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Original Article

Estrogen receptor β2 inhibits growth of lung transplantation tumors in mice by upregulating P38 mitogen-activated protein kinase

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Abstract: Objective: The purpose of this study was to further observe the function of ERβ2 in lung cancer, examining its relationship with p38MAPK *in vivo*. Methods: A549/H358/LTEP-a2 cells were used to construct nude mice transplanted tumor models by subcutaneous implantation. Moreover, hERβ2, iERβ2, hp38MAPK, hERβ2+SB, iERβ2+hp38MAPK, and empty groups were set. The weights of the mice and volumes of the transplanted tumors were measured each week. Growth curve was also measured. Expression of relevant proteins in the transplanted tumors was detected by IHC and WB. Results: Tumors in the hERβ2 group had slow growth, significantly more than that of empty and iERβ2 groups. The specific inhibitor of p38MAPK could reverse the effects of hERβ2 on tumor growth. Compared with the empty group, weights and transplanted tumor volumes of nude mice in the iERβ2 group had a significant increase. This effect could be reversed by increasing expression of p38MAPK. According to IHC and WB analysis, p38MAPK expression was significantly increased and bcl-2 expression was decreased in the hERβ2 group, compared to the empty group. p38MAPK specific inhibitor increased bcl-2 expression in the hERβ2 group, whereas in the iERβ2 group, the reverse effects were observed. p38MAPK was decreased and bcl-2 protein was significantly increased, which was also disturbed by p38MAPK. Conclusion: ERβ2 high expression may have the function of inhibiting the progression of lung cancer through the upregulation of p38MAPK and inhibition of bcl-2 expression.

Keywords: ERβ2 and p38MAPK, lung transplantation tumor, A549, H358, LTEP-a2, mice

Introduction

Lung cancer has become one of the most life threating diseases worldwide [1, 2]. At present, there are many studies concerning the prevention and treatment of lung cancer, but the overall treatment effects of lung cancer remain unsatisfactory. In recent years, more and more studies [3-5] have found that ER plays an important role in non-small cell lung cancer. Mechanisms regarding this disease are not clear. Some researches [6, 7] have reported that there is an important link between ER and EGFR. The latter belongs to the tyrosine kinase family and is expressed in a variety of cancer tissues. Compared to men, TKI treatment is more effective in women with lung cancer. Most studies [8-11] suggest that ER has two subtypes, ERα and β. The latter has at least five subtypes (ERβ1-5). In a previous study [12], ERβ2 had a good prognosis in lung cancer and its positive expression was significantly correlated with lower pTNM stage. This led to further exploration of its mechanism in lung cancer.

Recently, the roles of P38 mitogen-activated protein kinase (p38MAPK) and signaling pathways in lung cancer development have drawn attention. P38MAPK is one of the four major groups of MAPKs. It has six isoforms, namely p38α1, p38α2, p38β1, p38β2, p38γ, and p38δ. Some researches [13-15] have found that extracellular stimuli, such as cytokines and bacterial pathogens, can promote phosphorylation and activation of the Tyr site of P38MAPK, which can transduce signals to the nucleus promoter-associated target genes to promote transcription. P38MAPK is involved in a variety of biological processes, including promoting apoptosis, participating in inflammatory reac-

tions, and development of malignant tumors, targeting many different proteins to regulate transcription and translation. In a previous study, it was found that ERβ2 and P38MAPK were co-expressed in lung cancer. It was speculated that there is a close relationship between ERβ2 and P38MAPK in the development of lung cancer.

The present study will further explore possible internal relationships through animal experiments and make preliminary theoretical foundations, aiming to improve the development mechanisms of lung cancer.

Materials and methods

Materials

Lung adenocarcinoma cells A549, H358, and LTEP-a2 (preserved by the public experimental platform of the First Affiliated Hospital of Sun Yat-sen University) were used. Culture medium was RPMI 1640 (Gibco) with 5% fetal bovine serum (Hangzhou Sijiqing Bioengineering Materials Graduate School). Antibodies were mouse anti-human ERβ2 (MCA2279GT, Serotec), mouse anti-human p38MAPK (AN1020, Abgent, USA), mouse anti-human Ki67 (AO-1284a, Beijing Huaxia Ocean Technology Co., Ltd. Company), and mouse anti-bcl-2 (51005, Shanghai Kehui Biotech Co., Ltd.). The B-actin antibody and HRP-labeled rabbit anti-mouse IgG were purchased from Beijing Zhongshan Biotechnology Co., Ltd., and the ECL reagent was purchased from Jiangsu Biyuntian Institute of Biotechnology. PVDF membranes were purchased from Millipore, USA. Five-week-old female BALB/CA-nu mice were purchased from Guangdong Animal Experimental Center and were fed at the SPF level. During the experiment, the animals were kept in compliance with relevant regulations for the management and protection of experimental animals.

