Xihuang Pill inhibits the development of DMBA combined estrogen and progesterone induced breast precancerous lesions rats by PI3K/AKT/mTOR signaling pathway

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Research Article

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Abstract

Objective.

To study the inhibitory effect of Xihuang Pill on the development of DMBA combined estrogen and progesterone induced breast precancerous lesions rats by PI3K/AKT/mTOR signaling pathway, and to explore the effect of Xihuang Pill in preventing and treating breast cancer. *Method.* Establishment of a rat model of breast precancerous lesion with DMBA combined estrogen and progesterone sequential induction for 10 weeks. Xihuang Pill was administered by gavage continuously for 4 weeks. Take rat breast tissue and stain with hematoxylin- eosin (HE). The pathomorphological changes were observed with light microscope; TUNEL staining to detect cell apoptosis in breast tissue; Western blot was used to detect the protein expression of P-PI3K, P-AKT (S473), P-AKT (T308), PTEN, P-Tuberin/TSC2, P-Tuberin (p-S939), p-mTOR, P-4E-BP1 in breast tissues. The qRT-PCR was used to detect the gene expression of PTEN mRNA and VEGF mRNA. Immunohistochemistry was used to detect the protein expression of P-S6, p-p70s6k and VEGF. *Result.* Compared with the disease model group, the low, middle and high dose Xihuang Pill groups could significantly reduce the degree of breast pathology, and the number of apoptosis of breast precancerous lesions cells increased with the increase of Xihuang Pill dose; The expression levels of P-PI3K, P-AKT (S473), P-AKT (T308), p-mTOR, P-4E-BP1, p-S6, p-p70S6K, VEGF protein and VEGF mRNA dropped with the increase of Xihuang Pill dose. The expression levels of PTEN, P-Tuberin/TSC2, P-Tuberin (p-S939) protein and PTEN mRNA elevated with the increase of Xihuang Pill dose. *Conclusion.* Xihuang Pill can promote the apoptosis of breast precancerous lesion cells and reduce the proliferation of vascular endothelial cells, and then inhibit the progression of breast precancerous lesions. Its mechanism probably associated with the regulation of PI3K/AKT/mTOR pathway related gene protein expression.

1. Introduction

Cancer is one of the diseases that seriously threaten human health in the world. For women, the morbidity and mortality of breast cancer are rising year by year, which poses serious harm to women's health [1, 2]. The genesis and progression of breast cancer is a process of “normal cells → simple hyperplasia → atypical hyperplasia → in situ carcinoma → invasive carcinoma”[3]. Precancerous lesions are the necessary stage for the genesis and progression of breast cancer, and it includes simple hyperplasia, atypical hyperplasia and in situ carcinoma. So active intervention measures can block or even reverse the process of breast canceration[4]. Intervention of breast precancerous lesions and blocking its further development is the key to prevent and cure breast cancer. Modern research methods of breast cancer treatment include targeted therapy, chemotherapy, endocrine therapy and surgery[5], etc. These methods have achieved good curative effect to cure breast cancer in clinical, but there are many adverse reactions. Therefore, these methods are difficult for breast precancerous lesion patients to accept. Traditional Chinese medicine adheres to the academic thought of “preventive treatment of disease”. In the period of precancerous lesions, traditional Chinese medicine can intervene to block the progress of breast cancer. Precancerous lesions of breast belong to the category of “Ru Pi” of traditional Chinese medicine. Phlegm

toxin and blood stasis is the core pathogenesis of its occurrence and development. The classic prescription Xihuang pill (XHP) has the effects of reducing phlegm and resolving masses, decreasing swelling and relieving pain, promoting blood circulation and removing blood stasis, clearing heat and detoxicating. Through clinical practice, it can be used to cure breast precancerous lesions and prevent breast cancer with distinct curative effect[6, 7]. Our prophase research found that[8–10] XHP inhibited the activity of breast precancerous lesions MCF-10AT cells and induce it apoptosis by suppressing mTOR and VEGF protein expression of PI3K/AKT/mTOR signal pathway, and improve breast microcirculation and abnormal hemorheology in 7,12-dimethylbenzoanthracene (DMBA) combined estrogen and progesterone induced breast precancerous lesions rats. Breaking the balance between cell proliferation and apoptosis is the most important mechanism of breast cancer progression. PI3K/AKT/mTOR signal pathway is the main pathway to regulate the proliferation, growth, survival and apoptosis of breast cells. Its abnormal activation is very important for the occurrence and development of breast cancer. Therefore, inhibiting this signal transduction pathway has become the key to breast cancer target therapy and prevention of metastasis and recurrence. Based on this research, we hypothesized that XHP pharmacological mechanism for inhibits the development of breast precancerous lesions may be closely related to the PI3K/Akt/mTOR signaling pathway. For the purpose of proving this hypothesis, we establish the rat model of breast precancerous lesions by DMBA combined estrogen and progesterone sequential induction for 10 weeks in this study, and then oral XHP for 4 weeks to study the effect of XHP on PI3K/AKT/mTOR signaling pathway-induced rats breast cancer inhibition of pre-lesion, analysis of the mechanism of XHP prevention and treatment of breast cancer.

2. Materials And Methods

2.1 Animals and Drugs

Sixty SPF 6-week-old female Sprague Dawley (SD) rats (weighing 180 ± 20 g) were bought from Hebei Experimental Animal Center (Shijiazhuang, Hebei, China, License Number: 1705351). The animal experiment was approved by the medical ethics committee of Hebei University of traditional Chinese medicine and was in compliance with all regulatory guidelines. All methods were carried out in accordance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments). All the rats were allowed food and water ad libitum. and they entered the experiment after 1 week of adaptive feeding. XHP (Zhejiang Tianyitang Pharmaceutical Co., Ltd Division, lot No.: 1703011). DMBA (7, 12-Dimethylbenz [a]anthracen, TCI, CAS57-97-6). Estradiol benzoate injection (Ningbo No.2 hormone factory, lot No.: 110252511). Tamoxifen citrate tablets (Yangzijiang Pharmaceutical Group Co., Ltd., lot No.: 17041311). Progesterone injection (Ningbo No.2 hormone factory, lot No.: 110251670).

Preparation of DMBA: accurately weigh 7, 12-dimethylbenz [a] anthracen (DMBA), dissolve it in sesame oil at a ratio of 7 $mg{\cdot}ml^{-1}$, and put it in a water bath at 60℃ for ultrasonic oscillation dissolving for standby.

Preparation of XHP aqueous solution: according to the needs of the experiment, soak the XHP in a little distilled water the day before the experiment, and the drug particles can be soaked exactly. On the day of the experiment, crush the soft soaked XHP and prepare the concentrations with distilled water. XHP aqueous solutions of 270, 550, and 1370 $mg{\cdot}kg^{-1}$ are reserved for use.

Tamoxifen aqueous solution preparation: according to the experimental needs, tamoxifen aqueous solution of 4 $m{\cdot}kg^{-1}$ was prepared with distilled water.

2.2 Model and Drug Use

2.2.1 Animal Model Establishment

We have established the rat model of breast precancerous lesion as previously reported on the literature[9–11]. On the 1st day, DMBA dissolved in sesame oil was given to rats by gavage at the ratio of 1 $ml{\cdot}100g^{-1}$. From the second day, 5 days as a cycle (On days 1–3, rats were injected with estradiol benzoate 0.5 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$ into the inner side of hind legs. On the 4th day, rats were injected with progesterone 4 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$ into the inner side of hind legs. Observation on day 5th). After 12 consecutive cycles, we established the rat model of breast precancerous lesion.

