cur decreased the cell viability of GH3 and MMQ cells in both a dose- and time-dependent manner. After 48 h of drug treatment, the cell viability of Cur combined with BRC-treated group was significantly lower than that of BRC-treated group (**Fig. 1C**). These results suggested that Cur strengthens BRC inhibits the rat prolactinoma cell proliferation.

Cur strengthens BRC induces cell cycle G1 phase arrest

Flow cytometric analysis were used to analyze the cell cycle. GH3 and MMQ cells were treated with Cur (50μM), BRC (50μM) and Cur (50μM) combined with BRC (50μM) for 48 h. Cur and BRC treated cells exhibited G1 phase proportion increase with S phase proportion decrease respectively, combined treatment with Cur and BRC further increased ratio of G1/S phase (**Fig. 2A**). To further investigate how Cur regulates cell cycle, the cell cycle related protein were detected by western blot. In Cur and BRC treated cells, the expression levels of Cyclin D1, CDK4 and Cdc25A, which play key roles in G1-S entry, compared with control cells were decreased respectively. Moreover, the expression levels of Cyclin D1, CDK4 and Cdc25A in Cur combined with BRC-treated group were significantly lower than those of BRC-treated group in both cell lines (**Fig. 2B**). These data demonstrated that Cur effectively strengthened the BRC-induced cell cycle G1 phase arrest.

Cur strengthens BRC induces apoptosis in rat pituitary adenoma cells by upregulation of ERK1/2 and EGR1

Our previous studies have demonstrated that BRC mainly induce apoptosis through ERK/EGR1 pathway in prolactinoma [12]. To examine whether Cur further enhaced BRC-induced apoptosis in rat pituitary adenoma cells. The apoptotic ratio of GH3 and MMQ cells induced by Cur combined with BRC was detected by flow cytometry using Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining. GH3 and MMQ cells were treated with 50 μM Cur and BRC for 48 h. The Cur- and BRC-induced apoptosis rates were significantly greater than those of the control in both cell lines. Meanwhile, Cur enhanced the apoptosis rates of BRC-induced in GH3 and MMQ cells (**Fig. 3A**). Caspase-3 as a key molecule is involved in drug-induced apoptosis. In the early stages of apoptosis, caspase-3 is activated and eventually cleaved caspase-3 induces apoptosis [16]. Both Cur and BRC promoted the expression levels of cleaved caspase-3 in the both cell lines compared with control cells. Meanwhile, the cleaved caspase-3 expression levels of Cur combined with BRC-induced were significantly higher than those of BRC-induced in the both cell lines (**Fig. 3B**). Our previous results showed that the expression levels of ERK and EGR1 in the BRC treatment groups were remarkably higher than those of the control cells [12]. To detecte whether Cur strengthens BRC induces apoptosis by ERK/EGR1 pathway in rat pituitary adenoma

cells. ERK1/2 and EGR1 expression were detected in GH3 and MMQ cells by western blot. Compared with the control group, the p-ERK1/2 and EGR1 protein level in the Cur or BRC treated groups was significantly increased. Moreover, the expression levels of p-ERK1/2 and EGR1 protein in the Cur combined with BRC treatment group was significantly higher than those of BRC treatment group (**Fig. 3C**). In short, these results suggested that Cur accelerated BRC-induced apoptosis in rat pituitary cells via ERK/EGR1 signalling pathway.

Cur strengthens BRC induces autophagic cell death in rat pituitary adenoma cells

Prior studies have reported that over-activation of autophagy can induce autophagic cell death (ACD) [17,18]. Our previous results suggested that BRC induced autophagy in rat pituitary adenoma cells, while ACD was not involved in the BRC induced cell death [12]. To investigate whether Cur strengthens BRC induces autophagy in rat pituitary adenoma cells, GH3 and MMQ cells were treated with Cur (50μM), BRC (50μM) and Cur (50μM) combined with BRC (50μM) for 48h respectively. Autophagosome was analyzed by TEM. After 48 h of Cur or BRC exposure, the numbers of autophagosome were significantly increased in the cytoplasm in GH3 and MMQ cells. Compared with the BRC-treated group, the autophagosomes induced by Cur combined with BRC in the both cell lines were significantly increased (**Fig. 4A**). When autophagy was activated, the LC3 transforming from microtubule-associated protein light chain 3-I (LC3-I) to LC3-II is a classical hallmark of autophagy [19]. The LC3-I and LC3-II expression were detected in GH3 and MMQ cells by western blot. The conversion effect of LC3-I to LC3-II by Cur combined with BRC-induced in GH3 and MMQ cells was increased compared with BRC-treated group (**Fig. 4B**). To examine whether increased autophagy was responsible for Cur combined BRC-induced cell death, autophagy was inhibited through the chemical inhibition of autophagy by 3-Methyladenine (3-MA) in GH3 and MMQ cells. The autophagosomes induced by Cur combined with BRC were significantly reduced after treatment with 3-MA (5 mM) in both cell lines (**Fig. 5A**). Furthermore, 3-MA effectively inhibited the transformation rate of LC3-I to LC3-II by Cur combined with BRC-induced in both cell lines (**Fig. 5B**). On the other hand, 3-MA reversed the Cur combined with BRC-mediated inhibitory effect on both cell types at 48 h (**Fig. 5C**). Together, these results indicate that Cur enhanced BRC-induced autophagy in rat pituitary adenoma cells. ACD is involved in cell death in the prolactinoma cell lines in response to Cur combined with BRC treatment