Construction of the vector

Suzhou Hongxun Biotechnology Co., Ltd., performed gene synthesis (ERβ2, p38MAPK), constructed an overexpression vector with pLVX-IRES-ZsGreen1, and selected EcoRI and BamHI double enzyme digestion sites. Each gene introduced a restriction site forward 5'-GGAATTC Reverse 5'-CGCGGATCC. Each reagent was added for enzyme digestion reaction according to the following system: 4 μL of pLVX-IRES-ZsGreen1, 2 μl of 3.1 buffer (NEB), 1 μl each of EcoRI and BamHI, replenished to 20 μL, and the recovered pLVX-IRES-ZsGreen1 fragment was annealed to double after synthesis. A gene fragment with a cohesive end was subjected to the linking reaction and the linking system: 2 μl of a 10 × T4 ligase reaction solution, 4 μl of the recovered plasmid fragment, 6 μl of the gene fragment, 1 μl of T4 ligase, and 7 μl of H_2O. The linking system was placed on the PCR instrument. Overnight connection was at 16°C. Moreover, 10 μl of the ligation product was used for transformation and the feeling state was stbl3. The positive recombinant plasmid was selected by sequencing and the plasmid was extracted with the endotoxin plasmid extraction kit to perform subsequent cell transfection experiments. Wuhan Jingsai Company constructed the pGensil-ERβ2 low expression vector.

Cell transfection, identification, and culture

A549, H358, and LTEP-a2 were cultured in RPMI 1640 plus 5% fetal bovine serum. Glucan-encapsulated activated carbon adsorption was used to remove hormones from fetal bovine serum. The conditions were 37°C and 5% CO_2 routine culture. One day before transfection, A549 (H358, LTEP-a2 replicate) cells were seeded on a 24-well culture plate, after cell fusion reached 70-90%. pLVX-IRES-ZsGreen1-ERβ2 (hERβ2), pLVX-IRES-ZsGreen1-p38MAPK (hp38MAPK), and other high expression plasmids, along with pGensil-ERβ2 (iERβ2) low expression plasmids and empty plasmids, were transfected into cells, according to LipofectamineTM 2000 reagent operating instructions. After 48 hours of transfection, screening started and cells were passaged to 24-well plates. Un-transfected cells were passaged at the same density as controls. After adherence, G418 was added to the medium for screening. According to the cell death situation and nutrient status of the medium, the fluid was changed in time. After all cells in the control group died, resistant clones were obtained. Resistant clones were used to identify the WB protein. The single-cell suspension was prepared and inoculated in a 96-well plate. After proliferation, it was transferred to a 24-well plate to expand the culture. The stable transfer cell of hERβ2,

iERβ2, hp38MAPK, hERβ2+SB, iERβ2+hp38-MAPK, and empty was obtained.

Establishment of subcutaneous xenograft tumor models in nude mice

BALB/CA-nu nude female mice were 5 weeks old with a body weight of (19 ± 2) g. During the first week of conventional feeding, all nude mice were ovariectomized. They were inoculated 2 weeks after feeding. Single cell suspensions were inoculated subcutaneously into the right dorsal axillary of the nude mice (1×10^8 cells/body) and 7 groups were set up: hERβ2, iERβ2, hp38MAPK, iERβ2+hp38MAPK, hERβ2+SB203580 (specific inhibitor of p38-MAPK), empty (10 per group), and blank group (3). Each group was intraperitoneally injected with E2 and each dose was calculated as rat body weight. The standard was: E2 (20 μg/kg), hERβ2+SB203580 group: SB203580 was perfused through the stomach and calculated according to body weight, Standard: SB203580, 25 mg/Kg. After 5 consecutive weeks of observation, the mice were sacrificed or killed by cervical dislocation. Volumes of the transplanted tumors were estimated to be volume (mm^3) = Length (mm) × Width $(mm)^2/2$. The tumor growth curve was plotted. Calipers measured the sizes of the tumors. Body weights of the mice were measured with an electronic scale weekly before injection. After the animals were killed or died, the tumors were completely removed for further testing.

