Downregulation of estrogen receptor β inhibits lung adenocarcinoma cell growth

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Abstract. Estrogen receptor β (ERβ) is an important ER subtype in lung adenocarcinoma. However, the functions and mechanisms of ERβ have not been fully elucidated. The aim of the present study was to investigate the biological effects and relevant mechanisms of ERβ in lung adenocarcinoma. The protein expression of ERβ was found to be higher in lung adenocarcinoma tissues compared with that in adjacent non-cancerous tissues (n=75, P<0.001). Of note, ERβ protein expression was significantly correlated with tumor size (P=0.018), lymph node metastasis (P=0.041), clinical stage (P=0.041) and differentiation (P<0.001). In addition, ERβ protein expression in A549 cells was found to be higher compared with that in human bronchial epithelial cells (HBEs). Furthermore, knockdown of ERβ expression inhibited colony formation and cell invasion in vitro, whereas the number of metastatic tumors in the lungs of mice was decreased in vivo. Western blot analysis demonstrated that the expression of phosphorylated extracellular signal-regulated kinase (pERK), matrix metalloproteinase (MMP)-2 and MMP-9 was decreased by downregulation of ERβ. Therefore, ERβ may play an important role in lung adenocarcinoma progression via the MEK/ERK signaling axis, and it may represent a novel therapeutic target for lung adenocarcinoma in the future.

Introduction

Lung cancer is the most common malignant tumor and one of the main causes of cancer-related mortality worldwide (1). The 5-year survival rate is ~15%, and only 15-17% of patients diagnosed with distant metastasis survive for 1 year (2,3). Adenocarcinoma is one of the major subtypes of non-small cell lung cancer (NSCLC), and lung adenocarcinoma patients frequently develop metastases (4). It is estimated that >35% of patients with advanced lung adenocarcinoma develop distant metastases, resulting in shorter survival and poor quality of life (5). Conventional treatments, including chemotherapy, radiotherapy and bisphosphonates, have been shown to have limited efficacy (1). Hence, it is crucial to elucidate the mechanisms underlying lung adenocarcinoma progression.

Estrogen and progesterone receptors have been shown to play an important role in NCSLC, particularly lung adenocarcinoma (6). The incidence and mortality of lung cancer were found to be higher among women who receive hormone replacement therapy (7). The effects of estrogen are mediated via estrogen receptors (ERα and ERβ) (8). Estrogen receptors are consistently found in lung cancer tissues and cell lines (particularly adenocarcinoma), mostly in the form of ERβ (9). Hsu et al reported that estrogen promoted lung adenocarcinoma cell proliferation and migration via ERβ, and high expression of ERβ was identified as an adverse prognostic factor in patients with lung adenocarcinoma (9). However, the detailed mechanism underlying ERβ-mediated lung adenocarcinoma progression remains unclear.

The aim of the present study was to determine whether the expression of ERβ is higher in lung adenocarcinoma tissues, as well as observe the effects of its knockdown by lentivirus interference RNA on lung adenocarcinoma cell growth and invasion in vitro and in vivo.

Materials and methods

Immunohistochemical staining of ERβ. Tissue microarray (TMA) assays were obtained from Superchip (Shanghai, China) and included 75 cases of tissues from lung adenocarcinoma and adjacent normal tissues (array ID: HLug-Adel50Sur-02). The recorded clinicopathological information included age, sex, tumor size, tumor location and TNM stage. Experiments were performed as described previously (10,11). The tissue sections were de-paraffinized, rehydrated, and treated according to standard protocols (12). Polyclonal rabbit anti-ERβ antibody

