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Original Paper

Reproductive Hormones and Their Receptors May Affect Lung Cancer

Mengmeng Dou[a] Keyan Zhu[a] Zhirui Fan[b] Yuxuan Zhang[a] Xiufang Chen[c] Xueliang Zhou[b] Xianfei Ding[d] Lifeng Li[b] Zhaosen Gu[a] Maofeng Guo[e] Ming Yan[f] Xiaoming Deng[a] Peihong Shen[f] Shuling Wang[e]

[a]Department of Integrated Traditional and Western Medicine, [b]Department of Oncology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, [c]Department of Internal Medicine, Zhengzhou Xinhua Hospital of Traditional Chinese Medicine, [d]Department of General ICU, The First Affiliated Hospital of Zhengzhou University, [e]Department of Pathology, The Basic Medical Institute of Zhengzhou University, [f]Henan Cancer Hospital (Affiliated Tumor Hospital of Zhengzhou University), Zhengzhou, China

Key Words

Ovariectomized mice • Oestradiol • Testosterone • Lung cancer

Abstract

Background/Aims: In contrast to men, women have experienced a rapid increase in lung cancer mortality. Numerous studies have found that the sex differences in lung cancer are due to reproductive hormones. Experiments in female mice with and without ovariectomy were performed to explore the possible mechanism by which sex hormones (and their receptors) influence lung cancer. ***Methods:*** Twenty-four female C57BL/6 mice aged 56-62 days were randomly divided into the ovariectomized group and the control group. In the ovariectomized group, the bilateral ovaries were removed via the dorsal approach, while the control group underwent a sham operation with bilateral ovarian fat resection at the same sites. After 3 weeks of recovery, Lewis lung cancer cells were transplanted into these mice by subcutaneous inoculation of a tumour cell suspension to establish the ovariectomized lung cancer model. Beginning on the 6th day after subcutaneous inoculation, mouse weight and transplanted tumour volume were measured every 3 days. After 3 weeks, all the mice were killed by cervical dislocation, and we measured the tumour weight. Mouse serum and tumour tissues were removed. Then, the serum levels of E_2 (oestradiol) and T (testosterone) were detected by ELISA; the protein expression levels of AR (androgen receptor), ERα (oestrogen receptor α) and ERβ (oestrogen receptor β) were detected by Western Blot and IHC (immunohistochemistry); and the mRNA expression levels of AR, ERα and ERβ were detected by qRT-PCR (quantitative real-time polymerase chain reaction) in the ovariectomized and control groups. ***Results:*** Compared with the control group, both mouse weight and transplanted tumour volume increased rapidly in the ovariectomized group, and the transplanted tumour weight was significantly heavier in the ovariectomized group (1.83±0.40 and 3.13±0.43, $P<0.05$). E_2 and

M. Dou and K. Zhu contributed equally to this article.

Shuling Wang and Peihong Shen

Department of Pathology, The Basic Medical Institute of Zhengzhou University, Zhengzhou and Henan Cancer Hospital (Affiliated Tumor Hospital of Zhengzhou University), Zhengzhou 450000, (China); E-Mail wsl5869@163.com/ 13838122660@163.com

T serum levels decreased exponentially in the ovariectomized group, while the E_2/T ratio increased compared with the control group (E_2: 55.88±11.45 and 78.21±9.37; T: 0.82±0.14 and 1.46±0.16; ratio: 69.62±14.43±29.81 and 52.22±5.42; all P<0.05). The Western blot and IHC results indicated that AR, ERα and ERβ protein expression levels were obviously higher in transplanted tumour and lung tissues from the ovariectomized group, with particular increases in ERβ in transplanted tumour tissue and in ERα in lung tissue. The PCR results also showed markedly higher mRNA expression levels of AR, ERα and ERβ in the ovariectomized group, and in particular, ERβ in transplanted tumour tissue and ERα in lung tissue were significantly increased in the ovariectomized group. ***Conclusion:*** Ovariectomy decreased E_2 and T serum levels and increased the E_2/T ratio in mice, and this imbalance in the internal environment promoted the growth of transplanted tumours. Sex hormone disorder not only promoted transplanted tumour growth but also significantly reduced the protein and mRNA expression levels of sex hormone receptors. The metabolism of E_2 and T may affect the growth, proliferation and metabolism of lung cancer cells, and the mechanism by which sex hormones and their receptors influence lung cancer is worthy of further research.

