

PI3K/AKT phosphorylation activates ERR α by upregulating PGC-1 α and PGC-1 β in gallbladder cancer

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Abstract. The nuclear estrogen-related receptor- α (ERR α) is an orphan receptor that has been identified as a transcriptional factor. Peroxisome proliferator-activated receptor- γ (PPAR γ) coactivator-1- α (PGC-1 α) and PPAR γ coactivator-1- β (PGC-1 β) act as the co-activators of ERR α . Our previous study reported that activated ERR α promoted the invasion and proliferation of gallbladder cancer cells by promoting PI3K/AKT phosphorylation. Therefore, the aim of the current study was to investigate whether PI3K/AKT phosphorylation could enhance ERR α activity in a positive feedback loop. LY294002 and insulin-like growth factor I (IGF-I) were used to inhibit and promote PI3K/AKT phosphorylation, respectively. A 3X ERE-TATA luciferase reporter was used to measure ERR α activity. The present study found that LY294002 inhibited PI3K/AKT phosphorylation, decreased the proliferation and invasion of NOZ cells and suppressed the activity of ERR α . Conversely, IGF-I induced PI3K/AKT phosphorylation, promoted the proliferation and invasion of NOZ cells and enhanced the activity of ERR α . The protein expression levels of PGC-1 α and PGC-1 β were elevated and reduced by IGF-I and LY294002, respectively. Moreover, knockdown of PGC-1 α and PGC-1 β antagonized ERR α activation, which was enhanced by PI3K/AKT phosphorylation. Taken together, the present study demonstrated that PI3K/AKT phosphorylation triggered ERR α by upregulating the expression levels of PGC-1 α and PGC-1 β in NOZ cells.

Introduction

The incidence of gallbladder and biliary tract cancer has increased by 76% between 1990 and 2017 on a global scale (1). Due to the low early detection rate, gallbladder cancer (GBC) often undergoes local invasion and lymph node metastasis (2). Most patients with GBC are diagnosed at advanced stages and are unresectable (3). These patients tend to relapse despite having received standard chemotherapy and radiotherapy. Therefore, the overall survival of GBC is extremely low, ranging from 13.2-19 months (4,5). A recent study revealed that a value of 65 IU/ml CA 19-9 may be helpful in evaluating the prognosis of GBC (6). Currently, there is no effective chemotherapy or targeted therapy for the treatment of GBC. Novel immunotherapeutic drugs, such as immune checkpoint inhibitors of anti-programmed cell death protein-1 antibody and anti-programmed cell death-ligand 1 antibody, have shown limited efficacy in the clinical intervention of GBC (7,8). Therefore, additional efforts should be made to identify novel targets and to determine the in-depth mechanism to advance the understanding and the curative effect of GBC.

Estrogen-related receptor- α (ERR α) is a member of the orphan nuclear receptors (9) and belongs to the ERR family, which consists of ERR α , ERR β and ERR γ (10). ERR α was identified on the basis of the structural similarity between its DNA binding domain and human estrogen receptor (ER) α ; however, ERR α does not bind to natural estrogens or estrogen-like molecules (11). ERR α is involved in various biological processes and activities, including energy metabolism and cell proliferation and invasion, by binding to estrogen-related response elements and estrogen response elements (EREs) (12). A number of orphan nuclear receptors are activated by the peroxisome proliferator-activated receptor γ (PPAR γ) coactivator (PGC) family, including PGC-1 α , PGC-1 β and PGC-1 γ (13). In the absence of specific ligands, ERR α can be activated by PGC-1 family members, such as PGC-1 α (14) and PGC-1 β (15). Moreover, as wild-type PGC-1 α (PGC-1 α WT) can activate other receptors, such as ERR β and ERR γ , researchers have reported that some peptides (such as L3-09) can bind to ERR α specifically. Herein, the investigators replaced L2 and L3 motifs with L3-09 peptides to generate PGC-1 α 2x9, in an attempt to selectively activate

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ERR α (16). Moreover, a 3X ERE-TATA luciferase reporter was applied to measure the activity of ERs and ERRs, including ERR α (12).

As one of the most important signaling transduction pathways in mammalian cells, the PI3K/AKT signaling pathway functions to inhibit cellular apoptosis and promote proliferation by interacting with multiple downstream effectors (17). LY294002 has been proved to specifically inhibit the activity of the PI3K (18,19), whereas recombinant human insulin-like growth factor-I (IGF-I) can be applied to activate the PI3K/AKT signaling pathway (20). The binding of IGF-I to IGF-I receptor (IGF-IR) functions to induce receptor autophosphorylation and to elevate the tyrosine kinase activity of IGF-IR, thereby leading to the activation of the 85-kDa subunit of PI3K by recruiting and phosphorylating intracellular insulin receptor substrate-1 (21-23). AKT is then activated via recruitment to cellular membranes by the PI3K lipid (24). Previous studies have reported that ERR α triggered PI3K/AKT phosphorylation by enhancing the transcription of Nectin-4, thereby promoting the growth and metastasis of GBC (25,26).

The present study aimed to investigate whether PI3K/AKT phosphorylation could positively activate the activity and expression of the PGC-1/ERR α axis. To that end, LY294002 and IGF-I were used to specifically inhibit and trigger PI3K/AKT phosphorylation, respectively. Moreover, a 3X ERE-TATA luciferase reporter was applied to measure the degree of ERR α activation. XCT-790 is a specific inverse agonist of ERR α . PGC-1 α 2x9 and XCT-790 were used to specifically enhance and inhibit the activity of ERR α , respectively.

Materials and methods

Cell culture. The NOZ human GBC cell line was purchased from Shanghai Key Laboratory of Biliary Tract Diseases, and was cultured in William's medium E (Genom Biotech Pvt., Ltd.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) in the humidified incubator containing 5% CO₂ at 37°C.

