EXPERIMENTAL STUDY

Effect of Liuweidihuang pill and Jinkuishenqi pill on inhibition of spontaneous breast carcinoma growth in mice


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Abstract

OBJECTIVE: To investigate the preventing and treating action of Liuweidihuang pill (LP) and Jinkuishenqi pill (JP) on spontaneous breast carcinoma in mice.

METHODS: A model of spontaneous breast carcinoma was derived from 11.5-month-old female Kunming breeding mice following the delivery of several litters. The mice were randomly divided into five groups: model control group (C), Liuweidihuang pill high-dose group (LH; 4.6 g · kg⁻¹ · d⁻¹), Liuweidihuang pill low-dose group (LL; 2.3 g · kg⁻¹ · d⁻¹), Jinkuishenqi pill high-dose group (JH; 4.6 g · kg⁻¹ · d⁻¹) and Jinkuishenqi pill low-dose group (JL; 2.3 g · kg⁻¹ · d⁻¹). Cancer tissue volume was measured by water immersion. Histopathology was analyzed by hematoxylin and eosin staining. Vascular endothelial growth factor (VEGF), extracellular signal-regulated kinase (ERK) and cyclin D1 protein expression in cancer tissue was assayed by western blotting.

RESULTS: Compared with the control group, cancer tissue volume and weight were lower in the LP and JP groups, and survival time was longer. The expression of VEGF, ERK and Cyclin D1 were inhibited in the LP and JP groups (P < 0.05), and cell differentiation was increased. Tumor weights and volumes and VEGF, ERK and Cyclin D1 expression in LL or LH were significantly lower than in JL and JH (P < 0.01).

CONCLUSION: Both LP and JP could restrain cancer growth and promote cancer cell differentiation; moreover, LP was more effective than JP. The likely mechanism of action was via inhibition of VEGF, ERK and cyclin D1.

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Key words: Breast neoplasms; Pathology; Vascular endothelial growth factors; Extracellular signal-regulated MAP kinases; Cyclin D1; Liuweidihuang pill; Jinkuishenqi pill

INTRODUCTION

Breast cancer (BC) is a common malignancy in women and its mortality rate has increased over the past 30 years. The major treatment for breast carcinoma is still surgery, while chemotherapy, radiation therapy, and hormone and immune therapy serve as auxiliary ap-
proaches. However, these therapies often give rise to many complications, and seriously affect patient quality of life. Clinical practices using Traditional Chinese Medicine (TCM) have displayed significant advantages in managing breast carcinoma. Through regulating the Yin-Yang balance of the body, it can improve the quality of life for breast carcinoma patients by increasing survival and improving viscerum function and immunity. Breast cancer is caused by healthy Qi insufficient, long-term deficit of liver and kidney Qi and Yin-Yang unbalance. Clinical treatment often use fundoscopic (replenishing Qi and blood), nourishing liver and kidney, reinvigoration, meantime paying attention to the Yin-Yang balance, with emphasis on Yang; strengthening healthy Qi and eliminating pathogenic factors, with emphasis on strengthening healthy Qi. For strengthening healthy Qi, more attention should be paid to the balance between Yin and Yang, regulating Qi and blood with emphasis on Yang, so as to striving in Yang from Yin and striving in Yin from Yang.

Liuweidihuang pill (LP) and Jinkuishunqi pill (JP) are provided for this type of disease. LP is not cloying, and has the function of strengthening healthy Qi, dispelling exogenous pathogens, eliminating toxicants, and simultaneously applying purging-tonifying therapy. Treatment with JP, a classical Yang-reinforcing prescription, seeks to supplement Yang with the combination of appropriate Yin so as to achieve the effect of reinforcing Yang from Yin. The prescription is widely used by recent generations because of its significant effects.

The general principle of breast cancer prevention and treatment is to support immune function, disperse pathogens, nourish Yin and supplement kidney function. This study, based on TCM, explored the effect of NY and invigoring the kidney during the treatment of spontaneous breast carcinoma using Liuweidihuang Wan and a classical WY prescription, Jinkuishenqi pill. As cyclin D1, vascular endothelial growth factor (VEGF) and extracellular signal-regulated kinase (ERK) play very important roles in the occurrence and development of breast carcinoma, we also explored the effect of the different treatments on these targets.

MATERIALS AND METHODS

Reagents
Liuweidihuang pill (batch number 211021283) and Jinkuishunqi big honeyed pills (batch number Z11020054) were purchased from Beijing Tong Ren Tang Technologies Co., Ltd., (Beijing, China). Trizol was obtained from Invitrogen (Seattle, Washington City in USA). Horseradish peroxidase-labeled goat anti-rabbit IgG monoclonal antibody was from Beijing Zhongshan Golden Bridge Co., Ltd. (Beijing, China). Methanol and phosphate buffer were domestic analytical reagents.

