EUGENOL SUPPRESSES THE DEVELOPMENT OF ESTROGEN RECEPTOR-POSITIVE PRECANCEROUS BREAST LESIONS AND REGULATES ESTROGEN RECEPTOR-RELATED PROTEINS

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ABSTRACT

Introdution*: The breast precancerous lesions is a necessary stage of breast cancer, and most of drugs for the treatment of breast precancerous lesions have a lot of adverse reactions, so finding a new drug effects on precancerous lesions of breast cancer has important significance. Eugenol has therapeutic effect on tumor， However, the mechanism of the treatment is not clear, the purpose of this study is to evaluate eugenol anti-tumor effect on ER positive precancerous lesions of breast cancer.*

Material and methods*: 40 female SD rats were randomized into 5 groups, 8 in each. The groups were named Blank control group, Model group, Tamoxifen group, The low dose of Eugenol cream group (0.5mg) and The high dose of Eugenol cream group (1mg) respectively. The rats with breast precancerous lesions were induced by 7,12-dimethylbenz(a)anthracene (DMBA) combined with estrogen and progesterone injection. Drug interventions by tamoxifen(TAM) ointment and Eugenol cream were also launched during the model formation. The rats were executed after 70d. In MCF-10AT cell lines, we use 180μM eugenol to treat the cell lines, then observed 24 hours. And then the correlated indexes are detected by Western-blot and elisa.*

Results*: 1mg eugenol significantly delayed the pathology process of rat breast tissue models in precancerous lesions of breast cancer, meanwhile, 1mg eugenol reduced the level of serum estradiol(E2) and the reduce rate was 36%, increased progesterone(P) and the increase rate was 26%, in addition, the protein levels of ERα, GPER, p-AKT, GSK3-α, GSK3-β, p70s6k were decreased in the rat models and the decreased rates were respectively 31%, 53%, 57%, 58%, 56%, 93%, as well as MCF-10AT cell lines were treated with 180 μM eugenol, the decreased rates were respectively 35%, 60%, 63%, 57%, 42%, 68% ($P < 0.01$). However, in rat models the expressions of PTEN and ERβ protein were increased and the increase rates were 76%, 58% ($P < 0.05$), in MCF-10AT cell lines the increase rates of the expressions of PTEN and ERβ protein were 46%, 177%.*

Discussion*: eugenol is effective in the treatment of ER positive precancerous lesions of breast cancer, indicating that eugenol may be a new effective drug in treating ER positive precancerous lesions of breast cancer.*

Keywords*: Eugenol, estrogen receptor-positive, precancerous lesions of breast cancer, antitumor therapy, prevention.*

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Introduction

Breast cancer which is the most common tumor in women has been threatening to women's health. Recently, the incidence rate of breast cancer presents a dramatically rising trend. In China, breast cancer accounts for 15% of all female tumors(1). As we all know, precancerous lesions of breast cancer is a key stage in the process of breast tissue from lesions to cancer. At present, the cognition about the pathogenesis of precancerous lesions of breast cancer has not yet been clarified, but most scholars prefer to believe the imbalance of estrogen (estradiol) which is the initiating factor and key element that cause endocrine disorders and high sensitivity of breast tissues to hormone.

Precancerous lesions of breast cancer mainly show a relative or absolute increase in the level of estrogen, as well as the reduction or deficiency of progesterone, leading to the ratio imbalance of estrogen and progesterone ultimately. Moreover, local breast tissues present over-hyperplasia and even cystic lesion, carcinoma in situ, and develop to breast cancer at last when the body of the long-term influence of the imbalance relationship of estrogen and progesterone[2-4].

Most of the drugs for the treatment of breast precancerous lesions have a lot of adverse reactions. Tamoxifen is the main drug for the treatment of breast precancerous lesions, in 1970s, tamoxifen, which a nonsteroidal antiestrogen antitumor drug, began to be used in clinical, but it brings about much side effects, such as uterine endometrial hyperplasia, uterine endometrial tumor, thrombocytopenia, etc.[5]. However, percutaneous absorption of drugs can effectively reduce some adverse events of tamoxifen[6-7]. In addition, percutaneous administration can also inhibit breast hyperplasia, precancerous lesions and reduce local microcirculation blood flow velocity of breasts, so as to block the progression from precursor lesions to cancer[8].

