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Estrogen-related receptor-α promotes gallbladder cancer development by enhancing the transcription of Nectin-4

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Abstract

Estrogen-related receptor-α (ERRα) is a nuclear receptor of transcription factor that binds to estrogen responsive elements and estrogen-related responsive elements. Estrogen-related receptor-α is involved in metabolic processes and implicated in the progression and growth of several human malignancies. However, the biologic role and clinical significance of ERRα in gallbladder cancer (GBC) remains to be clarified. Here, we reported that ERRα protein expression was notably higher in GBC tissues than in cholecystitis tissues, and that the aberrantly higher ERRα expression was positively correlated with advanced TNM stage and indicated dismal prognosis of GBC ($P < .01$). In GBC cell lines NOZ and OCUG, the targeted depletion of ERRα retarded the growth and suppressed the migration and invasive capabilities of GBC cells, and inhibited epithelial-mesenchymal transition by decreasing the expression of mesenchymal markers and elevating the expression of epithelial markers. Moreover, ERRα knockdown inhibited tumor growth in nude mice and led to decreased expression levels of Nectin-4, p-PI3K p85α, and p-AKT. Overexpression of ERRα in the GBC-SD cell line showed exactly the opposite effect. The targeted inhibition of Nectin-4 antagonized GBC cell proliferation and invasion, which were induced by ERRα upregulation. Moreover, Nectin-4 depletion inhibited the ERRα-induced activation of the PI3K/AKT pathway. Chromatin immunoprecipitation analysis and dual-luciferase reporter gene assays showed that ERRα enhanced the transcription of *Nectin-4* by binding to the promoter of *Nectin-4*. In conclusion, our data indicated that ERRα could be a potential target for the genetic treatment of GBC.

KEYWORDS
ERRα, gallbladder cancer, invasion, Nectin-4, proliferation

Lei Wang, MengMeng Yang, and Xingmei Guo contributed equally to this work.

1 | INTRODUCTION

Although generally considered as a rare lesion, gallbladder cancer (GBC) is the most common malignancy in the human biliary tract.[1,2] Due to local invasion and lymph node metastasis, the therapeutic options for GBC are limited. The carcinogenesis of GBC silently progresses in the metaplasia-dysplasia-carcinoma sequence, making it hard to achieve early diagnosis.

Gallbladder cancer, as a highly lethal neoplasm, features late diagnosis, unsatisfactory treatment, and unfavorable prognosis with conventional therapy.[3-5] Thus, it is critical and urgent to determine novel prognostic biomarkers and to discover the underlying mechanisms of GBC progression, thereby identifying targeted therapeutics for GBC. Moreover, as the female-to-male ratio of GBC incidence rates ranges from 2 to 6,[6,7] and thus shows a female predominance, we intend to investigate the expression of several sex-specific genes and focus on estrogen-related receptor-α (ERRα).

Previous studies reported that ERRα is a negative prognostic factor in the setting of several types of cancers[8-10] that enhances cellular growth, migration, and invasion of tumor cells. Moreover, ERRα is a key hypoxic growth regulator and is involved in metabolic reprogramming.[10,11] Although ERRα is structurally similar to estrogen receptor,[12,13] it does not interact with estrogens but with estrogen responsive elements (EREs) and estrogen-related responsive elements (ERREs), and mediating the downstream pathways.[14] To our knowledge, the activity of ERRα is not under the regulation of any known estrogens,[15] yet it could be tested by 3xERE-TATA-luciferase reporter. Although no natural ligand has been confirmed for ERRα, a member of the orphan nuclear receptor family of transcriptional factors,[12,16] peroxisome proliferator-activated receptor-γ coactivator-1-α (PGC-1α) has been discovered as an agonist for ERRα. Nevertheless, it was reported that PGC-1α WT could activate several other transcription factors. Chang et al identified some peptides, including L3-09, that specifically target ERRα.[17] The researchers replaced both L2 and L3 motifs with L3-09 peptides to generate PGC-1α 2 × 9, so as to selectively activate ERRα. Their research provides an important and solid cornerstone for the identification of downstream pathways of ERRα.

In the current study, we found that ERRα expression was negatively correlated with the prognosis of GBC patients. Specifically, the knockdown of ERRα significantly weakened GBC cell proliferation and invasion in vitro and in vivo. Estrogen-related receptor-α enhanced GBC cell invasion by inducing epithelial-mesenchymal transition (EMT). Moreover, the knockdown of ERRα inhibited the expression of Nectin-4, a member of the nectin family that plays an important role in multiple pathophysiological characteristics.[18-21] In our previous study,[22] we determined that Nectin-4 is critical for GBC proliferation and invasion by activating PI3K/AKT signaling pathway. We predicted that ERRα binds to 2 EREs (ERE-4 and ERE-7) in the promoter of Nectin-4, in accordance with the results of JASPAR (http://jaspar.genereg.net), an open-access database of transcription factor-binding profiles. We undertook ChIP analyses and dual-luciferase reporter gene assays and found that ERRα enhances the transcription of Nectin-4 by binding to the promoter of Nectin-4 at ERE-4 and ERE-7.

2 | MATERIALS AND METHODS

2.1 | Patients and clinicopathologic data

Gallbladder cancer tissues were obtained from 59 GBC patients who were seeking treatment at the Department of Hepatopancreatobiliary Surgery, The Affiliated Wuxi No. 2 People's Hospital of Nanjing Medical University between 2011 and 2017. No patients had received radiotherapy or chemotherapy before surgery. Moreover, 59 cholecystitis patients who underwent laparoscopic cholecystectomy were enrolled and the cholecystitis tissues were collected. This study was approved by the ethics committee of Nanjing Medical University (ID no. NJMU-EC-2018-147), and written informed consent was obtained from all the enrolled subjects. Two experienced pathologists confirmed the diagnoses of GBC and cholelithiasis. The collected tissue samples were fixed in 4% formalin and embedded in paraffin for immunohistochemical (IHC) staining. These GBC patients were staged in accordance with the 8th edition of the American Joint Committee on Cancer Staging Manual.[23]

2.2 | Quantitative IHC assays

Immunohistochemistry was carried out and assessed according to a standard procedure.[24] The intensity of the immunoreaction was scored as follows: 0, no immunoreactivity; 1, weak immunoreactivity; 2, moderate immunoreactivity; and 3, strong immunoreactivity. The percentage of ERRα expression was scored in light of the following standard: 0, less than 5% immunoreactive cells; 1, 5%-24% immunoreactive cells; 2, 25%-49% immunoreactive cells; 3, 50%-74% immunoreactive cells; and 4, 75% or more immunoreactive cells. The total score was calculated as the sum of the extent and intensity parameters. The samples were further divided into negative (0-1), weak (2-3), moderate (4-5), and strong (6-7) staining. Only moderate and strong staining specimens were considered positive, and the other specimens were considered negative.

2.3 | Quantitative real-time PCR

Total RNAs were extracted from the cells using Trizol reagent (Takara). PrimeScript Reverse Transcriptase (Takara) was used to synthesize cDNA, in accordance with the manufacturer's instructions. The primers used for the detection of ERRα and GAPDH (internal control) expression were as follows: ERRα sense, 5′-CACTATGGTGTGGCATCCTG-3′ and antisense, 5′-CGCTTGGTGATCTCACACTC-3′; and GAPDH

sense, 5′-AGAAGGCTGGGG CTCATTTG-3′ and antisense, 5′-AGGGGCCATCCACAGTCTTC-3′. We operated the StepOnePlus Real-Time PCR system (Applied Biosystems) to amplify cDNA by use of the SYBR-Green method (Takara).

2.4 | Cell culture and chemicals

GBC-SD, NOZ, OCUG, and EH-GB1 cell lines were obtained from the Shanghai Key Laboratory of Biliary Tract Disease Research, Shanghai, China. GBC-SD, OCUG, and EH-GB1 cells were cultured in high-glucose DMEM (Gibco), and NOZ cells were cultured in William's medium E (Genom). All of the media were supplemented with 10% FBS (Gibco). All of the cells were cultured at 37°C in a humidified incubator with 5% CO_2.

2.5 | Immunofluorescence analysis

Paraformaldehyde (4%) was used to fix GBC cells. Subsequently, the cells were permeabilized in 0.5% Triton X-100 at room temperature and were then blocked using 1% BSA for 1 hour. The GBC cells were then incubated with anti-ERRα primary Ab at 4°C for 12 hours, followed by incubation with goat anti-rabbit IgG (Proteintech) at 37°C for 2 hours. The cells were counterstained with DAPI.

