Androgen Receptor(AR) and Estrogen Receptor α (ER α) Affect Cell Growth and Metastasis of Non-Small Cell Lung Cancer Through EGFR/PI3K/AKT Axis.

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Research

Keywords: Androgen receptor, Estrogen receptor α , EGFR/PI3K/AKT axis, Non-small cell lung cancer

Posted Date: February 12th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-181202/v1>

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Abstract

Background/Aims: Androgen receptor (AR), estrogen receptor α (ER α) signaling and their interaction with epidermal growth factor receptor (EGFR) signaling pathway is a potential therapeutic target in non-small cell lung cancer (NSCLC). To explore the cross communication between AR, ER α and EGFR signaling pathway, we used RNA silencing technology and sex hormones intervention in NSCLC cell line.

Methods: Model system was the well-characterized A549 adenocarcinoma NSCLC cells which can express AR and ERs well. We used different concentrations of testosterone (T), estradiol (E₂) intervention cells and small interfering RNA (siRNA) specifically targeting AR, ER α in A549 cells, then examined the expression of AR, ER α and EGFR/PI3K/AKT axis, followed by detection of cells proliferation, apoptosis, migration and invasion.

Results: After knocked down the expression of AR, ER α , the EGFR/PI3K/AKT axis was inhibited, and the proliferation decreased, apoptosis increased, migration and invasion decreased in A549 cells. 50 μ M T and 50 μ M E₂ intervention inhibited AR, ER α and EGFR/PI3K/AKT axis expression, and the proliferation decreased, apoptosis increased, migration and invasion decreased in A549 cells. 10 μ M T, 10 μ M E₂ and 100 μ M T, 100 μ M E₂ intervention promoted the expression of AR, ER α and EGFR/PI3K/AKT axis, which enhanced the activity and migration invasiveness of A549 cells.

Conclusions: These data provide a rationale for further investigation of the antitumor activity of clinical sex hormones therapy and the development of anticancer drugs targeting sex hormone receptors in the clinic. Future .

Background

Non-small cell lung cancer (NSCLC) is the most common cause of cancer mortality throughout the world(Sequist and Lynch, 2008). It remains of the most lethal types of cancer; a diagnosis of NSCLC portends a 5-year survival rate of only 18%(Siegel et al. , 2017). Thus, improvements in treatment strategies for NSCLC are of great interest. The "2017 China Cancer Registry Annual Report" shows that there are about 800,000 new cases of lung cancer in China each year, and there are about 700,000 deaths every year. Cytotoxic chemotherapy is traditionally the basis for the treatment of advanced NSCLC, but even the most radical regimens provides only about 20% -35% of the tumor response opportunities, and the average survival time has increased by 2-4 months(JH et al. , 2002, Sandler et al.). The high mortality rate of lung cancer patients is mainly due to delays in detection and diagnosis, resulting in the identification of disease at an advanced stage and limited treatment options provided to patients(Youlden et al. , 2008). For most patients, chemotherapy for the early stages of the disease is sensitive, but then it develops drug resistance and leads to relapse(Mostertz et al. , 2010). Since the introduction of EGFR tyrosine kinase inhibitors (EGFR-TKIs) 17 years ago in lung cancer clinical trials, they have had a huge impact on the treatment of NSCLC, but most patients will develop resistance within one year of taking the drug(Jackman et al. , 2010). Needless to say, there is no successful anti-cancer

drug can effectively prevent and reduce the progression of lung cancer. Therefore, there is an urgent need to develop new molecular mechanisms aimed at better understanding the role of potential anti-cancer chemicals and to further provide more effective basic therapeutic strategies.

Recent medical research shows that sex hormones are involved in the development of lung tissue, the formation of pulmonary inflammation and perhaps the molecular biology of lung cancer (SOwowski et al. , 2017, Siegfried, 2014, Zhang et al. , 2009). It has been found that Androgen receptor (AR), estrogen receptor α (ER α) and estrogen receptor β (ER β) are expressed in normal lung tissue and in lung tumors in human and mouse (Dou et al. , 2017, Raso et al. , 2009). A Chinese study showed that the expression of AR may be related to the disease progression and lymph node metastasis of lung cancer (Yan et al. , 2008). A study from the United States and Norway reported that the prognosis of NSCLC patients was correlated with the level of ER α (Olivo-Marston et al. , 2010). On the contrary, a Canada study showed that elevated ER β levels were associated with a higher survival rate in NSCLC patients (Navaratnam et al. , 2010). Therefore, reproductive hormones and their receptors may be potential biomarkers for predicting the occurrence, development, treatment and prognosis of NSCLC (Dou, Zhu, 2017).