2.2.2 Grouping and Administration methods

According to weight stratification and random grouping method, 60 rats were divided into normal control group (n = 10) and disease model group (n = 50). The administration methods were as follows: Normal control group: After disposable gavage of sesame oil 1 $mL{\cdot}100g^{-1}$ without DMBA, the rats are routinely fed; Disease model group: The model was reproduced in accordance with 2.2.1 Model Establishment method and fed routinely. After 10 weeks of successful modeling, rats in the disease model group were stochastically divided into 5 groups, 10 rats in each group: disease model group, tamoxifen group and XHP low, middle and high dose groups. The administration methods were as below: Disease model group: fed routinely. Tamoxifen group: tamoxifen (4 $mg{\cdot}kg^{-1}$) 1 $ml{\cdot}100g^{-1}$ was given by gavage one time a day for 28 days, fed routinely. XHP low, middle and high dose groups: Xihuang pills were given low (270 $mg{\cdot}kg^{-1}$), middle (550 $mg{\cdot}kg^{-1}$) and high-dose (1370 $mg{\cdot}kg^{-1}$) 1 $ml{\cdot}100\ g^{-1}$ by gavage, once a day for 28 days, fed routinely. At the end of the 14th week of animal experiment, the materials were collected. Before taking the material, the rats were fasted 24h, drank freely, and euthanized by anesthetic overdose of sodium pentobarbital (200 $mg{\cdot}kg^{-1}$, i.p.). Take 6 pairs of mammary glands and surrounding skin and subcutaneous tissue of rat chest and abdomen under sterile conditions, about 1.0 cm × 1.0 cm. Some tissue samples were fixed with 10% neutral buffered formalin and embedded in paraffin for HE and immunohistochemical and TUNEL staining. Some tissue samples were frozen in fluid nitrogen and kept them in -80°C for immunoblotting and RT-qPCR later on.

2.3 HE staining

The tissue is embedded in paraffin and the sections are deparaffinized. Then Hematoxylin stain, 10 minutes, tap water for 1 minute; 1% Hydrochloric acid ethanol (70% ethanol 99ml + concentrated

hydrochloric acid 1ml), 20 seconds, tap water for 10 minutes; Eosin stain, 10 minutes, tap water for 1 minute. Finally, to observe the pathological changes of breast tissue by transparent and sealed piece.

2.4 Immunohistochemical detection

Paraffin-embedded blocks were cut in 3μm sections. The sections were then routine dewaxed and rehydrated, followed by antigen retrieval for 5 min by sodium citrate buffer (pH 6.0) at 100°C, and cooled down to room temperature naturally, then each section treated with 100μL 3% H_2O_2 for 10 min in order to reduce endogenous peroxidase activity, and washed using PBS (at pH 7.4, 3 times for 3 min), and blocked with 100μL 2.5% normal goat serum for 30 min at room temperature prior to aspiration of the blocking solution, added with 100μL diluted primary antibody and incubated at room temperature for 1 h, washed using PBS (at pH 7.4, 3 times for 3 min), added with 100μL secondary antibody and incubated at room temperature for 30 min, washed using PBS (at pH 7.4, 3 times for 3 min), treated with 100μL diaminobenzidine (DAB) substrate–chromogen solution for 5 min and counterstained with Harris hematoxylin. Finally, the sections were differentiated in 1% acid alcohol, dehydrated and sealed with neutral gum, and then observed and photographed under a microscope. The above primary antibodies were as follows: Phospho-S6 Ribosomal Protein (Ser235/236) XP® Rabbit mAb (cat. no. 4858S; 1:400 dilution; CST); VEGF Antibody (cat. no. NB100-664; 1:50 dilution; Novus); Anti-phospho-p70 S6 Kinase (pThr389) antibody (cat. no. SAB4503957; 1:50 dilution; Sigma).

2.5 TdT-mediated dUTP nick end labeling (TUNEL)

First of all, paraffin-embedded breast tissue blocks were cut in 3μm sections, and each section was stained, parched chip, dewaxed and PBS rinsed. Then, 50μL TUNEL reaction mixed liquids (In situ cell death detection kit-POD, cat. no. 11684817910; Roche) was added to each section, and the reaction time was 1 h in a dark wet box for 37°C, followed by rinsing with PBS (3 min × 3 times) protected from light, (After stained with TUNEL reaction mixed liquids, the tissue showed green fluorescence under 488 nm wavelength light of fluorescence microscope). Remove PBS, add 50μL DAPI (cat. no. AR1177; BOSTER) to each section, incubate for 10 min at room temperature and rinsed 3 times in PBS, each time for 3 min. (After DAPI staining, the tissue showed blue fluorescence under 405nm wavelength light of fluorescence microscope, i.e. IF single staining). Finally, the fluorescent anti-quencher (cat. no. P0126; Biyuntian, China) was added and observed after sealing. TUNEL positive cells showed green labeling under fluorescence microscope, while TUNEL negative nuclei appeared blue with DAPI.

2.6 Reverse Transcription Real-Time qPCR (RT-qPCR)

20-50mg breast tissues were split and centrifuged by RNA-Solv Reagent (Omega), and total RNA was isolated, and the RNA concentration and quality were assessed at 260/280nm and 260/230nm using UV-Visible spectrophotometer (756MC, Shanghai Precision Scientific Instrument Corp., Shanghai, China). The ratios of OD260/OD280 were between 1.8 and 2.1. RNA concentration (μg/μl) = (OD260-OD320) × dilution ratio × 0.04.c). Total RNA (2μg) was reverse transcribed using PrimeScript® RT reagent Kit with gDNA Eraser (Perfect Real Time; Takara, Dalian, China) according to the manufacturers protocol. Then, quantitative real-time PCR (qRT-PCR) was performed using the Applied Biosystems 7500Fast Real-Time

PCR System and SYBR® Premix Ex Taq™ (Tli RNaseH Plus; TaKaRa, Dalian, China). The following primers were used: GAPDH (internal control), forward primer sequence: 5'-CAGGAAATGATGACCTCCTGAAC-3', reverse primer sequence: 5'-TGTTT TTGTAAGTATCTTGGTGCC-3', amplicon length was 80 bp; VEGF, forward primer sequence: 5'-GCAGATCATGCGGATCAAACC-3', reverse primer sequence: 5'-GCTCACAGTGAATGTGGTCACTTA-3', amplicon length was 136 bp; and PTEN, forward primer sequence: 5'-GCGTGCGGATAATGACAAGG-3', reverse primer sequence: 5'-AGCCTCTGGATTTGATGGCTC-3', amplicon length was 157bp. The PCR reaction conditions were as follows. Initial denaturation at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. The specificity of the PCR product was confirmed by melting-curve analysis. Relative mRNA expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method, and the formula was as follows[12, 13]: ΔCt = CT (target gene) - CT (internal reference); ΔΔCT = ΔCt experiment group – ΔCt control group.