Immunohistochemistry SP staining and scoring

All tissues were fixed in 4% paraformaldehyde and paraffin-embedded. Serial sections (4 um) were made. Randomly sampled from the front to the back at random distance, 10 samples were taken from each specimen. The primary antibody was: anti-ERβ2 (1:50), Anti-p38MAPK (1:250), anti-ki-67 (1:400), and anti-bcl2 (1:50). PBS, instead of the primary antibody, was used as a negative control. After sitting overnight at 4°C, rewarming at 37°C for 45 minutes, and washing with PBS 3 times, secondary antibodies were added, incubating at 37°C for 1 hour. PBS was washed 3 times for 5 minutes. DAB displayed color for 5-10 minutes. They were counterstained with hematoxylin and microscopical observation of brown indicated positive. Three pathological examinations were performed. Under a 400-fold microscope, 100 cancer cells were selected from each visual field. The semi-quantitative method was used to obtain the final score (1 to 16) through multiplying the staining intensity fraction by the percentage of positive cells. The percentage of positive cells was calculated according to the following method, ≤ 20%, 1; 20-50%, 2; 50-70%, 3; > 70%, 4; The staining intensity was as follows: negative, 1; weak, 2; 3; Strong, 4.

Western blotting

A total of 100 ul of RIPA lysate was added to 20 mg of tumor tissue, homogenized on ice, and centrifuged at 12000 r/min for 10 minutes at 4°C. The supernatant was separated and protein concentrations were detected. Protein was separated by 10% SDS-PAGE electrophoresis. The sample volume was 40 ug and the volume was 20 ul. After electrophoresis, a "sandwich" was prepared and the protein was electrotransferred onto the PVDF membrane after transfer. After blocking with 5% skim milk powder for 2 hours, primary antibodies anti-bcl-2 (1:900), anti-ERβ2 (1:500), anti-p38MAPK (1:1000), and anti-β-actin (1:10,000) were added. After washing the membrane, second antibody was added. They were incubated on the shaker at room temperature for 50 minutes. The membrane was washed with TBST 5 times, 5 minutes each time, observing the ECL exposure. Gelpro 32 analysis was used for gel image analysis.

Statistical analysis

GraphPad Prism 6.0 software was used. Experimental data are expressed as mean ± SEM. Moreover, t-test was used for statistical analysis of differences between groups, while one-way ANOVA was used for analysis among groups. $P < 0.05$ indicates statistical significance. "*" stands for "$P < 0.01$".

Results

P38MAPK interferes with ERβ2 inhibiting the growth of lung xenografts tumors

After 5 weeks of rearing (**Figure 1A-D**), the growth of xenograft tumors in the hERβ2 group was significantly slower, with the average volume increasing from 18.342 ± 2.3 mm^3 to 278.45 ± 30.3 mm^3. However, in the empty group, the average tumor volume in mice