Mutations in the EGFR gene are widely recognized alterations involved in the pathobiology of lung cancer and have a clinical impact on NSCLC therapy. As an important pathway of EGFR mediated signaling pathway, PI3K/AKT axis has recently been confirmed to be involved in the oncogene gene alteration of NSCLC (Fumarola et al. , 2014). Regarding the function of ERs and EGFR, the stimulation of ERs has been reported to increase the activity of the EGFR signal, and the signal of EGFR increases the activity of ERs. And there is no correlation between AR and EGFR has been reported so far.

In the present work, our aim was to evaluate whether reproductive hormones and their receptors may inhibit growth and metastasis of NSCLC cells. Model system was the well-characterized A549 adenocarcinoma NSCLC cells which can express AR and ERs well (Garon et al. , 2013, Kaiser et al. , 1996, Weinberg et al. , 2005). We used different concentrations of testosterone (T), estradiol (E₂) intervention cells and small interfering RNA (siRNA) specifically targeting AR, ER α in A549 cells to observe the effect on EGFR/PI3K/AKT axis and cell growth, metastasis of A549 cells. In addition, the mechanism of sex hormones and their receptors on the occurrence and development of lung cancer was also discussed.

Materials And Methods

Cell lines and reagents

A549 cell lines were obtained from the Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd (Shanghai, China) and cultured in DMEM/HIGH GLUCOSE medium (Hyclone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS, Ausbian, Australia) and 1% Penicillin-Streptomycin (Sigma, St. Louis, USA) at 37°C in a humidified atmosphere containing 5% CO₂. Once every four days, the cells were harvested through trypsinization with trypsin-EDTA (Sigma) and under appropriate dilution were cultivated to a future growth in fresh medium.

Gene silencing and sex hormones intervention

2×10^5 cells were seeded in 6-well plates (Corning, Cambridge, MA, USA) with 2ml medium. After cells have attached overnight and cells adhesion rate were about 50%, cultures were changed into serum-free medium. Small interfering RNA (siRNA) specifically targeting AR (GenePharma, Shanghai, China) and ER α (GenePharma) were prepared in DMEM (Hyclone) for 5 minutes and Lipofectamine-2000 (Lipo-2000, Invitrogen, Milan, Italy) were prepared in DMEM (Hyclone) also for 5 minutes, then mixed for 20 minutes before transferring to culture mixture. The preparation of negative control siRNA (NC-siRNA, GenePharma), which is not homologous with AR and ER- α genes, is the same as described previously. We also set up the Lipo-2000 transfection group to rule out its influence. After 5 h of incubation, the medium was removed, then replaced with fresh serum-containing medium and continue to culture. After another 48 h, cells were harvested for indicated detection. The siRNA sequences were: 5'-GCACCUCUCUCAAGAGUUUTT-3' (AR sense) and 5'-AAACUCUUGAGAGAGGUGCTT-3' (AR antisense); 5'-GGGCUCUACUUCAUCGCAUTT-3' (ER α sense) and 5'-AUGCGAUGAAGUAGAGCCCTT-3' (ER α antisense); 5'-UUCUCCGAACGUGUCACGUTT-3' (NC sense) and 5'-ACGUGACACGUUCGGAGAATT-3' (NC antisense).

5×10^5 cells were seeded in 10 cm cell culture dish (Corning). Testosterone (T) and estradiol (E $_2$) were obtained from Solarbio (Solarbio, Beijing, China). Due to their poor solubility, stock solutions of 10×10^{-3} M T and E $_2$ were freshly prepared in methanol. The final concentration of methanol in the medium did not exceed 1% (v/v) and this was found to have no effect on cell proliferation (Andreescu et al., 2005). Cells were prepared as before, after cells have attached overnight and cells adhesion rate were about 50%, add 0, 10, 50 and 100 μ M sex hormones in A549 lung cancer cells, after 2 days of incubation, cells were harvested for more research.

Protein extraction and western blotting

Total protein from each experiment A549 cells were washed twice with ice-cold PBS and isolated on ice in lysis buffer (150 μ l RIPA, 1mM PMSF, Solarbio) with phosphatase inhibitor cocktail (Solarbio)). Insoluble material was cleared by centrifugation at 12,000g for 5minutes. Protein concentration was estimated by using BCA protein assay kit (Solarbio).

