ERR α Expression in Ovarian Cancer and Promotes Ovarian Cancer Cells Migration in Vitro

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Abstract

Purpose: Ovarian cancer is one of the common gynecological malignancies, which is prone to metastasize and thus causes a high fatality rate. Estrogen-related receptor alpha (ERR α) is highly expressed in various malignant tumors. Our objective was to explore the impact of ERR α expression on the progression of ovarian cancer.

Methods: The correlation between ERR α expression level and clinical pathological parameters in ovarian cancer tissues were analysed via cancer public database CPTAC. The expression level of ERR α in ovarian cancer cells were confirmed by RT-qPCR and Western Blot methods. The cellular ERR α expression was up-regulated via lentivirus transfection and down-regulated via specific antagonist. The invasion and metastasis capabilities of ovarian cancer cells were observed by wound healing assay and trans-well chamber assay.

Results: The CPTAC database showed that the ERR α expression levels were higher in the late-stage and high-grade ovarian cancer tissues compared with those in early-stage and low-grade tissues. Ovarian cancer cells with higher expression level of ERR α had stronger invasion and metastasis capabilities in vitro. After up-regulating the ERR α expression level, the invasion and metastasis capabilities of ovarian cancer cells were enhanced, while down-regulation weakened. Moreover, there was a positive correlation between the percentages of wound closure and cellular ERR α mRNA expression level ($r=0.921$, $P<0.01$), and the cell invasiveness was also positively correlated with the cellular ERR α mRNA expression level ($r=0.926$, $P<0.01$).

Conclusions: Our results suggest that ERR α may play a positive role in the progression of ovarian cancer, and may serve as a promising predictive biomarker.

1. Introduction

Ovarian cancer is one of the most common gynecological malignancies. It has a high risk of invasion and metastasis, post-treatment recurrence and drug resistance. There are about 125,000 people die of ovarian cancer every year in the world (1), so it is a serious threat to women's lives and health. Ovarian cancer patients lack typical symptoms at the early stage, and about 70% of the cancer cases are diagnosed at the advanced stage, and the five-year survival rate is only 20–30% (2). Ovarian cancer is prone to metastasis, recurrence and poor prognosis, and thus present substantial challenges in its diagnosis and treatment. Therefore, there is an urgent need to find promising biomarkers or therapeutic targets for ovarian cancer, so as to increase the rate of early diagnosis and to overcome the difficulties of tackling the metastasis of ovarian cancer.

The estrogen-related receptor alpha (ERR α) is an orphan nuclear receptor (NR) that can perform biological functions without binding to a ligand. Nuclear receptor superfamily (NRS) is a family of transcription factors with a wide range of functions. Most of them can directly bind to genomic DNA in response to specific ligands, and control the expression of target genes under the action of co-activators or co-

inhibitors, so as to regulate the metabolism and homeostasis. ERR α coding gene is located at the 11q13 site of human chromosome and mainly contains 3 functional domains: N terminal domain (NTD), DNA binding domain (DBD) and ligand binding domain (LBD) (3). ERR α competes with estrogen receptor alpha (ER α) for binding to the same target genes, transcription factors and co-activator proteins, thereby interacting and bypassing the classic estrogen signaling pathway (4). Recently, many studies (5) have found that ERR α can affect the metabolic re-programming of tumor cells to meet the energy needs in the process of tumorigenesis and progression, which makes orphan nuclear receptor ERR α receive extensive attention in the field of cancer. ERR α have been reported to play a crucial role in tumorigenesis and progression of breast cancer (6), endometrial cancer (7), prostate cancer (8), colorectal cancer (9) and lung cancer (10). However, only few studies have reported the ERR α expression level of ovarian cancer and its biological function in ovarian cancer. Therefore, our purpose is to study the role of ERR α expression level in the progression of ovarian cancer.