Therefore, it is necessary to develop a kind of anti-tumor external drug with good effect and less adverse event. In recent years, more and more scholars to research natural antitumor drugs. Clove, a traditional Chinese medicine has been widely used for many years in clinical to treat hiccup, vomiting, nausea, diarrhea, abdominal pain, hernia, ringworm, etc. Eugenol is the main component of clove oil, is a kind of organic phenol, modern pharmacological research shows that it has anti-inflammatory, antipyretic and analgesic, anesthetic efficacy; also has antibacterial, antifungal, antioxidant, anticancer, avoid insect repellent and other activity, and metabolic side effects of small, low residue, so favored in the the pharmaceutical and cosmetics[9]. Food and Drug Administration (FDA) also has indicated that eugenol is one of the safe food ingredients with no toxic side effects, besides, eugenol can obviously inhibit ER positive breast cancer cell lines[10].

So in this study, we have concluded that eugenol has an obvious anti-tumor effect on ER positive breast precancerous lesions by researching eugenol act on breast precancerous lesion rats model and cell lines MCF-10AT.

Material and methods

Animals

40 healthy female Specific Pathogen-Free(SPF) Sprague-Dawley(SD) rats with no pregnancy, weighing 160-180g, were obtained from Guangdong Medical Laboratory Animal Center (Guangdong, China, Animal quality license number: SCXK (Yue) 2008-0002). The rats were housed under the condition of controlled temperature 25°C in SPF animal houses, animal experimental management center, Jinan University. All these rats were randomized into 5 groups, 8 in each. The groups were named Blank control group, Model group, Tamoxifen group, The low dose of Eugenol cream group (0.5mg) and The high dose of Eugenol cream group (1mg) respectively. After feeding with 1 week, the rats had regular breast depilation with the nipple as the center around 1cm of each pair of breasts by hair-removal cream. Modeling and drug intervention were started.

DMBA combined with estrogen and progesterone induced rat models of breast precancerous lesion and drug intervention

The blank control group were naturally fed and other groups using modeling agent DMBA(Tokyo Chemical Industry Co., Ltd., commodity code: D0677) with 70 mg/kg disposable gastric lavage[11]. After the second day, The rats were injected Estradiol Benzoate Injection (0.5mg/kg, per day) which were purchased in Shanghai General Pharmaceutical Limited by Share Ltd (Batch number: 13050) into the muscle of medial side of hind leg for 3 days, and injected Progesterone (4 mg/kg, per day) which were purchased in Baiyun Mountain pharmaceutical the Limited by Share Ltd (Batch number: 130316) from the fourth day, then observed a day, lasting 14 cycles (5 days for a cycle) in total. During the 14 cycles, the breasts of blank control group were smeared by cream excipient with 0.2g for each rat. Tamoxifen group was treated with 1% tamoxifen cream. Eugenol groups using the corresponding concentrations of eugenol cream agent with 0.2g for each rat, respectively. After 14 weeks, rats in each group were injected with 2% nembutal in abdominal, then killed the rats after the blood in abdominal aorta was obtained. Observation and judgment of breast tissues pathological changes using HE staining.

Cell culture and Drug treatment

MCF-10AT cell lines were originally obtained from the Karmanos Cancer Institute (KCI) of the United States. MCF-10AT cells were cultured in culture-flask with 5ml 5% horse serum (hyclone; Lot No.AYB6028) and complete DMEM/F-12 culture solution (GIBCO; Lot No.122958) under 37°C, 5%CO2 conditions. Cells in logarithmic growth phases were used to do experiment. 180μM PBS were added to blank control group, and the same dosage were put into tamoxifen group and eugenol group, then observed 24 hours.