2.6 | RNA interference

The shRNA sequence targeting human ERRα was 5′-GCGAGAGGAGUAUGUUCUA-3′, and the negative control sequence was 5′-TTCTCCGAACGTGTCACGT-3′. Both shRNAs were synthesized and inserted into the PGMLV-SC5 lentivirus core vector, which was constructed by Genomeditech. Concentrated viruses were transfected into NOZ and OCUG cells in serum-free medium. Twenty-four hours later, the serum-free medium was removed and replaced by the complete culture medium.

2.7 | Construction of plasmids and transfection

The full-length sequence of ERRα (NM_004451.4) was cloned into the pCDNA3.1 expression vector (Genomeditech). pGL3-Basic-3*ERE-TATA-luc containing triple AGGTCANNNTGACCT and plasmids with WT PGC-1α (pCDNA3.1(+)-3 × Flag-C-M-PGC-1α-WT) and mutant-type (MT) PGC-1α (pCDNA3.1(+)-3 × Flag-C-M-PGC-1α-2 × 9) were synthesized by Genomeditech following the instructions of a previous study.[17] The synthesized plasmids were transfected into cells combined with ViaFect Transfection Reagent (Promega), and Neomycin (Genechem) was used to select stable ERRα-expressing clones. The expression level was validated by western blotting. Empty vector-infected cells (MOCK) served as control.

2.8 | Antibodies and western blot analysis

Anti-ERRα Ab was obtained from Novus Biologicals, anti-Nectin-4 Ab was obtained from Abcam, anti-Lamin B1 Ab was purchased from Bosterbio, and anti-E-cadherin, anti-Vimentin, anti-PI3K p85, anti-p-PI3K p85α (phospho-Y607), anti-AKT, anti-p-AKT (Ser473), and β-actin Abs were acquired from Cell Signaling Technology. Proteins from cells were separated by SDS-PAGE and then transferred to PVDF membranes (Millipore). We blocked the membranes with 5% skimmed milk (5% BSA for the testing of p-PI3K and p-AKT) for 1 hour. The membranes were incubated with diluted primary Abs at 4°C for 12 hours. Afterward, the membranes were incubated with the secondary Ab for 1 hour at room temperature. Enhanced chemiluminescence (Millipore) was used to detect the targeted signal. β-Actin and Lamin B1 served as the loading controls for total protein and nucleoprotein, respectively. All assays were carried out in triplicate.

2.9 | In vitro tumorigenesis assays

Gallbladder cancer cell growth was assessed with CCK-8 (Dojindo). The absorbance value (OD) of the cells was measured at the wavelength of 450 nm using a Spectra Max 190. To evaluate the anchorage-independent growth of NOZ, OCUG, and GBC-SD cells, the cells were cultured in 6-well plates for 9 days, and then fixed with 4% paraformaldehyde for 30 minutes and stained with 0.1% crystal violet (Sigma) for 15 minutes. Colonies with more than 50 cells were counted.

2.10 | In vitro migration and invasion assays

Transwell filters (8 μm; BD Biosciences) were used in the assays. In total, 2×10^4 cells were diluted with 200 μL serum-free medium and planted in the upper chamber. For the invasion assay, the upper chamber was coated with Matrigel (BD Biosciences). A total of 500 μL complete culture medium with 15% FBS was added to the lower chamber. After incubation (20 hours for GBC-SD and OCUG cells; 16 hours for NOZ cells), cells that had migrated to the bottom of the membrane were fixed with 4% paraformaldehyde for 20 minutes and were stained with crystal violet for 20 minutes. Four fields of view were randomly captured using the camera.

2.11 | Nude mouse s.c. xenograft model

Twenty nude nu/nu mice (3-5 weeks, 12-16 g) were obtained from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). These mice were randomly and evenly divided into 4 groups (n = 5), including Lv-shERRα, Lv-shNC, ERRα-transfected, and MOCK. In each group, 2×10^6 cells were injected into the left axilla of each mouse s.c. We recorded tumor size each week. After 4 weeks, the mice were killed to harvest and weigh the tumors. We calculated the volume of the tumor according to the

following equation: tumor volume (mm^3) = 1/2 × $width^2$ × length, where the length and width represent the maximum diameter and the shortest diameter, respectively. The experiments were carried out following the protocols approved by the Nanjing Medical University Institutional Animal Care and Use Committee.

2.12 | Chromatin immunoprecipitation

The promoter of Nectin-4 was obtained from the website of NCBI. The sequences of EREs and ERREs are listed in Table S1. The JASPAR website was used to predict the potential binding sites for ERRα in the promoter of Nectin-4. Primers were designed to amplify these potential binding sites. The sequences of these primers are listed at Table S1. SimpleChIP Enzymatic Chromatin IP Kits from Cell Signaling Technology were used to carry out the ChIP assays following the manufacturer's instructions. NOZ cells were used to undertake the ChIP assay. An Ab against ERRα (rabbit mAb #13826; Cell Signaling Technology) was used to pull down cross-linked chromatin, and the isolated DNA fragments were amplified by the above primers. Mouse OGDH intron 1 primers (#13828; Cell Signaling Technology) and mouse MYT-1 promoter primers (#8985; Cell Signaling Technology) were used as positive control primers and negative control primers, respectively. DNA electrophoresis and quantitative PCR were used to detect the expression of aimed DNA fragments.

2.13 | Dual-luciferase reporter gene assay

Several transcription factors, including ERRα, can be activated by WT PGC-1α. MUT PGC-1α (PGC-1α 2 × 9) can specifically activate ERRα.[17] In the current study, pCDNA3.1(+)-3 × Flag-C-M-PGC-1α-WT and pCDNA3.1(+)-3 × Flag-C-M-PGC-1α-2 × 9 were used to activate ERRα, and pGL3-Basic-3*ERE-TATA-luc was used to detect the activity of ERRα. pGL3-Basic-Nectin4-WT-luc containing the WT promoter sequence −2000 to −1 of Nectin-4 was synthesized by Genomeditech. The ERE sequences in the Nectin-4 promoter were randomly mutated to obtain pGL3-Basic-Nectin4-MT-luc. Moreover, pGL3-Basic-Nectin4-WT-luc and pGL3-Basic-Nectin4-MT-luc were used to detect the transcriptional regulation of ERRα on Nectin-4. HEK-293T cells were plated in the 24-well plates. Lipo2000 (1.5 μL), pRL-TK (25 ng), reporter plasmids (250 ng) and plasmids with target genes (250 ng) were transfected into 293T cells. A luminometer was used to detect the activity of *Renilla* luciferase and firefly luciferase. The final results are presented as the ratios of firefly luciferase activity to *Renilla* luciferase activity. Empty vector-infected cells (MOCK) were used as control.

