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Preventive Acupuncture Intervenes Against Cell Proliferation in 7,12-Dimethylbenz(a)Anthracene-Induced Breast Cancer Model Rats Via the PTEN-PI3K/AKT Signaling Pathway

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Abstract:

Acupuncture as a safe and effective alternative therapy in traditional Chinese medicine (TCM) which has shown unique advantages as well as achieved efficacy in the field of clinical breast cancer prevention. However, there is a lack of experimental evidence regarding the mechanism of action of acupuncture on the process of breast cancer formation. 6-week-old female SD rats were subjected to a single intragastric administration of DMBA at 100mg/kg body weight (BW) to induce spontaneous breast cancer. Preventive acupuncture therapy starting the second week after DMBA administration performed thrice a week for 16 weeks. Tamoxifen (3 mg/kg BW, ig, qd) starting the day right after DMBA administration and lasting for 16 weeks served as a positive control treatment. After treatment, Tumor weight was monitored and immunohistochemical analysis of PI3K/Akt Signaling Pathway-related Proteins and proliferation index of the mammary tumors were performed. Average tumor weight per rat was significantly decreased in PA and tamoxifen treatment groups compared with Model group ($P<0.01$). Further immunohistochemical analysis revealed that expression of Ki67 and p-AKT in tumors from rats treated with PA or tamoxifen were significantly lower

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than those from Model group（P＜0.01）while expression of PTEN in tumors from rats treated with PA or tamoxifen were significantly higher than those from Model group（P＜0.01). Preventive acupuncture therapy can upregulation the expression of PTEN, regulate the PI3K/Akt signaling pathway-related proteins, inhibit the expression of proliferation index Ki67 in mammary tumors, so as to exert an inhibitory effect on DMBA-induced mammary tumorigenesis in female rats.

Keywords: *Preventive acupuncture, The PI3K/Akt signaling pathway, mammary tumors, Cell proliferation.*

I. INTRODUCTION

Breast cancer is the most common female malignancy with high morbidity and mortality worldwide. In 2020, the number of new cases of breast cancer has accounted for 11.7% of the overall cancer incidence [1], which is one of the four most prevalent cancers. Breast cancer is a serious threat to women's health and life, and has a great impact on society, economy, family and women's psychology. At present, the treatment of breast cancer in Western medicine is mainly based on anti-cancer treatment, which is mainly performed by surgical excision with radiotherapy and chemotherapy in clinical practice. However, the recurrence rate of such approach is 50%, and the adverse effects on patients are large and cause great pain. Therefore, it is urgent to explore the pathogenesis and find effective prevention and treatment methods.

The pathogenesis of breast cancer is influenced by many factors, and the pathogenesis is not yet clear. It has been shown that the development of breast cancer is closely related to cell proliferation and apoptosis [2-3], and the phosphatidylinositol 3-hydroxy kinase (PI3K)/serine/threonine kinase (Akt) signaling pathway is involved in and promotes the proliferation and differentiation of breast cancer cells [4]. PI3K, Akt and oncogenes (phosphataseand tensin hology deleted from chromosome 10, PTEN) and several proteins with different functions are involved in the process of breast cancer development and progression, and are important targets for the treatment of breast cancer [5-7].

It has been shown that breast cancer undergoes a spectrum of "normal → general hyperplasia → atypical hyperplasia → carcinoma in situ → invasive carcinoma" [8], and this process can be reversibly transformed under certain conditions. Therefore, it is more important to explore effective preventive treatments to stop or reverse breast cancer than to treat it after it has developed. In recent years, Chinese medicine, especially acupuncture therapy, has occupied an important position in the field of breast cancer prevention. Meridian differentiation preventive

