ERR α promotes gallbladder cancer development via activating PI3K/AKT signaling pathway mediated by Nectin-4

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Abstract

Background: Gallbladder cancer (GBC) is the most common malignant tumour of the bile duct with a poor prognosis. The estrogen-related receptor alpha (ERR α) is a nuclear receptor that has been associated with metabolic processes and cancer progression. Increased ERR α has been shown in endocrine-related cancer and non-endocrine-related cancer. Nevertheless, its role in GBC remains unknown. **Methods:** ERR α expression was analyzed by immunohistochemistry in 59 GBC samples. Its association with clinicopathologic characteristics was evaluated. The effect of ERR α on GBC cell proliferation and invasion was evaluated by loss- or gain-of-function assays in vitro and in vivo. The influence of ERR α on EMT biomarkers, Nectin-4 and PI3K/AKT signaling pathway was detected by western blotting. Inhibition of Nectin-4 was conducted to explore the potential mechanism of ERR α in GBC. **Results:** Our study reveals increased ERR α expression in GBC tissues vs. non-tumour adjacent tissues. ERR α expression is significantly positively correlated with high TNM stage, high invasion depth and lymph node metastasis, while negatively associated with prognosis. Targeted depletion of ERR α by lentivirus-mediated shRNA decreased cell proliferation in vitro and in vivo. ERR α promoted cancer cell migration and epithelial-mesenchymal transition by regulating the expression of EMT relevant genes. Ectopic expression of ERR α promoted GBC cell growth and invasion in vitro, while inhibition of Nectin-4 attenuated cell growth and invasion induced by ERR α . Moreover, ERR α overexpression activated the PI3K/AKT signaling pathway while this effect can be blocked by Nectin-4 depletion. Nectin-4 was involved in the oncogenic function of ERR α to activate PI3K/AKT signaling pathway in GBC. **Conclusions:** ERR α promotes GBC progression via activating PI3K/AKT signaling pathway mediated by Nectin-4. ERR α may be a potential prognostic factor and molecular therapeutic target for GBC.

Background

Gallbladder cancer (GBC) is the most common biliary tract malignancy with an increasing incidence worldwide^[1, 2]. Due to easier local infiltration and distant metastasis, gallbladder carcinoma has limited therapeutic options and a poor outcome with conventional therapy^[3-5]. An increasing need for novel prognostic biomarkers and targeted therapeutics based on epidemiology exists. The female-to-male (F/M) ratio of GC incidence rates varied greatly: it exceeded 5 in several high-risk areas as well as in low-risk areas, but was typically between 2 and 3^[6, 7]. Therefore, we investigated several sex-specific genes and found that Estrogen-related receptor α (ERR α) promotes human gallbladder tumor growth.

ERR α is structurally most related to the canonical estrogen receptor(ER)^[8, 9]. Unlike ERs, ERR α does not bind to naturally occurring estrogens, but functions by binding to estrogen-related responsive element(ERE) and estrogen-related responsive element (ERRE), thus regulating bioenergetic pathways required for cell- and tissue-specific functions^[10]. ERR α is one of the orphan members of the nuclear receptor superfamily, and, as such, acts a transcriptional regulator^[11]. In contrast to several other members of the NR family, no natural ligand has been, to date, identified for ERR α , which is thus referred to as “orphan”^[12]. This receptor promotes cellular proliferation, migration, invasion, resistance to hypoxia,

as well as metabolic reprogramming, which all contribute to cancer aggressiveness^[11, 13-15]. However, the expression and function of ERRα in gallbladder cancer remains unclear.

In this study, we demonstrated for the first time that a significant correlation exists between higher expression of ERRα and a worse prognosis in GBC patients. We showed that shRNA-mediated knockdown of ERRα markedly reduced gallbladder cancer cell growth and tumorigenicity *in vitro* and *in vivo*. And ERRα promoted GBC cell migration and invasion by inducing epithelial-mesenchymal transition (EMT). Conversely, upregulating ERRα promoted the migration and invasion ability of GBC cells. We also demonstrated that the Nectin-4 and PI3k/AKT pathway is involved in the oncogenic effects of ERRα in GBC cells.