2.7 Western blot

The rat breast tissue proteins were extracted with RIPA lysis buffer (R0020, Solarbio). and protein concentration was measured by BCA protein assay kit (PC0020; Solarbio). Then, each lysed sample was mixed with SDS-PAGE loading buffer at 4:1 ratio and boiled in a 100°C water bath for 5 min and centrifuged at 14,000 rpm for 5 min. 20μl of the protein samples were subjected to SDS-PAGE analysis and then transferred onto PVDF membranes (Millipore) at 100 V for 90 min. After transfer, membranes were blocked with 5% milk in TBST (T1081, Solarbio) for 1 h and then incubated with primary antibodies at 4°C overnight. The above primary antibodies were as follows: P-PI3K(SAB4503957, Absin), P-AKT(SER473)(4060S, CST), P-AKT(Thr308) (13038S, CST), P-mTOR(5536S, CST), PTEN(9188S, CST), P-Tuberin/TSC2(3617S, CST), Tuberin(p-s939)(ab52962, abcam), P-4E-BP1 (2855S,CST) and β-actin(Sc-47778, Santa). All primary antibodies were used at a concentration of 1:1000. Afterwards, the membranes were washed by TBST 3 times for 10 min each and incubated with secondary antibody (Goat anti-rabbit IgG-HRP or Goat anti-mouse IgG-HRP, Santa) 1:5000 dilutions for 1 h at room temperature, then washed with TBST 3 times for 10 min each. Then, the protein bands were detected using enhanced chemiluminescence (ECL).

2.8 Statistical Methods

All data were expressed as *mean ± standard deviation* and were analyzed using the SPSS 20.0 statistical software package. Comparisons between groups were performed by one-way ANOVA. For the differences in pathology data among the groups were were analyzed with the Wilcoxon rank-sum test. $P < 0.05$ was statistically significant.

3. Results

3.1 Effect of XHP on pathomorphology of breast tissue in breast precancerous lesions rats

The histological morphology and pathology results showed that there was no precancerous lesions and invasive cancer in the breast tissue of rats in the normal control group. But disease model group, Tamoxifen group, XHP low-dose, XHP middle-dose and XHP high-dose group with various levels of precancerous lesions and invasive carcinomas (Fig. 1). Kruskal-Wallis H test showed that the differences between the groups were significant (χ^2 = 358.58, P = 0.000.) and the degree of hyperplasia was the most serious in the disease model group (mean rank = 501.93). The results of Mean-Whitney U test showed that the degree of breast tissue hyperplasia in the disease model group was higher than that in the normal control group (Z = 14.529, P = 0.000); The degree of breast tissue hyperplasia in tamoxifen group and XHP low, middle and high dose groups was lower than that in disease model group (P < 0.05 or P < 0.01) (Table 1). It is suggested that XHP can prevent and reverse the pathomorphology changes of breast tissue in rats with precancerous lesions induced by DMBA combined estrogen and progesterone.

Table 1
Comparison of the pathological changes of breast tissue in rats of each group

Groups	Dose ($mg \cdot kg^{-1}$)	Breast number	No hyperplasia	General hyperplasia	Precancerous lesions	Invasive carcinomas
Normal control	-	120	110	10	0	0
Disease model	-	120**	0	7	95	18
Tamoxifen	4	$120^{\#\#}$	11	46	61	2
XHP low-dose	270	$120^{\#}$	4	15	89	12
XHP middle-dose	550	$120^{\#\#}$	10	28	75	7
XHP high-dose	1370	$120^{\#\#}$	9	44	62	5

Note: compared with the normal control group, $^{**}P < 0.01$; compared with the disease model group, $^{\#\#}P < 0.05$, $^{\#}P < 0.05$.

3.2 Effect of XHP on apoptosis of breast tissue cells in breast precancerous lesions rats

The result of the TUNEL assay revealed that there were no apoptotic or little in the normal control group (After DAPI staining, the nucleus showed blue fluorescence and the apoptotic cells showed green fluorescence under fluorescence microscope, that is, green fluorescence was TUNEL staining positive cells). Compared with the normal control group, the apoptosis rate of disease model group increased, and the difference was statistically significant (P < 0.05); Compared with the disease model group, tamoxifen

group and XHP low, middle and high dose groups significantly increased the apoptosis of breast tissue, and the difference was statistically significant ($P < 0.05$), and the effect of high-dose group of XHP was better than that of middle and low-dose groups. It is suggested that XHP can induce the apoptosis of breast tissue cells of breast precancerous lesions rats (Fig. 2–3).

3.3 Effect of XHP on protein expression of P-PI3K, PTEN, P-AKT (S473) and P-AKT (T308) in tissue cells of breast precancerous lesions rats

The protein levels of P-PI3K, PTEN, P-AKT (S473) and P-AKT (T308) were detected by Western blot analysis, the results showed that: Compared with the normal control group, the expression of P-PI3K, P-AKT (S473) and P-AKT (T308) protein in disease model group was significantly higher, while the expression of PTEN protein was significantly lower ($P < 0.05$). The XHP low, middle and high dose group had significantly lower expression of the P-PI3K, P-AKT (S473) and P-AKT (T308) protein and higher expression of the PTEN protein compared with the disease model group ($P < 0.05$). Therefore, the effect of XHP high-dose group was better than that of middle and low-dose groups (Fig. 4).

3.4 Effect of XHP on protein expression of P-Tuberin/TSC2, P-Tuberin (p-S939), p-mTOR and P-4E-BP1 in tissue cells of breast precancerous lesions rats

Application of Western-blot to detect cell protein expression levels of P-Tuberin/TSC2, P-Tuberin (p-S939), p-mTOR and P-4E-BP1in rat breast tissues. the results showed that: Compared with the normal control group, the protein expression of P-Tuberin/TSC2 and P-Tuberin (p-S939) in breast tissue cells were decreased, but the protein expression of p-mTOR, P-4E-BP1 were increased of the disease model group, the difference was statistically significant ($P < 0.05$). Compared with the disease model group, the protein expression of P-Tuberin/TSC2 and P-Tuberin (p-S939) in XHP low, middle and high dose groups were significantly increased, while the protein expression of p-mTOR, P-4E-BP1 were decreased, the difference was statistically significant ($P < 0.05$). In addition, the effect of XHP high-dose group was better than that of middle and low-dose groups (Fig. 5).

3.5 Effect of XHP on protein expression of p-S6, p-p70S6K, VEGF in tissue cells of breast precancerous lesions rats

The protein expression of p-S6, p-p70S6K and VEGF were detected with immunohistochemistry in breast tissue of rats in each group. Positive cell percentage was measured using ImageJ software. The results showed that: In the normal control group, there were little or no p-S6, p-p70S6K and VEGF protein expressed in breast tissue cells. Compared with the normal control group, the protein expression of p-S6, p-p70S6K and VEGF in breast tissue cells of the disease model group was increased, the difference was statistically significant ($P < 0.05$). Compared with the disease model group, the protein expression of p-S6, p-p70S6K and VEGF in the XHP low, middle and high dose group were decreased, the difference was statistically significant ($P < 0.05$). In addition, the effect of XHP high-dose group was better than that of middle and low-dose groups (Fig. 6–7).

3.6 Effect of XHP on PTEN mRNA and VEGF mRNA level of tissue cells of breast precancerous lesions in rats

Relative expression levels of PTEN mRNA and VEGF mRNA in each group were determined by qRT-PCR. It was found that PTEN mRNA in rats in the disease model group was significantly decreased compared with the rats in the normal control group ($P < 0.05$). indicating that there was a deletion of PTEN mRNA in the breast tissue of precancerous lesions rats induced by DMBA combined with estrogen and progesterone.

Compared with the disease model group, the level of PTEN mRNA expression was significantly increased in the XHP low, middle and high dose group ($P < 0.05$). It is suggested that XHP can up-regulate the expression of PTEN mRNA or prevent the loss of PTEN mRNA in breast tissue of rats with breast precancerous lesions induced by DMBA combined with estrogen and progesterone (Fig. 8).