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Inc., Corning, NY, USA) at a density of 1x10^4 cells/well and
briefly, A549 cells were seeded into 96‑well plates (Corning
all purchased from Abcam. Next, the membranes were
(dilution 1:2,000; cat. no. ab3577) and mouse monoclonal
(MMP)-2 (dilution 1:2,000; cat. no. ab37150), MMP -9
dilution 1:500; cat. no. ab76299), matrix metalloproteinase
kinase (ERK)1/2 (dilution 1:500; cat. no. ab176640), pERK1/2
Cell proliferation assay. The MTT assay (Sigma -Aldrich;
was determined by chemiluminescence (Pierce; Thermo
Irvine, CA, USA). Finally, the content of the target proteins
Cell lines and cell culture. The human lung adenoscarcoma
cell line A549, the human breast cancer cell line MCF -7 and
human bronchial epithelial (HBE) cells were preserved in our
laboratory. The A549, MCF-7 and HBE cells were maintained
in RPMI-1640 medium (C1187550B; Gibco, Thermo Fisher
Scientific, Inc., Waltham, MA, USA) supplemented with 10%
fetal bovine serum (FBS; Biochrom GmbH, Berlin, Germany).
The cells were incubated at 37˚C in a humidified atmosphere of
5% CO2/95% air.

Western blot analysis. Cells were lysed for protein extraction.
After being quantified, 25 µg of protein was subjected to 10%
SDS-polyacrylamide gel electrophoresis and transferred to
polyvinylidene fluoride membranes. The membranes were
then blocked in 5% fat-free milk and incubated overnight
at 4˚C with rabbit polyclonal antibodies against ERβ (dilution
1:2,000; cat. no. ab3577), extracellular signal-regulated kinase (ERK)1/2 (dilution 1:500; cat. no. ab176640), pERK1/2
(dilution 1:500; cat. no. ab76299), matrix metalloproteinase
(MMP)-2 (dilution 1:2,000; cat. no. ab37150), MMP -9
dilution 1:2,000; cat. no. ab38898) and mouse monoclonal
antibody against GAPDH (dilution 1:8,000; cat. no. ab8245),
all purchased from Abcam. Next, the membranes were
incubated with anti-rabbit/ mouse secondary antibodies
(cat. nos. GTX213110-10/GTX213111-01; GeneTex, Inc.,
Irvine, CA, USA). Finally, the content of the target proteins
was determined by chemiluminescence (Pierce; Thermo
Fisher Scientific, Inc.).

Cell proliferation assay. The MTT assay (Sigma-Aldrich;
Merck KGaA) was conducted to determine cell proliferation.
Briefly, A549 cells were seeded into 96-well plates (Corning
Inc., Corning, NY, USA) at a density of 1x10^4 cells/well and
treated with E2 (10 nM), DPN (10 nM) or PPT (10 nM) for 0, 24,
and 72 h, and added the same volume of phosphate-buffered
saline (PBS) as the control group. At each time-point, 20 µl
MTT (10 mg/ml) was added to each well and successively
incubated for another 4 h at 37˚C. After removing the super-
natant, 150 µl dimethylsulfoxide (S7020; Invitrogen; Thermo
Fisher Scientific, Inc.) was added for 10 min to dissolve the
formazan crystals. The absorbance was measured at 490 nm
with a microplate reader (Multiskan MK3; Thermo Fisher
Scientific, Inc.). Each experiment was performed in triplicate
and repeated three times.

Cell infection. Short hairpin RNAs (shRNA1: GCATGGAAC
ATCTGTCtAAA; shRNA2: GCTGAATGCCCCAGTGtTt;
shRNA3: GCCAAAGGGGtCCtCCACAgAA) targeting ERβ
(ERβ-GV248-RNAi NM_001437, target sequence: GCAAG
AGGGtcCtCCACAgAA) and control shRNA (NC-GV248,
target sequence: TtCtCCGAgACGtGtCAGt) were
obtained from Shanghai GeneChem Co., Ltd. (Shanghai,
China). A549 cells were infected with the ERβ-shRNA lentivirus to knock down ERβ expression, and NC-shRNA was
used at the same time as a negative control group, according
to the manufacturer's instructions. After 72 h, green fluores-
cent protein indicated that the rate of infection was ~90% at
a multiplicity of infection of 10. Stably transfected cells were
then selected with puromycin for 2 weeks.