Cur strengthens BRC induces ACD by downregulation of AKT and GSK-3β

To identify the mRNA and biological pathways associated with Cur combined BRC-treated the rat prolactinoma cells. MMQ cells were treated with Cur (50μM) combined BRC (50μM) for 48 h respectively. The heatmaps of the microarray data showed that the mRNA expression profiles after prolactinoma cells were treated with Cur combined BRC for 48 h. Upregulated (red spots) and downregulated (green spots) mRNAs showed significantly different expression levels between the control group and Cur combined BRC-treated group. Network-based visual analysis was used to analyze the mRNA-target interactions and functional associations **(Fig. 6A**). The AKT/GSK-3β pathway was predicted to be associated with Cur combined BRC-induced autophagy in MMQ cells according to the results of the network analysis. GSK-3β

as an important regulatory protein is involved in a variety of physiological processes and progression of tumor[20]. Some studies have shown that GSK-3β, as a downstream molecule of AKT, is involved in the regulation of autophagy[21]. Under physiological conditions, GSK-3β inhibits autophagy activation. GSK-3β is phosphorylated and inactivated to block autophagy inhibition when tumor cells are subjected to abnormal external stimuli[22]. Next, we identified the effect of Cur combined BRC on the AKT/GSK-3β pathway. We detected the expression levels of p-AKT and p-GSK-3β in GH3 and MMQ cells treated with Cur (50μM) or BRC (50μM) or Cur (50μM) combined BRC (50μM) for 48 h by western blotting. The results showed that the expression levels of p-AKT and p-GSK-3β was significantly lower in the both cell lines treated with Cur or BRC than in the control cells. We also found that the phosphorylation levels of AKT and GSK-3β were significantly lower in Cur combined BRC-treated group than in BRC-treated group (**Fig. 6B**). After shRNA transfection was used to downregulate GSK-3β (**Fig. 6C,D**), the LC3-B expression levels were increased in GH3 and MMQ cells compared with sh-ctr group by immunofluorescence staining and western blotting (**Fig. 6E,F**), the inhibitory effect on the proliferation of rat pituitary adenoma cells was weakened (**Fig. 6G**). These results showed that Cur can enhance suppress the activation of AKT/GSK-3β signalling pathway to induce ACD in rat prolactinoma cells.

Cur sensitizes prolactinoma to BRC in vivo

To further explore the effects of on rat pituitary cells in vivo, the inhibition efficacy of Cur combined BRC on tumor formation using subcutaneously transplanting GH3 cells in BALB/c nude mice was examined. After tumor formation, tumourigenic nude mice were randomly divided into three groups of 5 mice each. The tumor sizes and weight in the Cur combined BRC treatment group were significantly smaller than those in the control or BRC treatment group (**Fig. 7A-C**). Apoptosis was detected by TUNEL staining in the subcutaneously transplanted tumors, and the expression levels of Ki-67, p-ERK1/2, EGR1, p-AKT and p-GSK-3β in three groups were detected by immunohistochemistry (**Fig. 7D**). We found that the positive rate of tumor cell TUNEL staining in Cur combined with BRC treatment group was significantly higher than that in the other two groups. Compared with the other two groups, the positive rate of Ki-67 staining in Cur combined with BRC treatment group was significantly decreased. EGR1 and p-ERK1/2 expression levels were significantly higher in Cur combined with BRC treatment group than in the other two groups. Moreover, we also found that the expression levels of p-AKT and p-GSK-3β were significantly lower in Cur combined with BRC treatment group than BRC treatment group. Taken together, these results suggested that Cur enhanced BRC-induced apoptosis and ACD via activating the ERK1/2-EGR1 signaling pathway and inhibiting the AKT/GSK-3β signaling pathway in prolactinoma cells respectively (**Fig. 7E**).

Discussion

Medical therapy with DAs is very effective in the majority of prolactinomas. However, a subset of individuals with prolactinomas exhibit a varying degree of resistance to DAs, and the patients do not achieve rapidly normalization of PRL levels or substantial tumor size reduction. Other treatment options for DAs resistant prolactinoma including transsphenoidal surgical debulking, radiotherapy or

temozolomide (TMZ), it is still uncertain which treatment is optimal choice. Therefore, there is an urgent need for alternative treatment options for DA-resistant prolactinomas.

BRC as an only commercial DA is the most frequently used in treatment prolactinoma with the longest history of use in China. However, there were still some patients with prolactinoma who failed to respond satisfactorily to BRC[23, 24]. TMZ has been shown to be effective in aggressive pituitary tumors and carcinomas, including DAs resistant prolactinomas, but the long-term effects of treatment may not be maintained[25]. Moreover, some cases showed that after combined treatment with BRC and metformin, the tumor size of prolactinoma was significantly decreased during the three-month follow-up period[26]. Cur has been shown to be an effective anti-cancer agent in different tumor types[27]. Previous study showed that Cur inhibited pituitary tumour cell proliferation and induced apoptosis in vitro[28]. But, whether Cur was able to enhance the functions of BRC resistant prolactinomas in induction of anti-proliferation, apoptosis and autophagic cell death (ACD) still uncertain. In the our previous study, we made use of the rat prolactinoma cell line, GH3 and MMQ, to compare the intracellular apoptotic and autophagy protein activated by BRC. We found that MMQ cells are more resistant to BRC compare with GH3 cells[12]. Our results showed that Cur enhance the BRC-induced cell death in GH3 and MMQ cells with dosage- and time-dependent in present study. Cell cycle analysis showed that Cur enhance BRC-induced cell cycle arrest at G1/S phases in the two types cell. Moreover, our results have shown that Cur enhanced BRC-induced inhibition of cell cycle regulators such as Cyclin D1, CDK4 and Cdc25A, which are critically regulatory factors involved in promoting cells proliferation. These data demonstrated that Cur could enhance BRC-induced anti-proliferation in GH3 and MMQ cells.

In the past decades, apoptosis is also called programmed cell death(PCD), PCD as a distinct biochemical and genetic pathway was investigated in various cancer cells [29, 30]. Cancer cells that undergo excessive autophagy were triggered to cell death in complete absence and independent of caspase activation manner[31]. ACD and apoptosis as two main cell death cause were studied in present study. Previous study showed that BRC could inhibit PRL secretion and induce apoptosis in GH3 cells[32, 12]. We found that Cur was able to enhance the functions of BRC-induced apoptosis rates in GH3 and MMQ cells. The relative expression of cleaved caspase-3 in cells combined treatment with BRC and Cur was also significantly higher than that in BRC-treated cells. These results suggested that apoptosis is involved in the function of Cur combined with BRC induced cell death. In previous study, our results showed that when BRC-induced autophagy in GH3 and MMQ cells was inhibited using 3-MA, the cell viability of BRC treatment group did not change significantly[12]. In present study, we found that Cur can enhance the BRC-induced autophagy. Additionally, when Cur combined with BRC induced autophagy was inhibited, the cell viability in combined treatment with BRC and Cur group in the two cells was significantly increased. The results showed that ACD is involved in Cur combined with BRC induced cell death. Taken together, Cur enhanced BRC-induced cell death in rat pituitary adenoma cells through apoptosis and autophagy dual pathways.