Introduction

Lung cancer remains the leading cause of cancer-related death globally, and outcomes for patients diagnosed with advanced non-small cell lung cancer (NSCLC) are poor despite recent advances in treatment[1]. In 2016, there were 222,500 new cancer cases and 155,870 cancer deaths related to the lung and bronchus system in the United States. In contrast to the steady increase in survival for most cancers, that for lung cancer has been slow, with a current 5-year relative survival of 18%[2]. There are a number of factors associated with the poor prognosis, such as drug resistance[3] and gender and age differences[4]. For a majority of patients, the disease condition is initially chemosensitive but then relapses with acquired chemoresistance[3]. The low survival rate of patients suffering from lung cancer is caused mainly by delayed detection and diagnosis, resulting in the identification of disease at an advanced stage and leaving the patient with limited treatment options[5].

Although some molecular alterations[6, 7] are shared among various histologic subtypes of cancer, the majority of genomic alterations remain distinct[8]. Accumulating evidence[9-13] indicates that the presence and progression of lung cancer is affected by gender-dependent factors, especially oestrogens. Oestrogens affect cellular processes by binding to oestrogen receptors (ERs) and signalling in two different ways: genomic and non-genomic[14]. In genomic signalling, oestrogens bind to ERs, mainly ERα and ERβ, which causes ER dimerization, translocation to the nucleus and binding to specific DNA regions known as oestrogen response elements (EREs)[15, 16].

Some modern medical studies[14, 17, 18] have shown that sex hormones play an important role in the development of lung tissue, the formation of lung inflammation and perhaps the molecular biology of lung cancer. Many researchers found that the outcomes of patients with lung cancer were related to the use of anti-oestrogens[19]. Fan S et al also reported that ERβ activation in lung cancer cells promotes metastasis by increasing the expression of MMP-2, which is associated with cellular invasiveness[20]. Thus, sex hormones and their corresponding receptors may be potential molecular biomarkers that predict the occurrence, development, treatment, and prognosis of lung cancer.

Herein, we established a sex hormone deficiency model in C57BL/6 female mice by ovariectomy. After subcutaneous inoculation of a tumour cell suspension in all mice, body weight, transplanted tumour volume and weight, serum levels of oestradiol (E_2) and testosterone (T) and ER expression were determined to identify potential differences upon ovariectomy. In addition, the specific mechanisms by which sex hormones and their receptors influence the development of lung cancer were explored.

Materials and Methods

Animals and reagents

Twenty-four C57BL/6 female mice aged 56-62 days and weighing 20±2 g were purchased from Beijing Weitong Lihua Experimental Animal Technology Company (certificate number: SCXK 2012-0001). These 24 mice were randomly divided into the ovariectomized group and the control group. Pentobarbital sodium (no. 57330, 5 g/bottle) was from Merck (Germany). Benzylpenicillin sodium (1600 thousand units per bottle) was from Huabei Pharmaceutical Company. Instant PBS powder (LB12231), citrate buffer powder (LB00085), neutral balsam (20160308), Haematoxylin Stain reagent (SLBK4907V) and other reagents were purchased from Zhengzhou Saiboer Biotechnology Company. Enzyme-linked immunosorbent assay (ELISA) test kits for E_2 and T (KGE014 and KGE010) were purchased from R&D Systems (USA). Polyclonal antibodies against AR, ERα, and ERβ (ab133273, ab75635, and ab3577) were from Abcam (USA).

Generation of the ovariectomized mouse model

First, all female mice were weighed and then injected with 1% pentobarbital sodium (0.07 ml/10 g) to induce anaesthesia. After disinfecting the mouse back, we made a small approximately 1.5-cm incision on the left or right flank 0.5 cm from the intersection of the lines extending from the hind legs and the midline of the back. The skin and muscular layers were exposed sequentially, and when we pulled out the fat pad on the left or right side with forceps, the ovary and fallopian tube were exposed. The fat pad was returned to the abdominal cavity after ligating fallopian tube and removing the ovary. The two sides were treated in the same way. Lastly, we sutured the muscle layer and the epidermis continuously with absorbable sutures. The control group was treated similarly as the case group without removing the ovary or ligating the fallopian tube, namely, the fat pad was held with blunt forceps, a section of the adipose tissue was cut, and the layers were sutured continuously with absorbable sutures. To prevent infection, all mice were treated with penicillin (60,000 units per mouse) for seven days after surgery.