2. Materials And Methods

Dada acquisition and processing

The bioinformatics portal UALCAN (<http://ualcan.path.uab.edu>) was used to access ERR α protein expression levels in normal ovarian tissues and in ovarian cancer tissues. This resource for expression analysis uses data from the Clinical Proteomic Tumor Analysis Consortium (CPTAC) Confirmatory/Discovery datasets (proteomics by mass-spectrometry) (11). Protein data are expressed as Z-values, and representative of standard deviations from the median across samples for the given cancer type. Log2 spectral count ratio values from CPTAC were first normalized within each sample profile, then normalized across samples.

Cell lines and cell culture

Ovarian cancer cell lines HO8010 and its metastatic equivalent HO8910PM were purchased from the Type Culture Collection Centre of Chinese Academic of Science (Shanghai, China), and were cultured in 90% Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS, Gibco), 1% penicillin (100 IU/mL), and 1% streptomycin (100 IU/mL) in a 5%-CO₂ incubator at 37°C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated according to the manufacturer's protocol (Invitrogen, USA, Thermal). The quality and content of mRNA were assessed using a DNA Counter NanoDrop2000 (Thermo Fisher Scientific, Inc.). Those samples with an optical density (OD) 260/280 ratio > 1.8 were used in the experiments. The cDNA synthesis was performed by the Reverse Transcription Reagent Kit (abm, Vancouver, BC, Canada) according to the manufacturer's instructions. Cobas z480 System (Roche, Basel, Switzerland) and EvaGreen 2X qPCR Master Mix (abm, Vancouver, BC, Canada) were used for quantitative real-time PCR analysis. The specific primers used for qPCR were as follows: ERR α (forward: 5'-ACC GAG AGA TTG TGG

TCA CCA-3', reverse: 5'-CAT CCA CAC GCT CTG CAG TACT-3' (101 bp); glyceraldehyde 3-phosphate dehydrogenase (GADPH, control): forward 5'-GCA CCG TCA AGG CTG AGA AC-3', reverse 5'-TGG TGA AGA CGC CAG TGGA-3' (138 bp). The relative mRNA levels were calculated using the 2^{-(Delta Delta C(T))} Method (12).

Western blotting

Total protein was extracted from ovarian cancer cells using RIPA lysis buffer (Sig-ma-Aldrich, St. Louis, MO, USA) and low-temperature centrifugation at 10,000 × g for 10 min at 4°C. Total protein was quantified using a bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL, USA). 35 µg of whole cell protein was loaded per lane and separated via SDS-PAGE on a 12% gel at 110 V. Proteins were blotted onto nitrocellulose membranes. Blotted membranes were respectively incubated with the anti-human ERRα rabbit monoclonal antibody (1:500 dilution; Cell Signaling Technology, Beverly, MA, USA) and β-Actin rabbit monoclonal antibody (1:1000 dilution; Affinity Biosciences, no.AF7018) overnight at 4°C. The membranes were washed with Tris-buffered saline containing 0.04% Tween-20 (TBST), followed by incubation with HRP-labeled goat anti-rabbit IgG antibody (1:1000; Affinity Biosciences, no. S0001) for 1 h at room temperature. An enhanced chemiluminescence (ECL) detection system (Thermo Fisher, Waltham, MA, USA) was used to visualize the bands.

Construction of stable ERRα-expressing ovarian cancer cells by lentivirus transfection

A customized ERRα-overexpression lentivirus vector and lentivirus-negative control (plasmid sequence: Ubi-MCS-3FLAG-SV40-EGFP-IRES-puromycin) was obtained from Genechem Co., LTD. (Shanghai, China). Ovarian cancer cells (HO8910) were transfected with the ERRα lentivirus and screened/selected using puromycin (1 µg/ml; Genechem; China). The surviving cells were cultured into multiple monoclonal cell lines and were assessed for the expression of ERRα using PCR analysis. Three groups of cells were set: cells infected with ERRα-overexpression lentivirus vector (group OV-ERRα), lentivirus-negative control (group NC), and cells treated with DMSO as the control (group CON) (Fig. 1).