The expression level of VEGF mRNA of the disease model group was significantly increased compared with the normal control group ($P < 0.05$), which suggested that the expression of VEGF mRNA in the breast tissue of rats with precancerous lesions induced by DMBA combined with estrogen and progesterone is up-regulated. The expression level of VEGF mRNA in the XHP low, middle and high dose group were significantly decreased compared with the disease model group ($P < 0.05$). It is suggested that XHP can inhibit the protein synthesis of VEGF mRNA or down-regulate the expression of VEGF mRNA in breast tissue of breast precancerous lesions rats induced by DMBA combined with estrogen and progesterone (Fig. 8).

4. Discussion

Xihuang Pill (XHP) is a famous patented Chinese medicine, having therapeutic effect on tumor, it was first recorded in the "*waike zhengzhi quansheng ji*" of ancient Chinese Qing Dynasty. XHP is consisted of bezoar, musk, frankincense (vinegar) and myrrh (vinegar)[14]. It has the effects of promoting blood circulation and removing blood stasis, clearing heat and detoxicating, reducing phlegm and resolving masses, decreasing swelling and relieving pain[15]. During recent years, with the rapid grow of integrated traditional Chinese/complementary medicine and modern medicine, the function of XHP in breast cancer progression has been most extensively studied. Clinical studies show clearly that XHP combined with chemotherapy, radiotherapy or endocrine therapy could significantly improve the clinical efficacy, KPS, immune function and reduce the toxicity of chemotherapy or radiotherapy in breast cancer patients[14, 16, 17]. Animal experiments have also confirmed that XHP may inhibit breast cancer through the downregulation of estrogen receptor (ER) and progesterone receptor (PR) expression, inhibition of Bc1-2 expression, increastion of p53 gene expression and IL-2, IFN-γ, CD3+, $CD4^+$ and B7–1 levels[17, 18], XHP can inhibit the growth of breast cancer in 4T1 mice through upregulation of MEKK1, SEK1, JNK1, and AP-1 expression in Tregs, promoting Treg cell apoptosis in the tumor immune microenvironment[7]. In vitro experiments, XHP was found to inhibit the proliferation and migration, but advanced the apoptosis of

breast cancer cell lines of MCF-7, Hs578T and MDA-MB435 and breast precancerous lesions MCF-10AT cells.

Its mechanisms might be associated with regulating the protein and gene expression of Cyclin A, CDK2, Bcl-2, Bax, mTOR and VEGF[8, 19, 20]. Based on our research[8–10, 17], we hypothesized that XHP pharmacological mechanism for inhibiting the development of breast precancerous lesions could be closely interrelated to the PI3K/Akt/mTOR signaling pathway.

The current study used a model of 7,12-dimethylbenz[a]anthracene (DMBA) combination with estrogen and progesterone-induced breast precancerous lesion in rats. After confirming that the model was successfully established, XHP was administrated for treatment. subsequently, the specific molecular mechanisms of XHP were investigated. The pathomorphological analysis revealed that Xihuang pill could block and reverse the histopathological changes of breast tissue induced by DMBA combined with estrogen and progestogen. Moreover, the immunofluorescence TUNEL staining assay of breast tissue cells of breast precancerous lesions rats showed that the number of TUNEL positive cells in the XHP group increased compared with the normal control group. In addition, we found greater number of apoptotic cells in the XHP high-dose group than in the middle-dose and low-dose group, these findings revealed that XHP amplified apoptosis in a dose-effect manner. The imbalance between the proliferation and apoptosis of cells was closely associated with breast tumorigenesis and development. PI3K/Akt/mTOR pathway is an important intracellular signaling transduction pathway participate in cell multiplication, differentiation, apoptosis, cycle progression, motility, tumorigenesis, tumor growth and angiogenesis, which plays a major part in both the occurrence and progression of breast cancer[21, 22]. Therefore, inhibition of the PI3K/Akt/mTOR pathway is critical for breast cancer therapy. This signaling pathway involves four key molecules, PI3K, PTEN, Akt and mTOR[23]. PI3K is consisted of a regulatory subunit (p85) and a catalytic subunit (p110), and the activation of PI3K depends upon the activation of the p85 subunit. Once PI3K is activated, its catalytic subunit activates AKT through phosphorylation of AKT, and continuously activates mTOR through phosphorylation of mTOR[24]. AKT has two important phosphorylation sites: Thr308 and Ser473. The phosphorylation of Serine 473 is necessary for the complete activation of AKT[25]. mTOR is a key downstream mediator in the PI3K/Akt/mTOR signaling pathway that plays a key role in regulating cell growth and hyperplasia during breast cancer development[26]. PTEN is a negative regulator in the PI3K-Akt-mTOR signaling pathway, could dephosphorylate the PI3K to down-regulate the activation of PI3K-Akt-mTOR pathway[27]. In this study, we found that XHP could significantly reduce the protein expression of P-PI3K, P-AKT (S473) and P-AKT (T308) in breast precancerous lesion cells, and the higher the dose of XHP, the lower the expression of the three proteins, so there was a negative correlation; XHP could increase the protein expression of PTEN, which was positively related with the dose of XHP.

TSC2 is a potent mTOR suppressor, phosphorylation of TSC2 disturbs the formation of the TSC1-TSC2 complex, which negatively regulates MTOR activity[28]. Wen G Jiang et al. [29] researched the expression of TSC gene in breast cancer cells or tissues, and found that TSC was strongly positive expressed in normal breast epithelial cells. In invasive tumor tissues, the gene expression was decreased, and the

transcription level of TSC2 in normal tissues was apparently higher than that in tumor. the activation of mTOR promotes the phosphorylation of its downstream effectors, including phosphorylation of eIF4E binding protein 1 (4EBP1) and activation of p70 ribosomal S6 protein kinase (p70S6K), which phosphorylates S6 ribosomal protein, hus inducing cell proliferation, angiogenesis and metastasis[30]. Studies have shown that when eIF4E increases, the protein expression of VEGF also increases accordingly. Studies have shown that 4EBP1 could up-regulated the translation of VEGF mRNA, and promoted breast cancer angiogenesis[31]. Shao Bo et al. [32]studied the expression of p-S6 in breast tumor tissues, adjacent nontumorous tissues and benign breast lesion tissues by immunohistochemical method, and and discovered that p-S6 expression was up-regulated in breast tumor and related to the happening and progression of breast cancer. Lao Hai li et al. [33] studied the expression level of mTOR/p70S6K signaling pathway in in breast cancer, the investigation suggest that the mTOR/P70S6K signaling pathway is specifically activated in breast cancer and it might play an important role in the pathogenesis of breast cancer. Elevated expression of VEGF is a feature of invasive breast cancer[34]. In this project, the upstream regulatory factors (P-Tuberin/TSC2, P-Tuberin (p-S939)), downstream regulatory factors (P-4E-BP1, p-S6, p-p70S6K, VEGF) and p-mTOR protein which were related to PI3K/Akt/mTOR were analyzed. Western blot assays showed that compared with the disease model group, the expression of P-Tuberin/TSC2 and P-Tuberin (p-S939) protein was increased in XHP low, middle and high dose groups, while the expression of p-mTOR and P-4E-BP1 protein were decreased. The effect of high-dose group of XHP was better than that of middle and low-dose groups. Immunohistochemistry results displayed that the protein expression of p-S6, p-p70S6K and VEGF was significantly decreased, and the dose of XHP was negatively correlated with the protein expression, compared with the disease model group. This strongly suggests that XHP can up-regulates the upstream negative regulatory factors of mTOR, such as P-Tuberin/TSC2, P-Tuberin (p-S939), and down-regulates the downstream regulatory factors such as P-4E-BP1, p-S6, p-p70S6K, VEGF, etc., which can block PI3K/Akt/mTOR signaling pathway, thereby effectively inhibiting breast precancerous lesions.