Methods

Patients and clinicopathological data

This study was approved by the ethics committee of Nanjing Medical University. (ID no: NJMU-EC-2018-147), and all patients provided written informed consent. Cancer tissue specimens were achieved from 59 GBC patients with pathologically confirmed GBC after surgery between 2011 and 2017 at the department of hepatobiliary surgery, the Affiliated Wuxi No.2 People's Hospital of Nanjing Medical University. None of the patients received radiotherapy or chemotherapy before surgery. In addition, 59 cholecystitis patients undergoing simple cholecystectomy were studied as controls. All diagnoses of GBC, cholelithiasis, and lymph node metastasis were confirmed by two experienced pathologists separately, and all tissue samples were fixed in 4% formalin immediately after removal and were embedded in paraffin for immunohistochemical staining(IHC). The tissue samples were staged on the basis of the AJCC-TNM Classification of Malignant Tumors (7th edition).

Quantitative immunohistochemistry assays

IHC was performed and evaluated using a standard procedure^[16].The intensity of immunoreaction was scored as follows: 0, no staining; 1, weak staining; 2, moderate immunoreactivity and 3 for strong immunoreactivity. The percentage of ERRα expression was semi-quantitatively scored as follows: 0 for <5% immunoreactive cells; 1 for 5-25% immunoreactive cells; 2 for 25-50% immunoreactive cells; 3 for 50-75% immunoreactive cells and 4 for >75% immunoreactive cells. The final immunoreaction score was defined as the sum of extent and intensity parameters. The samples were classified as negative (0-1), weak (2-3), moderate (4-5), and strong (6-7) staining. For statistical purposes, only the final immunoreaction scores of moderate and strong were considered positive, and the others were negative.

Quantitative real-time PCR

Trizol reagent (Takara) was used to extract total RNA from cells. cDNA was synthesized by use of PrimeScript Reverse Transcriptase (Takara, Osaka, Japan) following the manufacturer's instructions. The primers used for detection of ERRα, and GAPDH (internal control) expression were listed as following:

ERR α (sense): 5'-CACTATGGTGTGGCATCCTG-3'; ERR α (antisense): 5'-CGCTTGGTGATCTCACACTC-3'; GAPDH (sense): 5'-AGAAGGCTGGGG CTCATTTG-3'; GAPDH (antisense): 5'-AGG GGC CAT CCA CAG TCT TC-3'. We operated StepOnePlus™ Real-Time PCR system (Applied Biosystems) to amplify cDNA using the SYBR-Green method (Takara).

Cell culture and chemicals

GBC-SD cell line was obtained from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China. NOZ, OCUg and EH-GB1 cell lines were obtained from the Health Science Research Resources Bank, Osaka, Japan. The GBC-SD, OCUg, EH-GB1 cells were maintained in DMEM (Gibco, Gaithersburg, MD, USA), and the NOZ cells in William's medium E (Lonza, Belgium, WI, USA) containing 10% fetal bovine serum (FBS) at 37 °C in a humidified 5% CO₂ incubator.

Immunofluorescence analysis

Cells were fixed in 4% paraformaldehyde and permeabilized in 0.5% Triton X-100 at room temperature. After blocking with 1% BSA for 1 h, the cells were incubated with anti-ERR α antibody at 4 °C overnight. Then, the cells were incubated for 30 min at 37 °C with Goat Anti-Rabbit IgG (Proteintech, Wuhan, China) and counterstained with DAPI.

RNA interference, construction of plasmids and transfection

The short hairpin RNA (shRNA) sequence targeting human ERR α was 5'-GCGAGAGGAGUAUGUUCUA-3'. The sequence of negative control shRNA was 5'-TTCTCCGAACGTGTACGT-3'. Both shRNAs were synthesized and inserted into the PGMLV-SC5 lentivirus core vector, containing a cytomegalovirus (CMV)-driven enhanced green fluorescent protein (EGFP) reporter gene. These lentiviruses were constructed by Genomeditech (Shanghai, China). NOZ and OCUg cells were infected with concentrated virus in serum-free medium. The supernatant was replaced with complete culture medium after 24 h. ERR α expression in the infected cells was validated by qRT-PCR and western blot assays after 120 h.

Construction of plasmids and transfection

The full-length sequence of ERR α (GenBank accession number NM_004451.4) was cloned into the pCDNA3.1 expression vector (Genomeditech, Shanghai, China) and transfected into GBC-SD cells using ViaFect™ Transfection Reagent following the manufacturer's instructions (Promega). Stable ERR α -expressing clones were selected for 2 weeks using neomycin (Genechem), and the expression level of ERR α was determined by western blot assays. Empty vector-transfected cells (MOCK) were used as control.