Previous results demonstrated that apoptosis as the predominant type involved in cell death of pituitary cell line in response to BRC treatment by ERK1/2-EGR1 pathway[12]. In present study, we found that Cur

was able to enhance the functions of BRC in induction of apoptosis by ERK1/2-EGR1 pathway. In order to explore the mechanisms of Cur combined with BRC induced ACD, we used a microarray to define the differences in relative gene expression and signaling pathways between control group and Cur combined with BRC treatment group in pituitary adenoma cells. The differential gene expression and interaction network was analysed. We found that the function of Cur combined with BRC induced autophagy was associated with AKT signaling pathway and GSK-3β expression. Previous study revealed that PI3K/AKT/GSK-3β signal pathway participates in the regulation of autophagy in oral cancer[21]. GSK-3β as an important regulatory protein is involved in the development and progression of various diseases, which is also involved in the regulation of autophagy in tumor cells[20]. Its activity is regulated by the two different specific phosphorylation sites. Tyr216 phosphorylation can increase kinase activity, whereas phosphorylation occurs at Ser9, the inactivation form of GSK-3β that relieve autophagy inhibition[33]. We analysed the levels of AKT and GSK-3β phosphorylation in Cur combined with BRC-treated GH3 and MMQ cells and found that the levels of p-AKT and p-GSK-3β were significantly decreased compared to those in the controls. The results of immunohistochemistry in the subcutaneous xenograft model were consistent with above results. We then downregulated the expression of GSK-3β by shRNA transfection and found that the cell viability of GH3 and MMQ cells was significantly reduced. These results suggested that Cur enhanced BRC induces ACD through inhibiting the AKT/GSK-3β signalling pathway.

Conclusions

In summary, the results of our present study provided the following conclusions. First, Cur enhanced BRC-induced anti-proliferation in GH3 and MMQ cells. Second, Cur enhanced BRC-induced cell death in rat pituitary adenoma cells through apoptosis and autophagy dual pathways. Third, Cur strengthened BRC induced apoptosis and ACD through activating the ERK/EGR1 and inhibiting the AKT/GSK-3β signaling pathway in prolactinoma cells respectively (Fig. 7E). Our study demonstrates that the underlying mechanisms of Cur sensitizes prolactinoma to BRC in rat prolactinoma cells to provide a novel therapeutic strategy for the medication treatment of DAs resistant prolactinoma. However, further research is still needed to reveal the precise mechanisms of GSK-3β regulates autophagy in DAs resistant prolactinoma cells.

Abbreviations

Cur: Curcumin; BRC: bromocriptine; CAB: cabergoline; DAs: Dopamine agonists; D2R: Dopamine 2 Receptor; ACD: autophagic cell death; 3-MA: 3-Methyladenine; TMZ: temozolomide; TEM:Transmission electron microscopy; CCK-8: cell counting kit-8; FITC: fluorescein isothiocyanate; PI: propidium iodide.

Declarations

Ethics approval and consent to participate

Animal experiments protocols were approved by the Institutional Animal Committee of Jinling Hospital.

Consent for publication

Not applicable

Availability of data and materials

The datasets during and analysed during the current study available from the corresponding author on reasonable request.

Competing of interests

The authors declare that they have no competing of interests.

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Authors' contributions

CT and JZ carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. FY and JY carried out the immunoassays. XC participated in the sequence alignment. XC participated in the design of the study and performed the statistical analysis. CM conceived of the study, and participated in its design and coordination and helped to draft the manuscript. CM revised the paper. All authors read and approved the final manuscript.