Using qRT-PCR to analysis the expression of PTEN mRNA and VEGF mRNA in breast tissue of rats. compared with the disease model group, the expression levels of PTEN mRNA in each dose group of XHP were significantly increased, while the expression of VEGF mRNA were decreased. It is suggested that XHP can prevent the loss of PTEN mRNA or up-regulate the expression of PTEN mRNA in breast tissue of breast precancerous lesions rats. However, the decrease of VEGF mRNA gene expression indicates that XHP can inhibit the transcription of VEGF mRNA or down-regulate the expression level of VEGF mRNA in breast tissue of breast precancerous lesions rats. The above results were consistent with that of PTEN by Western blot and VEGF by immunohistochemistry. Therefore, PTEN and VEGF genes are relatively antagonistic in the process of transcription and protein synthesis. PTEN gene activation can inhibit VEGF by interrupting PI3K/AKT signaling pathway to affect the synthesis of angiogenic factors, and then inhibit neovascularization of breast cancer.

In summary, we have put forward a new mechanism of XHP inhibit the progress of breast precancerous lesions rats induced by DMBA combined estrogen and progesterone. The PI3K/Akt/mTOR signaling pathways were involved in this process. Intervention on breast precancerous lesions rats with XHP

inhibits the proliferation and induces apoptosis through inhibiting the PI3K/AKT/mTOR signaling via upregulating the expression of proteins PTEN, P-Tuberin/TSC2, P-Tuberin (p-S939) and PTEN mRNA, and downregulating the expression of proteins P-PI3K, P-AKT (S473), P-AKT (T308), p-mTOR, P-4E-BP1, p-S6, p-p70S6K, VEGF and VEGF mRNA, suggesting XHP may become a promising anti-tumor drug by directly regulating the PI3K/AKT/mTOR pathway in pre-breast cancer.

Declarations

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Contributions

Conceived and designed the experiments: D. H. Li. Performed the experiments: D. H. Li, H. F. Fan, N. Guo, C. X. Sun, J. F. Dong. Analyzed the data: Y. F. Su, Y. T. Gu, J. Liu. Wrote the paper: H. F. Fan, D. H. Li.

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Ethics declarations

Competing interests
The authors declare no competing interests.

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Figures

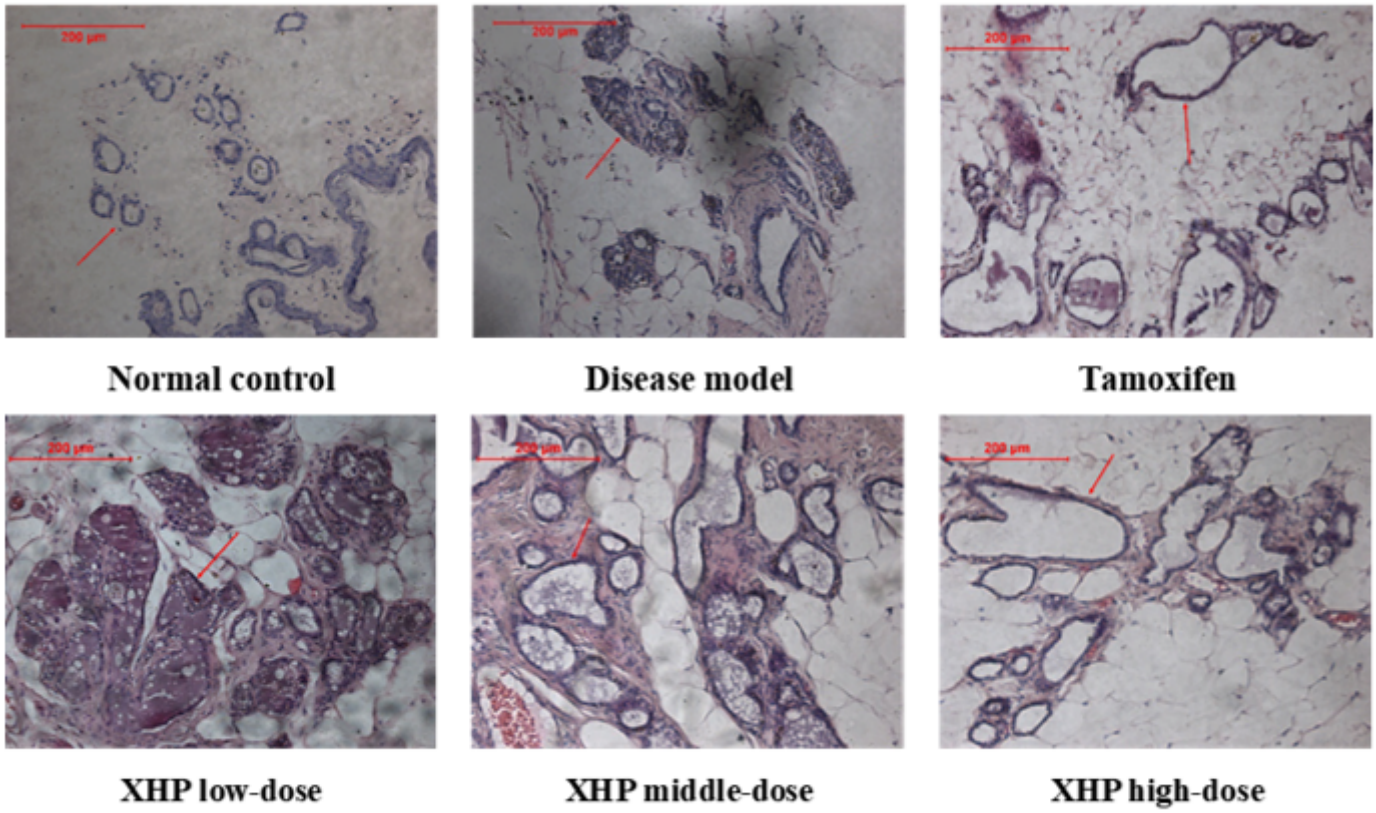

Figure 1

Histopathological changes of breast tissue in rats of each group (HE staining, ×100)