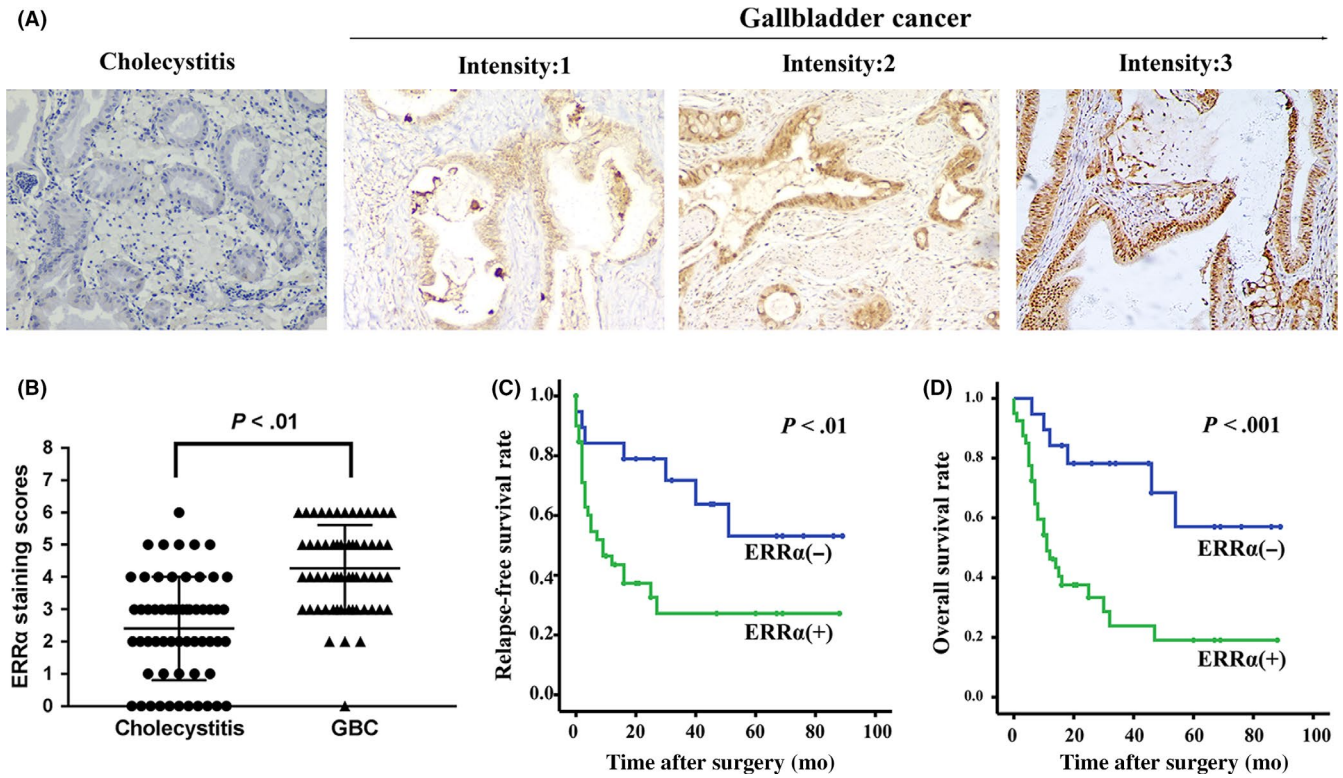

FIGURE 1 Estrogen-related receptor-α (ERRα) expression in gallbladder cancer (GBC). A, Immunohistochemical staining for ERRα protein expression. Representative images of cholecystitis and GBC patients with ERRα staining intensity of 1, 2, and 3. B, Scatterplots of the final immunoreaction score in GBC and cholecystitis tissues. Total score was calculated as the sum of the extent and intensity parameters. The expression of ERR protein in GBC tissues was significantly higher than that in cholecystitis tissues. C, D, Relapse-free survival (RFS) (C) and overall survival (OS) (D) curves of GBC patients grouped by ERRα expression. Moderate and strong staining specimens were considered positive, whereas other specimens were considered negative. Patients with positive ERRα staining have statistically poorer RFS and OS. All of the experiments were repeated 3 times

2.14 | Statistical analysis

IBM SPSS Statistics 19.0 was used for statistical analyses. The quantitative data are shown in the form of mean ± SD. The independent Student's *t* test was used to compare the means of 2 groups. The χ^2 test was used to examine the correlation between ERRα expression and clinicopathologic data. We used the Kaplan-Meier test for the univariate survival analysis and the Cox proportional hazards model was applied for the multivariate survival analysis. $P < .05$ was statistically significant. All results are representative of 3 replicates.

3 | RESULTS

3.1 | Estrogen-related receptor-α expression and clinical significance in GBC

We tested nuclear expressions of ERRα in the GBC and cholelithiasis specimens collected from the subjects using IHC (Figure 1A). A total of 67.80% (40/59) of the GBC patients showed positive ERRα staining (Table 1). The ERRα protein expression level in tumors was significantly higher than that in cholelithiasis tissues (Figure 1B, $P < .01$). Moreover, we found that higher ERRα levels were closely associated with more advanced TNM stages of GBC ($P < .01$), deeper invasion depth ($P < .01$), and lymph node metastasis ($P < .01$) (Table 1). More intriguingly, Kaplan-Meier analysis of relapse-free survival (RFS) and overall survival (OS) indicated that positivity of ERRα was correlated with shorter RFS and OS (Figure 1C,D, $P < .01$). The multivariate survival analysis showed that ERRα expression was negatively associated with prognosis of GBC patients (Table 2, $P < .05$).

3.2 | Estrogen-related receptor-α expression in GBC cell lines

Immunocytochemical staining revealed that ERRα protein was expressed in the nuclei of GBC cells (Figure 2A). We detected the expression of ERRα in 4 GBC cell lines by RT-PCR and found that ERRα expression was higher in NOZ and OCUG cells and relatively lower in EH-GB1 and GBC-SD cells (Figure 2B). Thus, to investigate the tumor-promoting effect of ERRα, we deliberately depleted ERRα in NOZ and OCUG cell lines and overexpressed ERRα in the GBC-SD

TABLE 1 Correlations between estrogen-related receptor-α (ERRα) expression and clinicopathologic features of gallbladder cancer

	Total cases	ERRα expression level		
Clinicopathologic features	*N*	Negative *N* (%)	Positive N (%)	*P* value
Gender				
Male	23	8 (34.8)	15 (65.2)	.735
Female	36	11 (30.6)	25 (69.4)	
Age (y)				
<60	17	4 (23.5)	13 (76.5)	.364
≥60	42	15 (35.7)	27 (64.3)	
Histology				
WD + MD	46	15 (32.6)	31 (67.4)	.900
PD + UD	13	4 (30.8)	9 (69.2)	
Resection margin				
Negative	50	17 (34)	33 (66)	.704
Positive	9	2 (22.2)	7 (77.8)	
Pathologic T stage				
Tis + T1 + T2	34	17 (50)	17 (50)	<.010*
T3 + T4	25	2 (8.0)	23 (92)	
Lymph node metastasis				
Absent	44	19 (43.2)	25 (56.8)	<.010*
Present	15	0 (0)	15 (100)	
TNM stage (AJCC)				
0-II	30	17 (56.7)	13 (43.3)	<.010*
III–IV	29	2 (6.9)	27 (93.1)	

AJCC, American Joint Committee on Cancer; MD, moderately differentiated; PD, poorly differentiated; UD, undifferentiated; WD, well differentiated.

*$P < .05$ was considered statistically significant.

TABLE 2 Univariate and multivariate analysis of prognostic factors in gallbladder cancer patients

		Univariate analysis		Multivariate analysis	
Variable	Unfavorable/favorable	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Gender	Female/male	0.825 (0.411-1.653)	.582	—	—
Age (years)	≥60/<60	1.083 (0.505-2.326)	.835	—	—
Histology	PD + UD/WD + MD	1.256 (0.567-2.778)	.569	—	—
Resection margin	R1/R0	2.724 (1.177-6.306)	.0140*	3.652 (1.438-9.273)	<.010*
Pathologic T stage	T3 + T4/Tis + T1 + T2	4.475 (2.186-9.162)	<.010*	—	—
Lymph node metastasis	Present/absent	2.320 (1.119-4.813)	.019*	—	—
TNM stage (AJCC)	III-IV/0-II	4.906 (2.337-10.297)	<.010*	3.237 (1.465-7.153)	<.010*
ERRα expression	Positive/negative	3.91 (1.602-9.542)	<.010*	2.869 (1.040-7.915)	.042*

AJCC, American Joint Committee on Cancer; CI, confidence interval; ERRα, estrogen-related receptor-α; HR, hazard ratio; MD, moderately differentiated; PD, poorly differentiated; R0, negative resection margin; R1, positive resection margin; UD, undifferentiated; WD, well differentiated; —,not included.

*Statistical significance, $P < .05$.