Treatment with ERRα specific antagonist XCT790

The XCT790 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Prior to treatment, the ovarian cancer cells (HO8910PM) were seeded in 6-well plates at a density of 1×10⁵ cells/well and cultured in 3 ml serum-free DMEM for 12 h to achieve adherence, and then in DMED supplemented with 10% fetal bovine serum, 1% penicillin (100 IU/mL), and 1% streptomycin (100 IU/mL). When the cell population reached about 80% (logarithmic phase), as we previously studied (13), ovarian cancer cells were treated with 10 µM XCT790 for 24 h, and then were collected for RNA and protein extraction. Similarly, prior to the cellular wound healing assay and trans-well chamber assay, cells were incubated with final concentrations of 10 µM XCT790 for 24 h.

In vitro wound healing assay

Ovarian cancer cells were seeded at density 1×10^5 and cultured in 6-well plates to 80–90% confluence and were serum-starved for 24 h. Two scratches were then introduced to the cell layer in each well using a 100–1,000 μ l tip. Following washing twice with PBS, the cells (HO8910, HO8910PM, group OV-ERR α , group NC) were incubated in DMED supplemented with 10% fetal bovine serum, 1% penicillin (100 IU/mL), and 1% streptomycin (100 IU/mL). The cells treated with ERR α specific antagonist XCT790 (group XCT790-treated) were incubated in DMED supplemented with fetal bovine serum, penicillin, streptomycin and 10 μ M XCT790. Images of the same regions were captured at 0 and 24 h following stimulation with light microscope (TE2000-U; Nikon, Japan); the paired images were analysed (the ratio of the difference in scratches area between 0 h and 24 h to the 0 h scratches area).

Trans-well chamber migration assay

After thawing overnight at 4°C on ice, Matrigel™ Basement Membrane Matrix (50 μ L; BD, USA) was added to a Millicell Hanging Cell Culture Insert (Millipore, USA) to coat the membrane and incubated at 37°C for 30 min. 200- μ L cell suspensions containing 0.5% FBS (5.0×10^5 cells/mL) were added to the insert, placed in 24-well plates containing 1,300 μ L of DMEM supplemented with 10% FBS. The plates were incubated for 24 h at 37°C. Then, non-invading cells on the top of the filter were removed with a cotton swab, and the filters were fixed with methanol and stained with crystalline violet. The filters were removed from the inserts and mounted onto slides for imaging and quantification as described in a previous study (14).

Statistical analysis

Statistical analysis was performed using the average results of three experiments under identical conditions. Numerical data are presented as the mean $\pm$ standard deviation. Differences between two means were compared by Student's t-test. A one-way analysis of variance was performed for multiple comparisons of groups, which was followed by the Fisher's least significant difference post hoc test, and associated parameters were further analysed using the Spearman's correlation test. Data were analysed using 19.0 for Windows (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. ERR α expression is correlated with clinicopathological parameters of ovarian cancer patients

UALCAN bioinformatics analysis was used to analyse protein levels of ERR α in normal ovarian tissues and in ovarian cancer tissues. The protein expression data was obtained from the mass-spectrometry proteomic profiles, generated by the Clinical Proteomic Tumor Analysis Consortium (CPTAC). ERR α expression level was significantly higher ($P < 1E-12$) in late-stage ovarian cancer [stage 3 ($n = 75$); stage 4 ($n = 16$)] compared to early stages [stage 1 ($n = 2$)], suggesting a role for ERR α in tumor progression

(Fig. 2A). In addition, the results showed that the expression level of ERR α was higher in Grade 3 ovarian cancer tissues [Grade 3 (n = 79)] compared to Grade 2 ovarian cancer tissues [Grade 2 (n = 6)], also suggesting a role for ERR α in tumor progression, $P < 1E-12$ (Fig. 2B).

3.2. The expression level of ERR α in ovarian cancer cell lines

Real-time PCR (RT-PCR) analysis revealed the relative mRNA expression level of ERR α in HO8910 cell lines was lower than its metastatic equivalent HO8910PM cells ($P = 0.002$; Fig. 3A).