Antibodies and western blotting

The anti-ERR α antibody was purchased from Novus Biologicals, Canada. The anti-Nectin-4 antibody were purchased from Abcam (Cambridge, MA, USA). The anti-Lumin B1 antibody were purchased from Boster

(California, USA). Anti-E-cadherin, -vimentin, -PI3K, -p-PI3K (Tyr607), -AKT, -p-AKT (Ser473), and β -actin antibodies were acquired from Cell Signaling Technology (Danvers). Proteins from cells were separated by SDS-PAGE and then transferred onto PVDF membranes (Millipore Corp., Billerica, MA, USA). The membranes were blocked in 5% skimmed milk (5% BSA for p-PI3K and p-AKT) and then incubated with at 4° C overnight with primary antibodies. After washing, the membranes were reacted with secondary antibody at room temperature. The blots were detected by enhanced chemiluminescence (Millipore, Billerica, MA). β -Actin and Lumin B1 were used as the loading controls for total protein and nucleoprotein respectively. All assays were performed in triplicates.

In vitro tumorigenesis assays

A Cell Counting Kit-8 (CCK-8; Dojindo) cell proliferation assay was performed to assess the proliferation of cells according to the manufacturer's instructions. The absorbance value was measured at 450 nm. A colony formation assay was performed to evaluate the anchorage-independent growth of NOZ, OCUg and GBC-SD cells. After culturing for 9 days, cells were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet (Sigma) for 15 min. The numbers of colonies (>50 cells/colony) were photographed and counted.

In vitro migration and invasion assays

Cell migration and invasion were performed using 8- μ m transwell filters (BD Biosciences, Franklin Lakes, NJ, USA). 2×10^4 cells were planted in the upper chamber with 200 μ l serum-free medium, while the lower chamber was filled with 500 μ l basal medium containing 15% FBS. For cell invasion assay, the upper chambers were pre-coated with 50 μ g/chamber of a solubilized basement membrane in the form of Matrigel (BD Biosciences). After 20 h of incubation, cells that migrated to the bottom of the insert were fixed with 4% paraformaldehyde for 20 min and stained with crystal violet for 20 min. Four random fields were chosen to capture images.

Nude mice subcutaneous xenograft model

Ten Nude nu/nu mice (3–5 weeks, 12–16g) were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). These mice were randomly divided into two groups (Lv-shNC and Lv-shERR α). 2×10^6 NOZ cells were subcutaneously injected into the left axilla of each mouse. Tumor size was monitored every week. Four weeks later, all the mice were executed by dislocation of the cervical vertebra and all implanted tumors were surgically collected. Tumor volume was calculated using the following formula: tumor volume (mm^3) = $1/2 \times \text{width}^2 \times \text{length}$, where the width and length were the shortest and longest diameters, respectively. All of the experiments were carried out followed by the protocols of the NJMU Institutional Animal Care and Use Committee.

Statistical analysis

IBM SPSS Statistics 19.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. The quantitative data were expressed as the mean $\pm$ standard deviation (SD). The independent Student's t-test was used to compare the means of two groups. The difference between ERR α expression and clinicopathologic parameters were performed using χ^2 tests. The Kaplan–Meier test was performed for univariate survival analysis. Multivariate analysis was determined by using the Cox proportional hazards model. Significant differences between groups were considered for $p < 0.05$. All of the results are representative of three independent experiments.

Results

Nuclear ERR α were highly expressed and associated with a poor outcome in GBC patients

To explore the role of ERR α in GBC progression, we tested nuclear ERR α expression in tumor samples from patients with GBC or cholelithiasis by using IHC(Fig. 1a). 67.80% (40/59) of the GBC patients showed positive nuclear ERR α staining(Table 1). ERR α expression level was significantly higher in tumor tissues than in cholelithiasis tissues (Fig. 1b, $P < 0.01$). A Clinicopathological correlation analysis of the 59 GBCs found that higher ERR α levels were associated with higher AJCC TNM stage($P < 0.01$), deeper invasion depth($P < 0.01$) and lymph node metastasis($P < 0.01$) (Table 1). More intriguingly, Kaplan–Meier analysis for relapse-free survival(RFS) and overall survival(OS) indicated that positivity of ERR α correlated with shorter RFS and OS(Fig. 1c and 1d, $P < 0.01$). Multivariate Cox regression analysis further revealed that ERR α expression was negatively correlated with post-operative survival(Table 2, $P < 0.05$), indicating ERR α expression level is an independent prognostic marker in GBC patients.