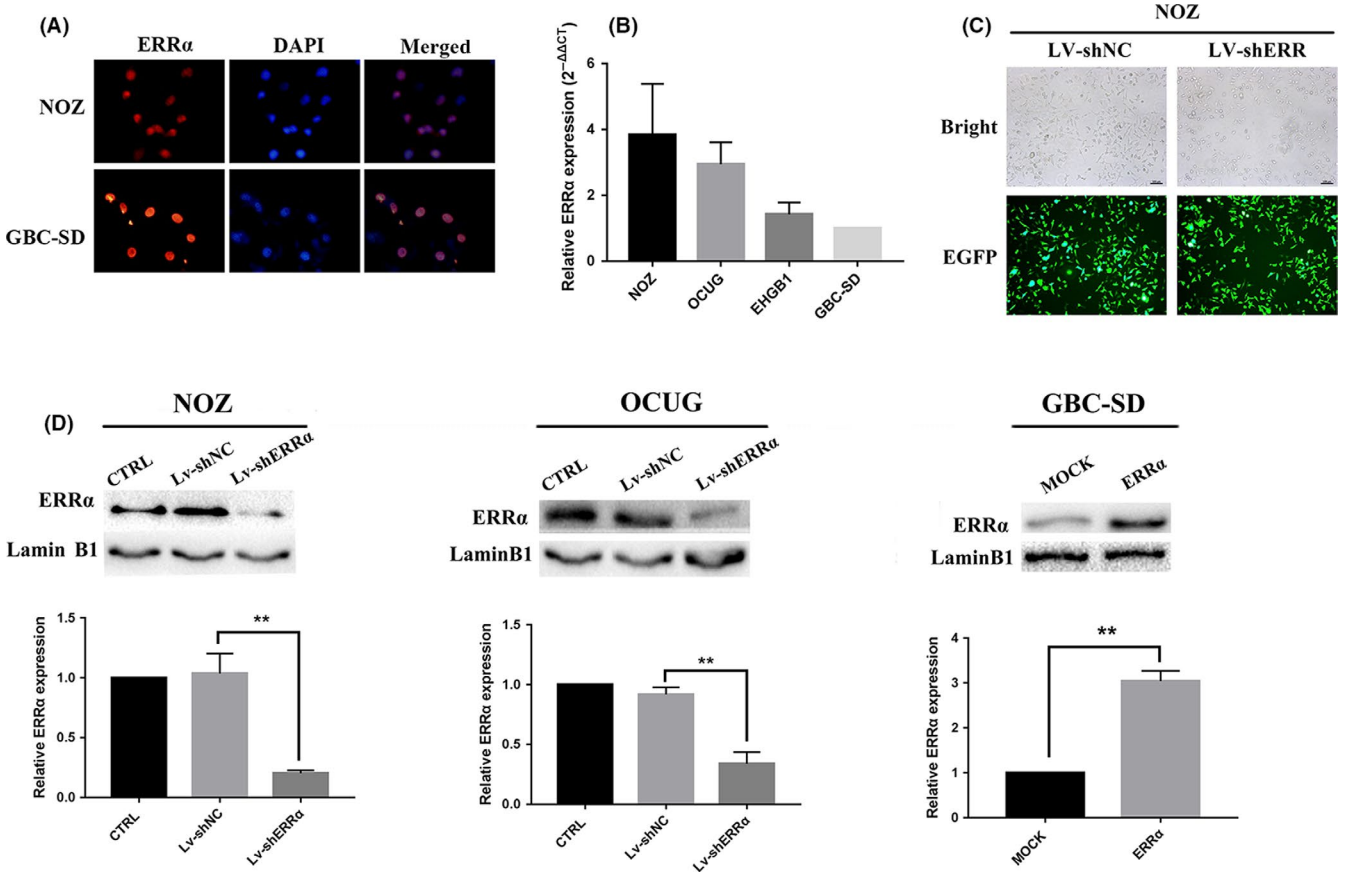

FIGURE 2 Cellular localization and relative expression of estrogen-related receptor-α (ERRα) in gallbladder cancer (GBC) cell lines. A, Immunofluorescence images of ERRα (red) and DAPI (blue) staining in NOZ and GBC-SD cells (100×). ERRα localized in the nucleus. B, mRNA expression of ERRα is relatively higher in NOZ and OCUG cells, and relatively lower in EH-GB1 and GBC-SD cells. C, Lentiviral transduction efficiencies were assessed in GBC cells (NOZ cells as representative) (100×). The lentiviral transfection efficiency in NOZ cells was higher than 95%. D, Protein expression of ERRα in ERRα-depleted NOZ and OCUG cells and ERRα-overexpressing GBC-SD cells. Immunoblots are quantified and listed in lower graphs. Lamin B1 was used as endogenous control for nucleoprotein. All of the experiments were repeated 3 times. $^{**}P < .01$

cell line. Seventy-two hours later, we used fluorescence microscopy to examine the viral transfection efficiency by detecting the level of EGFP. The lentiviral transfection efficiencies in all these cell lines were higher than 95% (Figure 2C, NOZ cells as representative). Western blot assay was carried out to confirm the transfection efficiency, and indicated that ERRα expression was effectively

suppressed by Lv-shERRα and overexpressed by the ERRα expression vector (Figure 2D).

3.3 | Effects of ERRα knockdown and overexpression on GBC cell growth in vitro and in vivo

We undertook CCK-8 and colony formation assays to evaluate whether ERRα can affect the proliferation of GBC cells. Compared with negative controls, the viability of NOZ and OCUG cells transfected with Lv-shERRα was significantly decreased (Figure 3A). As shown in Figure 3B, ERRα depletion significantly decreased the colony formation ability of NOZ and OCUG cells ($P < .01$). Conversely, compared with empty vector-transfected cells, ERRα overexpressed GBC-SD cells showed significantly increased viability (Figure 3C) ($P < .01$) and strengthened colony-forming abilities (Figure 3D) ($P < .01$). As shown in Figure 3E, the proliferation of ERRα-depleted xenografts was significantly suppressed compared with negative controls in nude mice ($P < .01$). Conversely, the overexpression of ERRα significantly stimulated the proliferation of xenografts (Figure 3F) ($P < .01$). Collectively, these results suggest that ERRα can enhance cell proliferation in vitro and in vivo.

3.4 | Estrogen-related receptor-α promotes GBC cell migration and invasion by inducing EMT

Transwell migration and Matrigel invasion assays were carried out to evaluate whether ERRα can affect GBC metastasis. As shown