Samples containing equal amounts of protein were subjected to SDS-PAGE using 10% polyacrylamide gel. The proteins were then transferred to PVDF membranes (Millipore, Bedford, MA, USA) electrophoretically at 100 V for 90 min at 4°C. The membrane was washed and blocked for 1 h in blocking buffer [5% skimmed milk in 50mM TBS with 0.2% Tween-20 (TBST)]. After washing with TBST, the membrane was incubated with AR (1:2000, D6F11) #4257 (Cell Signaling, Danvers, MA, USA), ER α (1:1000, D6R2W) #13258 (Cell Signaling), p-EGFR (1:4000, D7A5) #3777 (Cell Signaling), PI3k p85 (1:2000, 19H8) #4257 (Cell Signaling), AKT (1:4000, D9E) #4060 (Cell Signaling), and GAPDH antibody (1:4000, D16H11) #5174 (Cell Signaling) overnight at 4°C. This was followed by washing the membrane with TBST for three times and incubation with secondary antibodies for 2 h at room temperature. The

membrane was then washed three times with TBST and detected by ECL (Thermo, Meridian Rd., Rockford, USA) and exposed with Protein-simple automated protein analyzer (Protein-simple, USA).

RNA extraction and Real-time PCR

Total RNA was extracted from cells using TRIzol reagent (Ambion, Meridian Rd., Rockford, USA), and RNA concentration and purity were detected by a microplate reader (Eppendorf, Germany). A Prime Script™ RT reagent kit with gDNA Eraser (Takara, Japan) was used for reverse transcription; specifically, genomic DNA was removed at 42°C for 2 minutes, and the reverse transcription reaction was performed at 37°C for 15 minutes and 85°C for 5 seconds. Using cDNA as the template, Real-time PCR was performed with SYBR® Premix Ex Taq™ (Takara). GAPDH expression levels were used as the internal reference. The relative mRNA expression levels were quantitated by the $2^{-\Delta\Delta C_t}$ method. The primer sequences were: AR 5'-TGTACACGTGGTCAAGTGGGCCA-3' (forward) and 5'-GGGGCGAAGTAGAGCATCCTGGA-3' (reverse), ERα 5'-AGATGGTCAGTGCCTTGTTG-3' (forward) and 5'-CACTGAAGGGTCTGGTAGGA-3' (reverse), EGFR 5'-GAGAAAGAATACCATGCAGAAGG-3' (forward) and 5'-TCTGGTGGGTATAGATTCTGTGT-3' (reverse), PI3k 5'-CTGCAGTTCAACAGCCACAC-3' (forward) and 5'-ACAGGTCAATGGCTGCATCA-3' (reverse), AKT 5'-CTCTTTCCAGACCCACGAC-3' (forward) and 5'-GGACAGGTGGAAGAACAGC-3' (reverse).

Cell proliferation assay

Cells were seeded into 96-well plates (Corning) at 5×10^3 cells per well. The operation of gene silencing and sex hormones intervention were the same as described previously. After 48 h of continuous culture, cell proliferation in response to gene silencing and sex hormones intervention was determined using Cell Counting Kit-8 (Dojindo, Japan) according to the manufacturer's protocol.

Cell apoptosis assay

Cell apoptosis was detected by an Annexin V-FITC/PI Apoptosis Detection kit (KeyGEN, Jiangsu, China). Cells were seeded into 6-well plates (Corning) at the density of 2×10^5 cells per well. After incubation for 24 h at 37°C, the operation of gene silencing and sex hormones intervention were the same as described previously. After another 48 h, cells were collected and washed twice with ice-cold PBS and then resuspended, and aliquots of 1×10^5 cells were transferred into new 5 mL culture tubes in 500 µL of binding buffer. Then, 5 µL of Annexin V-FITC and 5 µL of propidium iodide were added to the resuspended cells. After incubation in the dark for 10 min at room temperature. Flow cytometry (Becton Dickinson, USA) was used to assess the apoptotic cells.

Cell migration assay

2×10^5 cells were seeded in 6-well plates (Corning) and the operation of gene silencing and sex hormones intervention were the same as described previously. After cells were completely covered with the plates, in a sterile environment, removed the lid from the 6-well plates, laid a sterilized plastic ruler across the top of the plates. Without removing the media, used a sterile 200 µl pipet tip guided by the sterilized plastic

ruler to make a scratch across the plates approximately 50 mm long. Then washed the plates twice with ice-cold PBS, and then photographed at 0,12,24,36 h respectively.

Cell invasion assay

Cell invasion assays were performed with the use of 8 µm pore Transwell inserts (Corning). The upper chambers of the Transwells were pre-coated with diluted Matrigel (BD Biosciences, Sparks, MD). A total of 10,000 cells were seeded onto the upper chamber in serum-free medium, and medium containing 10% serum was added to the lower chamber as a chemoattractant. After 24 h incubation, the upper surface of the insert was wiped with a cotton swab and cells that migrated to the lower surface were fixed by anhydrous methanol and stained with crystal violet. And then photographed with a 100x microscope and analyzed.