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acupuncture therapy is a kind of acupuncture therapy based on the theory of "preventive treatment of disease" in Chinese medicine, combining the acupoint theory and the circulation law of breast meridians, with main points of ST36, CV17, SP6. This therapy can prevent the malignant transformation of normal breast epithelial cells by stimulating the positive energy of the meridians. In addition, a previous study by our group found that preventive acupuncture under the guidance of meridian discrimination could not only reduce the incidence and number of tumors in spontaneous mammary tumors in female rats after denudation [9], but also reduce the incidence and number of tumors in DMBA-induced mammary carcinoma in rats and slow down the pathological evolution process [10]. However, most of the studies are still at the stage of clinical efficacy observation, and the mechanism of action of acupuncture in the treatment of breast cancer is not yet clear. Therefore, in this study, we investigated the mechanism of the effect of acupuncture on breast cancer through animal experiments, and proposed to confirm that acupuncture may affect the PTEN/PI3K/AKT pathway in rat mammary tumor tissues and regulate the expression of its related genes to effectively inhibit mammary tumor growth and achieve the goal of preventing breast cancer.

II. MATERIALS AND METHODS

2.1 Chemicals

7,12-Dimethylbenz(a)anthracene (DMBA) (purity≥95%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Tamoxifen citrate tablets were purchased from Yangzijiang Pharmaceutical Group Co., Ltd., lot No.:17041311.

2.2 Animals and treatment

2.2.1 Animals

Forty female SD rats (SPF, 6 weeks old, weighing 180–220 g obtained from Hunan Lake Scenery Company Limited, license number: SCXK (Hunan) 2016- 0002) were housed in polycarbonate cages at controlled temperature 24±2℃ under a 12h light/12h dark cycle (lights on at 7:00 am). The animals had free access to standard chow diet and water. All experimental procedures were carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals. All animal studies were approved by the Kunming Animal Care and Use Committee.

2.2.2 Grouping

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After a week of acclimatization, 40 rats were randomly divided into four groups with 10 rats in each group as follows: the Control group; the Model group; the Tamoxifen group and the PA group.

2.2.3 Establishment of animal model and treatment

Spontaneous mammary tumorigenesis was induced by a single dose of DMBA (100 mg/kg in 0.5 ml Sesam oil) through intragastric gavage on Day 0 in rats of the Model group, the Tamoxifen group and the PA group. Rats from the Control group were given an equal volume of sesame oil. Acupoints including Zusanli(ST36), Danzhong(CV17), Sanyinjiao(SP6) were located according to the atlas of acupoints in rat according to Xu et al [11].The rats were fixed on a self-made fixator, and a special acupuncture needle 75% ethanol was used for routine disinfection, followed by oblique puncture of SP6 at 45° downward and needle insertion of about 5 mm; direct puncture of ST36 and needle insertion of about 7 mm; horizontal puncture of CV17 at 15° downward and needle insertion of about 3 mm, and twirling and tonifying method for 27 turns. After acupuncture, each rat was observed for 20 minutes in a single cage, and those with needle detachment were timely supplemented. Preventive acupuncture therapy was applied to rats of the PA group every 3 days for 16 weeks. Rats of the Tamoxifen group received tamoxifen citrate dissolved in double diluted water through intragastric gavage at a dosage of 3mg/kg every day and lasted for 16 weeks. All these treatments started from the day after DMBA administration and lasted till the end of the experiment.

2.3 Necropsy and evaluation of mammary tumorigenesis

General status of the rats was monitored every day and body weight was recorded weekly. Mammary glands of all rats were observed and palpated every 3 days from the first day after DMBA administration till the end of the experiment. After 16 weeks of treatment and observation, all the rats were fasted overnight before sacrifice and autopsy of each animal was then carried out upon anesthesia with ketamine (50 mg/kg BW; *i.p.*). All the tumors of each experiment group were removed. The length, width and height of each tumor were measured by a caliper before they were weighed, counted and photographed. Finally, all the tumors were fixed in 10% neutral formalin solution for at least 24 hours before histopathological analysis.