ERR α expression in GBC cell lines

Immunocytochemical staining revealed that ERR α protein expressed in the nucleus of GBC cells (Fig. 2a). We detected the expression of ERR α in four GBC cell lines by RT-PCR and found that ERR α expression was overexpressed in NOZ and OCUg cells and gradually decreased in EH-GB1 and GBC-SD cells(Fig. 2b). Therefore, we chose high ERR α expression NOZ and OCUg cell lines to explore oncogenic function with Lv-shERR α and Lv-shNC. Conversely, low ERR α expression GBC-SD cell line was chosen to investigate oncogenic function with ERR α -expression vector and empty vector. The efficiencies of lentiviral transduction were examined by EGFP expression under a microscope at 72 h after transduction. Lentiviral transduction efficiencies in these cell lines were higher than 95% (Fig. 2c). Western blotting were performed to confirm transfection efficiency, indicating that ERR α expression was effectively suppressed by Lv-shERR α (Fig. 2d) and overexpressed by ERR α -expression vector(Fig. 2d).

Effects of ERR α knockdown and overexpression on GBC cell proliferation in vitro and in vivo

CCK-8 and colony formation assays were performed to assess whether ERR α affects GBC cell proliferation. As shown in Fig 3a, the viability of NOZ and OCUg cells transfected with Lv-shERR α was

significantly decreased compared with negative controls. Fig 3b revealed that the colony formation ability of NOZ and OCUg cells was significantly inhibited by ERR α depletion. ($P<0.01$). Furthermore, to explore the effect of silencing ERR α on gallbladder cancer growth in vivo, we injected subcutaneously ERR α -depleted and negative control NOZ cells into the left axilla of nude mice. Fig 3c showed that the tumor volume and weight of ERR α -depleted xenografts were significantly inhibited. Conversely, compared with empty vector-transfected cells, ERR α -transfected GBC-SD cells revealed increased growth rates (Fig 5a) ($P<0.01$), greater colony forming abilities (Fig 5b) ($P<0.01$). Collectively, our data show that ERR α plays a vital role in enhancing cell proliferation both in vitro and in vivo.

ERR α promotes GBC cell migration and invasion by inducing EMT

Transwell migration and matrigel invasion assays were performed to assess whether ERR α affects GBC metastasis. As shown in Fig 4a, the migrative and invasive capability of NOZ and OCUg cells transfected with Lv-shERR α was significantly decreased compared with negative controls ($P<0.01$). Conversely, compared with empty vector-transfected cells, ERR α -transfected GBC-SD cells revealed increased migrative and invasive capability (Figure 5c) ($P<0.01$). To explore whether ERR α enhanced the invasiveness of GBC cells through EMT processes, we detected EMT biomarkers by western blotting. After ERR α was depleted, NOZ cells expressed high levels of E-cadherin, which is characteristic of epithelial cells (Fig. 4B). However, the expression level of Vimentin (indicating a mesenchymal phenotype) was lower in ERR α -depleted NOZ cells (Fig. 4B). This indicates that ERR α promotes GBC cell migration and invasion by inducing EMT.

ERR α enhances GBC cell proliferation and migration via activating PI3K/AKT signaling pathway

As shown in Fig. 5g, in NOZ cell line, knockdown of ERR α downregulated the expression of p-PI3K and p-AKT while the expression levels of total PI3K and AKT were not changed. Conversely, compared to empty vector-transfected cells, overexpression of ERR α in GBC-SD cell line upregulated the expression of p-PI3K and p-AKT. Those results indicated that PI3K/AKT signaling pathway mediated the carcinogenesis of ERR α in gallbladder cancer.

ERR α activated PI3K/AKT signaling pathway by regulating Nectin-4

In our previous study, we have demonstrated that Nectin-4 promotes GBC cell proliferation and migration by activating PI3K/AKT signaling pathway. In the present study, we observed decreased Nectin-4 level in the NOZ cells of ERR α depletion and increased Nectin-4 level in the GBC-SD cells of ERR α overexpression (Fig. 5g). Inhibition of Nectin-4 attenuated cell growth and invasion induced by ERR α overexpression in GBC-SD cells (Figure 5d, 5e, 5f). Furthermore, depleting Nectin-4 in ERR α -overexpressed GBC-SD cells depressed the expression of p-PI3K and p-AKT. These results indicate that Nectin-4 mediates the influence of ERR α on the PI3K/AKT signaling pathway.