Statistical analysis

The quantitative data were shown as the mean $\pm$ standard deviation. All statistical analyses were performed using SPSS version 24.0, and differences between groups were analyzed by independent sample *t*-test. And $P < 0.05$ was considered significant.

Results

Differences in AR, ER α and EGFR, PI3k, AKT protein expression levels after gene silencing and sex hormones intervention in A549 cells

After Small interfering RNA (siRNA) specifically targeting AR and ER α in A549 cells for 48 h, according to the western blotting analysis, AR and ER α protein expression levels were significantly knocked down (Fig.1 A, B). We then examined the protein expression of EGFR and its downstream PI3K/AKT axis and found that the EGFR, PI3K, AKT protein expression levels were down-regulated (Fig.1 A, B). After testosterone (T) and estradiol (E₂) at concentrations of 0, 10, 50 and 100µM interfered with A549 cells for 48 hours, according to the western blotting analysis, we found that 10µM T and 10µM E₂ interventions could up-regulate AR and ER α expression and increase protein expression on EGFR/PI3K/AKT axis, while 50µM T and 50µM E₂ interventions inhibited the expression of AR and ER α and inhibited the expression of EGFR/PI3K/AKT axis compared to the Control group. However, compared with the 50µM T and 50µM E₂ groups, 100µM concentration of T and E₂ intervention increased the expression of AR and ER α again, and correspondingly increased the expression of EGFR/PI3K/AKT axis (Fig.1 C, D).

Difference in AR, ER α and EGFR, PI3k, AKT mRNA expression levels after gene silencing and sex hormones intervention in A549 cells

Total RNA was extracted from cells, and the relative AR, ER α and EGFR, PI3k, AKT mRNA expression levels were shown in Fig.2. After gene silencing in A549 cells for 48 h, compared with the control group, Lipo group and NC-siRNA group respectively, AR and ER α mRNA expression levels in the AR-siRNA group

and ER α -siRNA group were significantly knocked down [AR: (0.96 $\pm$ 0.03),(0.99 $\pm$ 0.02),(1.02 $\pm$ 0.03) vs (0.08 $\pm$ 0.01) and ER α : (1.07 $\pm$ 0.06),(1.01 $\pm$ 0.04),(1.05 $\pm$ 0.07) vs (0.14 $\pm$ 0.02); P <0.001; Fig.2 A,B]. In addition, the expression levels of EGFR, PI3k, and AKT in the AR-siRNA group and the ER α -siRNA group were significantly lower than those in control, Lipo, and NC-siRNA groupS[EGFR: (0.42 $\pm$ 0.04) vs (1.03 $\pm$ 0.02), (0.98 $\pm$ 0.04), (1.01 $\pm$ 0.02); PI3k: (0.27 $\pm$ 0.02) vs (1.01 $\pm$ 0.01), (1.04 $\pm$ 0.04), (1.08 $\pm$ 0.02); AKT: (0.38 $\pm$ 0.06) vs (1.01 $\pm$ 0.02), (1.07 $\pm$ 0.04), (1.04 $\pm$ 0.04); P <0.001, Fig.2 A and EGFR: (0.39 $\pm$ 0.05) vs (1.05 $\pm$ 0.07), (0.98 $\pm$ 0.04), (0.96 $\pm$ 0.06); PI3k: (0.31 $\pm$ 0.02) vs (1.09 $\pm$ 0.09), (1.04 $\pm$ 0.02), (0.96 $\pm$ 0.02); AKT: (0.34 $\pm$ 0.05) vs (1.08 $\pm$ 0.07), (0.99 $\pm$ 0.04), (0.96 $\pm$ 0.03); P <0.001, Fig2 B].

After sex hormones intervened in A549 cells for 48 h, compared with the control group, 10 μ M T and 10 μ M E₂ groups could promote the expression levels of AR and ER α [(1.05 $\pm$ 0.05) vs (1.32 $\pm$ 0.05), P <0.01, Fig.2 C ; (1.06 $\pm$ 0.04) vs (1.18 $\pm$ 0.04), P <0.05, Fig.2 D], while 50 μ M T and 50 μ M E₂ groups inhibited the expression levels of AR and ER α [(1.05 $\pm$ 0.05) vs (0.31 $\pm$ 0.04), P <0.001, Fig.2 C ; (1.06 $\pm$ 0.04) vs (0.34 $\pm$ 0.05), P <0.001, Fig2 D]. When the concentrations of T and E₂ increased to 100 μ M, the expression levels of AR and ER α were enhanced again compared to 50 μ M T and 50 μ M E₂ groups[(0.67 $\pm$ 0.02) vs (0.31 $\pm$ 0.04), P <0.001, Fig.2 C; (0.87 $\pm$ 0.03) vs (0.34 $\pm$ 0.05), P <0.001, Fig.2 D]. Changes in expression levels of EGFR, PI3k, and AKT are consistent with changes in AR and ER α expression levels (Fig.2 C, D).