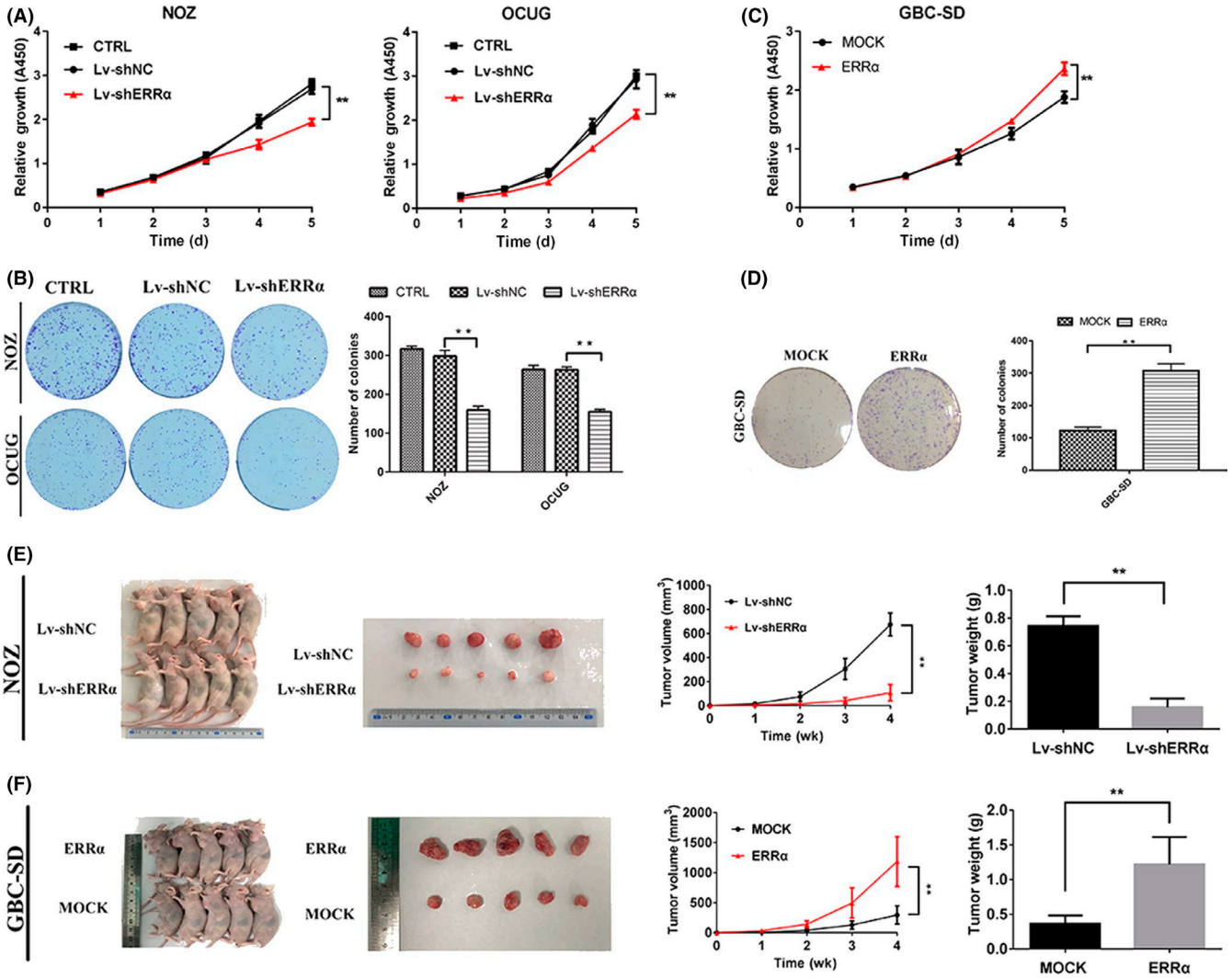

FIGURE 3 Effect of estrogen-related receptor-α (ERRα) on the proliferation, colony formation, and tumor formation of gallbladder cancer (GBC) cells. A, C, Compared with negative controls, the viability of NOZ and OCUG cells transfected with Lv-shERRα was significantly decreased, whereas ERRα-overexpressing GBC-SD cells showed significantly increased viability. B, D, ERRα depletion significantly decreased the colony formation ability of NOZ and OCUG cells. Conversely, compared with empty vector-transfected cells, ERRα-overexpressing GBC-SD cells showed strengthened colony formation ability. E, Proliferation of ERRα-depleted xenografts was significantly suppressed compared with negative controls in nude mice ($P < .01$). F, Overexpression of ERRα significantly stimulated the proliferation of xenografts. All data are presented as mean ± SD and all of the experiments were repeated 3 times. $^{**}P < .01$

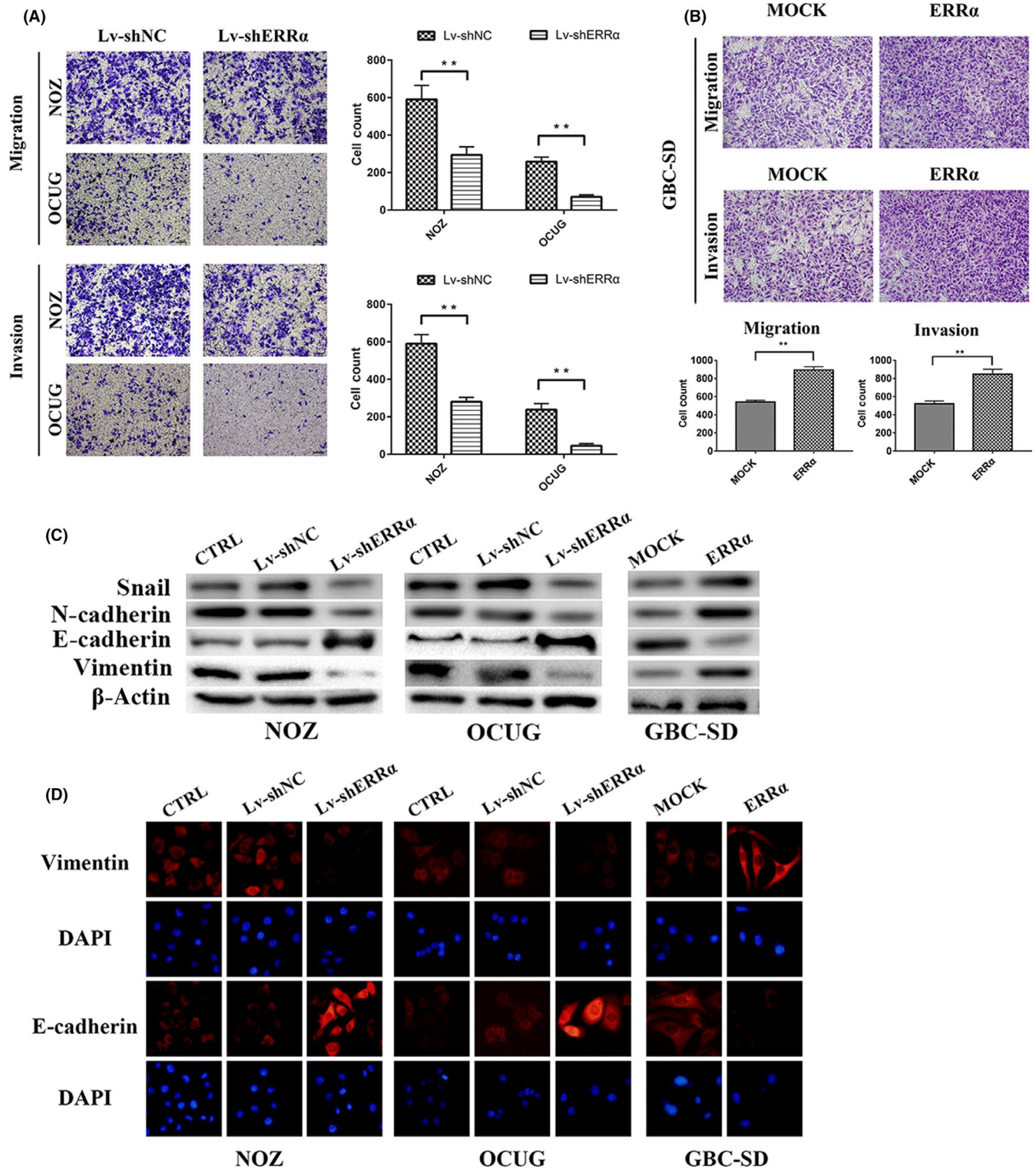

FIGURE 4 Influence of estrogen-related receptor-α (ERRα) on gallbladder cancer (GBC) cell migration and invasion. A, Migrative and invasive capabilities of NOZ and OCUG cells transfected with Lv-shERRα were significantly inhibited, compared with negative controls. B, ERRα-transfected GBC-SD cells showed greater migrative and invasive capabilities compared with empty vector-transfected cells. C, In NOZ, OCUG, and GBC-SD cells, the expressions of epithelial marker E-cadherin and mesenchymal markers Vimentin, N-cadherin, and Snail were detected by western blotting. D, In NOZ, OCUG, and GBC-SD cells, the expressions of Vimentin and E-cadherin were detected by immunofluorescence assay. Cells were counterstained with DAPI. All data are presented as mean ± SD and all the experiments were repeated 3 times. $^{**}P < .01$

in Figure 4A, the migrative and invasive capabilities of NOZ and OCUG cells transfected with Lv-shERRα were significantly inhibited, compared with negative controls ($P < .01$). Conversely, ERRα-transfected GBC-SD cells showed greater migrative and invasive capabilities compared with empty vector-transfected cells (Figure 4B) ($P < .01$). To investigate whether ERRα enhanced the invasiveness of GBC cells through EMT processes, we used western blotting and cell immunofluorescence assays to detect the expression of EMT biomarkers. In the ERRα-depleted NOZ and OCUG cells, we observed notably higher levels of E-cadherin, which is the characteristic of epithelial cells (Figure 4C,D). However, the expression levels of Vimentin, N-cadherin, and Snail, which indicate a mesenchymal phenotype, were lower in ERRα-depleted NOZ and OCUG cells (Figure 4C,D). On the contrary, ERRα-overexpressing GBC-SD cells showed remarkably downregulated expression of E-cadherin, as well as upregulation of Vimentin, N-cadherin, and Snail (Figure 4C,D). These findings indicate that ERRα promotes GBC cell migration and invasion by inducing EMT. The quantification of these immunoblots is provided in Figure S1A.