Discussion

Gallbladder cancer is a relatively rare neoplasm with a poor prognosis. In China, the incidence of GBC rises year after year^[6]. Late diagnosis and deprived prognosis are major problems for treatment of gallbladder carcinoma. The interplay of genetic susceptibility, lifestyle factors and infections in gallbladder carcinogenesis is still poorly understood. GBC is several-fold more common in women than in men^[7]. However, estrogen-related receptor α (ERR α) has not been linked to GBC.

The orphan receptor estrogen-related receptor α (ERR α) is a member of the nuclear receptor superfamily of ligand-regulated transcription factors. ERR α is structurally most related to the canonical estrogen receptor and has been shown to modulate estrogen signaling in breast cancer^[9]. Unlike ERs, ERR α does not bind to naturally occurring estrogens, but functions by binding to estrogen-related responsive element (ERE) and estrogen-related responsive element (ERRE)^[10]. ERR α has also been proved to be involved in the carcinogenesis of ERR α in human oral squamous cell cancer^[17], human prostate cancer^[18] and human colon cancer^[11]. Several mechanisms have been proposed to account for the carcinogenesis of ERR α in different tumors. Kressler D et al demonstrated that the activity of ERR α is regulated by the expression level and activity of its obligate coregulators, peroxisome proliferator activated receptor gamma coactivator-1 alpha and beta (PGC-1 α or PGC-1 β)^[19, 20]. By a chemical genomic approach, Chang CY et al found that activation of the Her2/IGF-1 signaling pathways and subsequent C-MYC stabilization upregulate the expression of peroxisome proliferator-activated receptor gamma coactivator-1 beta (PGC-1 β), thus upregulating the activation of ERR α in breast cancer^[21]. ERR α has been shown to transcriptionally regulate the expression of several genes, such as WNT11, CCNE1, OPN and OPG, involved in cell cycle, metastasis and metabolism^[22]. In the present study, we validated that the high expression of ERR α in GBC tissues correlated with high TNM stage, high invasion depth and lymph node metastasis. ERR α was further identified as an unfavorable independent prognostic factor for GBC, indicating that ERR α might play a role in GBC development.

In the present study, a series of assays *in vitro* and *in vivo* showed that downregulating ERR α repressed GBC cell growth and colony formation capability, indicating its role in GBC cell proliferation. Conversely, upregulating ERR α promoted the proliferation of GBC cells. Moreover, GBC cell migration and invasion ability were inhibited by ERR α knockdown. Conversely, upregulating ERR α promoted the migration and invasion ability of GBC cells, suggesting that ERR α might be involved in EMT. EMT plays a vital role in cancer invasion and metastasis. During this process, epithelial cells lose their properties and acquire mesenchymal phenotypes. Mesenchymal phenotype cells exhibit increased expression of mesenchymal-related markers, such as vimentin, and decreased expression of epithelial-related markers such as E-cadherin^[23]. In our study, knockdown of ERR α induced EMT by increasing epithelial marker E-cadherin and decreasing mesenchymal marker Vimentin, indicating that ERR α promotes GBC metastasis by inducing EMT. The above results indicate ERR α could serve as a potential diagnostic biomarker and therapeutic target in GBC patients. To get greater insights into the mechanisms of the pro-oncogenic function of ERR α , we found that the protein expressions of Nectin-4 and PI3K/AKT signaling pathway were reduced after downregulating ERR α .

Nectin-4 belongs to the nectin family which has various physiological and pathological functions in humans. Nectin-4 plays a role in the formation and maintenance of adherens junctions in cooperation with cadherins^[24-27]. Other scholars have revealed that nectin-4 is re-expressed as a tumour-associated antigen with pro-oncogenic properties in various carcinomas[28-30]. In our previous study^[31], Nectin-4 is critical for GBC proliferation and invasion via activating PI3K/AKT pathway. In the present study, we found that the expression of Nectin-4 was elevated after overexpressing ERR α in GBC cells. Inhibition of Nectin-4 attenuated cell growth and invasion induced by ERR α upregulation in GBC cells. Moreover, Nectin-4 depletion blocked ERR α -induced activation of the PI3K/AKT signaling pathway. We hypothesize that Nectin-4 was involved in the pro-oncogenic function of ERR α to activate PI3K/AKT signaling pathway in GBC, thus forming ERR α –Nectin-4–PI3K/AKT axis to promote the growth and invasion of GBC. This axis could be a new promising prognostic biomarker and specific therapeutic target.