Inoculating mice with Lewis lung cancer transplanted tumours

Tumour-bearing mice with Lewis lung cancer were decapitated and then soaked in 75% medical-grade alcohol. After 5 minutes, we placed the mouse on a clean bench, removed the tumour, eliminated necrotic tissue and normal tissue, and selected tumour sections in good condition. The selected tumour sections were cut into small pieces with scissors and then ground with a glass homogenizer in normal saline at a 1:3 ratio of tumour (g) to normal saline (ml). Then, the mixture was filtered through a 200 mesh screen to prepare a single cell suspension, and the percentage of viable cells was greater than 95% based on 0.2% trypan blue staining. Then, we adjusted the single cell suspension to a concentration of 1×10^7 cells per ml. After 3 weeks of recovery from surgery, each mouse in the ovariectomized and control groups was inoculated subcutaneously with 0.3 ml of the single cell suspension under the right axillary fold. On the 5^{th} day after inoculation, the subcutaneous node was palpable. In addition, beginning on the 6^{th} day, we measured the longest (a) and shortest diameters (b) of the transplanted tumours and weighed all mice every 3 days. We calculated the tumour volume using the following formula: $V\ (mm^3)=1/2\bullet ab^2$. The growth curve was generated based on the arithmetic mean of the weight or volume in each group. After 21 days, all the mice were weighed, blood was drawn, and the mice were killed by decapitation.

Enzyme linked immunosorbent assay (ELISA)

On the 21^{st} day after inoculation, all the mice in the ovariectomized and control groups were weighed. After rapidly removing the eyeball with sterile ophthalmic forceps, blood samples were collected into sterile tubes. Then, all the samples were allowed to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 ×g. The upper yellow-clear serum was removed, labelled and stored at ≤ -20°C. Based on the operational guidelines for ELISA kits (KGE014 and KGE010; R&D Systems, USA), we reconstituted the E_2 and T standards and generated the standard curves. According to the optical density (OD) of each well at 450 nm and the standard curve, the serum levels of E_2 and T could be calculated.

RNA extraction and qRT-PCR

Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, USA), and RNA concentration and purity were detected by a UV spectrophotometer. A cDNA kit (Thermo, USA) 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, PCR was performed with FastStart Universal SYBR Green Master Mix (Roche, USA). GAPDH expression levels were used as the internal reference. The following primer sequences were used: GAPDH 5'-GTTCCTACCCCCAATGTGTCC-3' (forward) and 5'-TAGCCCAAGATGCCCTTCAGT-3' (reverse); ERα 5'-CAGCAGTAACGAGAAAGGAAACA-3' (forward) and 5'-TCGGCGGTCTTTCCGTATG-3' (reverse); ERβ 5'-TGATGATGTCCCTCACGAAGC-3' (forward) and 5'-AGAACGAGGTCTGGAGCAAAG-3' (reverse); and AR 5'-AAATGGGACCTTGGATGGAGA-3' (forward) and 5'-TTTCCCTTCAGCGGCTCTTT-3' (reverse). The relative mRNA expression levels were quantitated by the $2^{-\Delta\Delta Ct}$ method. Each experiment was repeated independently three times.

Western blot analysis

When each mouse was killed by decapitation, the transplanted tumour and lung tissue were dissected and stored in liquid nitrogen. To extract total protein, we first ground the stored tissue with liquid nitrogen and then added a mixture of 10 μl of phenylmethanesulfonyl fluoride (PMSF) and 990 μl of RIPA, with continued grinding for 30 minutes. The obtained mixture was centrifuged for 15 minutes at 12000 ×g at 4°C, and the supernatants containing total protein were harvested. The bicinchoninic acid (BCA) method was used to detect the protein concentration. Laemmli sample buffer was added, and the samples were boiled. The proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with subsequent transfer onto a polyvinylidene difluoride (PVDF) membrane. The membranes were incubated overnight at 4°C with antibodies against AR (1:1000, ab133273), ERα (1:1000, ab75635), ERβ (1:500, ab3577) or β-actin (1:1000, GB13001-1). After subsequent incubation with secondary antibodies, the membranes were subjected to chemiluminescence detection and exposed on X-ray films. Band density was analysed by ImageJ2x software. Each experiment was repeated independently three times.