Chemicals. LY294002 (cat. no. S1105) was purchased from Selleck Chemicals to inhibit PI3K phosphorylation. Recombinant human IGF-I (cat. no. 291-G1) was acquired from R&D Systems, Inc. to promote PI3K phosphorylation. XCT-790 (cat. no. HY-10426) was purchased from MedChemExpress to inhibit the activity of ERR α . The concentration gradient was set to detect the values of IC₅₀ or half maximal effective concentration (EC₅₀) of the chemicals in NOZ cells. Inhibition curves with concentration gradients ranging from 0.1-20 μ M (treatment for 72 h at 37°C) and 0.6-40 μ M (treatment for 72 h at 37°C) for LY294002 and XCT-790, respectively, were drawn to determine the IC₅₀ values of LY294002 and XCT-790 in NOZ cells. Based on the IC₅₀ values, the final concentrations of 7 μ M LY294002 and 6 μ M XCT-790 were cocultured with NOZ cells at 37°C for 72 h in indicated experiments. The activation curve with concentration gradients ranging from 0.1-100 ng/ml (treatment for 72 h at 37°C) for IGF-I was drawn to determine the EC₅₀ value. Based on the EC₅₀ value, 13 ng/ml IGF-I was cocultured with NOZ cells at 37°C for 72 h in indicated assays.

Cell Counting Kit (CCK)-8 assay. The viability of GBC cells was determined using a CCK-8 assay (Dojindo Molecular

Technologies, Inc.) according to the manufacturer's protocol. The cells were seeded into the 96-well plate at the density of 1x10³ cells each well. Then, 24 h later, 10 μ l CCK-8 solution and 90 μ l complete medium were co-cultured with NOZ cells for 2 h at 37°C. The absorbance value (optical density) of NOZ cells was detected on a microplate reader at the wavelength of 450 nm (Bio-Tek Instruments, Inc.).

Colony formation assay. The biological effects of LY294002 and IGF-I on the colony formation ability of NOZ cells were tested. In brief, the NOZ cells were seeded into 6-well plate at a density of 500 cells each well. After 6 h, 7 μ M LY294002 and 13 ng/ml IGF-I were added into the medium for co-incubation with NOZ cells for 72 h at 37°C. Subsequently, LY294002 and IGF-I were removed, leaving the NOZ cells cultured at 37°C with the medium for 1 week. The cloning foci were fixed using 4% PFA (paraformaldehyde) for 20 min and were stained using 0.1% crystal violet for 20 min, both at room temperature. The colonies with >50 cells were counted under a light microscope (magnification, x20).

Transwell invasion assay. The 8- μ m Transwell filters (BD Biosciences) and 24-well Transwell chambers were used to detect the invasive capacity of cells. In total, 70 μ l 1 mg/ml Matrigel (BD Biosciences) was added onto the upper chamber at 37°C overnight. Then, the upper chamber with Matrigel-coated membrane was seeded with 4x10⁴ NOZ cells in 200 μ l serum-free medium. Moreover, 500 μ l basal medium containing 15% FBS was added into the lower chamber. Following the 20-h co-culturing in an incubator containing 5% CO₂ at 37°C, the cells that invaded to the lower layer were fixed using 4% paraformaldehyde for 20 min and were then stained using crystal violet for another 20 min, both at room temperature. In total, five random fields were chosen to count the invaded cells using a light microscope (magnification, x20) in order to determine the invasive capacity of NOZ cells. The assays were carried out in triplicate.

Antibodies and western blot analysis. Primary antibodies, including rabbit anti-PI3K p85 (1:1,000; cat. no. 4257), anti-AKT (1:1,000; cat. no. 4691) and anti-phosphorylated (p)-AKT (Ser473; 1:2,000; cat. no. 4060) were purchased from Cell Signaling Technology, Inc. Rabbit anti-ERR α primary antibody (1:500; cat. no. NBP1-47254) was purchased from Novus Biologicals, LLC. Rabbit anti-PGC-1 α (1:500; cat. no. ab191838), PGC-1 β (1:1,000; cat. no. ab176328) and p-PI3K p85 α (p-Y607; 1:1,000; cat. no. ab182651) were purchased from Abcam. Goat anti-rabbit HRP-conjugated secondary antibody (1:5,000; cat. no. S0001) was obtained from Affinity Biosciences.

Total proteins were extracted from each group of cells using RIPA lysis buffer (Cell Signaling Technology, Inc.), and a BCA protein quantification kit (Thermo Fisher Scientific, Inc.) was used to quantify the concentration of protein. A total of 30 μ g protein was separated via 10-15% SDS-PAGE and the proteins were then transferred onto PVDF membranes (MilliporeSigma). For the testing of non-phosphorylated antibody, 5% non-fat dry milk was used to block the PVDF membrane at room temperature for 1 h; for the testing of phosphorylated antibody, 5% BSA (Suzhou Yacoo Science Co., Ltd.) was used to block the membranes