As a member of the nuclear receptor superfamily of transcription factors, ERR α plays its role by regulating the transcription of targeted genes. It functions by binding to estrogen-related responsive element(ERE) and estrogen-related responsive element (ERRE). Bioinformatics analysis reveals that representative ERE exists in the promotor region of Nectin-4. Taken together, ERR α may regulate the transcription of Nectin-4. For the time to come, dual-luciferase reporter assay system would be used to evaluate the effect of ERR α on the transcription activity of Nectin-4.

Conclusion

This study uncovers for the first time that ERR α enhances GBC cell proliferation and invasion both in vitro and in vivo. In GBC cells, ERR α upregulates Nectin-4 to activate the PI3K/AKT signaling pathway. ERR α –Nectin-4–PI3K/AKT axis may be a potential prognostic factor and molecular therapeutic target for GBC.

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of Nanjing Medical University and written informed consent was obtained from all patients. All the animal experiments were approved by Nanjing Medical University (NJMU) Institutional Animal Care and Use Committee.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LW, MMY and HHJ conceived and designed the study. LW, MMY, XMG and ZYY performed the experiments and analyzed data. YJ provided patient materials and analyzed the data. LW MMY and HHJ wrote the paper. XMG and YJ edited the manuscript. HHJ supervised the study. All the authors read and approved the final manuscript.

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Figures

Figure 1

ERRα overexpression in GBC. a IHC staining for ERRα protein expression. Representatives of cholecystitis and GBC with ERRα staining score 1, 2 and 3. b Scatterplots of final immunoreaction score in GBC tissues and matched nontumor counterparts. The final immunoreaction score was defined as the sum of extent and intensity parameters. c and d represent RFS and OS curve of GBC patients grouped by ERRα expression.

Figure 2

Cellular localization and relative expression of ERRα in GBC cell lines. **a** Immunofluorescence images of ERRα (red) and DAPI (blue) staining in NOZ and GBC-SD cells (100×). **b** mRNA expression of ERRα in GBC cell lines NOZ, OCUg, EH-GB1 and GBC-SD. **c** Lentiviral transduction efficiencies were observed in NOZ cells (100×). **d** Western blot analysis of ERRα expression in ERRα-depleting NOZ and OCUg cells and ERRα overexpressing GBC-SD cells. LuminB1 was used as the loading control.

Figure 3

Influence of ERRα on GBC cell proliferation, colony formation and tumor growth. a The cell growth rates of treated GBC cells were measured by CCK-8 proliferation assays at different time points. b The colony formation assays were conducted and the numbers of colonies were calculated. c Effect of ERRα silencing on the tumor growth in nude mice by injecting NOZ cells treated by Lv-shNC and Lv-shERRα. All results (mean ± SD) are from three separate experiments; **P < 0.01.

Figure 4

Influence of ERRα on GBC cell migration and invasion. a Migration and invasion assays of CTRL, Lv-shNC, Lv-shERRα transfected cells. The number of invaded cells was calculated and is depicted in the bar chart. b The protein expression levels of E-cadherin, Vimentin in the indicated cells were determined by Western blot.

Figure 5

The role of Nectin-4 in ERRα-induced PI3K/AKT activation and GBC cell proliferation and migration. a-c CCK-8 proliferation, colony formation, migration and invasion assays were conducted to detect the influence of ERRα overexpression on GBC cell proliferation and invasion. d-f The GBC-SD cells overexpressing ERRα were cotransfected with Nectin-4, and the cell grow rate, colony formation and metastasis ability were examined. g NOZ and GBC-SD cells were treated as indicated. ERRα, Nectin-4, Phosphorylated PI3K (Tyr607)/total PI3K, Phosphorylated AKT (Ser473)/total AKT were determined by western blot. LuminB1 and β-actin were used as the loading controls.

Supplementary Files

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- [Tables12.pdf](#)