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Figures

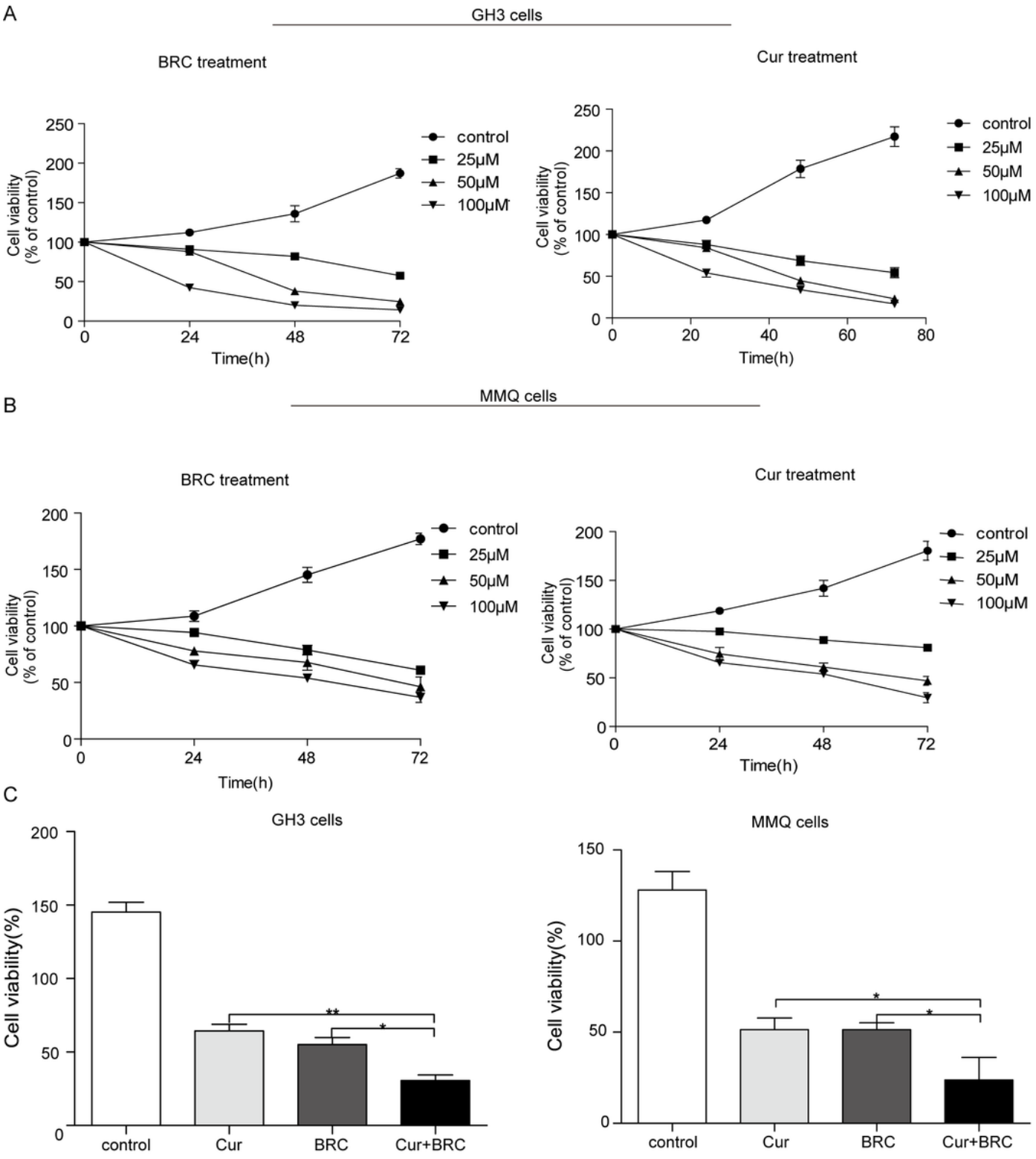

Figure 1

Cur and BRC decreased the viability of GH3 and MMQ cells. A, B: Cell survival was determined by the CCK8 assay. GH3 and MMQ cells were treated with Cur and BRC at concentrations of 0, 25, 50, and 100 μM, respectively, for 24, 48, and 72 h. C: GH3 and MMQ cells were treated with Cur, BRC and Cur combined with BRC at a concentration of 50 μM for 48 h, and cell viability was tested with the CCK8 kit (**p<0.01, *p < 0.05).

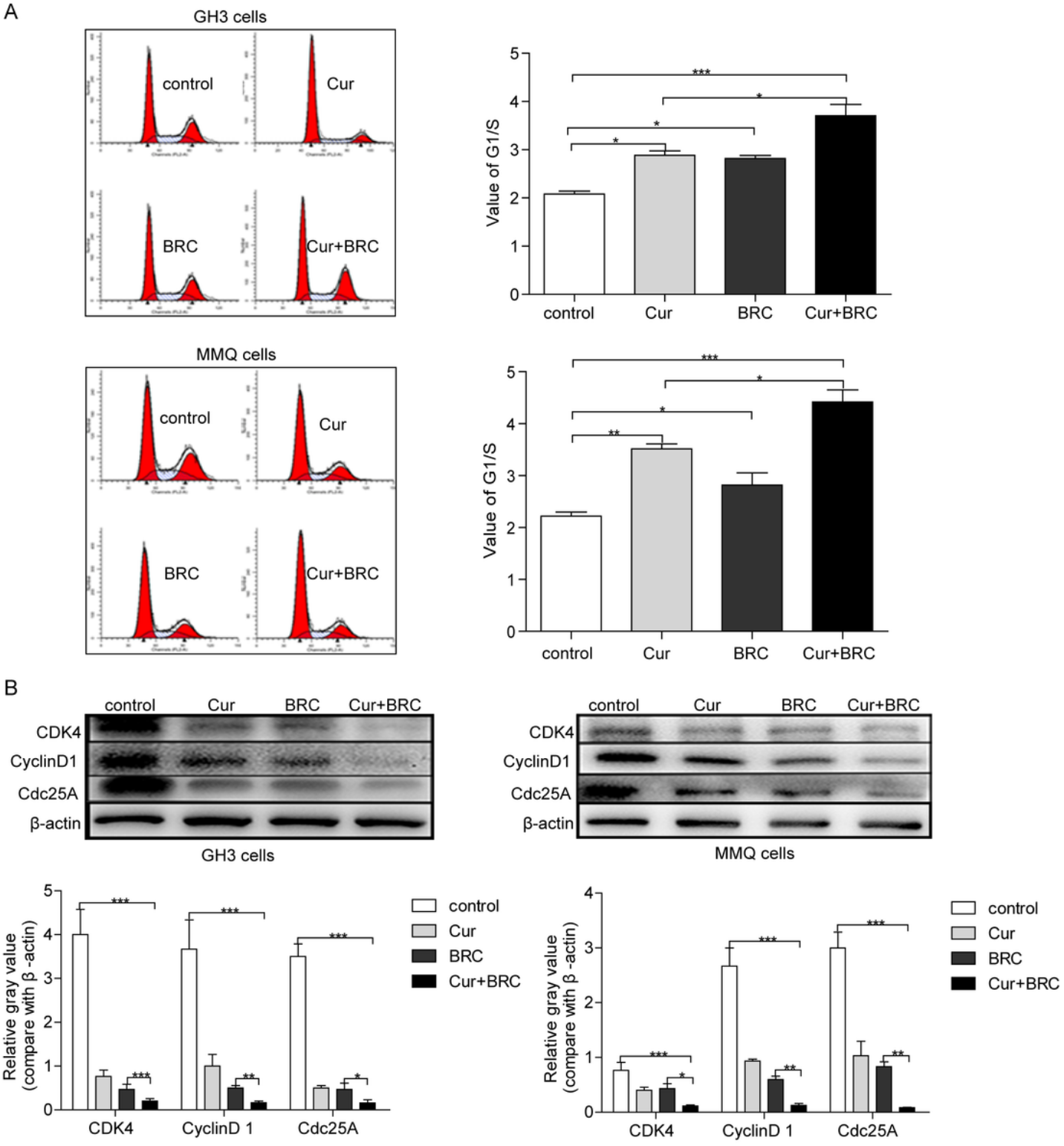

Figure 2

Cur enhances BRC-induced cell cycle G1 phase arrest. A: GH3 and MMQ cells were treated with DMSO (vehicle control), Cur (50 μM), BRC (50 μM) and Cur (50 μM) combined with BRC (50 μM) for 48 h. Cur could enhance BRC induced the proportion of G1/S phase (***p<0.001, **p<0.01, *p < 0.05). B: The expression levels of Cyclin D1, CDK4 and Cdc25A were decreased in drug treated cells compared with