Changes of cell proliferation and apoptosis after gene silencing and sex hormones intervention in A549 cells

After knocked down of AR and ER α expression, proliferation of A549 cells was inhibited compared with control, Lipo, and NC groups[(0.99 $\pm$ 0.05) vs (1.55 $\pm$ 0.01), (1.52 $\pm$ 0.02), (1.51 $\pm$ 0.02), P <0.001; (1.05 $\pm$ 0.03) vs (1.53 $\pm$ 0.02), (1.53 $\pm$ 0.01), (1.51 $\pm$ 0.05), P <0.001; Fig.3 A]. After sex hormones intervened in A549 cells for 48 h, compared with the control group, 10 μ M T and 10 μ M E₂ groups promoted cell proliferation[(1.66 $\pm$ 0.03) vs (1.36 $\pm$ 0.01), P <0.001; (1.63 $\pm$ 0.04) vs (1.44 $\pm$ 0.03), P <0.01; Fig.3 B], while 50 μ M T and 50 μ M E₂ groups inhibited cell proliferation[(0.67 $\pm$ 0.04) vs (1.36 $\pm$ 0.01), P <0.001; (0.70 $\pm$ 0.02) vs (1.44 $\pm$ 0.03), P <0.01; Fig.3 B]. When the concentrations of T and E₂ increased to 100 μ M, the proliferation of A549 cells was enhanced compared to 50 μ M T and 50 μ M E₂ groups[(1.01 $\pm$ 0.04) vs (0.67 $\pm$ 0.04), P <0.01; (1.06 $\pm$ 0.04) vs (0.70 $\pm$ 0.02), P <0.001; Fig.3 B].

Compared with Control group, Lipo group and NC group, knocked down of AR and ER α expression increased the apoptosis of A549 cells[(42.11 $\pm$ 1.26) vs (5.52 $\pm$ 0.22),(5.96 $\pm$ 0.65),(6.06 $\pm$ 0.54), P <0.001; (39.92 $\pm$ 0.62) vs (5.52 $\pm$ 0.22),(5.96 $\pm$ 0.65),(6.06 $\pm$ 0.54), P <0.001; Fig.3 C]. In the sex hormone intervention experiment, 10 μ M T and 10 μ M E₂ intervention groups showed decreased apoptosis of A549 cells compared to the control group[(1.58 $\pm$ 0.30) vs (5.52 $\pm$ 0.22), P <0.01; (3.27 $\pm$ 0.24) vs (5.52 $\pm$ 0.22), P <0.05; Fig.3 C]. While 50 μ M T and 50 μ M E₂ intervention groups promoted apoptosis of A549 cells compared with the control group[(36.41 $\pm$ 1.79) vs (5.52 $\pm$ 0.22), P <0.001; (37.46 $\pm$ 1.04) vs (5.52 $\pm$ 0.22), P <0.001; Fig.3 C]. When the concentrations of T and E₂ increased to 100 μ M, the apoptosis of A549 cells

also showed decreased compared to 50 μ M T and 50 μ M E₂ groups (8.79 $\pm$ 0.61) vs (36.41 $\pm$ 1.79) , $P < 0.001$; (9.46 $\pm$ 0.5) vs (37.46 $\pm$ 1.04), $P < 0.001$; Fig.3 C].

Changes of cell migration and invasion after gene silencing and sex hormone intervention in A549 cells

Knocked down of AR and ER α expression result in the poor migration and invasion compared to control, Lipo, and NC groups (Fig.4 A, B; $P < 0.001$). In the sex hormone intervention experiments, compared with the control group, 10 μ M T and 10 μ M E₂ intervention might promote cell migration and invasion, while 50 μ M T and 50 μ M E₂ intervention inhibited cell migration and invasion (Fig.4 A,B; (Fig.4 A, B; $P < 0.05$, $P < 0.001$). When the concentrations of T and E₂ increased to 100 μ M, the migration and invasion of the cells were enhanced compared with the concentration of 50 μ M (Fig.4 A, B; $P < 0.001$).