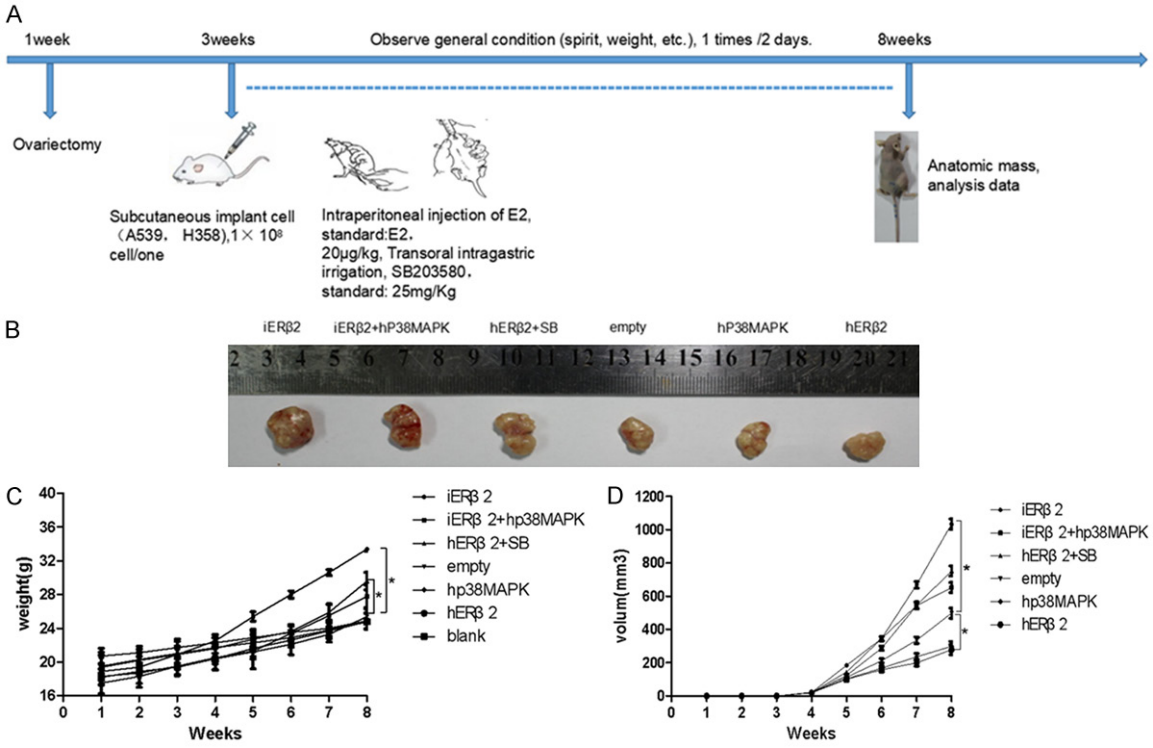

Figure 1. ERβ2 inhibits the growth of mice xenografts tumor by p38MAPK. A: It is the entire mouse transplant tumor model construction and rat feeding process. The entire experimental process was 8 weeks. The blank control group was not listed. Results of the A549 xenograft tumor models were selected (results of the other two cell lines were similar and not listed). B: The average size of the transplanted tumors in each group was compared. C, D: According to the calculated weight per week and the volume of the transplanted tumor, a growth curve was drawn.

increased from 19.43 ± 3.5 mm^3 to 498.21 ± 34.53 mm^3. The iERβ group (22.56 ± 4.2 mm^3-1034.56 ± 35.33 mm^3) showed statistical significance. However, the inhibitory effects of hERβ2 and the promotion effects of iERβ2 were interfered with by p38MAPK. In the iERβ2+hp38MAPK group and hERβ2+SB group, tumor volume was significantly reversed. Compared with the empty group, the tumor volume of the iERβ2+hp38MAPK group was (21.42 ± 2.2 mm^3-652.4 ± 23.43 mm^3). In the hERβ2+SB group, the tumor volume was (21.21 ± 3.4 mm^3-752.3 ± 45.3 mm^3). The weight growth of mice was slightly different. Compared with the empty group, only mice with iERβ2 and hERβ2+SB had a significant increase, while the other groups showed no significant changes.

ERβ2 can upregulate p38MAPK and interfere with expression of bcl-2 and ki67

After IHC analysis of transplanted tumor tissues (**Figure 2A**), each protein expression score was used as a semi-quantitative indicator. Compared with the empty group (**Figure 2B**), expression of p38MAPK protein was significantly increased in the hERβ2 group. In the iERβ2 group, expression of p38MAPK was significantly decreased. Further analysis revealed that expression of bcl-2 and ki67 in the hERβ2 group decreased, whereas iERβ2 showed the opposite change. Similar results were also observed in the WB test (**Figure 3A**, **3B**). In the hERβ2 group, expression of p38MAPK was significantly increased and expression of bcl-2 was decreased. In the hERβ2 group, the inhibitor of p38MAPK improved expression of bcl-2. At the same time, in the iERβ2 group, p38MAPK decreased and bcl-2 expression increased. Its effects may also be affected by p38MAPK.

Discussion

Most large-scale epidemiological analyses have shown that incidence of lung cancer and mortality rates have increased yearly, worldwide. This trend is particularly evident in Asia, especially in China. The age of lung cancer has