Animals
Three-hundred and forty female Kunming breeding mice (aged 11.5 months, average weight 22 g) were provided by the animal center of Jiangxi breeding mice (production license No. SCXK-2011-0001). The Animal Ethics Committee of National Research Institute for Familly Planning Beijing approved the animal experimentation protocols, and all animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals established by the Chinese Council on Animal Care. The animals were housed in accordance with the guidelines for care and use of animals in scientific research-registered animal facilities. The animals were maintained in cabin-type isolators at standard environmental conditions (22-25 °C, 40%-70% humidity) with a 12:12 h dark/light photo cycle. Trained technicians palpated the mammary glands of all animals every 3 days and noted the location and size of all nodules using standard techniques. At 11-18 months, 20%-30% of animals eventually developed a mammary tumor.

Following tumor development, each mouse was distributed to one of five groups using a random number table, as follows: control group (C), Liuweidihuang pill high-dose group (LH; 4.6 g·kg⁻¹·d⁻¹), Liuweidihuang pill low-dose group (LL; 2.3 g·kg⁻¹·d⁻¹), Jinkuishunqi pill high-dose group (H; 4.6 g·kg⁻¹·d⁻¹) and Jinkuishunqi pill low-dose group (JL, 2.3 g·kg⁻¹·d⁻¹). The stock concentration of LP and J was 24 g/100 mL, and was administered via intragastric administration. The mice in the control group were given physiological levels of saline to the agonal stage (symptoms: skeletonization, dispirited demeanor, significantly reduced dietary intake, slow reactions, decreased heart rate, faint breath or the occurrence of periodic breath) and then killed, recording the period between the beginning of the tumor and euthanasia. Tumors were then excised and weighed. Final diagnosis of the breast cancer was determined by histopathological analysis. The final volume of cancer tissue was measured by the water immersion method.

The inhibitory cancer ratio was calculated as follows: Inhibitory cancer ratio = P inhibitory = (Muskel – Munwell) / Muskel × 100%

Histopathology
Mammary tumors were collected in 10% buffered formalin. Fixed and paraffin-embedded tissues were cut at 5-μm thickness, stained with hematoxylin and eosin following standard procedures, and examined under a light microscope.
Western blotting
Protein was extracted from fresh-frozen specimens from 14-16 mouse mammary carcinomas and 15 normal mammary glands. Five sections of each sample were placed in 2-mL Eppendorf safe-lock tubes and immersed in Laemmli buffer for lysis. After incubation on ice for 20 min, tissue lysates were clarified for 10 min at 12 000 × g at 4 ℃, denatured at 95 ℃ for 5 min, and stored at -80 ℃ until needed. For electrophoresis, protein extracts from fresh-frozen mammary were subjected to Sodium dodecyl sulfate poly-acrylamide gelelectrophoresis (SDS-PAGE) on 8% polyacrylamide gels according to Zheng et al.18 Proteins were transferred to nitrocellulose membranes and blocked in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), plus 5% skim milk overnight at 4 ℃. The membranes were then incubated with VEGF, ERK, or cyclin D1 monoclonal mouse anti-human antibody at 1:1000 dilution (Santa Cruz) in PBS-T plus 2% skim milk for 2 h, washed five times with PBS-T, and incubated for 1 h with peroxidase-conjugated goat anti-rabbit secondary antibody (1:1000) in PBS-T plus 2% skim milk. After washing the membrane five times with PBST, immunoreactivity was visualized by incubation with an enhanced chemiluminescence (ECL) and an image was taken using an Alpha gel imager (GE Healthcare, Fairfield, CT, USA). Measurements of gray-scale value covering the total area of the bands were integrated. The background was subtracted.

Statistical analysis
Data analysis was carried out using SPSS 15.0 software (SPSS Institute, Chicago, IL, USA). One-way analysis of variance was used to compare differences. Chi-square tests were employed to analyze categorical data. P values of < 0.05 were considered statistically significant.

RESULTS
Characteristics of tumor-bearing mice
Mammary tumors were observed only in female breeding mice, mostly after delivering several litters. The age of tumor occurrence was about 12 months (range, 10-15 months). Unmated females did not develop tumors during their life span. Among 300 breeding females, 65 females developed mammary adenocarcinomas (incidence rate, > 20%). The tumors were unilateral and were observed only in 2nd or 5th or both 2nd and 5th mammary glands. Neoplastic masses, which reached approximately 10%-20% of the animal’s body weight at approximately 1.5 weeks, were multi-lobular with ulceration on the skin surface. On palpation, fluid was observed in each lobe. Within 1-5 weeks, the tumors grew rapidly, but tumor growth decelerated with lengthening time, at which body weight began to decline. At the agonal stage, mice were killed; the average body weight was around 33 g and tumor size was approximately 18 cm³.