Discussion

There is no doubt that lung cancer is still the number one cause of cancer deaths of the world today, and its morbidity and mortality are increasing every year (Siegel, Miller, 2017). NSCLC accounts for nearly 85% to 90% of lung cancer cases (Wakelee et al. , 2014), and EGFR is expressed in up to 80%-90% of NSCLC (Laskin and Sandler, 2004). Sex hormones may signal through their receptors, and their receptors are involved in a variety of malignancies, including hormone-dependent tumors such as breast cancer (Harbeck and Gnant, 2017) and prostate cancer (Latil et al. , 2001). Endocrine therapy is the main treatment for adjuvant treatment of patients with hormone-sensitive breast cancer and prostate cancer, such as ovarian castration in breast cancer and androgen deprivation therapy in prostate cancer (Rachner et al. , 2018, Roche et al. , 2011). Lung cancer has long been considered a non-hormone-dependent tumor. However, experimental and population-based evidence has been steadily accumulating that steroid hormones are fundamentally involved in the biology of the lung (Schwartz et al. , 2015, Siegfried, 2014), which provided the possibility of hormones-treated lung cancer and their receptors-targeted intervention for lung cancer therapy. Thus, we designed this experiment to observe the effects of sex hormones and their receptors on non-small cell lung cancer, and selected the EGFR/PI3K/AKT signaling pathway closely related to the development of lung cancer as the research object.

In the gene silencing experiment, the Lipo group and the NC group were set to exclude the influence of Lipo-2000 and NC-siRNA intervention on the experimental results. Western blotting and qPCR confirmed that AR and ER α were significantly knocked down in A549 cells (AR: (0.96 $\pm$ 0.03), (0.99 $\pm$ 0.02), (1.02 $\pm$ 0.03) vs (0.08 $\pm$ 0.01) and ER α : (1.07 $\pm$ 0.06), (1.01 $\pm$ 0.04), (1.05 $\pm$ 0.07) vs (0.14 $\pm$ 0.02); $P < 0.001$; Fig.1 A,B; Fig.2 A,B), we then detected the protein and mRNA expression levels of EGFR/PI3K/AKT axis closely related to lung cancer, and found that EGFR/PI3K/AKT axis was inhibited (Fig.1 A,B; Fig.2 A,B; $P < 0.001$). Cell proliferation and apoptosis assays showed that A549 cells decreased activity and increased apoptosis after AR and ER α were knocked down (Fig.3 A,C; $P < 0.001$). Cell migration and invasion assays also showed reduced migration and invasion of A549 cells after knockdown of AR and ER α (Fig.4 A, B). Garon EB¹⁷ reported anti-estrogen fulvestrant adds to effects of EGFR inhibitors, and sensitivity to fulvestrant correlates with greater baseline ER α gene expression. Some reports indicated that ER β

expression in lung cancer as a negative prognosticator in women, and patients expressing high levels of AR have an unfavorable prognostic outcome compared with low AR expressing patients(Mah et al. , 2011, Skjefstad et al. , 2016, Stabile et al. , 2011). Thus, we speculate that AR and ER α promote the development of NSCLC through EGFR/PI3K/AKT axis, and they could be treatment targets in NSCLC, as it has been successfully used in breast cancer and prostate cancer(Lancet, 1998, Rachner, Coleman, 2018, Roche, Niu, 2011).