There was no significant difference in ERR α mRNA expression level between ovarian cancer HO8910 cells and the lentivirus-negative control group ($P = 0.077$), while cells infected with ERR α -overexpression lentivirus vector (OV-ERR α group) had higher ERR α mRNA expression level compared with those untreated HO8910 cell lines ($P = 0.001$, Fig. 3B). After treating with ERR α specific antagonist XCT790, the relative mRNA expression level of ERR α was lower in XCT790-treated group compared with those untreated HO8910PM cells ($P < 0.001$, Fig. 3C). A similar tendency was observed for ERR α protein expression in these ovarian cancer cells. The protein expression level of ERR α in HO8910 was lower than its metastatic equivalent HO8910PM (0.637 ± 0.023 vs. 0.876 ± 0.105 , $P = 0.018$; Fig. 4A). The protein expression levels of ERR α in ovarian cells HO8910, lentivirus-negative control group (NC group) and OV-ERR α group were 0.637 ± 0.023 , 0.669 ± 0.043 and 1.713 ± 0.035 . (HO8910 vs. NC group, $P = 0.318$ and HO8910 vs. OV-ERR α group, $P < 0.001$, respectively; Fig. 4B). After treating with ERR α specific antagonist XCT790, the protein expression levels of ERR α were lower in XCT790-treated group compared with those untreated HO8910PM cells (0.378 ± 0.028 vs. 0.871 ± 0.110 , $P = 0.002$, Fig. 4C).

3.3. Ovarian cancer cells with different ERR α expression levels differ in metastasis and invasion

The wound healing assay (Fig. 5A) results showed that the percentages of wound closure exhibited by HO8910 and its metastatic equivalent HO8910PM were $24.3\% \pm 2.4\%$ and $36.3\% \pm 2.4\%$, respectively ($P = 0.007$). The percentages of wound closure exhibited by ovarian cells HO8910, NC group (lentivirus-negative control group) and OV-ERR α group (cancer cells infected with ERR α -overexpression lentivirus vector) were $24.3\% \pm 2.4\%$, $22.8\% \pm 2.0\%$, and $35.3\% \pm 0.6\%$ (HO8910 vs. NC group, $P = 0.534$ and HO8910 vs. OV-ERR α group, $P = 0.003$, respectively). After treating with ERR α specific antagonist XCT790, the percentages of wound closure were lower in XCT790-treated group compared with those untreated cancer cells ($14.9\% \pm 1.0\%$ vs. $36.3\% \pm 2.4\%$, $P < 0.001$) (Fig. 5C).

The results obtained with the invasion assay (Fig. 5B) were consistent with those obtained with the wound healing assay. Transmembrane cell counts exhibited by HO8910 and its metastatic equivalent HO8910PM were 112.333 ± 12.284 and 265.667 ± 30.663 , respectively ($P = 0.003$). The transmembrane cell counts exhibited by ovarian cells HO8910, NC group (lentivirus-negative control group) and OV-ERR α group (cancer cells infected with ERR α -overexpression lentivirus vector) were 112.333 ± 12.284 , 120.333

± 9.978 , and 482.333 ± 24.500 , respectively (HO8910 vs. NC group, $P = 0.514$ and HO8910 vs. OV-ERR α group, $P < 0.001$). After treating with ERR α specific antagonist XCT790, the transmembrane cell counts exhibited by XCT790-treated group were less than those untreated HO8910PM cells (107.667 ± 10.339 vs. 265.667 ± 30.663 , $P = 0.002$) (Fig. 5D).

Additionally, Spearman's correlation analysis showed that there was a positive correlation between the percentages of wound closure and cellular ERR α mRNA expression level ($r = 0.921$, $P < 0.01$, Fig. 6A), and the cell invasiveness was also positively correlated with the cellular ERR α mRNA expression level ($r = 0.926$, $P < 0.01$, Fig. 6B).