control group. Compared with BRC-treated cells, the expression levels of Cyclin D1, CDK4 and Cdc25A were significantly reduced in Cur combined with BRC treatment group (***p<0.001, **p<0.01, *p < 0.05).

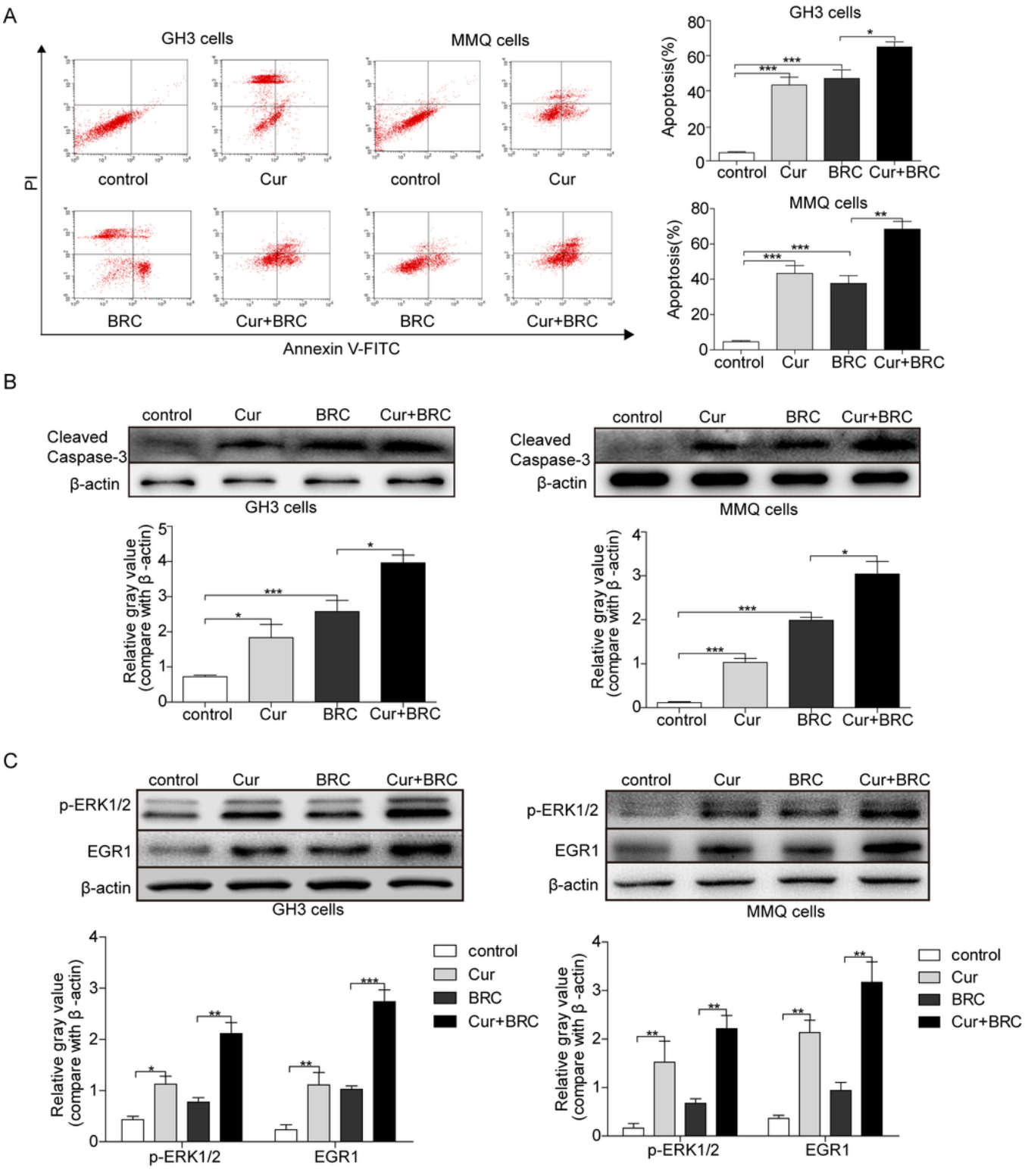

Figure 3

Cur strengthens BRC-induced apoptosis in rat pituitary adenoma cells by upregulation of ERK1/2 and EGR1. A: GH3 and MMQ cells were treated with CAB (50 μM), BRC (50 μM) and Cur (50 μM) combined with BRC (50 μM) as indicated for the apoptosis assay by Annexin V-FTIC and PI double staining. B, C:

GH3 and MMQ cells were treated with Cur (50 μM), BRC (50 μM) and Cur (50 μM) combined with BRC (50 μM), and the proteins of cleaved caspase-3, p-ERK1/2 and EGR1 were analysed by Western blot (***p<0.001, **p<0.01, *p < 0.05).