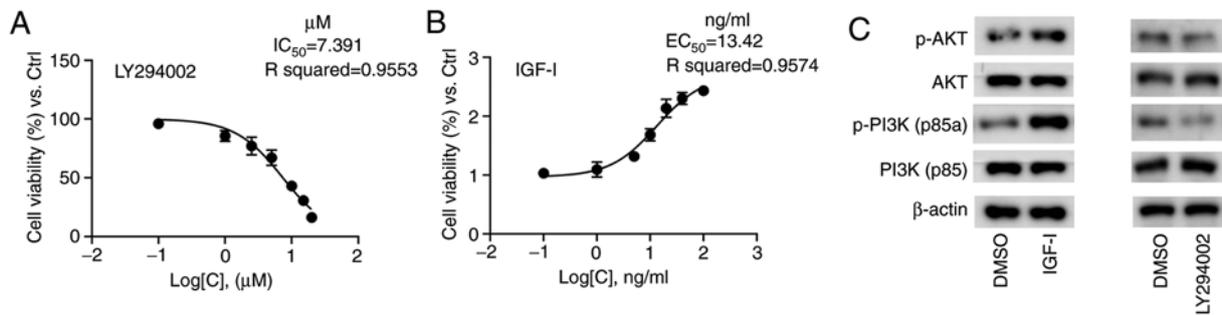


Figure 1. Sensitivity and dosage of LY294002 and IGF-I in NOZ cells. (A) The IC₅₀ value of LY294002 against NOZ cells was 7.39 μM, as calculated by the inhibition curve. (B) The EC₅₀ value of IGF-I against NOZ cells was 13.42 ng/ml, according to the calculation of the activation curve. (C) IGF-I notably elevated the expression levels of p-PI3K p85a and p-AKT, whereas LY294002 markedly inhibit the expression levels of p-PI3K p85a and p-AKT. The expression levels of the total PI3K and the total AKT were unaffected by IGF-I or LY294002. All of the experiments were repeated three times. IGF-I, insulin-like growth factor I; p-, phosphorylated; Ctrl, control.

at room temperature for 1 h. The incubation with primary antibody at 4°C lasted 12 h, followed by the 2-h co-incubation with HRP-conjugated secondary antibody (1:5,000) at room temperature. The intensities of the signals were determined using a Gel Doc 2000 system (Bio-Rad Laboratories, Inc.) after being visualized with an electrochemiluminescence kit (Wuhan Boster Biological Technology Ltd.).

RNA interference. The short hairpin (sh)RNA sequences to specifically knockdown ERRα, PGC-1α and PGC-1β were 5'-GCGAGAGGAGUAUGUUCUA-3', 5'-GAUGUGAAC GACUUGGAUACA-3' and 5'-UGUAGUUCUGUACAACUUCGG-3', respectively. The sequence for negative control (scrambled sequence) was 5'-TTCTCCGAACGTGTCACGT-3'. All sequences were constructed by Genomeditech Biotechnology, and were inserted into the PGMLV-SC5 lentivirus core vector (Genomeditech Biotechnology). In serum-free medium, the concentrated viruses with a MOI of 40 were then infected into the NOZ cells using ViaFect™ transfection reagent (Promega Corporation) following the manufacturer's instructions at 37°C. The supernatant was replaced with complete culture medium after 24 h. Subsequent experimentations were performed after 120 h.

Construction of plasmids and transfection. pGL3-Basic-3X ERE-TATA-luc that contains triple the AGGTCANN TGACCT, plasmids with WT PGC-1α [pCDNA3.1(+)-3 x Flag-C-M-PGC-1α-WT] and mutant-type (MT) PGC-1α [pCDNA3.1(+)-3 x Flag-C-M-PGC-1α-2x9] were synthesized by Genomeditech Biotechnology, in accordance with the protocol described in a previous study (16). A total of 2 μg constructed plasmids were then transfected into the NOZ cells using ViaFect Transfection reagent at 37°C. The supernatant was replaced with complete culture medium after 24 h. The expression level was analyzed via western blot analysis after 120 h. Moreover, the empty vector-infected cells (Mock-transfected) were used as the control.

Dual luciferase reporter gene assay. pGL3-Basic-3X ERE-TATA-luc was applied to detect ERRα activity. pRL-TK plasmids (25 ng; Genomeditech Biotechnology) containing PGC-1α WT or PGC-1α 2x9 (250 ng) and pGL3-Basic-3X ERE-TATA-luc (250 ng) were transfected into NOZ cells

using 1.5 μl Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 48 h. The activity of *Renilla* luciferase and firefly luciferase was detected on a luminometer using a SEAP Reporter Gene assay kit (Abcam; cat. no. ab133077). The empty vector-infected cells were used as the internal control. Finally, the results were expressed as the ratio of firefly luciferase activity/*Renilla* luciferase activity.

Statistical analysis. Quantitative data are presented as the mean ± SD based on triplicated experiments. An unpaired Student's t-test was used to compare the inter-group difference between two groups using GraphPad Prism 8.0 software (GraphPad Software, Inc.) for statistical analyses. Comparative data among multiple groups were analyzed using one-way ANOVA followed by Tukey's test, using SPSS 19.0 for Windows (IBM Corp.). The suppression curves for IGF-I, LY294002 and XCT-790 were plotted according to the results of seven differential concentrations. P<0.05 was considered to indicate a statistically significant difference.

Results

Sensitivity of NOZ cells to IGF-I and LY294002. The concentration gradients ranging from 0.1-20 μM were set to draw the inhibition curve, which demonstrated that the IC₅₀ value of LY294002 was 7.39 μM in NOZ cells (Fig. 1A). Similarly, the activation curve for IGF-I was drawn to determine that the value of EC₅₀ was 13.42 ng/ml (Fig. 1B). The final concentrations of 7 μM for LY294002 and 13 ng/ml for IGF-I were applied in the subsequent assays, and no obvious cytotoxicity was observed. The results of western blot analysis revealed that the protein expression levels of p-PI3K p85a and p-AKT were notably elevated in the NOZ cells cultured with IGF-I (Fig. 1C), indicating that IGF-I effectively activated the PI3K/AKT signaling pathway via PI3K/AKT phosphorylation. Conversely, LY294002 markedly reduced the expression levels of p-PI3K p85a and p-AKT (Fig. 1C), suggesting that LY294002 effectively diminished the PI3K/AKT signaling pathway via PI3K/AKT dephosphorylation.

Consistently, the proliferative capacity (Fig. 2A), colony formation ability (Fig. 2B) and the invasive capacity (Fig. 2C) of NOZ cells were significantly enhanced by IGF-I, but were significantly inhibited by LY294002.