4. Discussion

This study is the first to explore the impact of ERR α expression level on the progression of ovarian cancer. Cancer public database analysis showed that the expression levels of ERR α were related to the clinic stage and pathological grade of ovarian cancer tissue. Specifically, the expression levels of ERR α were higher in the late-stage and high-grade ovarian cancer tissues compared with those in early-stage and low-grade tissues. The in vitro experiments results showed that ovarian cancer cells with strong invasion and metastasis capability have higher ERR α mRNA and protein expression levels. In addition, after up-regulating the ERR α expression level, the invasion and metastasis of ovarian cancer cells was enhanced, while down-regulation weakened. It can be seen that ovarian cancer cells with different invasion and metastasis capabilities have different expression levels of ERR α , and ovarian cancer cells with different ERR α expression levels also have different invasion and metastasis capabilities. Using Spearman's correlation analysis, we found that both the percentages of wound closure and the cell counts of invasion were significantly positively correlated with the cellular ERR α mRNA expression level. In conclusion, that the expression level of ERR α in ovarian cancer cells is related to its invasion and metastasis capability. These results were consistent with the results of cancer public database analysis, so it suggested that ERR α may play a positive role in the progression of ovarian cancer. However, further studies need to involve more samples to confirm this conclusion.

ERR α is involved in the tumorigenesis and is considered as a potential biomarker in various cancers. Compared with normal tissues, ERR α expression levels were increased in breast cancer (15), prostate cancer (16), endometrial cancer (17) and colorectal cancer (18). High expression level of ERR α not only has the potential to become a diagnostic biomarker, but also is related to the progression of cancers. Lu et al. (19) studies show that ERR α can activate the pS2 promoter in breast cancer cell lines, which significantly increases the synthesis of Estrogen, and the abnormal increase of estrogen can lead to tumorigenesis. Chang et al. (20) reported that the activation of the Her2/IGF-1R signaling pathways and subsequent C-MYC stabilization upregulate the expression of PGC-1 β that can increase the expression of ERR α , and thus promoting the proliferation of breast cancer cell lines. Cai et al. (21) found that ERR α also stimulate glycolysis under normoxia and activate promoters of many genes encoding glycolytic enzymes in breast cancer cells, suggesting that ERR α can also promote the proliferation of breast cancer cells without involving estrogen signaling pathway. ERR α is not only involved in the proliferation of cancer

cells, but also in the invasion and metastasis. Huang et al. (17) reported that ERR α can trigger the migration and invasion of endometrial cancer cells via up regulation of TGFB1. Jia et al. (22) studies showed that bisphenol S can induce the expression of miR-10b in PC12 cells via ERR α . The upregulated miR-10b inhibited the expression of KLF4, which can suppress the migration and invasion of cancer cells. Zhang et al. (10) found that activation of NF- κ B/IL-6 is involved in ERR α induced migration and invasion of non-small cell lung cancer (NSCLC) cells, suggesting that ERR α might be a potential target for NSCLC treatment.

Ovarian cancer is one of the three common malignant tumors in gynecology, and it is also the cancer with the highest mortality rate in gynecology. Its lack of early diagnostic biomarkers, prone to metastasize, and prone to relapse are the causes for its high mortality and poor prognosis. Therefore, searching for cancer-related tumor biomarkers is of great significance for studying the pathogenesis of ovarian cancer and for exploring new treatment targets. Although many studies have reported ERR α is involved in the tumorigenesis and progression of various cancers, only a few studies have explored the effect of ERR α on ovarian cancer. Sophia Sn Lam et al. (23) reported that ERR α functions in epithelial-mesenchymal transition and in subsequent stem cell traits responsible for the acquisition of high degree of aggressiveness and potential for metastasis that are characteristic of ovarian cancer, which suggests ERR α activation as a mechanism of tumor aggressiveness and imply that targeting ERR α may be a promising approach in ovarian cancer treatment. Our study is the first to suggest that the expression levels of ERR α is related to the invasion and metastasis of ovarian cancer cells and that the ERR α may play a positive role in the progression of ovarian cancer, which provides a reference for further study on the mechanism of ovarian cancer progression and molecular targeted therapy.