Western-blot detection

Total protein in rat mammary tissues and MCF-10AT cell were extracted by RIPA, BCA protein assay kit quantitative were used to calculate the capacities of protein samples, then using SDS-PAGE electrophoresis.

After electrophoresis, taking the proteins into PVDF membrane, then closed by 5% evaporated skimmed milk for 2 h. After 2 h, the first antibodies: Anti-Estrogen Receptor α(clone E115) (Cat.#04-227) which was purchased from Millipore, ERβ(E101) Antibody (Cat.#BS2429) which was purchased from Bioworld, GPER(Cat.ab39742) which was purchased from Abcam, Phospho-Akt(Ser473)(Cat.#9271), PTEN(138G6)(Cat.#9559), GSK-3α(Cat.#4337), GSK-3β(Cat.#9315), p70S6K(Cat.#2708) which were purchased from cell signaling technology in USA (1:1000) were added and put them for rotary shaker for slow shaking at 4°C for the night. On the second day, washing the PVDF membrane 10 times for 5 min per one time in rotary shaker , then added Goat anti-Rabbit IgG-HRP (1:3000) which was purchased from ASBIO and incubated at room temperature for 2 h. After that, repeated the washing method above. Protein bands were visualized by using an ECL kit and densitometric analysis of the western blot results was performed with Image J analysis software.

Elisa

The serum from extracted abdominal aortic blood was obtained by 4°C,4500rpm/min centrifugation after static for half an hour, and then ELISA was used to detect the levels of E2 and P in the serum of rats. Adding samples into 96 well plate, then incubated for 30 min at 37°C after to sealing film. Subsequently, Removed liquid and patted dry, then added 50μl enzyme standard reagent and incubated for 30 min at 37°C, after that, remove liquid and pat dry again. At last, mixing the chromogenic agent which avoided light for 15min at 37°C with additional 50μl stop solution, then were detected by Microplate Reader under 450 nm wavelength.

Statistical analysis

The experimental data was analyzed by SPSS 13.0. Results of measurement were expressed by mean ± SD. Differences between two groups were assessed by Mean-Whitney Test and t-Test. Unordered variable comparisons were carried out by Kruskal-Wallis. Ordinal variables were used factorial design variance analysis of two factors and two levels. A P value of less than 0.05 was considered statistically significant.

Results

Morphological observation of mammary gland in rats[12]

The experimental results showed that the disease model groups of rats displayed precancerous lesions ($P<0.05$) when compared with the blank control group, indicating that DMBA combined with estrogen and progesterone can better induce breast precancerous lesions in rat models (Table1).

Table 1: The hyperplasia degree of breast tissue in rats.

Group	Number of teats	Non-Proliferation	General hyperplasia	Precancerous lesions	Invasive carcinomas
Control	96	88	8	0	0
Model*	96	0	6	74	16
Tamoxifen▲	96	9	20	62	5
Eugenol(0.5mg)▲	96	2	12	70	12
Eugenol(1mg)▲	96	10	16	63	7

Note: Comparative hyperplasia degree of breast tissue in rats of each group, $\chi 2=240.430$, $P=0.000$, according to $\alpha=0.05$ level, it suggested that there were differences in each group, there was statistical significance. Wilcoxn tested by comparison of two independent samples, *=compared with control group ($P<0.05$), ▲= compared with model group ($P<0.05$)

In addition, during the course of building models, the rats showed the various stages developmental features of breasts, including normal development of breast tissues, normal hyperplasia, precancerous lesion and invasive carcinoma. After the therapeutic interventions in tamoxifen and eugenol cream with different doses, we found that the grade of rats with precancerous lesions of breast cancer in tamoxifen group and eugenol group have decreased ($P < 0.05$), indicating that tamoxifen and eugenol can reduce the pathological lesions of rat mammary tissues (Figure 1), further

to reduce the incidence of breast cancer in rats, indicating eugenol and tamoxifen have an effect on anti precancerous lesions of breast cancer.