Reverse transcription-quantitative polymerase chain reaction
(RT-qPCR) analysis. Total RNA of infected A549 cells was
isolated from cells using TRIzol reagent (Invitrogen; Thermo
Fisher Scientific, Inc.), according to the manufacturer's instruc-
tions. Total RNA (500 ng) was then reverse-transcribed into
cDNA (Takara Bio, Inc., Otsu, Japan). RT-PCR was performed
using SYBR Premix Ex Taq™ II (Takara Bio, Inc.) and gene
expression was quantified using the cycle quantification (Cq)
method. The PCR primers were as follows: ERβ forward, GAT
cATTGCTCtCCTGtGAC and reverse, CACtCtTCtCCGt
TCAGtTt; GAPDH forward, AGCAGGTGtCtATACAtA
TAc and reverse, TGGAGGGACTAAAGAGGAGAGGt.

Colony formation assay. The infected A549 and control cells
in the logarithmic growth phase were harvested and plated into
6-well plates (cat. no. A1098Z01; BioExcellence International
Tech Co., Ltd.) at 500 cells/well. After incubation for 8 days,
cell colonies (>50 cells) were stained with 0.25% crystal violet
solution and their number was manually counted. Each experi-
ment included three independent biological replicates and each
was performed in triplicate.

Cell invasion assay. The invasive potential of the cells was
measured using 8-µm pore size Transwell inserts (Corning,
Inc.). The infected A549 and control cells were resuspended
in serum-free RPMI-1640 and then seeded in triplicates in
the upper chamber covered with 70 µl Matrigel (diluted in
1:8; Corning, Inc.). Medium (500 µl) containing 10% FBS was
added to the bottom chamber to serve as the chemoattractant.
After 24 h, cells that had migrated to the lower chamber were
fixed with 95% ethyl alcohol and then stained with 0.5% crystal violet solution. Finally, the number of invading cells
was counted in five random fields per sample and the mean
was calculated. Each experiment included three independent
biological replicates and each was performed in triplicate.
In vivo experiments. An experimental model of A549 cell lung metastasis was constructed to study the effects of ERβ on lung adenocarcinoma in vivo. A total of 18 female NOD/SCID mice, aged 4 weeks and weighing 20-25 g, were purchased from Beijing HFK Bioscience Co. (Beijing, China). They were housed in a specific pathogen-free (SPF) laboratory animal environment (temperature, 22˚C; ventilation rate, 15/h; light/dark cycle, 12/12 h; food was sterilized with Cobalt-60 irradiation and water was autoclaved, and access to the food was ad libitum; tumor size not exceed 2.0 cm) by professional breeders and randomly divided into three groups (6 mice/group). A549, A549-ERβ-shRNA and A549-NC-shRNA cells (1x10^6/200 µl) were harvested, resuspended in PBS and injected via the tail vein. After 3 days, E2 (0.1 mg/kg) was subcutaneously injected once a week in a volume of 100 µl per mouse. When the experimental mice developed symptoms such as lameness, joint stiffness, decreased exercise capacity, paraplegia, or an experiment for 42 days, the experiment required termination. The mice were sacrificed humanely in a transparent euthanasia device (ventilated 10% of isoflurane for another 3 min after the mice were dead). The lungs were then excised and weighed; the number of the metastatic lesions larger than 0.5 mm in diameter on the surface of the lungs was counted, fixed in 10% formalin, embedded in paraffin, and sectioned for H&E staining.