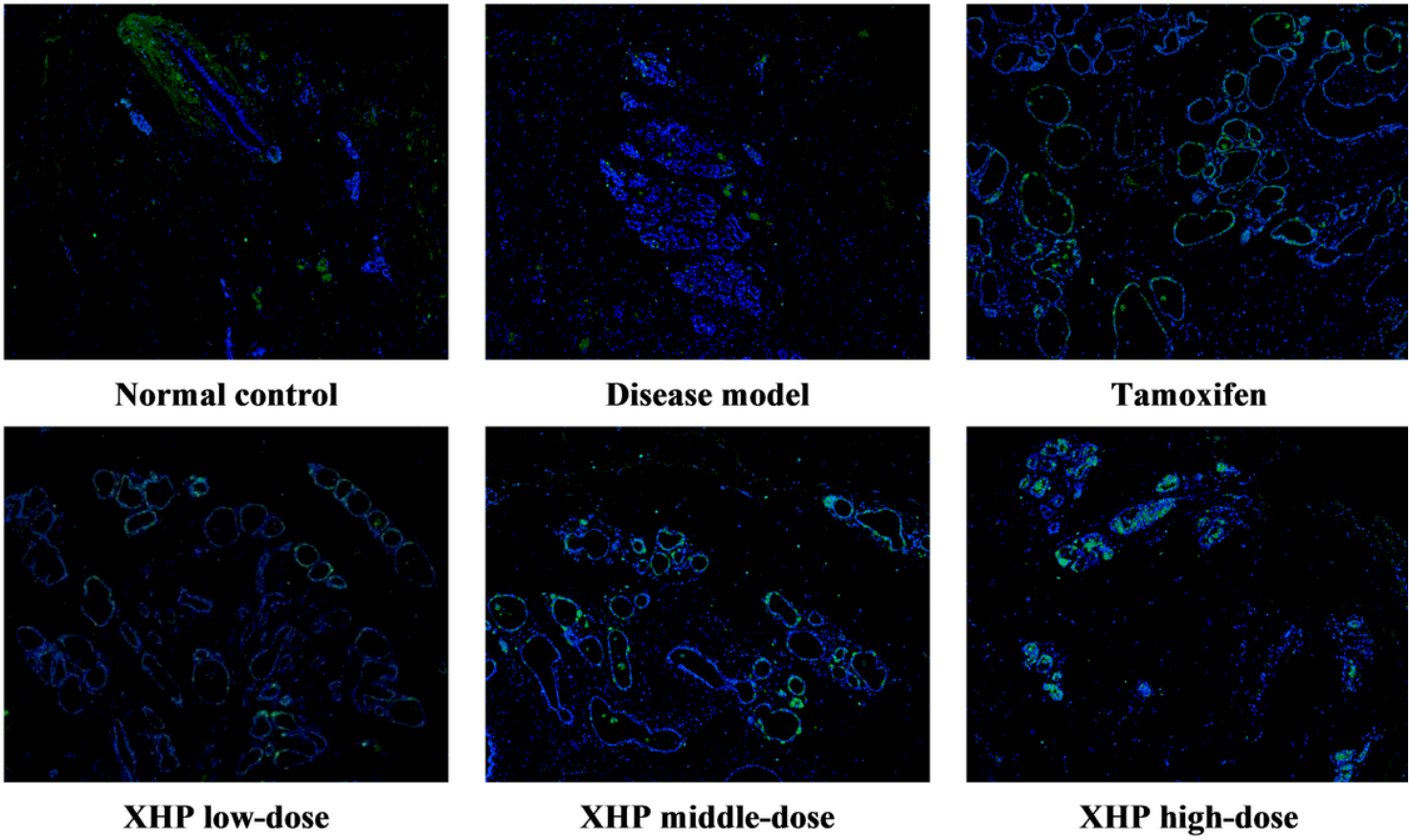

Figure 2

Apoptosis of breast tissue of rats in each group (TUNEL, ×100)

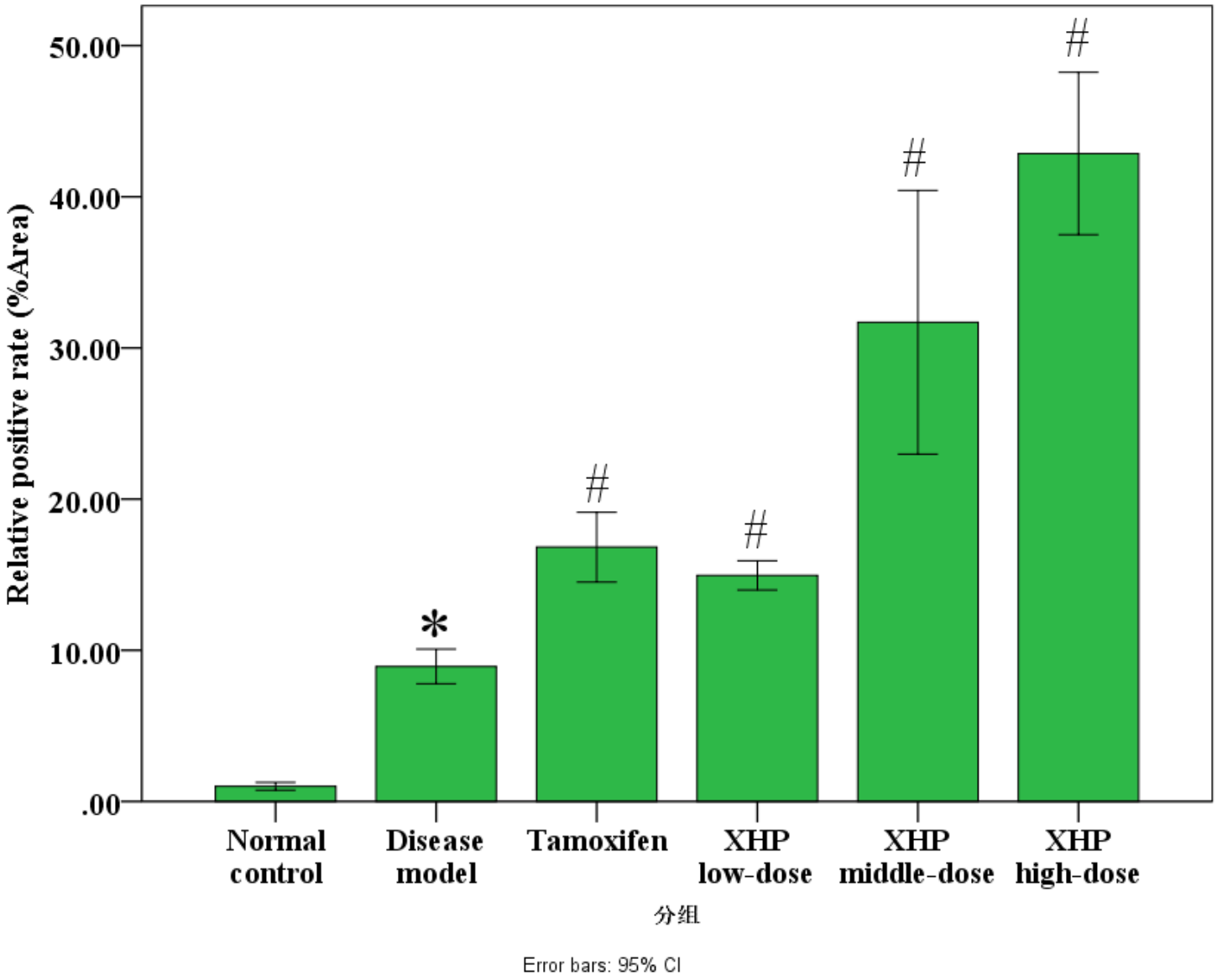

Figure 3

Relative the apoptosis changes of breast tissue in rats of each group. compared with the normal control group, *P<0.05; compared with the disease model group, #P<0.05. (n=10 in each group).