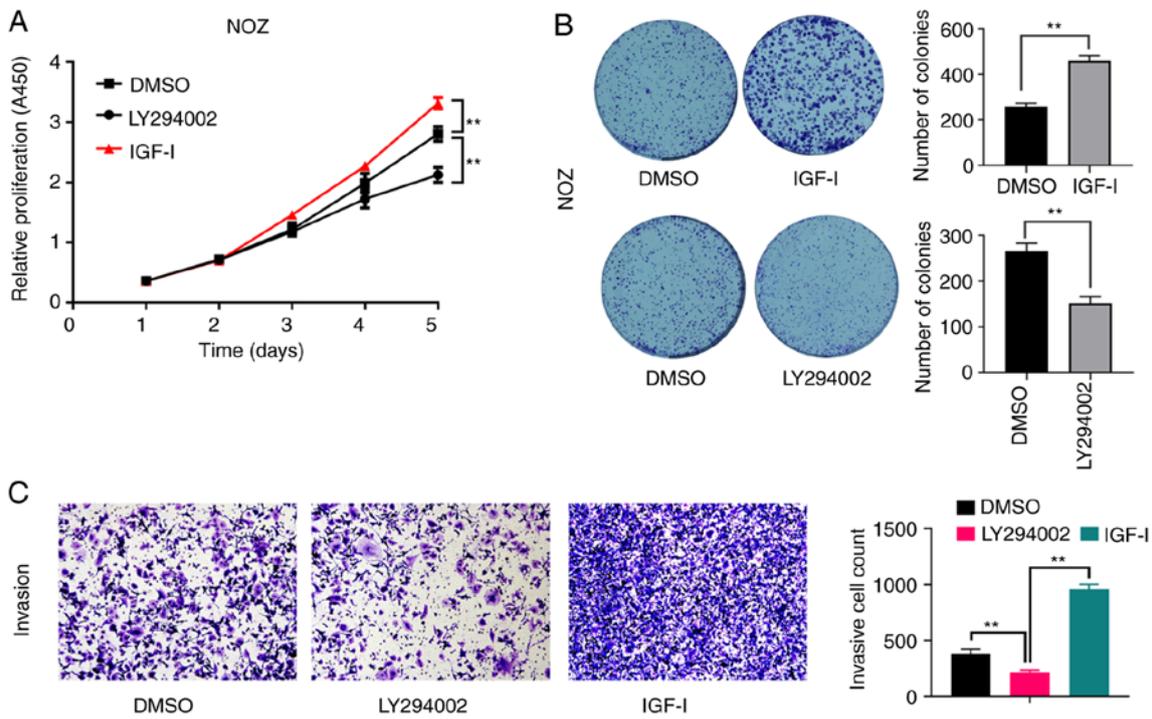


Figure 2. Biological effect of LY294002 and IGF-I on the proliferative and invasive capacity of NOZ cells. (A) A Cell Counting Kit-8 assay was performed to examine cell viability. (B) Colony formation assays. (C) Migration and invasion assays for LY294002 and IGF-I treated NOZ cells. Magnification, x20. All of the experiments were repeated three times. ** $P < 0.01$. IGF-I, insulin-like growth factor I; ERE, estrogen response element.