Statistical analysis. Statistical analyses were conducted using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Wilcoxon rank sum test was used for immunohistochemical total scores analysis and the p-ERK/ERK ratio analysis was conducted by t-test. The other data were analyzed by one-way analysis of variance (ANOVA) with least significant difference (LSD) test as the post hoc test. P-values <0.05 were considered to indicate statistically significant differences.
Expression of ERβ in lung adenocarcinoma patient samples and cell lines. Immunohistochemistry was used to evaluate the association of ERβ expression and pathological characteristics with a TMA that consisted of 75 paired lung adenocarcinoma specimens and corresponding normal samples. The expression of ERβ was observed in the cytoplasm. The correlations of ERβ expression with clinicopathological characteristics in the lung adenocarcinoma patients are presented in Table I and Fig. 1. We observed that the protein expression of ERβ was higher in lung adenocarcinoma tissues compared with that in adjacent non-cancerous tissues (P<0.001). Notably, ERβ protein expression was significantly correlated with tumor size (P=0.018), lymph node metastasis (P=0.041), clinical stage (P=0.041) and tumor differentiation (P<0.001). The results indicated that the expression of ERβ may be associated with the occurrence and progression of lung adenocarcinoma.

Furthermore, ERβ expression was compared among lung adenocarcinoma cells (A549), breast cancer cells (MCF-7) and HBE cells. MCF-7 cells were used as the positive control, and ERβ expression was found to be higher in A549 compared with that in MCF-7 and HBE cells (Fig. 2). The results indicated that ERβ is an important functional ER subtype in A549 cells.

E2 and DPN promote A549 cell proliferation. The effects of E2, DPN and PPT on A549 and MCF-7 cells were investigated. MTT assay was conducted to evaluate cell proliferation. As shown in Fig. 3A, the proliferation of cells treated with E2 and DPN was markedly increased compared with that in the control groups while PPT which was effective for the
AMCF-7 cells (Fig. 3B) did not obviously promote A549 cell proliferation; the E2-induced A549 cell proliferation may be mediated via ERβ more than ERα.

Expression of ERβ decreases in cells stably transfected by lentivirus RNA interference. The lentivirus RNA interference technique was used to downregulate the expression of ERβ. Following cell infection and antibiotic screening for 2 weeks, the infection efficacy was confirmed by RT-PCR (Fig. 4A) and western blot analysis (Fig. 4B and C). ERβ was stably decreased by shRNA and the ERβ-GV248-RNAi#3 was the most effective one, thus we named it as ERβ-shRNA for the following tests. The results indicated that ERβ expression was suppressed by ERβ-shRNA.
Downregulation of ERβ expression inhibits colony formation and invasion of A549 cells in vitro. The effects of ERβ-shRNA on A549 cells were examined. As shown in Fig. 5A-D, colony formation and cell invasion assays demonstrated that ERβ-shRNA inhibited A549 cell proliferation and invasion compared with NC-shRNA and control (P<0.05). Mechanistically, ERβ knockdown suppressed the expression of pERK, MMP-2 and MMP-9 (Fig. 5E; P<0.05). These findings indicate that ERβ is a functional mediator. Therefore, knockdown of ERβ expression inhibited A549 cell proliferation and invasion via downregulation of pERK, MMP-2 and MMP-9.

Downregulation of ERβ expression suppresses lung metastasis of A549 cells in vivo. To investigate whether downregulation of ERβ expression may serve as a therapeutic target for lung adenocarcinoma, an experimental lung metastatic mouse model was constructed. In vivo lung metastasis assay demonstrated that downregulation of ERβ expression was associated with fewer metastatic tumors and lower lung weight compared with the control (Fig. 6). These results indicated that downregulation of ERβ may inhibit tumor growth and lung metastasis in vivo.

Discussion

Estrogen and ERs are considered to play an important role in lung carcinogenesis (14). Several studies have demonstrated that ERβ is the predominant ER in lung cancer tissue and tumor cell lines, particularly adenocarcinoma (15,16). It was previously reported that estrogen promotes lung adenocarcinoma cell proliferation and migration via ERβ (11). However, the detailed mechanism underlying ERβ-mediated lung adenocarcinoma progression remains unclear. The present study was designed to investigate the biological effects and mechanism of action of ERβ in lung adenocarcinoma.