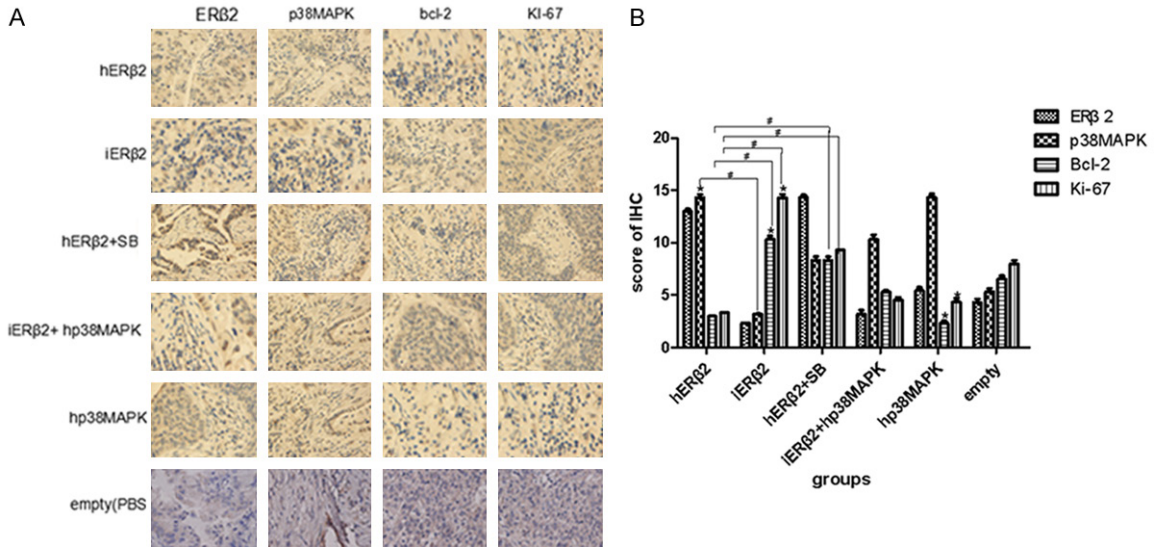

Figure 2. IHC detection of ERβ2, p38MAPK/bcl-2/ki-67 expression in xenografts tumors. A: In accordance with the method of SP detection and evaluation methods, each protein expression score was a semi-quantitative indicator, with brown as positive and PBS as a negative control (SP × 200). B: Compared with the empty group, p38MAPK protein expression was significantly increased in the hERβ2 group, while p38MAPK expression was significantly decreased in the iERβ2 group. Further analysis revealed that expression of bcl-2 and ki67 in the hERβ2 group decreased, whereas iERβ2 showed the opposite change.

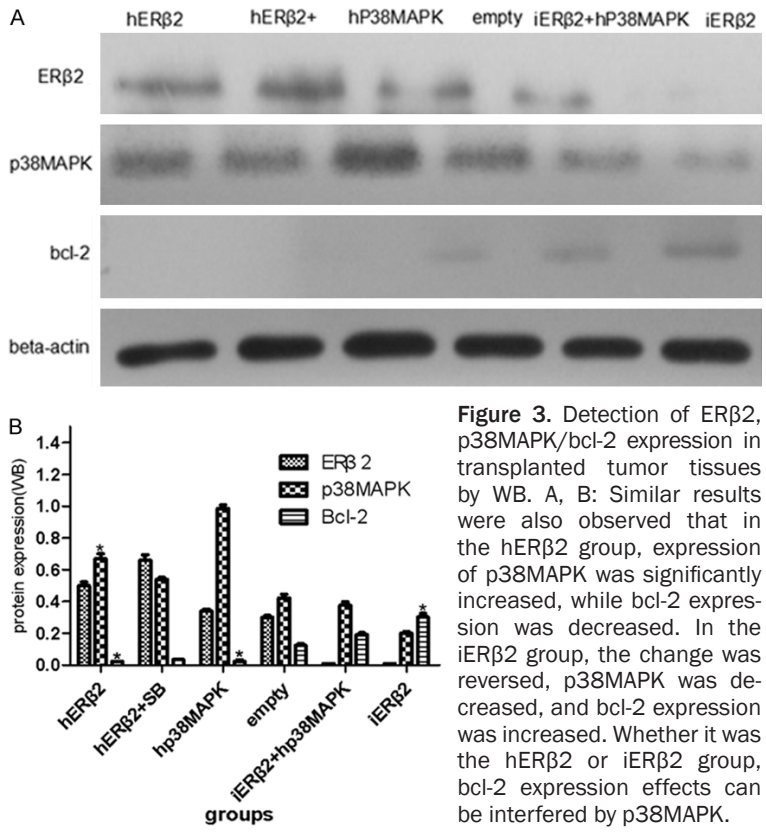

Figure 3. Detection of ERβ2, p38MAPK/bcl-2 expression in transplanted tumor tissues by WB. A, B: Similar results were also observed that in the hERβ2 group, expression of p38MAPK was significantly increased, while bcl-2 expression was decreased. In the iERβ2 group, the change was reversed, p38MAPK was decreased, and bcl-2 expression was increased. Whether it was the hERβ2 or iERβ2 group, bcl-2 expression effects can be interfered by p38MAPK.

a decreasing trend as well [1, 16, 17]. Lung cancer has strong invasiveness and distant lymph node metastasis, thus it is difficult to completely eradicate the disease. This is the main reason for the higher recurrence rates and poor prognosis of lung cancer at this stage. To make a breakthrough in the treatment of lung cancer, in-depth research on the pathogenesis of lung cancer is necessary. The present experiment explored the roles of ERβ2 in lung cancer, finding that it may play important roles in the prevention and treatment of lung cancer.