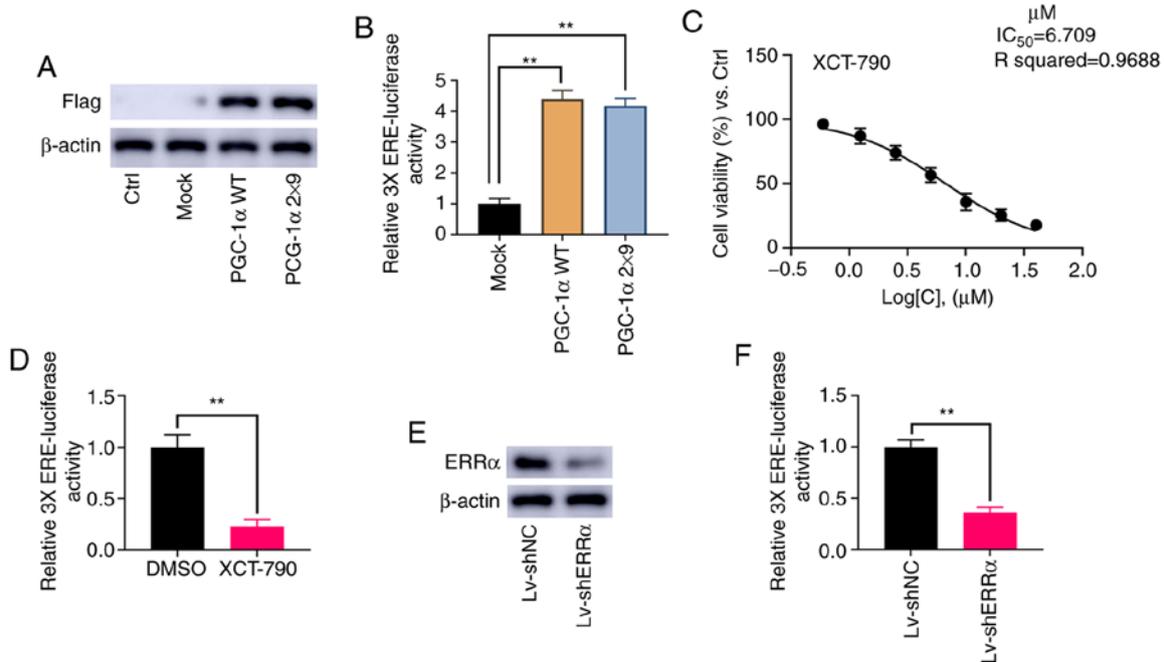


Figure 3. Detection of ERR α activation. (A) Overexpression of PGC1- α WT and PGC1- α 2x9 were validated via western blotting. (B) The activity of 3X ERE luciferase reporter was enhanced by PGC1- α WT and PGC1- α 2x9, compared with that of Mock. Empty vector-infected cells (Mock) were used as the control. (C) IC_{50} value of XCT-790 against NOZ cells was 6.709 μ M, according to the calculation of the inhibition curve. (D) The activity of 3X ERE luciferase reporter was reduced by the treatment of 6 μ M XCT-790. (E) Knockdown of ERR α was validated using western blotting. (F) The activity of 3X ERE luciferase reporter was diminished in the ERR α -knockdown cells. All of the experiments were repeated three times. ** $P < 0.01$. WT, wild-type; ERE, estrogen response element; ERR, estrogen-related receptor; PGC1- α , peroxisome proliferator-activated receptor- γ coactivator-1- α ; Ctrl, control; NC, negative control; sh, short hairpin RNA.

Detection of ERR α activation. PGC-1 α can activate ERR α , as well other receptors (16). To specifically and selectively activate ERR α , the current study followed the protocol

described by Gaillard *et al* (16) and Chang *et al* (27), replacing both L2 and L3 motifs in WT PGC-1 α with L3-09 peptides to generate PGC-1 α 2x9. PGC-1 α and PGC-1 α 2x9

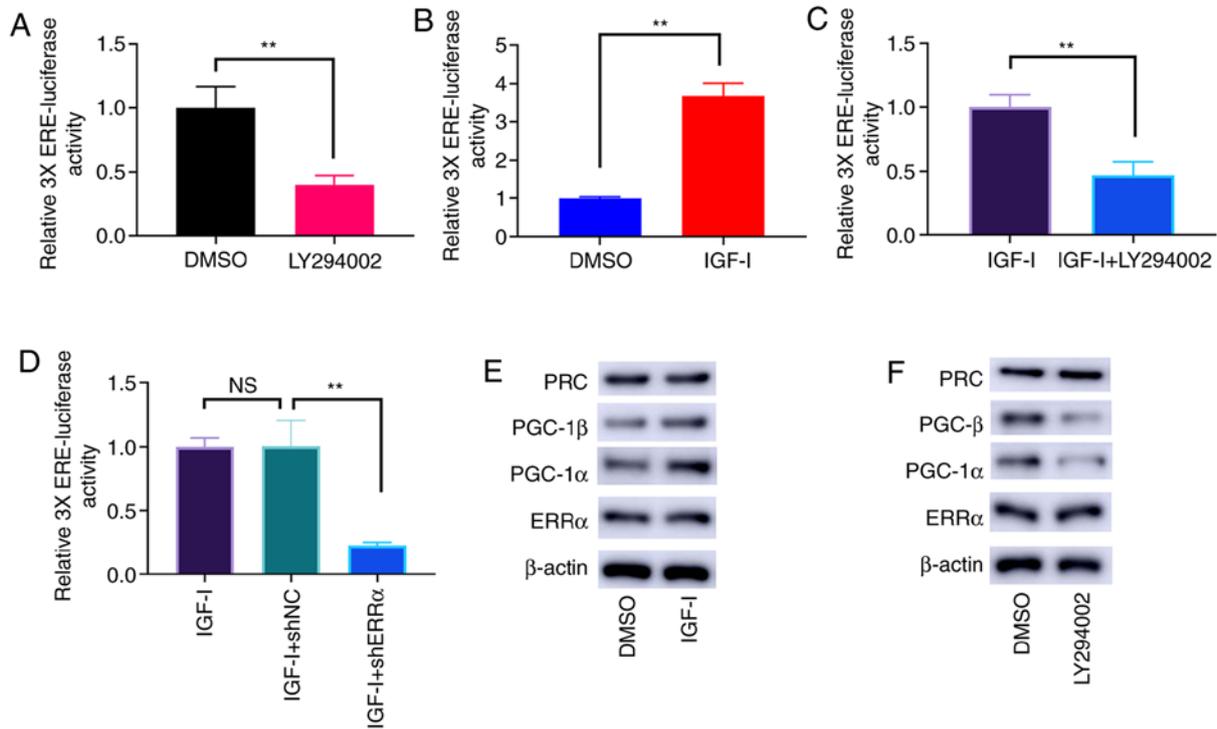


Figure 4. PI3K/AKT phosphorylation activates ERR α . (A) The inhibition of the PI3K/AKT phosphorylation by LY294002 effectively decreased the activity of ERR α . (B) PI3K/AKT phosphorylation induced by IGF-I effectively enhanced the activity of ERR α . (C) LY294002 treatment antagonized the upregulated ERR α activity induced by IGF-I. (D) The knockdown of ERR α antagonized the upregulated ERR α activity caused by IGF-I. (E) Protein expression levels of PGC-1 α and PGC-1 β were notably elevated by IGF-I. The expression levels of PRC and ERR α were not affected. (F) The protein expression levels of PGC-1 α and PGC-1 β were reduced by LY294002, while those of PRC and ERR α were not altered. All of the experiments were repeated three times. **P<0.01. IGF-I, insulin-like growth factor I; ERE, estrogen response element; ERR, estrogen-related receptor; PGC1-I, peroxisome proliferator-activated receptor- γ coactivator-1; PRC, PGC-related coactivator; NC, negative control; sh, short hairpin RNA.

were successfully overexpressed in NOZ cells (Fig. 3A). As shown in Fig. 3B, the relative activity of 3X ERE TATA dual luciferase reporter was significantly increased by PGC-1 α and PGC-1 α 2x9 (P<0.01).