2.4 Immunohistochemical analysis

Tissue sections of mammary tumor were subjected to immunohistochemical analysis using standard procedures. Immunohistochemical procedures were optimized by testing different antigen retrieval methods and negative controls. Rabbit polyclonal antibodies against PTEN,

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p-AKT were all purchased from Servicebio Technology Co. Ltd. Wuhan, China. Positive staining of tumor cells was quantitatively evaluated by IMAGE-PRO PLUS 6.0 and expressed as mean optical density. Positively stained areas were compared between different experiment groups. For the evaluation of cellular proliferation in the mammary tumors, Ki67 (Servicebio Technology Co. Ltd., Wuhan, China) immunohistochemical staining were performed and positively stained cells were counted on each tissue section at 5 random HPF. The percentage of Ki67 positive cells was assessed as the number of positive nuclei per 100 cells. The PTEN staining positive score was calculated by referring to the method designed by Xing et al[12]. Five high-power fields (×400) were randomly taken from each section under a light microscope, and the percentage of positive cells and staining intensity were scored in each field, and 1, 2, 3, and 4 points were scored according to the proportion of positive cells to the total number of cells ≤ 25%, 26% to 50%, 51% to 75%, and > 75%, respectively; positive cell staining was scored 0, 1, 2, and 3 points according to no color, weak (yellowish), moderate (brownish-yellow), and strong (brownish-brown), respectively. The values corresponding to the percentage of positive cells and staining intensity were summed as integral scores.

2.5 Statistical Analysis

Statistical analysis was performed using the SPSS Statistics 19 Software (IBM), and GraphpadPrism5.0 software was used to sort and generate statistical maps; Results are presented as means ± standard deviation (SD) for each experimental group, one-way analysis of variance was used, t-test was used for differences, and Statistical significance of differences was considered at a p value < 0.05.

III. RESULTS

3.1 Effect of preventive acupuncture on mammary tumor growth in DMBA-induced breast cancer model rats

As shown in the photos of Fig 1a, mammary tumors from each experiment group were dissected upon sacrifice and arranged in order of size. There were 15 tumors in total from rats in model group while 6 and 8 tumors were obtained from rats of Tamox group and PA group respectively. As shown in the Fig 1b, the mean tumor mass of rats in both tamoxifen and preventive-acupuncture groups decreased significantly compared with the model group ($P<0.01$), suggesting that preventive acupuncture has an inhibitory effect on the growth of DMBA-induced mammary tumors.

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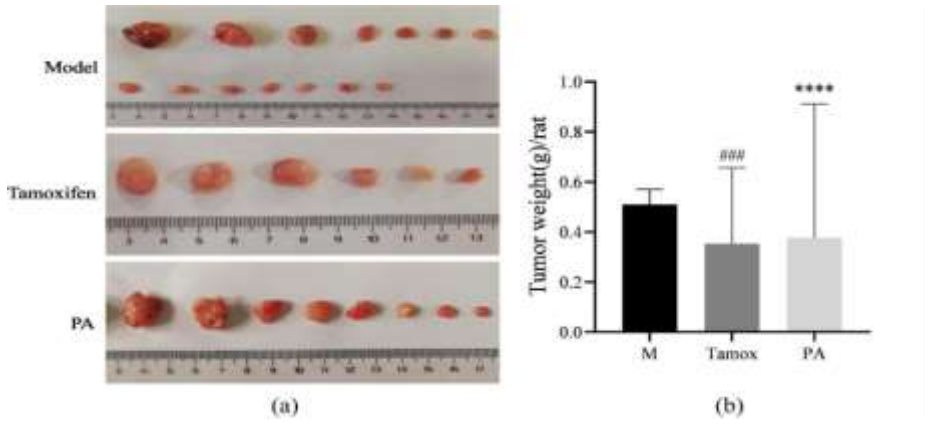

Fig 1 Effect of preventive-acupuncture on the mammary tumor growth of DMBA-induced breast cancer model rats. (a) Photographs of all tumors dissected from rats of the Model, Tamoxifen and PA group arranged in order of size.; (b) Effect of Preventive Acupuncture on average tumor weight per rat in individual experiment group of DMBA-induced rats. M: the model group; Tamox: the tamoxifen group; PA: the preventive acupuncture group. Data are represented as means ± SD (n = 8 for Model and n=10 for Tamoxifen and PA group). ####P＜0.001, ****P＜0.0001, compare with the model group.