To determine the association between ERβ and clinicopathological characteristics in lung adenocarcinoma, immunohistochemistry was used to evaluate the expression of ERβ in TMA, which included 75 tumor and adjacent normal tissues from patients with lung adenocarcinoma. A higher ERβ expression was detected in lung adenocarcinoma specimens compared with adjacent non-cancerous tissues. Notably, ERβ protein expression was found to be significantly correlated with tumor size (P=0.018), lymph node metastasis (P=0.041), clinical stage (P=0.041) and tumor differentiation (P<0.001).
suggesting that ERβ plays an important role in the occurrence and development of lung adenocarcinoma. Our findings were similar to those of Luo et al., who reported that ERβ overexpression promotes the progression of NSCLC (16).

Our findings in vitro were in agreement with the TMA results. ERβ expression was higher in the A549 cell line compared with that in HBE cells. ERβ was identified as the important ER subtype in lung adenocarcinoma cells. MTT assay was used to investigate whether the effects of E2 are mediated via ERα or ERβ. The results indicated that E2 induced lung adenocarcinoma cell proliferation via ERβ. These results are supported by the findings of Fan et al. (17) and Warner and Gustafsson (18), who reported that ERα was not the main mediator of transcriptional responses to E2 in NSCLC cells, but ERβ was more likely to be the primary type in lung tumor tissues and cell lines, and that estrogen-dependent responses in NSCLC cells are principally mediated by ERβ. Furthermore, we demonstrated that downregulation of ERβ expression inhibited colony formation and invasion of A549 cells. In vivo, we constructed an experimental lung metastatic mouse model. The lung metastasis assay demonstrated that downregulation of ERβ expression was associated with fewer metastatic tumors and lower lung weight compared with the control group. Our results are consistent with those of Fan et al., who reported that estrogen and DPN promote lung metastasis of A549 cells (17). Fan et al.'s study revealed that estrogen induced lung cancer metastasis through the ERβ/MMP-2 axis. However, in the present study, MMP-9 was identified as a novel target gene of ERβ. Thus, ERβ may promote lung cancer metastasis not only through MMP-2, but also through MMP-9. The development of lung adenocarcinoma is a complex multistep process. The ERK signaling pathway plays an important role in lung cancer cell proliferation.
and invasion. Therefore, the protein expression of ERK and pERK was determined in A549-ERβ-shRNA cells. The results revealed that downregulation of ERβ expression decreased pERK expression levels. This finding suggested that downregulation of ERβ expression inhibited the proliferation and invasion of A549 cells through suppressing the phosphorylation of ERK. ERs also regulate gene expression through binding to other transcription factors, such as the activator protein 1 (AP-1) (19), the most important structural components of which are c-Jun and c-Fos. In addition, MMP is crucial for malignant tumor metastasis (20,21), particularly MMP-2 and MMP-9.

Taken together, the findings of the present study indicate that ERβ may promote lung adenocarcinoma growth and metastasis through the MEK/ERK signaling pathway and MMP-2/MMP-9 expression, and therefore, hold promise as a novel therapeutic target for lung adenocarcinoma.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HZ designed the study, WC and BX performed the immunohistochemical assay and all the in vitro experiments and collected the data. HP, LH and WS conducted the animal experiments and collected the data. PC, LD, ZZ and LL analyzed the data and performed the relative statistical analysis. ZZ and LL provided guidance during the study. WC contributed to the writing of the manuscript. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All animal studies strictly abide by the Regulations on Animal Experimentation formulated by the Laboratory Animal Center of the Fourth Military Medical University (The Air Force Medical University) (Xi’an, China) and this study was approved by the Animal Experimental Ethical Inspection Committee of this Center (no. 20170803).

Competing interests

The authors declare that they have no competing interests.

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