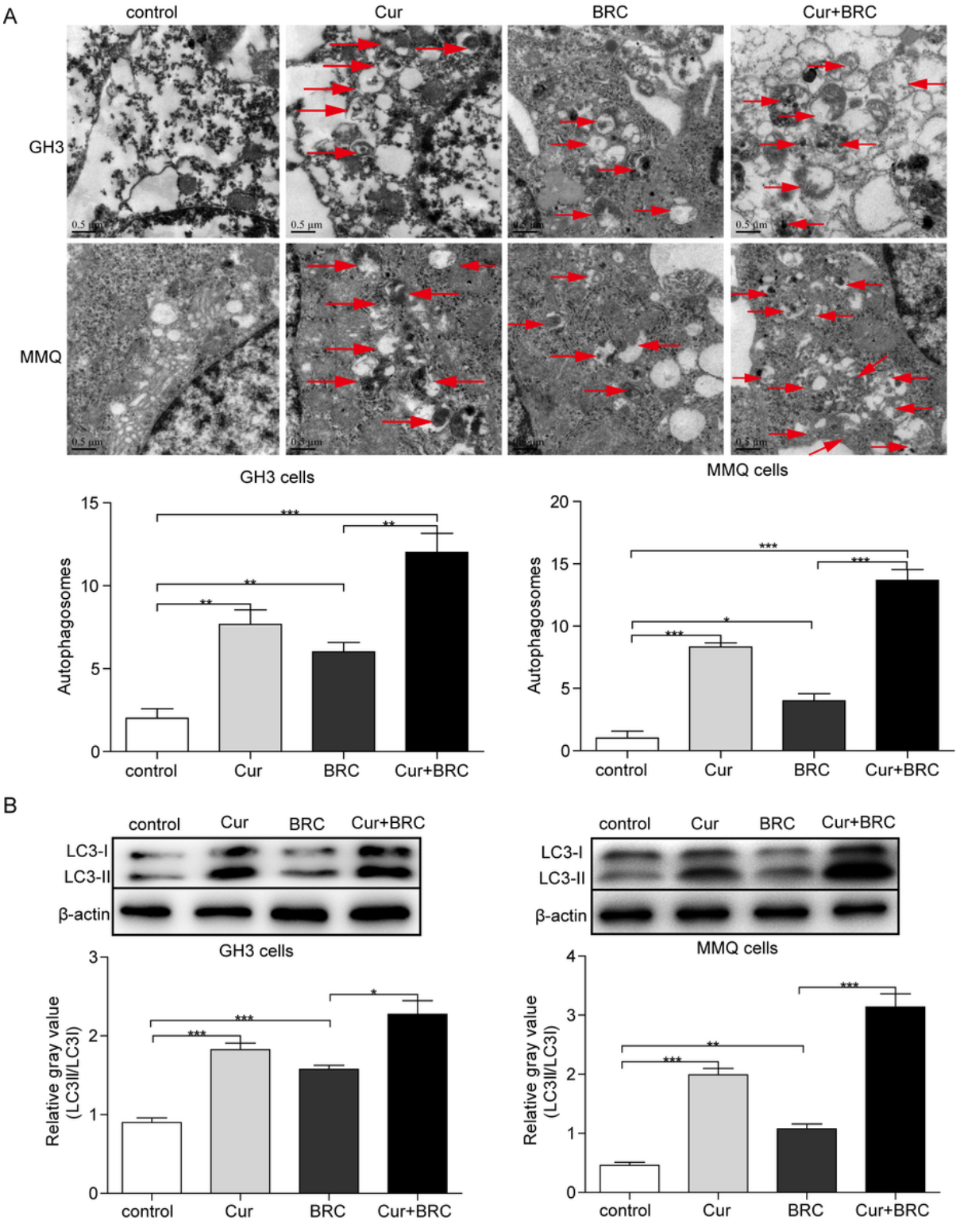

Figure 4

Cur strengthens BRC-induced autophagy in rat pituitary adenoma cells. A: Electron microscopy images with enlargements. Arrows show autophagosomes in GH3 and MMQ cells were treated with Cur (50 μM),

BRC (50 μM) and Cur (50 μM) combined with BRC (50 μM). B: The proteins of LC3-I and LC3-II were analysed by Western blot in GH3 and MMQ cells were treated with drug (***p<0.001, **p<0.01, *p < 0.05).