3.2 Effect of preventive acupuncture on proliferation index of Mammary Tumors

As shown in Fig 2a, Ki67 positive cells were detected by immunohistochemistry in brown color, and a large number of positive Ki67 expression was seen in the mammary tissues of the model group, and a significant decrease in the number of Ki67 expression positive cells was seen in the mammary tissues of the tamoxifen group as well as the preventive acupuncture group. Percentage of Ki67 positive cells in tumor tissue sections were shown in Fig 2b, the Ki67 positive cell expression rate was analyzed by Image-Pro Plus 6.0 software, and it was found that the Ki67 positive cell rate was significantly lower in the breast tissue of each treatment group compared with the model group ($P < 0.01$), as shown in Fig 2. These data suggest that preventive acupuncture treatment may inhibit tumor cell proliferation by decreasing Ki67 expression.

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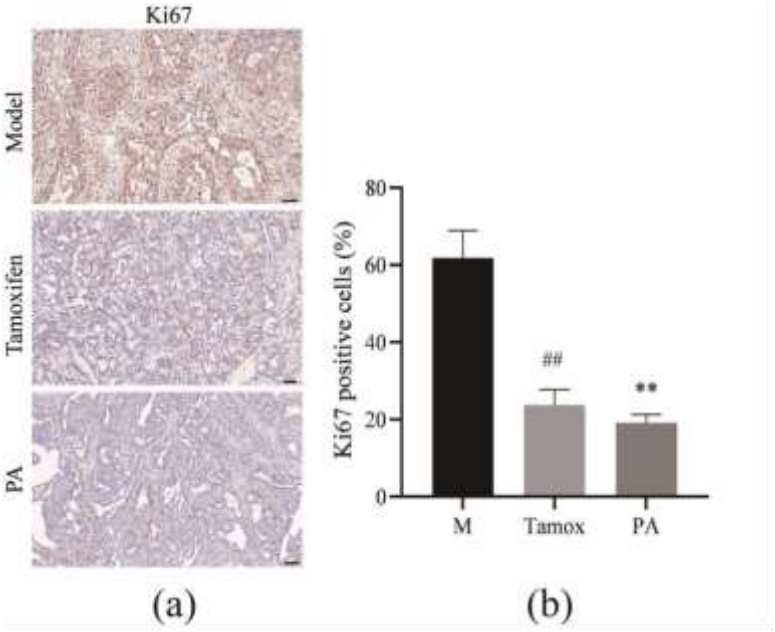

Fig 2 Effect of preventive-acupuncture on Ki67 expression in mammary tissue of rats with DMBA-induced breast cancer. The expression level of Ki67 in rat mammary tissue was detected by immunohistochemistry, brown color shows positive expression. (a) shows the immunohistochemical staining of Ki67 in mammary tumor tissue sections of rats in each group; (b) Percentage of Ki67 positive cells in tumor tissue sections were numerated under 5 random HPF and calculated by Ki67 positive nucleal staining cells/total cells. Data is expressed as mean ± SD (n=5). ## $P < 0.01$, **$P < 0.01$, compare with the model group.

3.3 Effect of preventive acupuncture on PTEN expression in mammary tissues of rats with DMBA-induced breast cancer

As shown in Fig 3a, immunohistochemical staining detected a small number of PTEN-stained cells in the mammary tumor tissues of the model group, and the staining was shallow. In the tamoxifen group and the preventive acupuncture group, the number of PTEN-stained cells was significantly increased and the staining was darker. The PTEN staining positive score were shown in Fig 3b, the staining scores showed that the positive expression scores of breast tissues in both tamoxifen and preventive acupuncture treatment groups were significantly higher compared with the model group ($P < 0.01$ for tamoxifen group; $P < 0.001$ for preventive acupuncture treatment group), and the increase was more significant in the preventive acupuncture group ($P < 0.001$). It is suggested that preventive acupuncture treatment may affect tumor cell proliferation by upregulating PTEN expression.