In this study, the ability to inhibit the growth of lung xenografts tumors in the high expression ERβ2 group was significantly higher than that in the empty group. IHC showed that expression of KI-67 in the hERβ2 group was significantly decreased. When expression of ERβ2 was decreased, the opposite phenomenon was observed. The growth of transplanted tumors was significantly accelerated and the

body weights of mice increased significantly during the same period, suggesting that ERβ2 may play a defensive role in the progression of lung cancer. In this study, by removing the ovaries and artificially feeding E2 to control the amount of E2 in the body, it was ensured that there were no significant differences in the amount of E2 in each group. Excluding external disturbance factors, an interesting role of ERβ2 in lung cancer progression was observed. Consistent with the fact that studies [12] have followed up the prognosis of several hundred patients, it was found that ERβ2 may have a good prognosis.

Traditional studies [18-20] have suggested that the mechanisms of ERβ may be like that of estrogen receptor α (ERα), namely ER/p38-MAPK signaling pathways, with ER binding DNA directly or through transcription factor (AP-1/NF-Kβ) binding to DNA. Other pathways may be its biological mechanism. The present study also found that ERβ2 can upregulate expression of p38MAPK, further confirming that ERβ2 inhibits the growth of lung xenografts tumors by p38MAPK specific inhibitor (SB) interference. In the iERβ2 group, the tumor growth effect was interfered by high expression of p38MAPK. These phenomena directly confirm that the function of ERβ2 in inhibiting tumor growth may be achieved through regulation of p38MAPK.

Many studies [21-24] have confirmed that p38MAPK can participate in a wide range of biological processes by regulating expression of different target proteins, including the promotion of apoptosis, inflammatory reaction, and malignancy. This study found that high expression of ERβ2 and p38MAPK reduced Bcl-2 protein expression, while ERβ2 downregulation of bcl-2 expression was interfered with by p38MAPK specific inhibitors. A similar phenomenon was observed in low expression of ERβ2. Expression of bcl-2 was increased, while the latter was also interfered by p38MAPK. These phenomena may suggest a common signaling pathway for ERβ2/p38MAPK/bcl-2.

Bcl-2 protein [25-27] is the encoded product of bcl-2 proto-oncogene, a cell survival promoting factor. It is a membrane integrin, molecular weight 26 kDa, located in mitochondria, endoplasmic reticulum, and continuous perinuclear membranes. It is widely expressed in embryonic tissues. The Bcl-2 protein family is a special family. At present, 25 Bcl-2 family homologous proteins have been discovered. Some of them promote apoptosis, such as Bad, Bid, and Bax. Some members prevent apoptosis, such as Bcl-2, Bcl-x, and Bcl-w. Bcl-2 [28-30] can prevent cytochrome c from the mitochondria released to cytoplasm, inhibiting apoptosis. Combined with the experimental data above, it was speculated that at least one of the possible reasons why ERβ2 inhibits the progression of lung xenografts tumors is via upregulation of p38MAPK and the decrease of the inhibitory apoptotic protein (bcl-2), resulting in slow cell growth. This conclusion can be further verified by downregulation of ERβ2 and inhibition of p38-MAPK. The latter two observed a faster growth of transplanted tumors and an increase in bcl-2 expression.

In summary, the present study was conducted to verify that ERβ2 inhibits the growth of lung tumors in a nude mice xenograft tumor model. Repeated experiments with three cells (A549, H358, and LTP-a2) were conducted. This function may be implemented by upregulating p38MAPK and inhibiting Bcl-2 expression. The aim of this study was to discover the new role of ERβ2 in inhibiting lung tumors, revealing the possible mechanisms of ERβ2 exerting a new role and laying a preliminary theoretical basis for targeting endocrine therapy for lung cancer.

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Disclosure of conflict of interest

None.

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