Immunohistochemistry

All the transplanted tumour and lung tissues from mice in the case and control groups were removed on ice after the mice were killed by decapitation. Then, 4-μm formalin-fixed, paraffin-embedded sections were created from these tissues. In brief, endogenous peroxidase was quenched with 3% hydrogen peroxide, and sections were incubated with normal goat serum for 30 minutes to block nonspecific antibody binding sites. Then, the sections were incubated with antibodies against AR, ERα or ERβ (ab133273, ab75635, and ab3577; Abcam, USA) overnight at 4°C and then with a biotin-free HRP-labelled polymer. DAB solution and haematoxylin were used to visualize the positive reactions. Immunohistochemical scoring was based on the staining intensity and the percentage of positively stained cells. The staining intensity was scored as follows: 0, no staining; 1, weakly stained; 2, moderately stained; and 3, strongly stained. The percentage of positively stained cells was scored as follows: 0, 0%; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, > 75%. The final staining score was the product of the staining intensity score and the percent positive score: -, final staining score < 3; +, final staining score = 3; ++, final staining score = 4; and +++, final staining score ≥ 5. We classified positive expression as a final staining score ≥ 4 and negative expression as < 4 in the mouse tumour and lung tissues. Each experiment was repeated independently three times.

Statistical analysis

The data were analysed by SPSS version 17.0. The rates of positive expression of AR, ERα and ERβ in lung and tumour tissues from the two groups were analysed by the χ^2 test. The median mouse body weight, transplanted tumour weight, transplanted tumour volume and E_2 and T serum levels were analysed by the *t* test. The data are presented as the mean ± standard deviation, and $P < 0.05$ was considered significant.

Results

Differences in mouse survival status, body weight, and transplanted tumour volume and weight

Compared with the control mice, the ovariectomized mice were timid, easily frightened and less active, and their hair colour was not as bright. On the 5th day after inoculation of the single cell suspension, a subcutaneous node was palpated. Beginning on the 6th day, we measured the longest (a) and shortest diameters (b) of the transplanted tumour and the mouse body weight every 3 days. We calculated the tumour volume ($V=1/2 \bullet ab^2$, in mm^3)

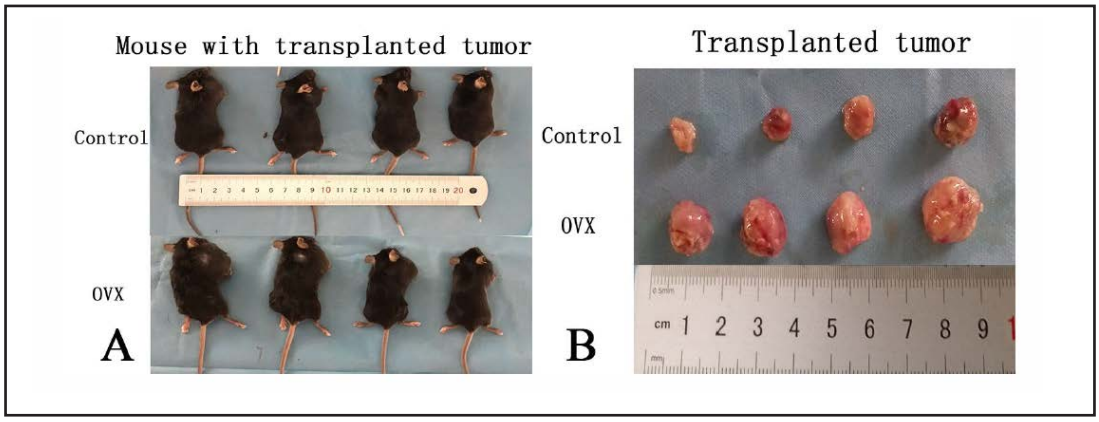

Fig. 1. Comparison of mice with transplanted tumours and the excised tumours. A: Typical pictures of mice with transplanted tumours in the control and ovariectomized groups. B: Typical pictures of excised tumours in the control and ovariectomized groups. Control: mice treated with partial bilateral fat pad removal near the ovary; OVX: ovariectomized, mice treated with bilateral ovary resection.