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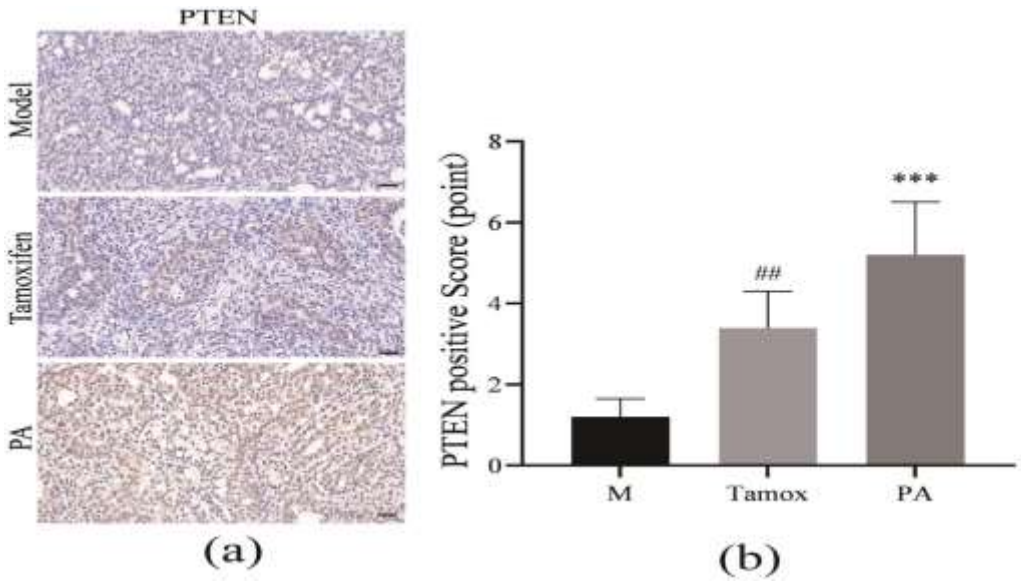

Fig 3 Effect of each preventive acupuncture on PTEN expression in mammary tissue of rats with DMBA-induced breast cancer. The PTEN expression level of rat mammary tissue was detected by immunohistochemistry, and the brown color shows PTEN staining. (a) Shows the PTEN immunohistochemical staining of mammary tumor tissue sections of rats in each group; (b) Shows the PTEN staining positive score. Data is expressed as mean ± SD (n=5). ## $P < 0.01$, ***$P < 0.001$, compare with the model group.

3.4 Effect of preventive acupuncture on p-AKT expression in mammary tissues of rats with DMBA-induced breast cancer

The p-AKT immunohistochemical staining of rat mammary tumor tissue sections in each group were shown in Fig 4a, immunohistochemical staining detected p-AKT staining in the mammary tumor tissues of the model group, with a higher number of positive cells and darker staining. p-AKT stained cells were significantly reduced in the mammary tumor tissues of the tamoxifen group as well as the preventive acupuncture group, and the staining was lighter. The number of p-AKT positive cells under 5 random HPF were shown in Fig 4b, compared with the model group, the number of p-AKT-positive cells in breast tissue was significantly reduced in both the tamoxifen group and the preventive acupuncture treatment group ($P<0.0001$). These results indicate that preventive acupuncture treatment may promote tumor cell proliferation by inhibiting p-AKT expression.