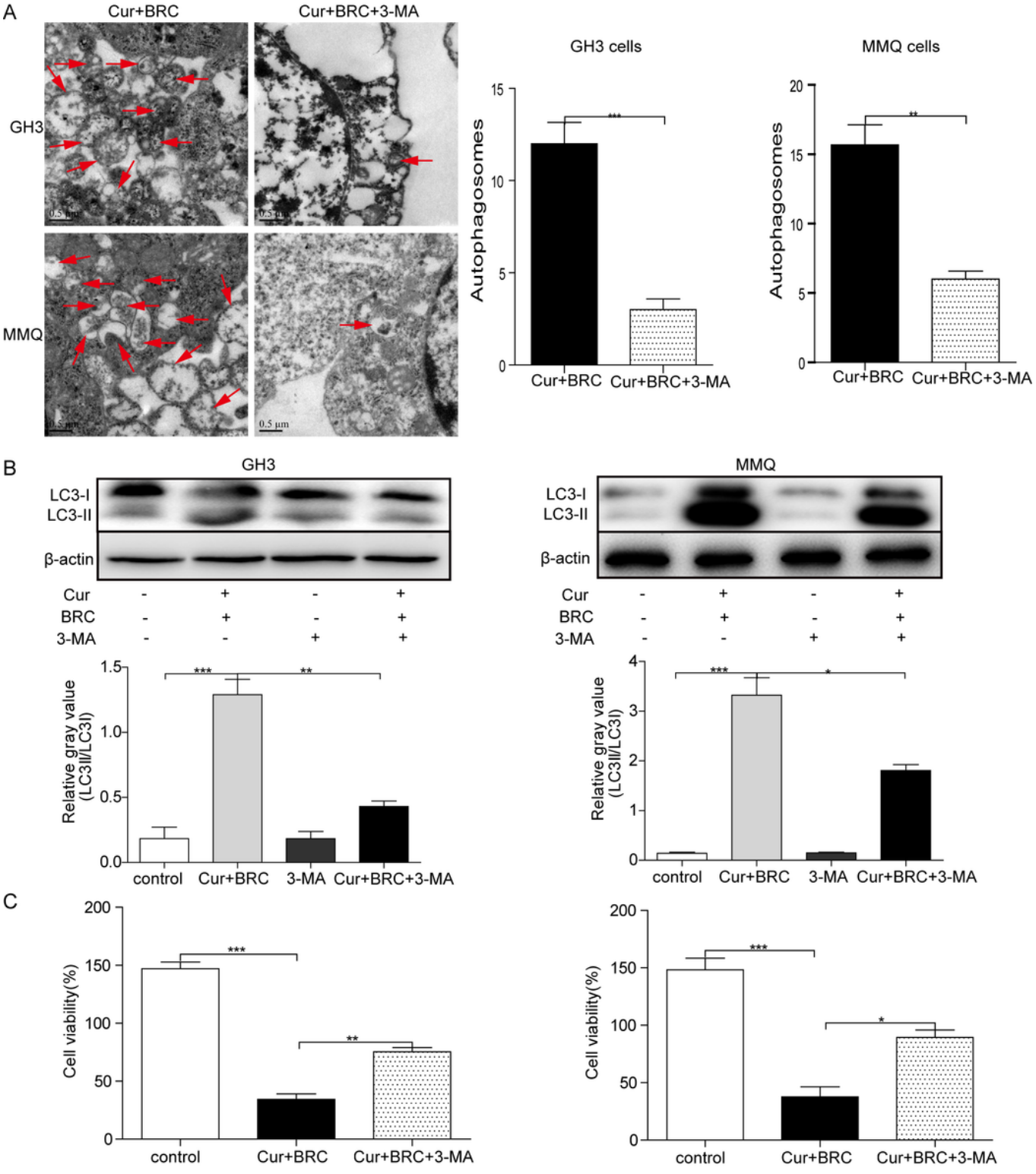

Figure 5

Cur promotes BRC-induced autophagic cell death in prolactinoma cells. A: Electron micrographs of GH3 and MMQ cells treated with Cur (50 μM), BRC (50 μM) and Cur (50 μM) combined with BRC (50 μM) for 48 h, with or without 3-MA (5 mM). B: Western blot analysis of LC3-I and LC3-II in GH3 and MMQ cells

with drug treatment and with or without 3-MA (5 mM). C: GH3 and MMQ cells were treated with Cur (50 μM), BRC (50 μM) and Cur (50 μM) combined with BRC (50 μM) for 48 h, with or without 3-MA (5 mM), and cell viability was tested with the CCK8 kit (***p<0.001, **p<0.01, *p < 0.05).

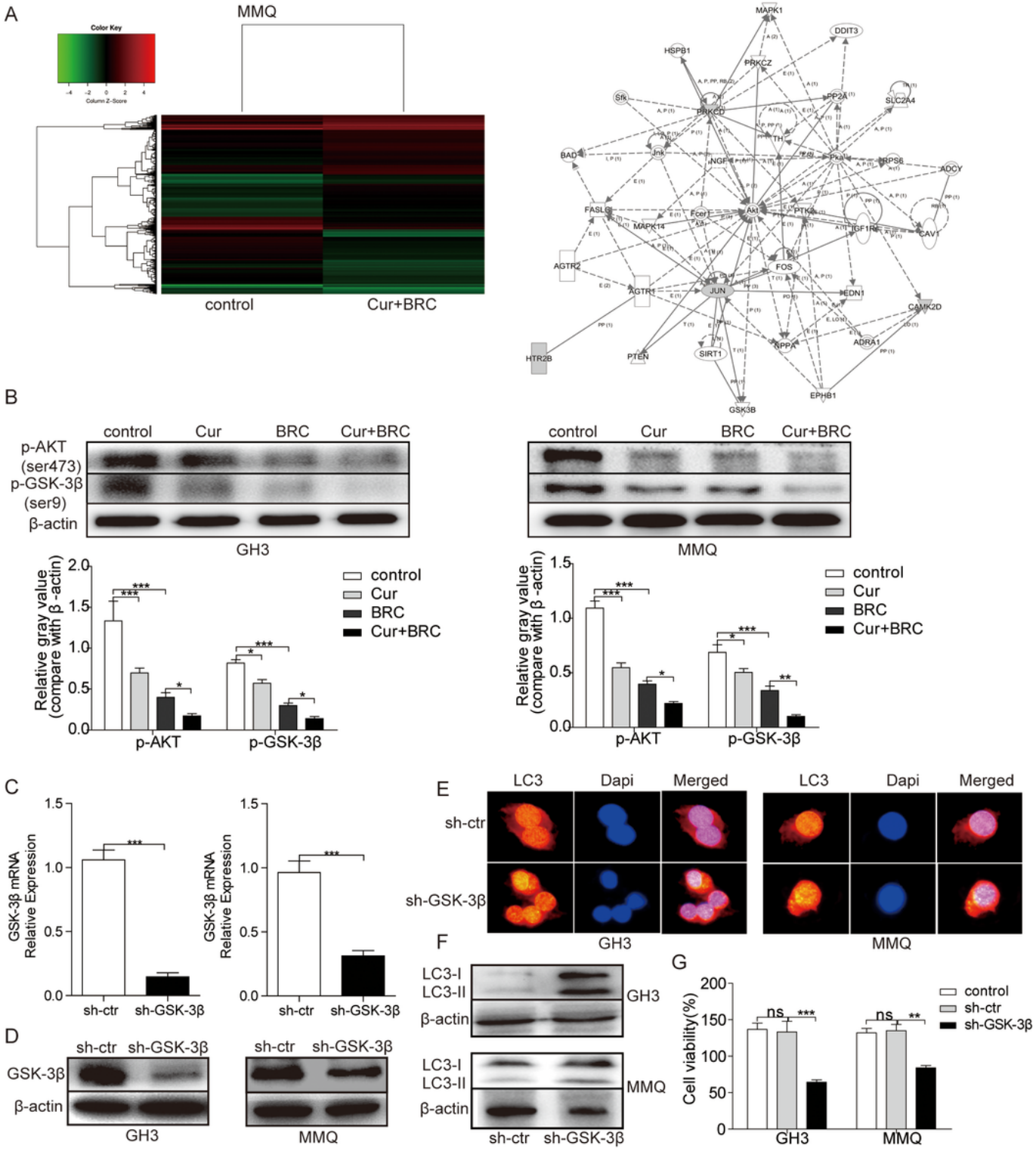

Figure 6

Cur enhances BRC-induced autophagic cell death via inhibiting AKT/GSK-3β signaling pathway in prolactinoma cells. A: Differential mRNA expression in MMQ cells treated with or without Cur (50 μM)