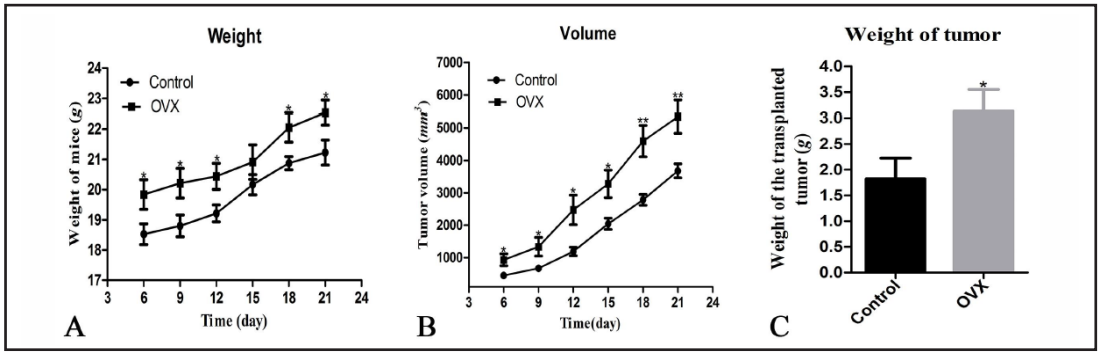

Fig. 2. Curves of mouse body weight and transplanted tumour volume and a comparison of transplanted tumour weight. The growth curves were generated based on the arithmetic mean of the weight or volume in each group. A. Curves of mouse body weight beginning at the 6th day after inoculation with the single cell suspension. B. Curves of the transplanted tumour volume. C. Transplanted tumour weight. Control: mice treated with partial bilateral fat pad removal near the ovary; OVX: ovariectomized, mice treated with bilateral ovary resection; *P<0.05, **P<0.01.

and generated a growth curve. The transplanted tumours were generally larger in the ovariectomized group than in the control group (Fig. 1). The growth curves showed that the transplanted tumours in the ovariectomized group grew faster than those in the control group (P<0.05). In addition, the tumour weight differed significantly between the two groups (1.83±0.40 and 3.13±0.43, P<0.05) (Fig. 2).

Serum levels of oestradiol (E_2) and testosterone (T) in the two groups

Three weeks after inoculation of the single cell suspension, blood samples were rapidly removed via retroorbital bleeding from all the mice in the two groups. The blood samples were allowed to clot for 30 minutes at room temperature and then centrifuged for 15 minutes at 1000 ×g. Based on the operational guidelines for the ELISA kits (KGE014 and KGE010; R&D Systems, USA), the serum levels of E_2 and T were calculated according to the OD and the E_2 and T standard curves. During the ovariectomy operation, 1 mouse died. During the tumour formation process, tumours in 2 mice in the control group and 1 mouse in the ovariectomized group did not grow. Thus, there were 10 available blood samples from each group. The analysis showed that the serum levels of E_2 and T in the ovariectomized group decreased exponentially, whereas the E_2/T ratio increased at certain times (55.88±11.45

Table 1. Serum oestradiol and testosterone levels in the control and OVX groups ($\bar{x}\pm s$). Control: mice treated with partial bilateral fat pad removal near the ovary; OVX: ovariectomized, mice treated with bilateral ovary resection; *P<0.005.

Group	n	E_2 (pg.ml^{-1})	T (ng.ml^{-1})	E_2/T
Control	10	78.21±9.37	1.46±0.16	52.22±5.42
OVX	10	55.88±11.45*	0.82±0.14*	69.62±14.43*