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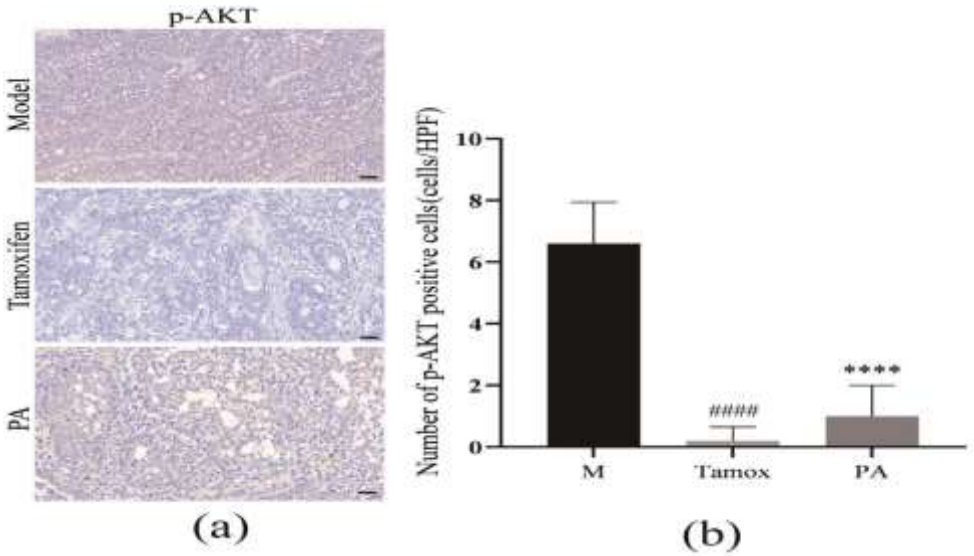

Fig 4 Effect of preventive-acupuncture on p-AKT expression in mammary tissue of rats with DMBA-induced breast cancer. The expression level of p-AKT in rat mammary tissue was detected by immunohistochemistry, and the brown color shows p-AKT staining. (a) shows the p-AKT immunohistochemical staining of rat mammary tumor tissue sections in each group; (b) shows the number of p-AKT positive cells under 5 random HPF. Data is expressed as mean ± SD (n=5). #### $P < 0.0001$, **** $P < 0.0001$, compare with the model group.

IV. DISCUSSION

Breast cancer is a malignant tumor that occurs in the glandular epithelial tissue of the breast and is currently a disease with a high mortality rate among breast diseases. At the same time, it often brings serious physical and psychological harm to patients because of the specificity of its lesion site [13]. Dimethylbenzanthracene (DMBA) is a polycyc1icaromatic hydro-carbons (PAHs) compound, which is a potent carcinogenic agent [14]. Cao et al. [15] showed that the DMBA-induced breast cancer model had poorly differentiated and highly malignant cells, and the biological behavior of the tumor cells and the pathomorphology of the tumor were similar to those of human breast cancer. In this study, we explored the preventive and blocking effects of meridian-discriminative preventive acupuncture on breast tumorigenesis using the DMBA-induced spontaneous mammary tumor model in rats, and further explored the possible mechanisms of its action on breast cancer prevention and treatment from the PI3K-AKT cell proliferation pathway, providing a modern molecular biological basis for acupuncture prevention and treatment of breast cancer, with the aim of exploring new ways of traditional Chinese medicine in cancer prevention and treatment.