As a specific inverse agonist of ERR α , XCT-790 can inhibit the activation of ERR α (28). The results demonstrated that the IC₅₀ value of XCT-790 in NOZ cells was 6.71 μ M (Fig. 3C), and therefore, a final concentration at 6 μ M XCT-790 was applied in subsequent assays. As presented in Fig. 3D, 6 μ M XCT-790 significantly inhibit the activation of 3X ERE TATA dual luciferase reporter (P<0.01). Moreover, it was found that the knockdown of ERR α significantly reduced the activation of 3X ERE TATA dual luciferase reporter (P<0.01; Fig. 3E and F). These results indicated that the relative 3X ERE TATA luciferase activity was consistent with the activity of ERR α .

PI3K/AKT phosphorylation triggers the ERR α activity. The dephosphorylation of PI3K/AKT by LY294002 led to the lower activities of ERR α (Fig. 4A). Conversely, PI3K/AKT phosphorylation induced by IGF-I enhanced the activities of ERR α (Fig. 4B), and this effect was offset by LY294002 (Fig. 4C) and ERR α knockdown (Fig. 4D). Nevertheless, the protein expression level of ERR α was not affected by PI3K/AKT phosphorylation. As potential coactivators of ERR α , PGC-1 α and PGC-1 β expression was notably elevated by PI3K/AKT phosphorylation (Fig. 4E). Conversely, dephosphorylation of PI3K/AKT by LY294002 reduced the protein expression levels of PGC-1 α and PGC-1 β (Fig. 4F). However, the protein

expression level of PGC-related coactivator (PRC) was not affected by PI3K/AKT phosphorylation (Fig. 4E and F).

PGC-1 α and PGC-1 β mediate the activation of ERR α enhanced by IGF-I. As shown in Fig. 5A and B, PGC-1 α and PGC-1 β were effectively knocked down by Lv-shPGC-1 α and Lv-shPGC-1 β . Moreover, the loss of PGC-1 α and PGC-1 β antagonized the increased ERR α activity caused by IGF-I (Fig. 5C and D). Similarly, the enhanced cell viability caused by IGF-I was antagonized by the knockdown of PGC-1 α and PGC-1 β and the treatment of LY294002 (Fig. 5E-G). The effect of LY294002 treatment and the knockdown of PGC-1 α and PGC-1 β also antagonized the increased colony formation and invasive ability of NOZ cells (Fig. 6A and B). Therefore, the activation effect of PI3K/AKT on ERR α was attributable to its ability of elevating PGC-1 α and PGC-1 β expression.

Discussion

The vast majority of GBC cases are diagnosed at the advanced stages, and the low 5-year survival rate of patients with advanced GBC is aggravated by low sensitivity to chemoradiotherapy and targeted therapy (29). Moreover, the molecular mechanisms that underlie the onset and progression of GBC continue to defy the medical community (30). Thus, additional efforts are required to develop novel effective targeted therapies, which are considered to be the key to improve the prognosis and the quality of life of patients with GBC.