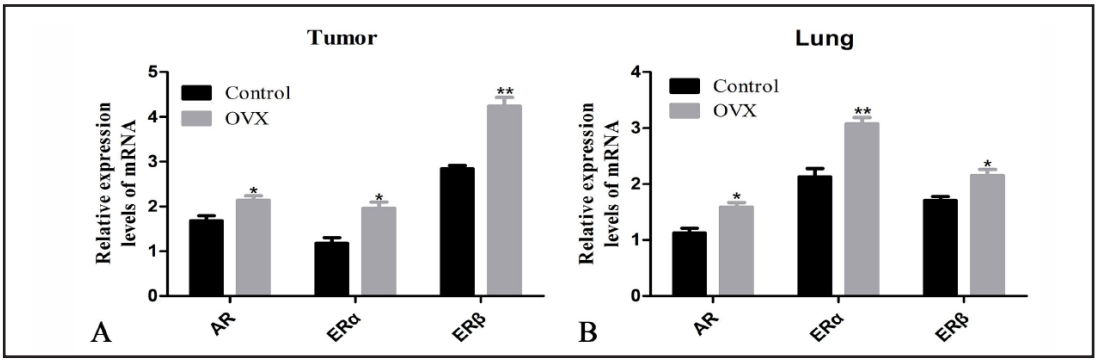

Fig. 3. Relative AR, ERα and ERβ mRNA expression levels in transplanted tumour and lung tissues from the control and OVX groups. Relative mRNA expression levels in transplanted tumour tissues from the two groups. B. Relative mRNA expression levels in lung tissues from the two groups. Control: mice treated with partial bilateral fat pad removal near the ovary; OVX: ovariectomized, mice treated with bilateral ovary resection; *P<0.05, **P<0.01.

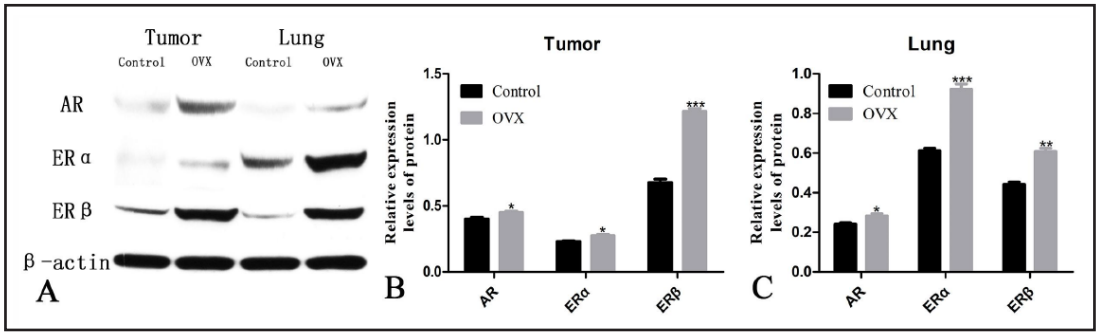

Fig. 4. Relative protein expression levels of AR, ERα and ERβ in transplanted tumour and lung tissues from the two groups. A, B, C. The protein expression levels were higher in the ovariectomized group than in the control group, especially for ERβ in transplanted tumour tissue and ERα in lung tissue. Control: mice treated with partial bilateral fat pad removal near the ovary; OVX: ovariectomized, mice treated with bilateral ovary resection; *P<0.05, **P<0.01, ***P<0.001.

and 78.21±9.37, 0.82±0.14 and 1.46±0.16, 69.62±14.43±29.81 and 52.22±5.42, P<0.05) compared with the control group (Table 1).

Differences in AR, ERα and ERβ mRNA expression levels in transplanted tumour and lung tissues

Total RNA was extracted from tissues, and the relative AR, ERα and ERβ mRNA expression levels in transplanted tumour and lung tissues are shown in Fig. 3. Expression levels in transplanted tumour tissues were higher in the ovariectomized group than in the control group, especially for ERβ mRNA (P<0.01). Furthermore, expression levels were higher in lung tissues from the ovariectomized group than in those from the control group, with a significant increase in ERα mRNA expression levels (P<0.01). The higher mRNA expression levels of AR, ERα and ERβ in the ovariectomized group may be associated with the low serum levels of E_2 and T, which led to the imbalance in the murine microenvironment.