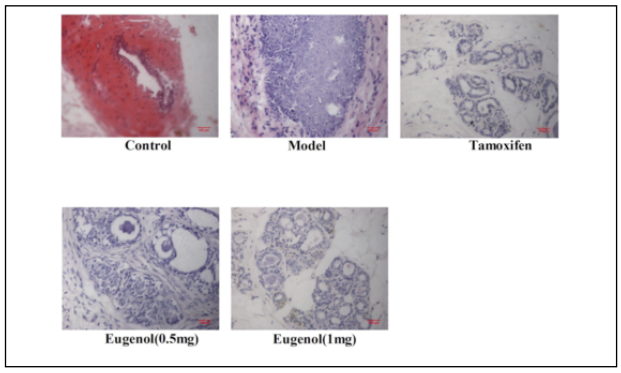

Figure 1: The pathological changes of breast tissue in rats of each group (HE×200).

The levels of E2 and P in rat models of precancerous lesions of breast cancer with the intervention of eugenol

ELISA results showed that compared with the blank control group, the level of E2 in rats of disease model groups increased significantly ($P < 0.05$) and P had a trend of reduction ($P < 0.05$), demonstrating that DMBA combined estrogen and progesterone can induced endocrine disorders of rats in breast precancerous lesions. Besides, the level of serum E2 and P showed descending and increasing tendency respectively in different degrees under the intervention of tamoxifen and eugenol ($P < 0.05$), indicating eugenol can regulate the endocrine disorders of estrogen and progesterone in rat models of precancerous lesions of breast cancer (Figure 2).

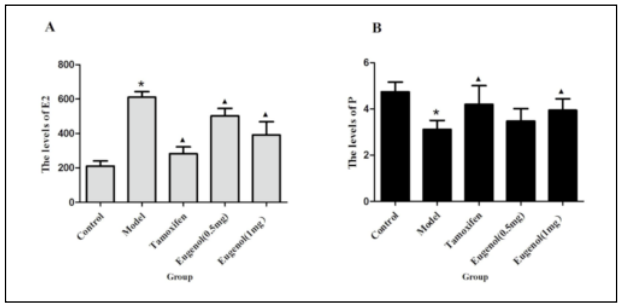

Figure 2: The serum levels of estradiol (E2) and progesterone(P) in rats. A=the levels of E2 in rats. B=the levels of P in rats.*=compared with control group(P<0.05). ▲=compared with model group(P<0.05).

Effects of eugenol on the rat breast tissues related protein in precancerous lesions

Western-blot technology was used to detect the key protein expressions of eugenol act on rat breast tissues in precancerous lesions. The results showed the protein expressions of ERα, GPER, p-AKT, GSK3-α, GSK3-β, p70s6k in model groups were increased ($P < 0.05$), and rose by 1.06, 0.77, 1.59, 2.65, 1.30, 5.94 times respectively, while the protein expressions of PTEN, ERβ were decreased ($P < 0.05$) and the decreasing trend achieved by 0.33, 0.57 times respectively, when compared with the blank control group. The protein expressions of ERα, GPER, p-AKT, GSK3-α, GSK3-β, p70s6k in tamoxifen group and eugenol group, when compared with the model group, were decreased ($P < 0.05$) and the decreasing were reached by 0.31, 0.53, 0.57, 0.58, 0.56, 0.93 times respectively, while the protein expressions of PTEN, ERβ were increased ($P < 0.05$), and rose by 0.76, 0.58 times respectively(Figure 3). All this results mentioned above showed Eugenol is effective against ER positive breast cancer in precancerous lesions.