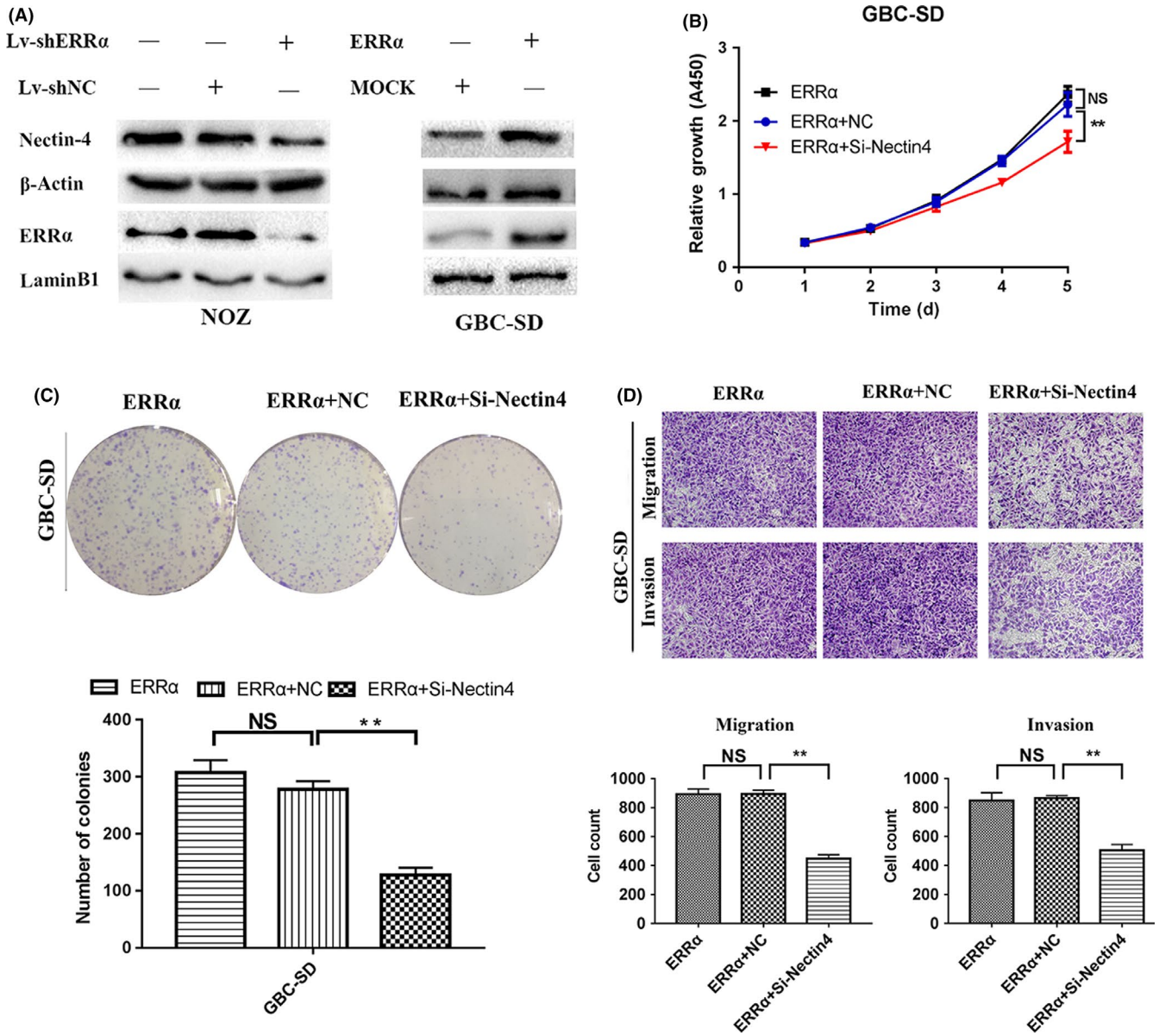

FIGURE 5 Role of Nectin-4 in estrogen-related receptor-α (ERRα)-induced gallbladder cancer (GBC) cell growth and invasion. A, NOZ and GBC-SD cells were treated as indicated. ERRα and Nectin-4 were detected by western blotting. Expression of Nectin-4 was decreased in NOZ cells with ERRα depletion; vice versa, the expression of Nectin-4 was increased in GBC-SD cells with ERRα overexpression. Lamin B1 and β-actin were used as internal references. B-D, GBC-SD cells overexpressing ERRα were cotransfected with Si-Nectin-4. Compared with negative controls (NC), when cotransfected with Si-Nectin-4, the abilities of cell growth, colony formation, migration, and invasion were significantly inhibited in ERRα-overexpressing GBC-SD cells. All data are presented as mean ± SD and all experiments were repeated 3 times. $^{**}P < .01$. NS, not significant

3.5 | Nectin-4 mediates the oncogenic function of ERRα

We observed increased Nectin-4 levels in GBC-SD cells with ERRα overexpression (Figure 5A). Conversely, the expression of Nectin-4 was decreased in the NOZ cells with ERRα depletion (Figure 5A). Nectin-4 inhibition attenuated cell growth, colony formation, migration, and invasion induced by ERRα overexpression (Figure 5B5). The immunoblots were quantified and the results are shown in Figure S1B.

3.6 | Estrogen-related receptor-α enhances transcription of Nectin-4

After analyzing the sequence of human *Nectin-4* promoter, we found 7 potential EREs (from ERE1 to ERE7) in the promoter of *Nectin-4* (Figure 6A). We scanned the promoter of *Nectin-4* using the JASPAR website and predicted 2 potential binding sites for ERRα, including "AATGACCTTGG" and "TCCAGGTCACA" (Figure 6A, red arrows). The relative scores for these 2 motifs were 0.95 and 0.88, respectively. In the ChIP assay, we designed 2 pairs of primers (primer 1 for the "AATGACCTTGG" motif, primer 2 for the "TCCAGGTCACA" motif). The results of ChIP-DNA electrophoresis and ChIP-qPCR confirmed that ERRα can bind to both motifs (Figure 6B,C).

Subsequently, we undertook dual-luciferase reporter gene assays to explore whether ERRα promotes the transcription of *Nectin-4*. First, we activated ERRα by using PGC-1α WT and mutant PGC-1α (PGC-1α 2 × 9). However, PGC-1α WT could also activate other nuclear transcription factors. In light of Chang's instructions,[17] we mutated PGC-1α to obtain PGC-1α 2 × 9, which could be used to specifically activate ERRα without activating the other transcription factors. Luciferase reporter plasmid (3 × ERE) was used to detect ERRα activity in 293T cells. The expressions of ERRα, PGC-1α WT, and PGC-1α 2 × 9 were validated by western blotting (Figure 7A). The immunoblots were quantified and listed in Figure S1C. The enhanced activity of 3 × ERE luciferase reporter by PGC-1α WT, PGC-1α 2 × 9 and ERRα overexpression (Figure 7B) indicated that the activity of ERRα was elevated by PGC-1α WT, PGC-1α 2 × 9, and ERRα overexpression. Correspondingly, the activity of WT *Nectin-4* promoter luciferase reporter was significantly enhanced by ERRα activation (Figure 7C-E). We mutated all of the EREs in the *Nectin-4* promoter and found that the activity of mutant *Nectin-4* promoter luciferase reporter was unaffected by ERRα activation (Figure 7C-E). Taken together, the results of ChIP assays and dual-luciferase reporter gene assays indicate that ERRα

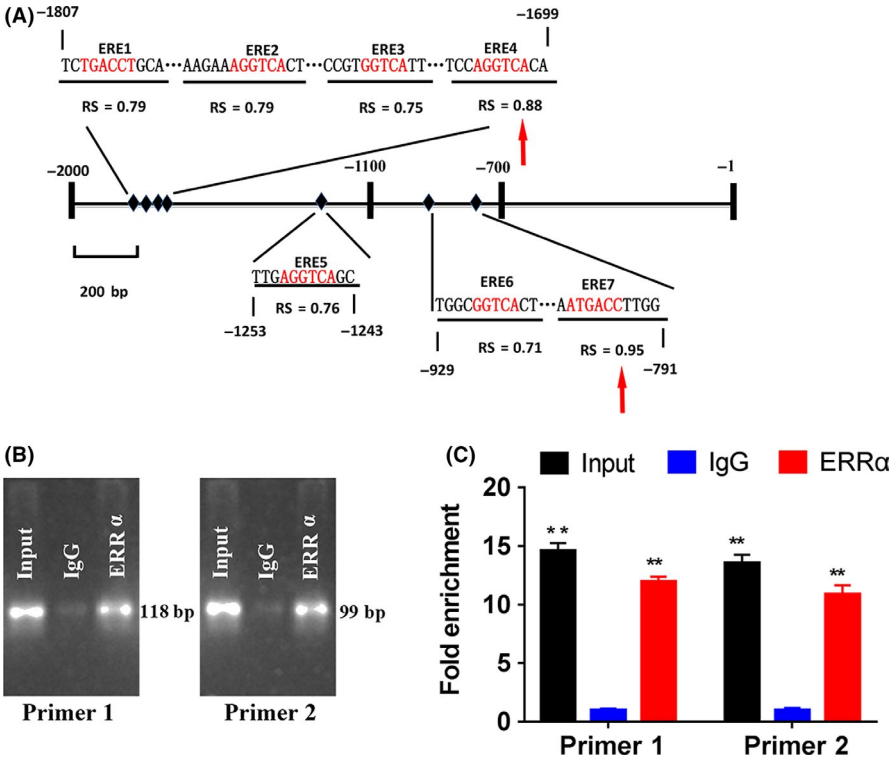

FIGURE 6 Bioinformatics analysis and ChIP assay. A, Seven estrogen responsive elements (EREs) were found in the promoter of *Nectin-4*. We scanned the promoter of Nectin-4 using the JASPAR website and predicted 2 potential binding sites for ERRα, including AATGACCTTGG and TCCAGGTCACA (red arrows). Relative scores (RS) for these 2 motifs were 0.95 and 0.88, respectively. B, C, Primer 1 and primer 2 were designed to amplify the AATGACCTTGG motif and the TCCAGGTCACA motif, respectively. Results of ChIP-DNA electrophoresis and ChIP-quantitative PCR confirmed that estrogen-related receptor-α (ERRα) can bind to both motifs. Input refers to amplified Nectin-4 from total chromatin; IgG, chromatin fragments pulled down by anti-IgG antibody. All data are presented as mean ± SD and all the experiments were repeated 3 times. RS, relative score. $^{**}P < .01$