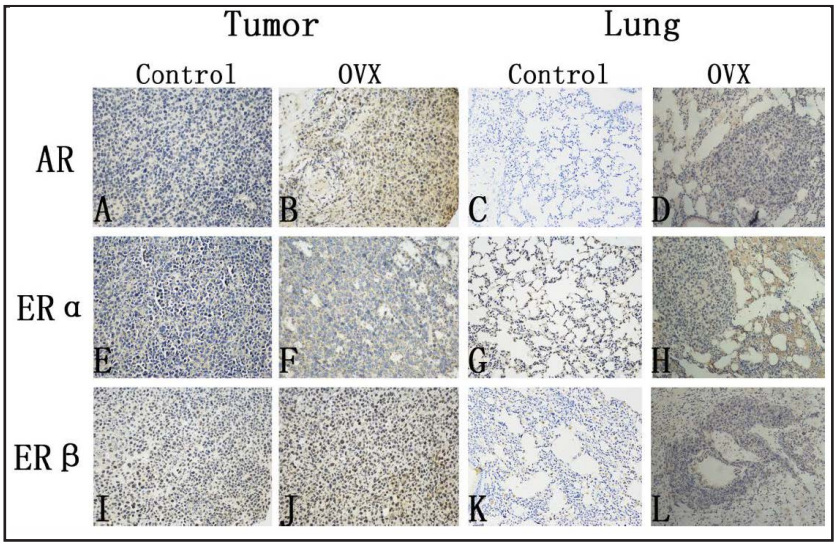

Fig. 5. AR, ERα and ERβ protein expression levels in transplanted tumour and lung tissues from the two groups (IHC, ×200 magnification). A-D. AR protein expression levels. E-H. ERα protein expression levels. I-L. ERβ protein expression levels. The protein expression levels in transplanted tumour and lung tissues were significantly higher in the ovariectomized group than in the control group. In addition, ERβ protein expression in tumour tissue and ERα protein expression in lung tissue were obviously high. Control: mice treated with partial bilateral fat pad removal near the ovary; OVX: ovariectomized, mice treated with bilateral ovary resection.

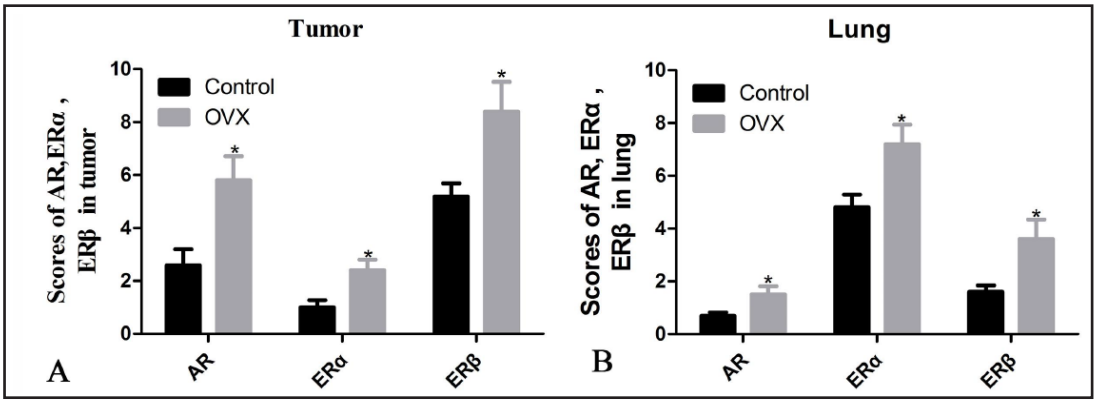

Fig. 6. AR, ERα and ERβ staining score by IHC analysis. The staining score was based on the staining intensity and the percentage of positively stained cells in transplanted tumour tissue (A) and lung tissue (B). Control: mice treated with partial bilateral fat pad removal near the ovary; OVX: ovariectomized, mice treated with bilateral ovary resection; *P<0.05.

Differences in AR, ERα and ERβ protein expression levels in transplanted tumour and lung tissues

According to the Western blot analysis, ERβ protein expression levels were highest in transplanted tumour tissues in both the control and ovariectomized groups (P<0.0001; Fig. 4). In lung tissues, ERα protein expression levels were highest in the ovariectomized group (P<0.0001). The expression levels of AR, ERα and ERβ were clearly higher in the ovariectomized group than in the control group. The IHC results showed that AR, ERα and ERβ staining was more evident in the ovariectomized group than in the control group (Fig. 5). Staining score analysis supported these observations and revealed that the ERβ protein expression score was highest in transplanted tumour tissue from the ovariectomized group (P<0.05; Fig. 6). In addition, in lung tissue, the ERα expression score was the highest (P<0.05). The rates of high expression of AR, ERα and ERβ in lung and tumour tissues from the two groups indicated that tumour ERβ expression and lung ERα expression were significantly different in the ovariectomized group compared with the control group (both P<0.05, Table 2). Therefore, Western blot and IHC results revealed high expression of AR, ERα and ERβ in transplanted tumour tissue and lung tissue from ovariectomized mice.