Declarations

Funding

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets analyzed during the current study are available in the UALCAN database (<http://ualcan.path.uab.edu>).

Code availability

Not applicable.

Authors' contributions

WH was a major contributor in writing the manuscript and performing the experiments. LC was a contributor in methodology and validation. PS was a contributor in conceptualization, supervision and funding acquisition. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

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Figures

Figure 1

Up-regulation of ERRα mediated by lentivirus vector transfection. (A) HO8910 cells treated with DMSO were observed by optical microscope (100X); (B) HO8910 cells treated with DMSO were observed by fluorescence microscopic (200X); (C) HO8910 cells infected with lentivirus-negative control were observed by optical microscope (100X); (D) HO8910 cells infected with lentivirus-negative control were observed by fluorescence microscopic (200X); (E) HO8910 cells infected with ERRα-overexpression lentivirus vector

were observed by optical microscope (100X); (F) H08910 cells infected with ERRα-overexpression lentivirus vector were observed by fluorescence microscopic (200X).

Figure 2

Protein expression level of ERRα in ovarian cancer tissues. Z-values represent standard deviations from the median across samples for the given cancer type. Log2 spectral count ratio values from CPTAC were first normalized within each sample profile, and then normalized across samples (Student's t-test). (A) Plots showing correlations between ERRα protein expression with clinical tumor stages; (B) Plots showing correlations between ERRα protein expression with pathological tumor grades. CPTAC, Clinical Proteomic Tumor Analysis Consortium. UALCAN website that used two-sided Student's t-test provided P-values, indicated by asterisk (*). P-value < 0.05 was defined as statistically significant.

Figure 3

Expression level of ERRα mRNA in ovarian cancer cells. (A) There was higher expression level of relative ERRα mRNA in ovarian cancer HO8910PM cells than in HO8910 cell lines. (B) The ERRα mRNA in HO8910 cell lines transfected with ERRα-overexpression lentivirus vector (OV-ERRα) showed an up-regulation of ERRα, while those treated with lentivirus-negative control (NC Group) did not; (C) The ERRα mRNA in HO8910PM cell lines treated with 10 μM XCT790 for 24 h showed a down-regulation of ERRα induced by XCT790. All experiments were conducted in triplicate. *P-value<0.05 was defined as statistically significant.

Figure 4

Expression level of ERRα protein in ovarian cancer cells, a similar trend to the mRNA expression. (A) There was higher expression level of relative ERRα protein in the ovarian cancer HO8910PM cells than in HO8910 cell lines. (B) The ERRα protein in HO8910 cell lines transfected with ERRα-overexpression lentivirus vector (OV-ERRα) showed an up-regulation of ERRα, while those treated with lentivirus-negative control (NC Group) did not; (C) The ERRα protein in HO8910PM cell lines treated with 10 μM XCT790 for

24 h showed a down-regulation of ERRα induced by XCT790. As indicated, *P-value <0.05 was defined as statistically significant.

Figure 5

The invasiveness and metastatic capacities of ovarian cancer cells. (A) Wound healing assay (10X). (B) Trans-well chamber assay (10X). (C) The percentages of wound closure exhibited by ovarian cancer HO8910PM cells was higher than HO8910 cell lines. The HO8910 cells transfected with ERRα-

overexpression lentivirus vector (OV-ERRα group) had a higher percentage of wound closure than its control group, while the HO8910PM cell lines treated with 10 μM XCT790 for 24 h showed a lower percentage of wound closure than its control group. (D) Transmembrane cell counts exhibited by HO8910PM cells were more than HO8910 cell lines. The OV-ERRα group had more transmembrane cell counts than its control group, while the HO8910PM cell lines treated with 10 μM XCT790 for 24 h had less, a similar trend to wound healing assay. As indicated, *P-value<0.05 was defined as statistically significant.

Figure 6

Spearman's correlation analysis. (A) Correlation between the percentages of wound closure and cellular ERRα mRNA expression level ($P < 0.01$). (B) Correlation between the cell counts of invasion and the cellular ERRα mRNA expression level ($P < 0.01$).