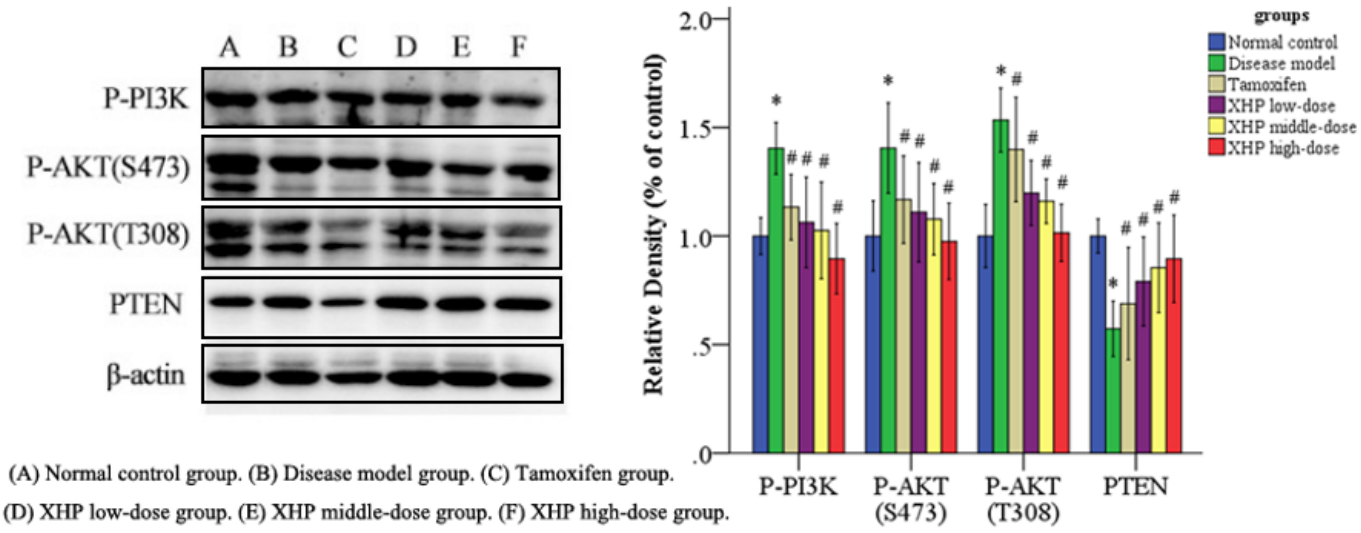

Figure 4

Expression of P-PI3K, P-AKT (S473), P-AKT (T308) and PTEN protein in breast tissue of rats in each group. Compared with the normal control group, *P<0.05; compared with the disease model group, #P<0.05. (n=10 in each group).

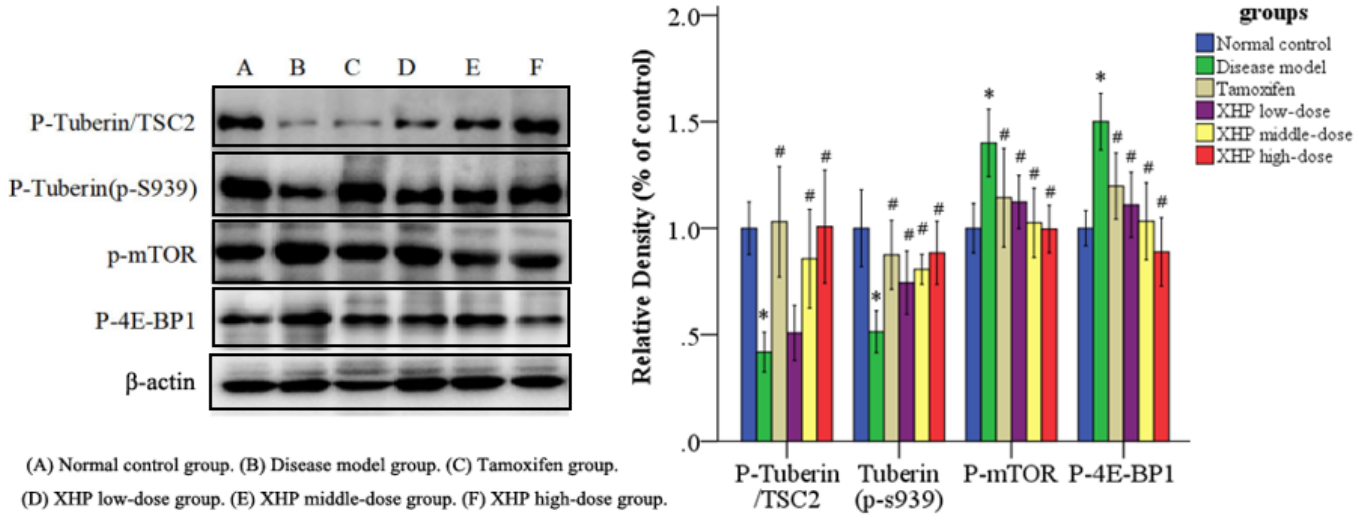

Figure 5

Protein expression of P-Tuberin/TSC2, P-Tuberin (p-S939), p-mTOR and P-4E-BP1 in breast tissue of rats in each group. Compared with the normal control group, *P<0.05; compared with the disease model group, #P<0.05. (n=10 in each group).

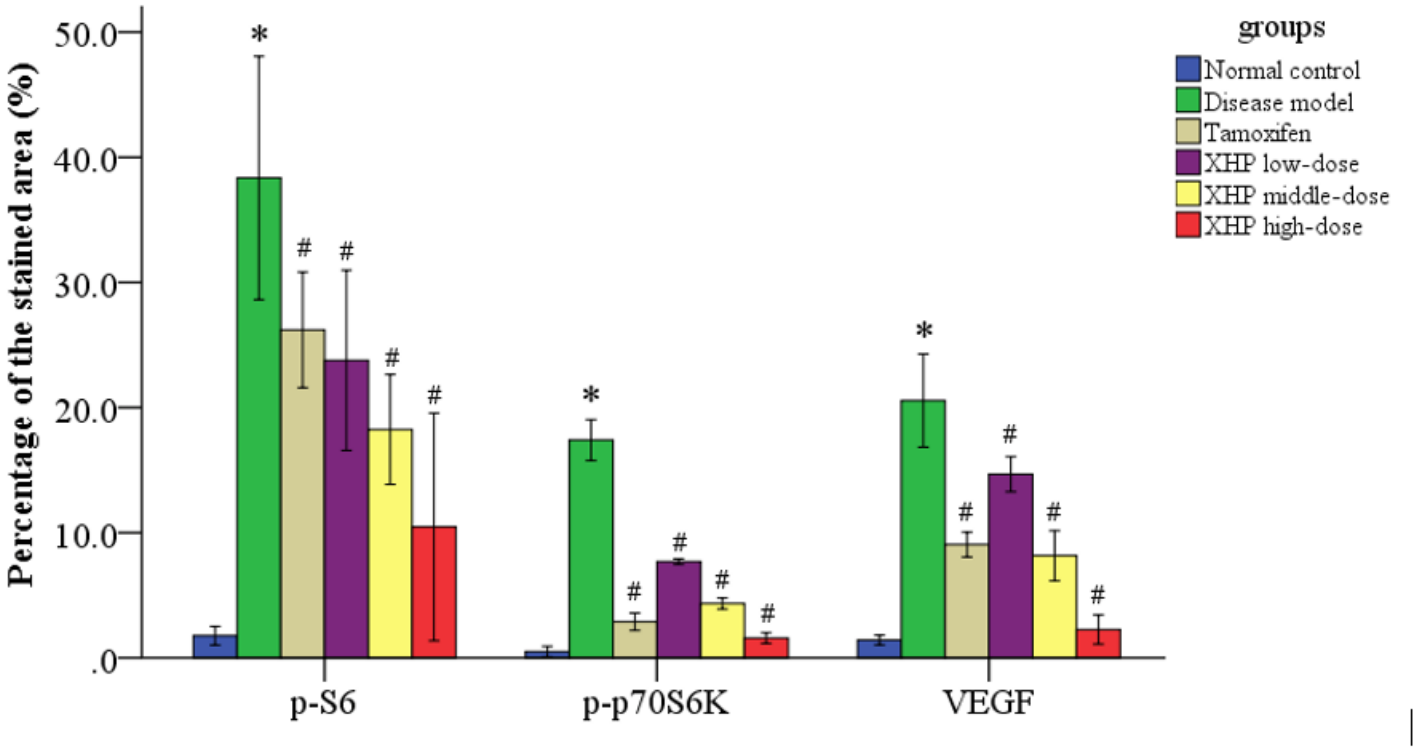

Figure 6

Protein expression of p-S6, p-p70S6K and VEGF in breast tissue of rats in each group. Compared with the normal control group, *P<0.05; compared with the disease model group, #P<0.05. (n=10 in each group).