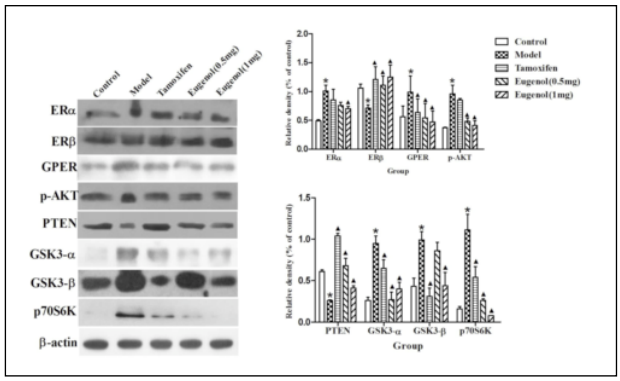

Figure 3: The protein expression levels of ERα, ERβ, GPER, p-AKT, PTEN, GSK3-α, GSK3-β, p70s6k in rats. *=compared with control group(P<0.05). ▲=compared with model group(P<0.05).

Effects of eugenol on related protein in MCF-10AT cell lines

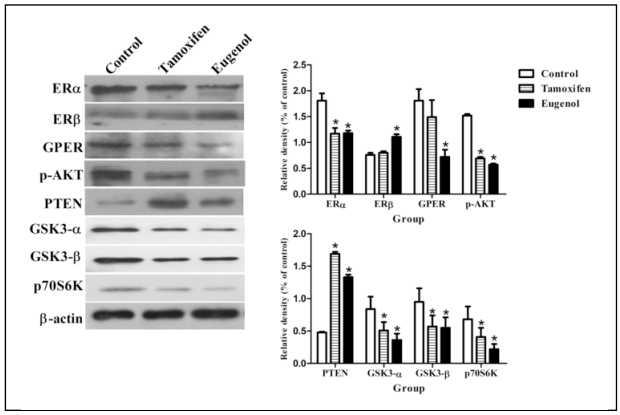

Figure 4: The protein expression levels of ERα, ERβ, GPER, p-AKT, PTEN, GSK3-α, GSK3-β, p70s6k in MCF-10AT cell lines. *=compared with control group(P<0.05). ▲=compared with model group(P<0.05).

The results of Western blot showed that: the protein expressions of ERα, GPER, p-AKT, GSK3-α, GSK3-β, p70s6k in tamoxifen group and eugenol group, compared with the blank control group, were decreased ($P < 0.05$) and the decreasing were reached by 0.35, 0.6, 0.63, 0.57, 0.42, 0.68 times, respectively, while the protein expressions of PTEN, ERβ were increased ($P < 0.05$), and rose by 0.46, 1.77 times, respectively (Figure 4), indicating Eugenol is effective on ER positive precancerous lesions of breast cancer.

Discussion

Breast tissue is one of the main target organs of female hormones. The physiological and pathological changes of mammary glands are closely related to the regulation of hormones. All these is stimulated by the comprehensive effect of hypothalamic-pituitary-ovarian axes and other endocrine hormones. Estrogen (E) and progesterone (P), as an important hormone in women, play important roles in the physiological and pathological processes of breasts. 80% breast tumors was ER positive which is a directive sex hormone biomarkers[13]. In animal models, ER positive breast tumors have influence on the differentiation capability, chemotherapy and endocrine therapy response and disease prognosis.

For ER / PR positive breast cancer, nowadays, 60% will use endocrine drugs to treat, such as tamoxifen, toremifene, letrozole, etc[14]. But some ER positive breast tumors are not sensitive to endocrine therapy[15]. GPER which is called as G protein coupled estrogen receptor, also known as G protein-coupled receptor 30 (GPR30), combined with estrogen or estrogen analogues, can activate multiple protein kinases, then quick start gene regulation and transcription. GPER, a new estrogen receptor, which is highly expressed in estrogen related tumors, such as breast cancer, endometrial cancer, and so on. GPER has a corresponding fast signal transduction and transcriptional regulation[16, 17]. GPER participates in the regulation of reproductive, nerve, endocrine, immune and cardiovascular systems, as well as involves in important biological reactions such as metastasis of breast cancer[18-19].