combined with BRC (50 μM). The mRNA-target interactions and functional associations of Cur (50 μM) combined with BRC (50 μM)-treated MMQ cells were analysed using network-based visual analysis analysis. B: Western blot analysis of p-AKT and p-GSK-3β in GH3 and MMQ cells treated with Cur (50 μM), BRC (50 μM) and Cur (50 μM) combined with BRC (50 μM) for 48 h. C, D: QRT-PCR and Western blot analysis of GSK-3β expression after GH3 and MMQ cells were transfected with the control (sh-ctrl) or the shRNA against GSK-3β for 72 h. E, F: Immunofluorescent labeling and Western blot analysis of LC3-B expression after GH3 and MMQ cells were transfected with the control (sh-ctrl) or the shRNA against GSK-3β for 72 h. G: CCK8 analysis of cell viability after GH3 and MMQ cells were transfected with the control (sh-ctrl) or the shRNA against GSK-3β for 72 h (***$p<0.001$, **$p<0.01$, *$p < 0.05$).

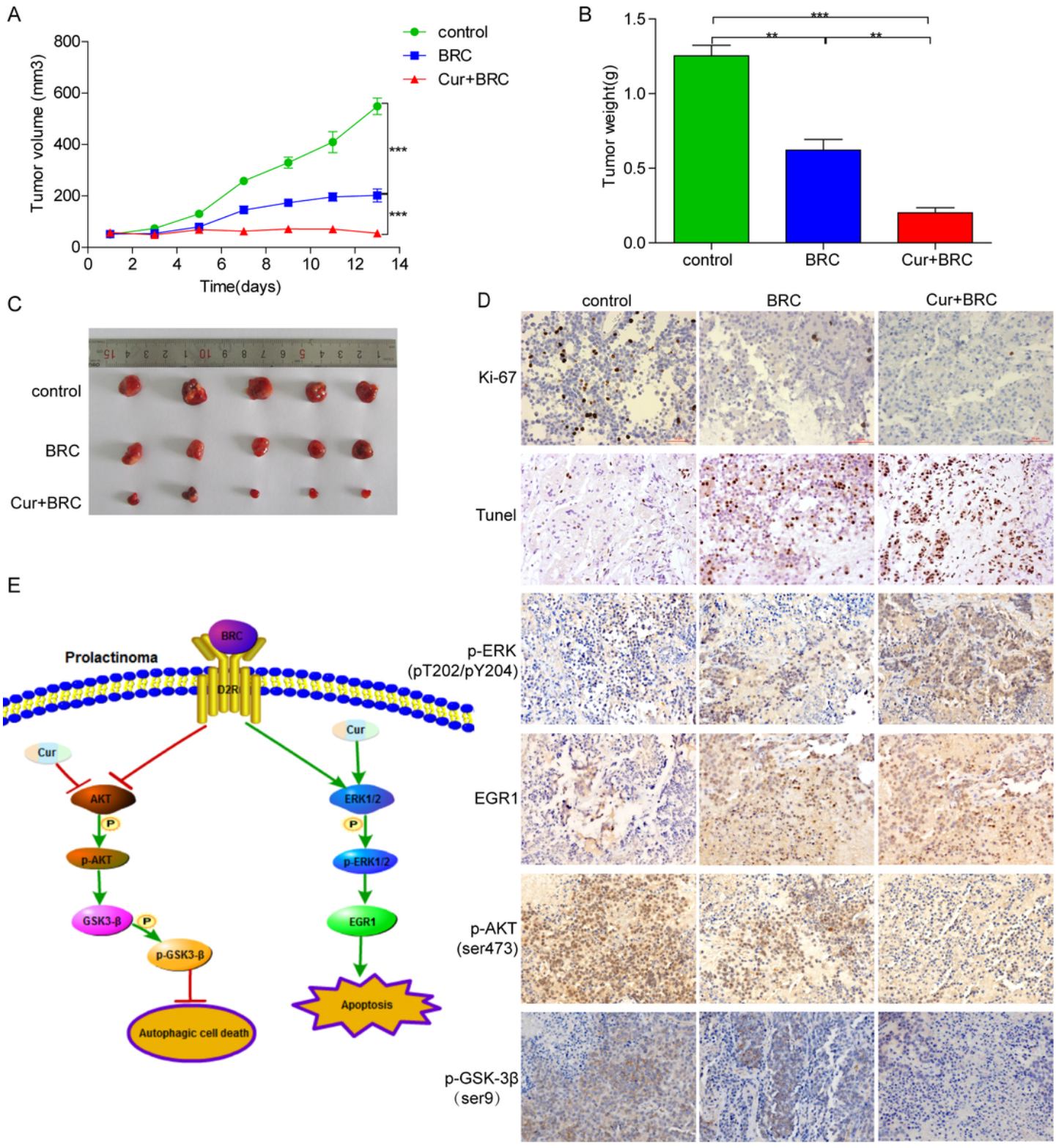

Figure 7

Subcutaneous xenograft model and drug treatment in vivo. A, B: After tumor formation, the nude mice were randomly divided into a control group, BRC-treated group and Cur combined with BRC-treated group (n =5). The tumor volume and weight of each group were assessed (***p<0.001, **p<0.01). C: Representative images of xenograft tumors in nude mice. D: The TUNEL staining and immunohistochemical analyses of Ki-67, p-ERK1/2, EGR1, p-AKT and p-GSK-3β in tumor samples of each

group. Scale, 50 μm. E: The proposed mechanism of Cur strengthens BRC-induced cell death in prolactinoma cells