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PI3K-Akt signaling pathway, as one of the important intracellular signaling pathways, is dysregulated in expression in many human tumors. It was found that PI3K-Akt signaling pathway is activated up to 70% in breast cancer [16], and the abnormal PI3K/Akt signaling pathway in breast cancer patients is associated with PTEN inactivation and phosphorylated Akt level, downstream biomolecules and molecular typing, and other factors. It plays a key role in promoting cell proliferation and inhibiting apoptosis in vivo by affecting the activation status of multiple downstream effector molecules. The PI3K/Akt signaling pathway has been reported to be mainly involved in cell proliferation and differentiation [17], which is regulated by various factors, including PTEN, CTMP, SHIP2, etc. PTEN, as a tumor suppressor gene, can negatively regulate the PI3K/Akt pathway by catalyzing the dephosphorylation of PIP3 to produce PIP2, which antagonizes the activity of PI3K. Akt activation further activates its downstream factors, such as Bcl-2 family, E2F, glycogen synthase kinase 3 (GSK3) and S6 protein kinase, to regulate cell proliferation, apoptosis and angiogenesis, etc. Therefore, PI3K/Akt signaling pathway is an important intracellular signaling pathway involved in cell proliferation, differentiation and metabolism, and is widely used in the study of clinical disease mechanisms. In addition, it has been shown that the PI3K/Akt signaling pathway has an inhibitory effect on the initiation of programmed death in tumor cells, promotes cell survival and inhibits the proliferation of tumor cells, and also has a significant inhibitory ability on normal cell apoptosis [18]. Liu et al [19] showed that the PI3K/Akt signaling pathway is involved in the development and progression of breast cancer and promotes cancer cell invasion and migration by regulating the expression of related proteins. Ki67 is a nuclear antigen associated with cell proliferation, mainly located in the nucleus, and is a non-histone protein, a class of proliferating cell nuclear antigens associated with the cell cycle, whose antigen expression varies with the cell cycle. MacCallum et al. [20] showed that this protein binds to other proteins and DNA and RNA in a complex manner, and presumably has a structural and spatial role in the nucleus. It suggests that Ki67 is a key factor in ribosome synthesis in cell division and essential for cell proliferation. Chen et al. [21] concluded from Mate data analysis that Ki67 is an important marker that can recognize nuclear proteins present in the nuclear matrix of proliferating cells and can effectively assess the proliferative activity of tumor cells and is currently the most reliable indicator of tumor aggressiveness and tumor prognosis.

The mechanism of acupuncture treatment has been a hot research interest for domestic and international scholars. With the help of new technologies and methods, the research in this field is now gradually penetrating to the cellular, molecular and genetic levels. Signal transduction is a rapidly developing discipline with multi-pathway, multi-link, multi-level and network characteristics, penetrating into different areas of life disciplines. PI3K-AKT signaling pathway is one of the intracellular pathways that promote cell survival and is widely involved in apoptosis

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and survival, therefore, the PI3K-AKT signaling pathway has become a targeted entry point for drug therapy in the study of therapeutic tumors [22]. The field of acupuncture research has also introduced the doctrine of signaling pathways, with a gradually increasing focus on the PI3K-AKT pathway, a large number of scholars have pointed out that acupuncture can exert therapeutic effects on many diseases by regulating this PI3K-AKT pathway [23]. "Preventive acupuncture is a typical application of TCM therapy for treating diseases before they occur. Our team created the meridian differentiation preventive acupuncture under the guidance of the theory of "preventive treatment of disease", with meridian differentiation as the main principle, meridian theory as the main basis, and combining acupuncture point theory and the circulation law of the breast meridians. This treatment method uses the main acupuncture points of ST36, CV17 and SP6 to prevent the malignant transformation of normal breast epithelial cells. the results of this study showed that the mean tumor mass of the meridian-identified preventive acupuncture group was significantly decreased; the expression of Ki67 and p-AKT was decreased and the expression of PTEN was increased in the meridian-identified preventive acupuncture group by immunohistochemical detection, indicating that meridian-identified preventive acupuncture has a significant inhibitory effect on PI3K-AKT signaling pathway-related proteins, which reduces the proliferation of mammary tumor cells by inhibiting their activation, thus the effect of the inhibition of PTEN -PI3K-AKT signaling pathway-related proteins is significant.

V. CONCLUSION

In conclusion, our study demonstrated the meridian differentiation preventive acupuncture has the effect of inhibiting the growth of breast tumors and delaying the onset of breast tumors, specifically by upregulating the expression of PTEN, regulating PI3K/Akt signaling pathway-related proteins, and inhibiting tumor cell proliferation, so as to exert an inhibitory effect on DMBA-induced mammary tumorigenesis in female rats.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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