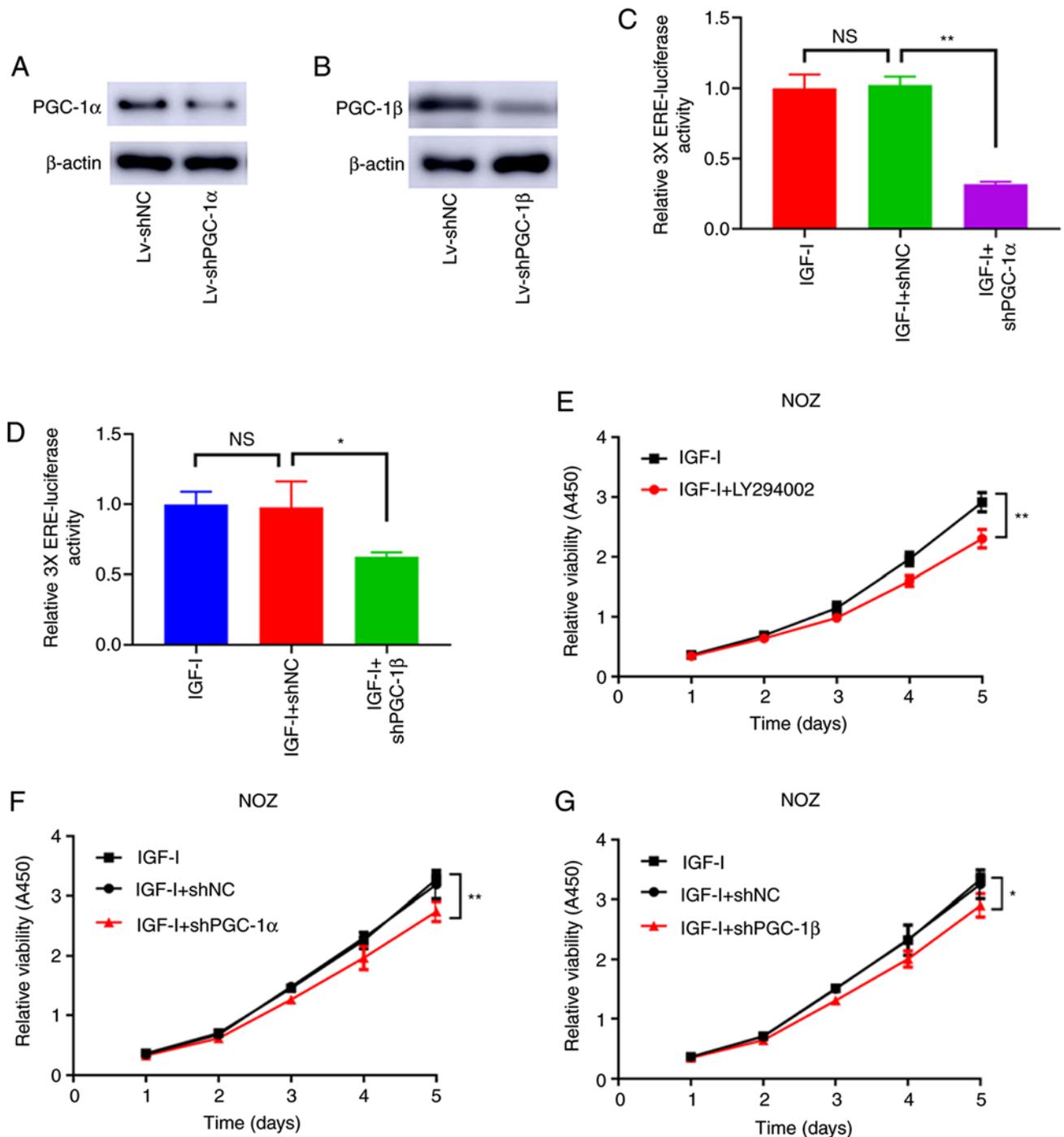


Figure 5. Knockdown of PGC-1 α and PGC-1 β attenuates the effect of IGF-I on ERR α . (A) Lv-shPGC-1 α and (B) Lv-shPGC-1 β effectively knocked down PGC-1 α and PGC-1 β , respectively. Knockdown of (C) PGC-1 α and (D) PGC-1 β attenuated the upregulated activity of ERR α caused by IGF-I. (E) LY294002 treatment and knockdown of (F) PGC-1 α and (G) PGC-1 β reduced NOZ cell viability that was enhanced by IGF-I. All of the experiments were conducted in triplicate. * $P < 0.05$, ** $P < 0.01$. IGF-I, insulin-like growth factor I; ERE, estrogen response element; ERR, estrogen-related receptor; PGC1-I, peroxisome proliferator-activated receptor- γ coactivator-1; NC, negative control; sh, short hairpin RNA; NS, not significant.

Our previous study reported that ERR α enhanced the transcription of Nectin-4, thereby triggering the PI3K/AKT signaling pathway to promote the growth and metastasis of GBC (25). As an orphan nuclear receptor in the nucleus, ERR α bears structural resemblance to ER α . Nevertheless, ERR α cannot be activated by estrogen (11). The majority of the genes under the regulation of ERR α are distinct from those mediated by ER α . The PGC-1 family serves as co-activators to activate ERR α , which, once activated, can

regulate the expression levels of genes that are involved in the tricarboxylic acid cycle, lipid metabolism and oxidative phosphorylation (27). Accumulating evidence has shown that ERR α may be involved in a wide variety of cancer types (31). Therefore, in-depth examination into the molecular mechanisms that affect the activity of ERR α could shed light on ERR α targets. For example, in a recent study, Yang *et al* (15) revealed that F-box and leucine-rich repeat protein 10 increased ERR α enrichment at the promoter region of its