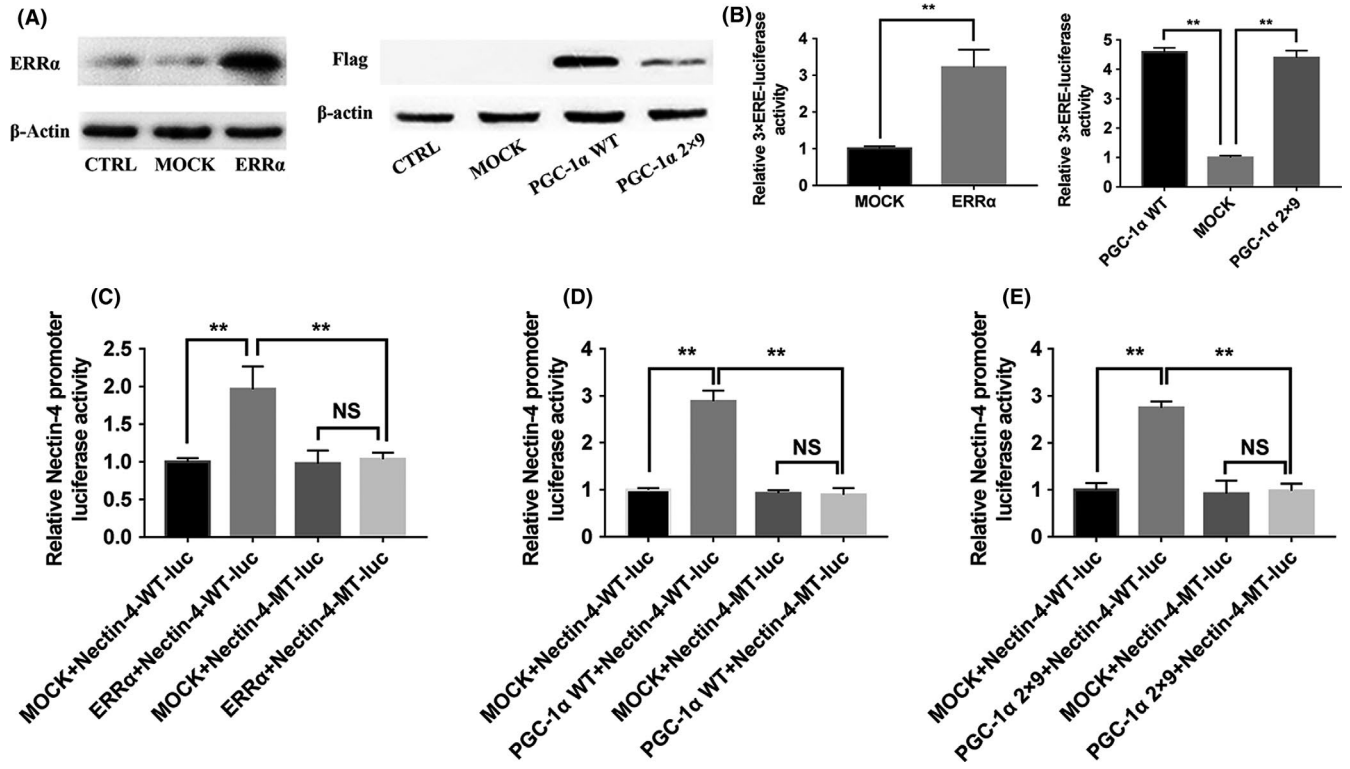

FIGURE 7 Activated estrogen-related receptor-α (ERRα) promotes the transcription of *Nectin-4*. A, Expression of ERRα, peroxisome proliferator-activated receptor-γ coactivator-1-α (PGC-1α) WT, and mutant PGC-1α 2 × 9 were validated in western blotting. As a coactivator, PGC-1α WT could activate ERRα and several other transcription factors. PGC-1α 2 × 9 was introduced to selectively activate ERRα. B, 3 × ERE luciferase reporter was used to detect the activity of ERRα. Enhanced activity of 3 × ERE luciferase reporter by PGC-1α WT, PGC-1α 2 × 9, and ERRα overexpression indicated that the activities of ERRα were elevated by PGC-1α WT, PGC-1α 2 × 9, and ERRα overexpression. C-E, Activity of WT Nectin-4 promoter luciferase (luc) reporter was significantly enhanced by ERRα, PGC-1α WT, and PGC-1α 2 × 9 compared with MOCK. All of the estrogen responsive element sequences in the Nectin-4 promoter were randomly mutated to obtain mutant Nectin-4 promoter luciferase reporter. Activity of mutant Nectin-4 promoter luciferase reporter was unaffected by ERRα, WT PGC-1α, and PGC-1α 2 × 9. Empty vector-infected cells (MOCK) served as the control. All data are presented as mean ± SD and all the experiments were repeated 3 times. $^{**}P < .01$, compared with MOCK. NS, not significant

promotes the transcription of *Nectin-4* by binding to the promoter of Nectin-4.

3.7 | Estrogen-related receptor-α activates the PI3K/AKT signaling pathway by regulating Nectin-4

In our previous study, we showed that Nectin-4 enhances GBC cell proliferation and migration by activating the PI3K/AKT signaling pathway. The present study suggested that ERRα knockdown suppressed the expression of Nectin-4, p-PI3K p85α, and p-AKT in NOZ cells (Figure 8A). Conversely, ERRα overexpression induced the expression of Nectin-4, p-PI3K p85α, and p-AKT in GBC-SD cells (Figure 8B), and that such effect could be antagonized by depleting the level of Nectin-4 (Figure 8B, immunoblots quantified on the right histogram).

4 | DISCUSSION

As the most common neoplasm in the human biliary system, gallbladder cancer has a poor prognosis and has shown certain geographic distribution.[6] The knowledge about the associated interplay of lifestyle factors, genetic susceptibility, and infections with the carcinogenesis of gallbladder remains scant. Currently, the diagnosis of gallstones and the removal of gallbladder are regarded as the keystone to the early prevention of gallbladder cancer, yet most patients can only be diagnosed with advanced gallbladder cancer. The incidence of GBC shows a female predominance, with female-to-male incidence ratios ranging generally from 2 to 6.[7] Nevertheless, the biological function of ERRα in the etiology and development of GBC remains to be clarified.