Samartzis et al.[20] indicated that the expression of GPER in breast cancer has two different immune group patterns of the cytoplasm and nucleus by evaluating the association for clinical pathological parameters and patient's overall survival rate in primary breast invasive carcinoma, which may reflect different biological characteristics. Prossnitz et al.[21] found that when GPR30 was completely silenced in MCF-7 cells, it could reduce the 17β-estradiol (E2) to inhibit the TGF-β pathway, in addition, it also induced mitogen activated protein kinase (MAPK) activation, interfere the activation of Smad protein to block the development of breast cancer. Wang et al.[22] showed that GPER mediates the effects of estrogen in different tissues under physiological and pathological conditions. The SiRNA interference of GPER can inhibit cell proliferations of breast and ovarian cancer. This study has showed that eugenol can reduce the serum estrogen level in rat models of breast precancerous lesions and effectively regulate estrogen receptor, such as ERα, ERβ, GPER by intervene the rat models and MCF-10AT cell lines of precancerous lesions of breast cancer, indicating eugenol can inhibit ER positive precancerous lesions of breast cancer.

The high expression of AKT protein in breast cancer is closely related to the ineffective treatment of anti estrogen[23,24]. Zhou et al.[25], study 165 invasive breast cancer patients, compared with normal breast epithelial cells, breast fibroadenoma, mammary hyperplasia in immunohistochemistry, the results showed P-AKT protein increase straightly from normal mammary epithelial cells hyperplasia to dysplasia, then tumor invasion, besides, the survival analysis also indicated that Akt phosphorylation decreased disease free survival. PTEN function may be loss during the course of mammary gland carcinogenesis, causing the accumulation of the second messenger PIP3, leading to excessively activate AKT.

At present, found that PTEN gene mutated and the protein absence in a variety of tumors, suggesting that PTEN plays an important role in occurrences and development of tumors. It is a common molecular event that PTEN presents low expression in breast cancer[26], while PTEN deletion has an important influence on the treatment of ER positive breast cancer[27].

Recent studies have found that the role of GSK-3β in cancer is getting more and more concerns[28-29]. Other studies have suggested that the rise of GSK-3β protein is coordinated with E2 ascending, meanwhile GSK-3β regulated E2 in promoting cell proliferation and differentiation [30]. So GSK-3β has an important effect on the increase

of estrogen receptor[31]. Bachelder et al.[32] had found that normal GSK-3β activity is a necessary factor to maintain the structure of mammary epithelial cells. Dembowy et al.[33] have suggested that the imbalance of GSK-3α and GSK-3β make mammary gland significantly increased, Kim et al.[34] had indicated GSK-3 inhibitors can inhibit the growth of breast tumor cells by reducing the expression of GSK-3α and GSK-3β. P70S6K over-expression can make cell growth abnormal and aberrant regulation, bringing about unlimited cell proliferation, and finally come into being tumor. P70S6K can activate 40S ribosomal protein S6 to regulate the synthesis of protein, P70S6K also stimulates the HER2 receptor, estrogen receptor and PI3K Akt signaling pathway in breast cancer, as well as participates in breast carcinoma proliferation and invasion. Segatto et al.[35] had indicated that p70S6K can induce apoptosis and inhibit survival in breast cancer cell lines. Baxi et al.[36] had reported the phosphorylation of p70S6K can prevent cell cycle and reduce cell proliferation in breast cancer. Segatto et al.[37] had showed the loss of p70S6K activity can obviously reduce the incidence rate of breast tumors in the nude mice by observing p70S6K antagonistic effect in breast cancer microenvironment. This study has indicated eugenol could decrease the protein expressions of p-Akt, GSK-3 and P70S6K which are closely related with estrogen in rat models and MCF-10AT cell lines of precancerous lesions of breast cancer, as well as increased the protein expression of PTEN, so as to prevent the precancerous lesions of breast cancer.

In summary, this research results have to show eugenol is effective in the treatment of ER positive precancerous lesions of breast cancer, indicating that eugenol may be a new effective drug in treating ER positive precancerous lesions of breast cancer. So it is necessary to do further study for eugenol to evaluate the long-term effect on breast precancerous lesions.

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