In the sex hormone intervention experiment, sex hormone intervention with different concentration gradients was set to observe the effects of different concentrations of sex hormones on lung cancer. By Western blotting and qPCR assays, we found that 10 μ M T and 10 μ M E₂ promoted the expression of AR and ER α (1.05 $\pm$ 0.05) vs (1.32 $\pm$ 0.05), P <0.01, Fig.2 C ; (1.06 $\pm$ 0.04) vs (1.18 $\pm$ 0.04), P <0.05, Fig.2 D, and 50 μ M T and 50 μ M E₂ decreased the expression of AR and ER α (1.05 $\pm$ 0.05) vs (0.31 $\pm$ 0.04), P <0.001, Fig.2 C ; (1.06 $\pm$ 0.04) vs (0.34 $\pm$ 0.05), P <0.001, Fig2 D, when the concentration of T and E₂ increased to 100 μ M, the expression of AR and ER α were promoted again(0.67 $\pm$ 0.02) vs (0.31 $\pm$ 0.04), P <0.001, Fig.2 C; (0.87 $\pm$ 0.03) vs (0.34 $\pm$ 0.05), P <0.001, Fig.2 D. Western blotting and qPCR results of the EGFR/PI3K/AKT axis showed consistent with AR and ER α changes (Fig.1 C, D; Fig.2 C, D; P <0.001). Cell proliferation and apoptosis assays showed that 10 μ M T and 10 μ M E₂ promoted cell growth and reduced apoptosis (Fig.3 B, C; P <0.05, P <0.01, P <0.001), 50 μ M T and 50 μ M E₂ inhibited cell growth and promoted apoptosis (Fig.3 B, C; P <0.001), when the concentrations of T and E₂ were increased to 100 μ M, the activity of the cells was enhanced and apoptosis was decreased (Fig.3 B, C; P <0.001). Cell migration and invasion assays showed that 10 μ M T and 10 μ M E₂ promoted cells migration and invasion, and 50 μ M T and 50 μ M E₂ inhibited cells migration and invasion, when T and E₂ concentrations were increased to 100 μ M, cells migration and invasion were promoted again(Fig.4 A,B). A recent study reported higher T predict increased incidence of lung cancer over the next decade in older men(Chan et al. , 2017). Marsaud V reported that the effect of E₂ on ER α expression in lung cancer was also related to intervention time(Marsaud et al. , 2003). Our animal experiments have shown that the internal environment of sex hormone imbalance promotes the development of mouse lung cancer xenografts¹¹, and Clinical studies also support the conclusion that there is an imbalance in sex hormones and their receptors in patients with lung cancer(Xu et al. , 2016, Zhou et al. , 2002). Combined with our experimental data, sex hormones may have a positive effect in the treatment of lung cancer, but the dose and time of administration of sex hormones still need to be explored, which is also the requirement to maintain environmental balance and stability in the body.

Although platinum-based chemotherapy has been clearly established in the treatment of lung cancer, there is still debate about the choice of chemotherapy regimen(Wakelee, Kelly, 2014). And in recent years, EGFR-TKIs have played an active role in the treatment of advanced lung cancer, but most patients will develop resistance within one year of taking the drug(Jackman, Pao, 2010). All of these indicate that it is necessary to explore treatments for lung cancer other than chemotherapy and to find new therapeutic targets for lung cancer. New data from randomized, prospective clinical trials show a significant impact of hormone replacement therapy (HRT) in lung cancer(Chlebowski et al. , 2009). Furthermore, a

prospective cohort study confirmed an increased, dose-dependent lung cancer risk among women who received HRT(Slatore et al. , 2010). Both Mah V(Mah, Marquez, 2011) and Marquez-Garban DC(Marquezgarban et al. , 2011) reported that estrogen and ERs can activate the Src family of nonreceptor tyrosine kinases, MAPKs (mitogen-activated protein kinases) and PI3K, which affected cancer cell growth, proliferation and apoptosis. A study from Lee SH(Lee et al. , 2015) showed an interaction between androgen and the PI3K/AKT pathway in prostate cancer. And the PI3K/AKT signalling pathway mediates the process of EMT and has attracted widespread attention as a potential target for the prevention and treatment of metastatic tumours(Bakin AV, 2000, Xu Q, 2014). And the project leader has achieved good clinical efficacy by using traditional Chinese medicine Bushenshugan formula to treat lung cancer by enhancing the patient's gonadal function(Fan Z, 2018, Xuehong Miao, 2015, Yuan Liu, 2011). Thus, it is worthwhile to study what signaling pathways of sex hormones and their receptors to promote the development of lung cancer, and future work should also evaluate sex hormones and their receptors antitumor activity in more clinic trials.

Declarations

Acknowledgements

We thank all the staff of Professor Zang Mingxi's laboratory in Zhengzhou University for their great help in the completion of this research, including the provision of all experimental equipment, some experimental supplies and technical guidance.

Funding: Supported by grants from the National Natural Science Foundation of China(81473497, U1804181), the Social Research Project of Henan Provincial Science and Technology Department (132102310106, 2018020408, 182102311187, 2018020396), and the Natural Science Foundation of Henan Provincial Department of Education (12A320077).

Ethics approval: Not applicable.

Consent to participate: Not applicable.

Consent for publication: Its publication has been approved by all co-authors, if any, as well as by the responsible authorities—tacitly or explicitly—at the institute where the work has been carried out.

Availability of data and material: We guarantee that all experimental data and material provided are available.

Code availability: All statistical analyses were performed using SPSS version 24.0.

Disclosure Statement: None.

Authors' contributions: K. Zhu, X. Li and Z. Gu contributed equally to this work.