Estrogen-related receptor-α is a member of the nuclear receptor family that serves as transcription factors. It bears structural resemblance to the canonical estrogen receptors,[13] yet ERRα does not respond to naturally existing estrogens. Instead, ERRα functions by binding to EREs and ERREs.[14]

Estrogen-related receptor-α has also been proved to be involved in the carcinogenesis of multiple human malignancies, such as oral squamous cell cancer,[25] prostate cancer,[26] and colon cancer.[8] Investigators have proposed the different mechanisms regarding the pathogenesis of ERRα in different tumors. Both PGC-1α and PGC-1β are the obligate coregulators of ERRα.[27] Chang et al uncovered that,

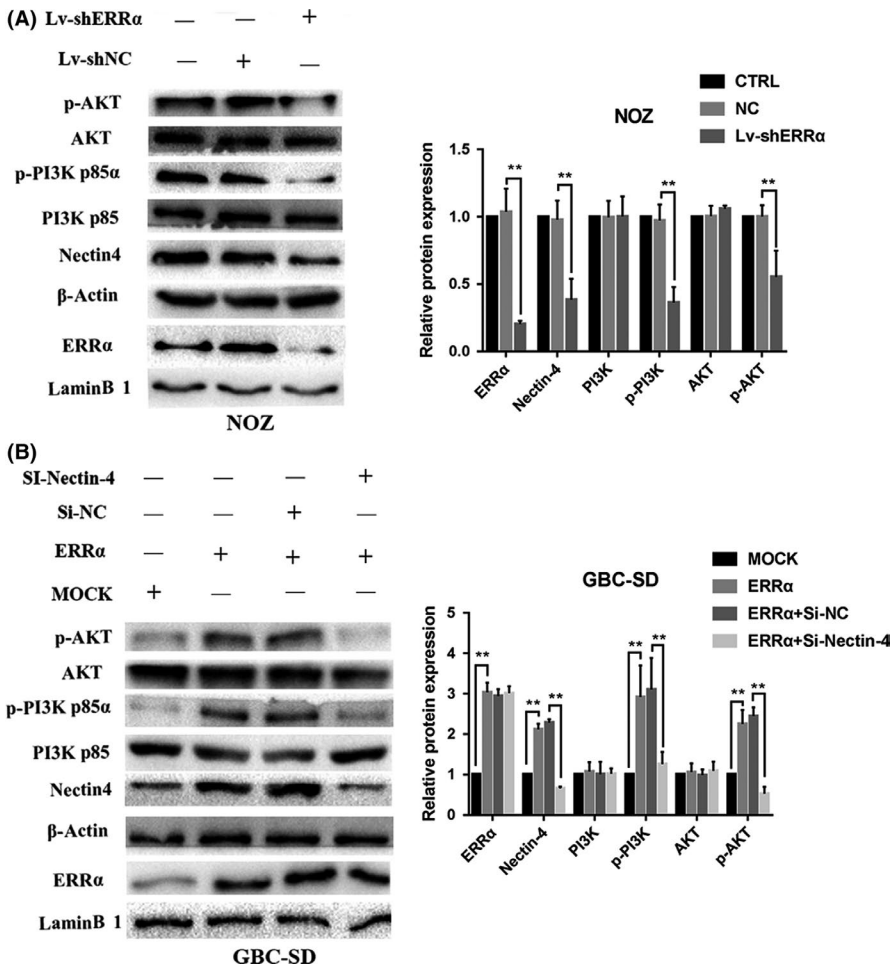

FIGURE 8 Estrogen-related receptor-α (ERRα) activates the PI3K/AKT signaling pathway by regulating Nectin-4. A, ERRα knockdown suppressed the expression of Nectin-4, p-PI3K p85α, and p-AKT in NOZ gallbladder cancer cells. Immunoblots are quantified on the histogram. B, ERRα overexpression induced the expression of Nectin-4, p-PI3K p85α, and p-AKT in GBC-SD cells, and the effect could be antagonized by depleting the level of Nectin-4. Immunoblots are quantified on the histogram. All data are presented as mean ± SD and all the experiments were repeated 3 times. $^{**}P < .01$

in breast cancer, the activation of ERRα can be upregulated by the activity of PGC-1, whose expression can be regulated by the human epidermal growth factor receptor 2 (Her2)/insulin-like growth factor 1 (IGF-1) signaling pathway.[16] In addition, ERRα can mediate the transcription of genes, including *WNT11*, *OPN*, *OPG*, and *CCNE1*, all of which play vital roles in proliferation, metastasis, and metabolism of cancer progression.[28] In the current study, we reported that the aberrantly high expression of ERRα in GBC tissues is correlated with advanced TNM stage, high invasion depth, and lymph node metastasis. Furthermore, ERRα was validated as an unfavorable independent prognostic factor for GBC, suggesting that ERRα might promote the development of GBC and exacerbate the prognosis.

In the present study, we found that the downregulation of ERRα repressed GBC cell growth, colony formation, migration, and invasion, whereas the upregulation of ERRα promoted GBC cell proliferation and invasion. Moreover, ERRα knockdown suppressed EMT by increasing the expression of epithelial marker E-cadherin and decreasing the expression of mesenchymal markers Vimentin, N-cadherin, and Snail, suggesting that ERRα might promote GBC cell invasion by inducing EMT, which is reported as an essential program that is activated during cancer invasion and metastasis.[29] Through EMT, the transformed epithelial cells can access mesenchymal traits that contribute to metastasis. Thus, cancer cells with mesenchymal phenotype can infiltrate the endothelial barriers and thereby enter blood and lymphatic circulations. Recent evidence suggested that the association between EMT regulators and cancer stem cells provides the molecular and cellular explanation for EMT and cancer metastasis.[30] In the course of EMT, the expression of mesenchymal-related markers will be upregulated, whereas the expression of epithelial-related markers would be downregulated.[31] Our data indicate that ERRα could serve as a potential diagnostic biomarker and therapeutic target for GBC. To gain greater insight into the mechanisms of the pro-oncogenic function of ERRα, we found that the protein expression of Nectin-4 and the PI3K/AKT signaling pathway were reduced after downregulating ERRα.

Nectin-4 is a member of the nectin family that shows multiple pathophysiological characteristics. Combined with cadherins, Nectin-4 helps to form and sustain adherens junctions.[18-21] Other researchers revealed that Nectin-4 promotes tumor progression in different carcinomas.[32-34] In our previous study,[22] we determined that Nectin-4 is critical for GBC proliferation and invasion by activating the PI3K/AKT pathway. In this study, we found that *Nectin-4* expression was elevated after overexpressing ERRα in GBC cells. The targeted inhibition of Nectin-4 attenuated cell proliferation and invasion induced by ERRα upregulation in GBC cells. Moreover, Nectin-4 depletion blocked the ERRα-induced activation of the PI3K/AKT pathway. The expression of genes in the PI3K/AKT pathway are known as the most frequently fluctuated in human

lesions, and the aberrant activation of the PI3K/AKT pathway is associated with cellular transformation, tumorigenesis, and cancer progression, as well as drug resistance.[35] Hence, it is hypothesized that Nectin-4 participates in the pro-oncogenic function of ERRα to activate the PI3K/AKT signaling pathway in GBC, thereby forming the ERRα-Nectin-4-PI3K/AKT axis to promote the proliferation and migration of GBC cells. This axis could be applied as a potential prognostic marker and target for the clinical intervention of GBC. As one of the nuclear transcription factors, ERRα regulates the transcription of targeted genes by binding to EREs and ERREs. Bioinformatics analysis revealed the existence of 7 representative EREs in the promotor region of *Nectin-4*. Therefore, ERRα could regulate the transcription of *Nectin-4*. In this study, we undertook dual-luciferase reporter gene assays to confirm this hypothesis. As ERRα has no natural ligand, it can be activated by its coactivator PGC-1α. Chang et al constructed a plasmid with PGC-1α WT to successfully activate ERRα, as well as other nuclear transcription factors. Therefore, they mutated PGC-1α and obtained PGC-1α 2 × 9, which can specifically activate ERRα. The mutant sequence was a gift from Professor Chang. We synthesized plasmids with PGC-1α WT and PGC-1α 2 × 9. The ERRα activities were detected by the 3 × ERE luciferase plasmid, and it was found that the ERRα activity was significantly increased by these plasmids, thereby achieving specific activation of ERRα. The activity of WT *Nectin-4*-luc was significantly increased after activation of ERRα. To verify whether ERRα regulates *Nectin-4* by targeting EREs in the promoter, we randomly mutated all EREs in the promoter of Nectin-4 and found that the activity of mutant *Nectin-4*-luc was not affected by ERRα. To explore which ERE ERRα binds to, we scanned the promoter of Nectin-4 on the JASPAR website and predicted 2 potential binding sites for ERRα, AATGACCTTGG and TCCAGGTCACA. These 2 motifs contain ERE-7 and ERE-4, respectively. The ChIP assay results confirmed that ERRα can bind to both motifs. Therefore, we speculated that ERRα specifically binds to ERE-7 and ERE-4 in the promoter of *Nectin-4*, thus promoting the transcription of *Nectin-4*. To our knowledge, the current study is the first to report that ERRα enhances the proliferation and invasion of GBC cells both in vitro and in vivo. Moreover, ERRα promotes the transcription of *Nectin-4* to activate the PI3K/AKT signaling pathway in GBC cells. Therefore, ERRα could be a promising prognostic marker and molecular target for developing novel therapeutic strategies of GBC.

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DISCLOSURE

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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