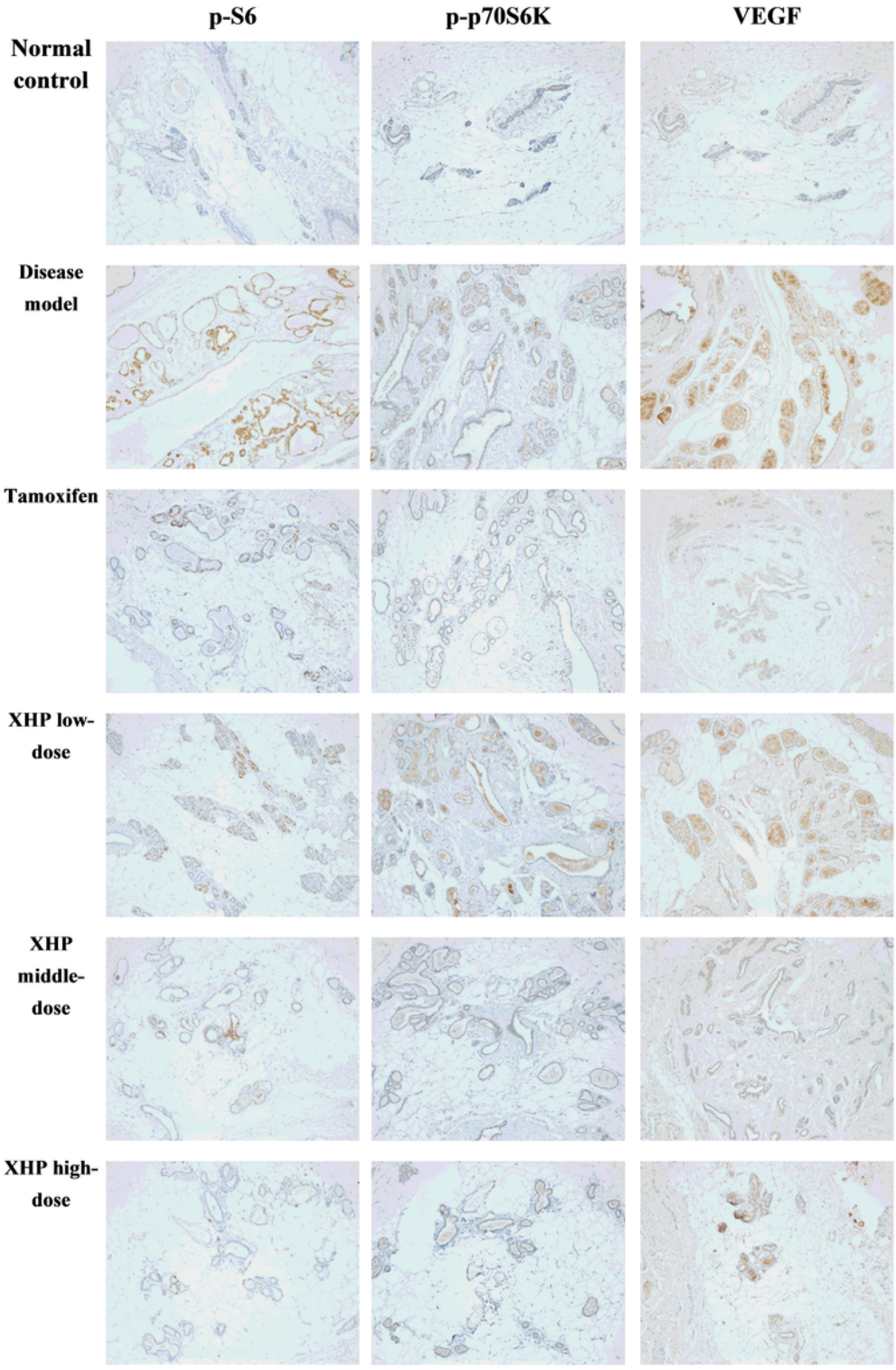

Figure 7

Protein expression of p-S6, p-p70S6K and VEGF in breast tissue of rats in each group (IHC, ×100).

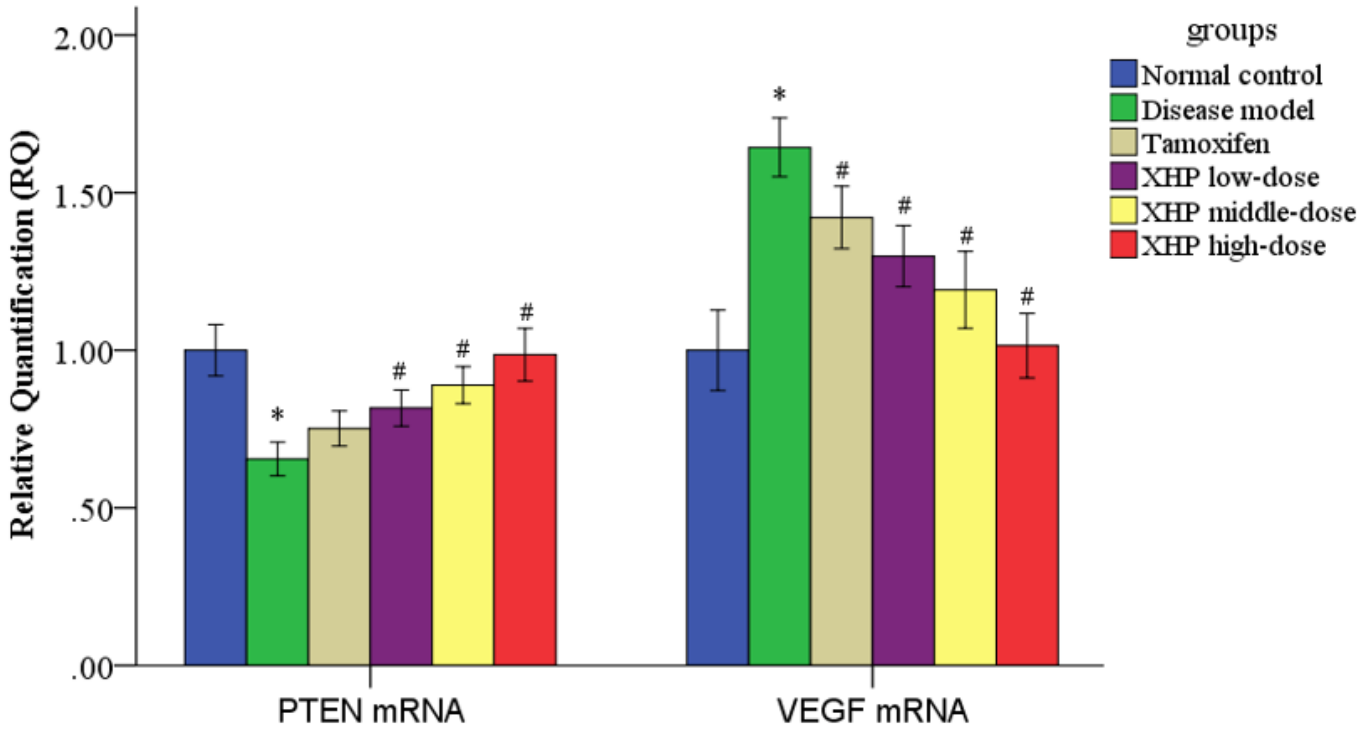

Figure 8

Relative expression levels of PTEN mRNA and VEGF mRNA in breast tissue of rats in each group