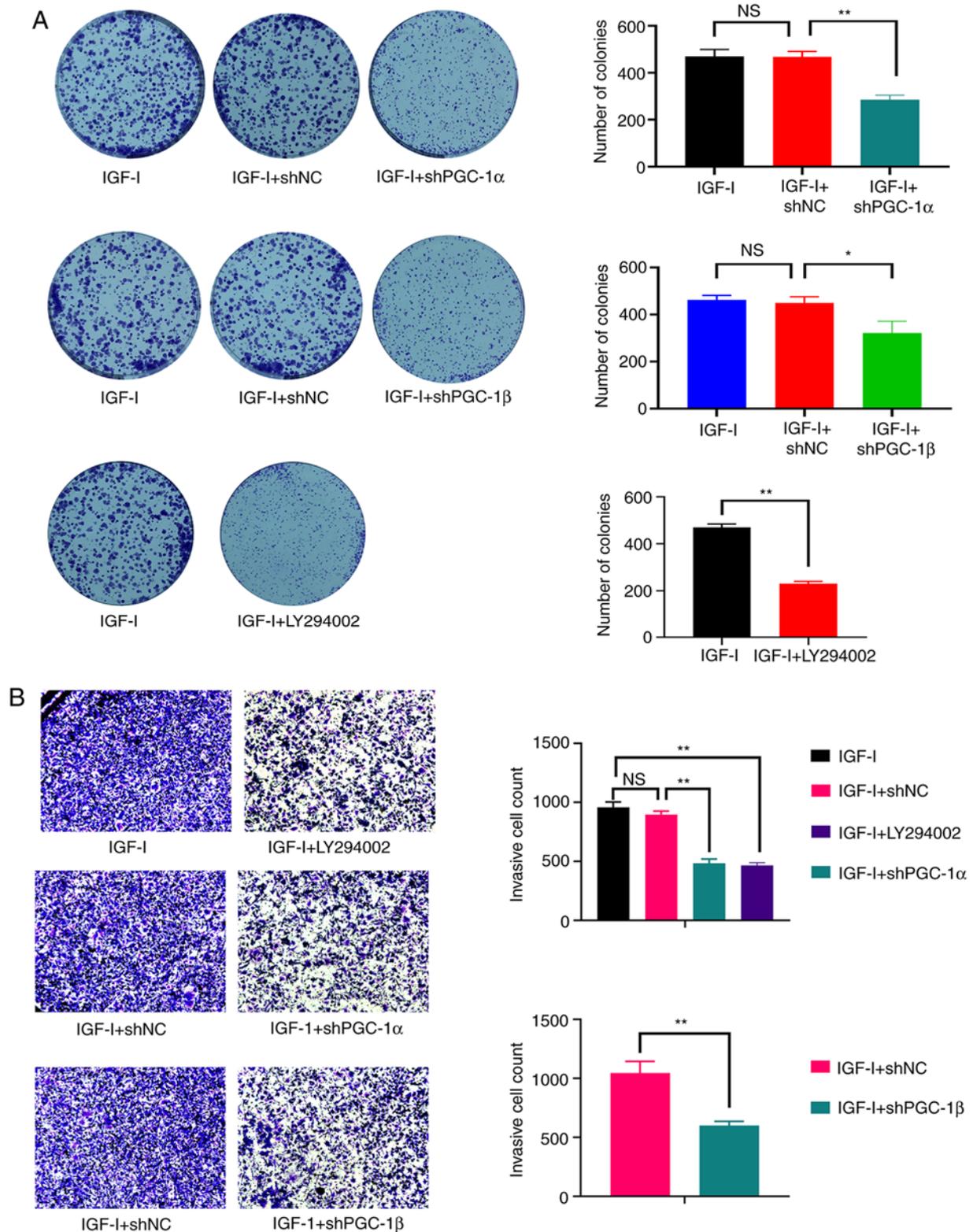


Figure 6. LY294002 treatment and the knockdown of PGC-1 α and PGC-1 β antagonize the effect of IGF-I on NOZ cell colony formation and invasion. (A) The cell colony formation ability elevated by IGF-I was significantly decreased by LY294002 and the knockdown of PGC-1 α and PGC-1 β . (B) The elevated invasive capacity of NOZ cells by IGF-I was antagonized by LY294002 and the knockdown of PGC-1 α and PGC-1 β . Magnification, x20. All of the experiments were conducted in triplicate. *P<0.05, **P<0.01. IGF-I, insulin-like growth factor I; PGC1-1, peroxisome proliferator-activated receptor- γ coactivator-1; NC, negative control; sh, short hairpin RNA; NS, not significant.

target genes by promoting the mono-ubiquitylation of ERR α . However, additional, novel pathogenesis mechanisms are yet to be elucidated.

The primary aim of the present study was to validate whether PI3K/AKT phosphorylation affects and regulates ERR α activity in GBC cells to form a positive feedback loop.

To that end, IGF-I and LY294002 were used to enhance and inhibit PI3K/AKT phosphorylation in NOZ cells, respectively. The present results demonstrated that the bioactivity of ERR α was upregulated and downregulated, respectively, and hence a positive feedback loop of ERR α /PI3K/AKT could be established.

The genes in the PI3K/AKT pathway show the highest frequency of aberrant expression in human cancer (17,32). The activated PI3K/AKT pathway functions to enhance the transformation, proliferation and invasion of cancerous cells. Moreover, the aberrant overexpression or activation of PI3K/AKT has been reported in various malignancies, including GBC, and is associated with an improved proliferative capacity and invasive potential of cancerous cells (17). Therefore, the PI3K/AKT signaling pathway is an ideal target to provide a promising approach for the prevention and clinical therapy of cancer cases. The PI3K/AKT signaling pathway exerts an anti-apoptotic effect mainly by influencing a variety of downstream effector molecules, such as CREB regulated transcription coactivator 1, ribosomal protein S6 kinase B1, S6 Rb and eukaryotic translation initiation factor 4E (17,32). At present, the PI3K/AKT signaling pathway and its related genes can be suppressed by applying gene intervention methods or via the treatment of small-molecule compound drugs. Blocking the activation of a variety of downstream anti-apoptotic effector molecules and promoting cell apoptosis are regarded as effective means to treat cancer (33). In the present study, it was found that PI3K/AKT phosphorylation activated ERR α , but does not promote the amplification of ERR α , which indicated that the activity of ERR α depends on the binding state rather than the total amount. The abundant factors in the ERR α /PI3K/AKT circuit are regarded as potential targets for the targeted therapy of GBC. Therefore, a novel combination therapy using the antagonist of ERR α and the inhibitors of PI3K/AKT signaling has a promising prospect to improve the prognosis of patients with GBC.

The present study demonstrated that PGC-1 α and PGC-1 β were downstream targets of the PI3K/AKT signaling pathway, and that the PGC-1 family acted as the nuclear transcription co-activator that mediates multiple cellular pathways, among which the regulation of metabolism (34) and tissue-specific functions (13,35-37) are most prominent. The PGC-1 family consists of PGC-1 α , PGC-1 β and PRC (13). The PGC-1 family serves a critical role in the regulation of mitochondrial biogenesis and bioenergetics. Furthermore, PGC-1 co-activators are essential to sustain tumor survival and growth (38). PGC-1 α activity is regulated by a number of post-translational modifications, such as methylation, phosphorylation and acetylation (39). PGC-1 α and PGC-1 β bind to multiple nuclear transcription factors or hormone receptors, including ER, ERR and thyroid hormone receptor. The presence of PGC-1 α and PGC-1 β is required for the activity of ERR α (36). In NOZ cells, the phosphorylated PI3K/AKT function could elevate the activity of PGC-1 α and PGC-1 β , and thereby enhance ERR α activity.

In summary, the present study reported the sensitivity and dosage of LY294002 and IGF-I in inhibiting and activating the PI3K/AKT signaling pathway in NOZ cells, respectively. The experimental results of dual luciferase reporter gene assay indicated that ERR α was positively regulated by PI3K/AKT

phosphorylation. Furthermore, PGC-1 α and PGC-1 β were shown to mediate the activation of ERR α stimulated by PI3K/AKT phosphorylation. Thus, the combined inhibition of multiple targets in the positive feedback loop of ERR α /PI3K/AKT may present significant potential to provide promising anti-cancer solutions.

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Availability of data and materials

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

Authors' contributions

LW, MY and HJ designed the study, analyzed the data, performed the experiments and wrote the manuscript. LW and HJ performed the critical revision of the manuscript and supervised the study. All authors read and approved the final manuscript. LW and HJ confirm the authenticity of all the raw data.

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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