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Figures

Figure 1

Relative protein expression of AR, ERα, EGFR, PI3K and AKT after gene silencing and sex hormones intervention in different groups. A, B: After knocked down of AR and ERα expression, the expression of EGFR/PI3K/AKT axis decreased. C, D: After sex hormones intervention for 48 h, compared with the control group, 10μM T and 10μM E2 intervention up-regulated the expression of AR, ERα and EGFR/PI3K/AKT axis, and 50μM T and 50μM E2 interventions down-regulated the expression of AR, ERα and EGFR/PI3K/AKT axis; when the concentrations of T and E2 were increased to 100μM, the expression of AR, ERα and EGFR/PI3K/AKT axis were up-regulated again compared to 50μM T and 50μM E2 groups. Control: cells without any intervention; Lipo: cells were transfected only with Lipo-2000; NC: cells were transfected with NC-siRNA; AR: cells were transfected with AR-siRNA; ERα: cells were transfected with ERα-siRNA; 10, 50 and 100μM T and E2: cells were intervened with 10, 50 and 100μM T and E2 respectively.

Figure 2

Relative AR, ERα, EGFR, PI3K and AKT mRNA expression levels after gene silencing and sex hormones intervention in different groups. A, B: After the mRNA expression of AR and ERα were knocked down, the mRNA expression of EGFR/PI3K/AKT axis decreased. C, D: After sex hormones intervention for 48 h, compared with the control group, 10μM T and 10μM E2 intervention up-regulated the mRNA expression levels of AR, ERα and EGFR/PI3K/AKT axis, and 50μM T and 50μM E2 interventions down-regulated the mRNA expression levels of AR, ERα and EGFR/PI3K/AKT axis, when the concentrations of T and E2 were increased to 100μM, the mRNA expression levels of AR, ERα and EGFR/PI3K/AKT axis were up-regulated again compared to 50μM T and 50μM E2 groups. Control: cells without any intervention; Lipo: cells were transfected only with Lipo-2000; NC: cells were transfected with NC-siRNA; AR: cells were transfected with AR-siRNA; ERα: cells were transfected with ERα-siRNA; 10, 50 and 100μM T and E2: cells were intervened with 10, 50 and 100μM T and E2 respectively. *P<0.05, **P<0.01, ***P<0.001: compared with Control group; ΔΔΔP<0.001: compared with 50μM T and 50μM E2 groups.

Figure 3

Cells proliferation and apoptosis after gene silencing and sex hormones intervention. A: After knocked down of AR and ERα expression, the proliferation of cells was inhibited compared to Control, Lipo and NC groups respectively. B: After sex hormones intervention for 48 h, compared with the control group, 10μM T and 10μM E2 intervention promoted cells proliferation, and 50μM T and 50μM E2 intervention inhibited cells proliferation, when the concentrations of T and E2 were increased to 100μM, the proliferation of

cells were enhanced compared to 50 μ M T and 50 μ M E2 groups. C: Knocked down of AR and ER α expression promoted cells apoptosis; 10 μ M T and 10 μ M E2 intervention inhibited cells apoptosis and 50 μ M T and 50 μ M E2 intervention promoted cells apoptosis, when the concentrations of T and E2 were increased to 100 μ M, the apoptosis of cells were inhibited again. Control: cells without any intervention; Lipo: cells were transfected only with Lipo-2000; NC: cells were transfected with NC-siRNA; AR: cells were transfected with AR-siRNA; ER α : cells were transfected with ER α -siRNA; 10, 50 and 100 μ M T and E2: cells were intervened with 10, 50 and 100 μ M T and E2 respectively. *P<0.05, **P<0.01, ***P<0.001: compared with Control group; $\Delta\Delta\Delta$ P<0.001: compared with 50 μ M T and 50 μ M E2 groups.

Figure 4

Cells migration and invasion after gene silencing and sex hormone intervention. A, B: Knocked down of AR and ERα expression inhibited cells migration and invasion; 10μM T and 10μM E2 intervention might promote cells migration and invasion; 50μM T and 50μM E2 intervention inhibited cells migration and invasion, when the concentrations of T and E2 were increased to 100μM, the migration and invasion of cells were enhanced again. Control: cells without any intervention; Lipo: cells were transfected only with

Lipo-2000; NC: cells were transfected with NC-siRNA; AR: cells were transfected with AR-siRNA; ER α : cells were transfected with ER α -siRNA; 10, 50 and 100 μ M T and E2: cells were intervened with 10, 50 and 100 μ M T and E2 respectively. *P<0.05, ***P<0.001: compared with Control group; $\Delta\Delta\Delta$ P<0.001: compared with 50 μ M T and 50 μ M E2 groups.