Table 2. AR, ERα and ERβ expression in transplanted tumour and lung tissues from the control and OVX (ovariectomized) groups.

Tissue type		n	AR expression		*P*	ERα expression		*P*	ERβ expression		*P*
			Positive	Negative		Positive	Negative		Positive	Negative	
Tumour	Control	10	3	7	0.36	4	6	0.37	4	6	0.02
	OVX	10	5	5		6	4		9	1	
Lung	Control	10	3	7	0.64	4	6	0.02	6	4	0.33
	OVX	10	4	6		9	1		8	2	

Discussion

For half a century, the incidence and mortality of lung cancer have been increasing, and lung cancer is the leading cause of cancer-related death in most developed countries[2]. As the number of female smokers continues to increase, the ratio of male to female patients with lung cancer has risen rapidly. Oestrogens can signal through either ERα or ERβ; these receptors are involved in numerous kinds of malignant tumours, including hormone-dependent tumours such as breast cancer[21] and prostate cancer[22]. However, many studies[23-25] have found that oestrogens and ER have significant effects on lung development and physiology, which provided a potential molecular mechanism for the gender differences in lung cancer. Thus, we performed this study to explore the previously unknown regulators of lung cancer.

Bilateral ovariectomy[26] was used to decrease sex hormones in mice in the ovariectomized group, and mice in the control group underwent partial removal of perinephric fat near the bilateral ovaries. Next, all mice were inoculated with a single cell suspension of Lewis lung cancer cells from C57BL/6 mice. Then, we observed significant differences between the control and ovariectomized groups in terms of body weight curves and transplanted tumour volume. In addition, the transplanted tumour weight of mice in the ovariectomized group was heavier than that in the control group (1.83±0.40 and 3.13±0.43, $p<0.05$). The serum analysis showed that the serum levels of E_2 and T in the ovariectomized group decreased exponentially, while the E_2/T ratio was increased at certain time points compared with the control group (E_2: 55.88±11.45 and 78.21±9.37; T: 0.82±0.14 and 1.46±0.16; ratio: 69.62±14.43±29.81 and 52.22±5.42, $p<0.05$). Further analysis of AR, ERα and ERβ mRNA and protein expression levels in tumour and lung tissues revealed higher expression in the ovariectomized group, particularly regarding ERβ in tumour tissue and ERα in lung tissue. Therefore, ovariectomy decreased the serum levels of E_2 and T and increased the E_2/T ratio in mice, and the resulting imbalance in the internal environment promoted the growth of transplanted tumours. ERα and ERβ played key roles in this process.

Clinical studies[27, 28] support the conclusion that there is an imbalance in sex hormones and their receptors in patients with lung cancer. E_2 levels were much higher in patients with lung cancer than in a normal control group, while T levels were lower in patients. In addition, several studies[29-31] have now reported that exposure to hormone replacement therapy (HRT) is associated with negative effects on lung cancer survival. Ganti et al[29] performed a retrospective chart review of women diagnosed with lung cancer and collected data including age, past history of cancer, HRT use and overall survival. They found that overall survival was significantly higher in patients without HRT compared with those who received HRT. HRT may affect the endocrine environment in such a way that promotes lung cancer.

The mechanism of lung tumourigenesis is complex, and lung cancer is mainly caused by various carcinogenic factors that cause gene changes and induce normal cells to become cancerous. However, it is not known how gene polymorphisms affect E_2 metabolism[32]. The body of clinical trials suggests that the patients with lung cancer who respond well to EGFR

TKIs are mainly female, not male[33], which may indicate potential cross-talk in signalling between EGFR and ER in lung cancer[34, 35]. Other studies found that abnormal ER expression is related to mutations in other genes. Both Mah V[16] and Marquez-Garban DC[36] reported that oestrogen and ER can activate the Src family of nonreceptor tyrosine kinases, MAPKs (mitogen-activated protein kinases) and PI3K (phosphatidylinositol-3 kinase), which affected cancer cell growth, proliferation and apoptosis. Thus, it will be worthwhile to study what affects the overexpression of ER-related receptors and subsequently promotes the formation of malignant tumours.

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Disclosure Statement

None.

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