Cancer tissue features following Liuweidihuang pill and Jin Kui Shun Qi pill treatment
Excised tumor masses revealed multi-lobes, and each lobe had multiple solid nodules enclosing a central necrotic core with foul-smelling inflammatory exudates and clotted blood. The tumor masses resembled luminal/tubular epithelial-like morphology with well-differentiated (Grade 1) lobes. Tumor masses in the control group were soft and irregular-shaped, with solid nodules enclosing a central area of necrotic tissue with foul-smelling inflammatory exudates and clotted blood. The mice in the LL-dose group had mostly gray or white, relatively hard tumor tissue. There were few blood vessels on the surface of the tumors with false membranes into the tumor, and while there was less necrotic tissue than in the control group tumors, it resembled bean curd residue. In the LH-dose group, tumor size was relatively larger, and the tumor tissue was softer and brown or grey, with fewer blood vessels on the tumor surface than the control group. Coffee-like fluid was contained in tumors. Tumor tissue in JL doses was hard, off-white, with less vascular distribution of the surface mass, and pseudocapsule formation, while in JH, tumors were mainly brown and off-white, with a brown fluid in their core. The results revealed that treatment effectiveness in the low-dose groups of LP and JP was better than those of the high-dose groups.

Pathological analysis of cancer tissue after Liuweidihuang pill and Jin Kui Shun Qi pill treatment
The mammary gland tissue of normal mice stained with hematoxylin eosin (HE) revealed that the cells were of similar size, and many breast ducts were identified by microscopy. Each lobe had lobules showing acinar or cystic patterns or a combination of acinar, cystic and inflammatory patterns. The tubules were lined by single or multiple layers of pleomorphic epithelial cells. Some regions of the tubules were filled with amorphous inflammatory exudates appearing as a cystic pattern. Tumor cells were pleomorphic and were spindle-, round-, polygonal- or signet ring-shaped, and were small to giant-sized with normal or foamy cytoplasm and eccentric or centrally-located nuclei. The neoplastic epithelial cells had indistinct borders, and contained scant to moderate amounts of cytoplasm and pleomorphic nuclei with mitotic figures. Multiple areas of necrosis with infiltration of neutrophils appeared between and within lobules (Figure 1).

Under high magnification, the ducts appeared as monolayer columnar structures. The cancer cells in the control group were spread in flakes or nests, with a few exhibiting a crypt-like pattern, and an increased karyoplasmic ratio was evident; the neoplastic epithelial cells had pleomorphic nuclei with mitotic bodies. The preliminary diagnosis was invasive ductal carcinoma (Fig-
ure 1 A1, A2). LH cells were spread in slices, and were of varying size with irregularly formed ducts demonstrating secretion (Figure 1 B1, B2). In contrast, while LL cells were also spread in slices of different sizes, they formed ducts that were arranged back-to-back, had a larger karyoplasmic ratio and the nuclei were pleomorphic (Figure 1 C1, C2). The LH cells were spread in slices, with a large necrotic patch; ducts were of different sizes and shapes, and the nuclei were large and deeply stained and exhibited obvious atypia (Figure 1 D1, D2). The JL cells demonstrated a nested distribution with different sized and shaped ducts and slight necrosis, and large, deeply stained nuclei with obvious atypia (Figure 1 E1, E2). The results showed that the breast ducts of spontaneous breast cancer mice were protected and cell differentiation was improved after treatment with LP and JP, moreover LL treatment group had the best effect on pathological changes.

**Cancer tissue volume, weight, and survival time after Liuweidihuang pill and Jinkuishunqi pill treatment**

Tumor volumes of LH and JL cells were significantly smaller compared with the control ($P < 0.05$ and $P < 0.01$, respectively), while tumor size in the JH cells was larger than that of the LL cells, although this was not statistically significant ($P > 0.05$; Figure 2A).

Tumor weights in the LL and JL were significantly lower compared with the control group ($P < 0.01$), while those in the LH and JH were not significantly different compared with the control group ($P > 0.05$). Tumor weights in the LL and LH were higher than those of the JL and JH, but this was not statistically significant ($P > 0.05$; Figure 2B). Moreover, the cancer inhibition ratio in the LL was obviously higher than that of the JH (Figure 2C).

After treatment, the survival time of cancer-bearing mice was prolonged. Among these groups, survival in the LL was longer than that of the control ($P < 0.05$),...
but that of the LH and JP compared with the control was not statistically significant ($P > 0.05$; Figure 2D). These results indicated that NY treatment was better than JP treatment.

**Effect of Liuweidihuang pill and Jinkuishunqi pill treatment on VEGF, cyclin D1 and ERK protein expression in cancer tissue**

VEGF, Cyclin D1 and ERK protein expression in cancer tissues was analyzed by western blotting. Compared with the control group, VEGF, Cyclin D1 and ERK expression in the LL or LH was significantly lower ($P < 0.01$), and was also significantly lower in the JL ($P < 0.05$). Furthermore, VEGF expression in the JH and JL was significantly lower compared with the control ($P < 0.05$), but Cyclin D1 changes in the JH were not statistically significant ($P > 0.05$; Figure 3 A, B, C). Compared with the LL or JH, VEGF, Cyclin D1 and ERK protein expression levels in the LL and JH were significantly lower ($P < 0.01$).

**DISCUSSION**

According to TCM, lack of healthy Qi and insufficiencies in Qi and blood are the main causes of breast cancer. Moreover, emotional injury, anxiety, and rage are important factors in the progression of this disease. As a result, breast cancer patients exhibit symptoms such as liver-Qi stagnation, and liver and kidney Yin deficiency. Therefore, the use of TCM to treat breast cancer through regulating Yin and Yang, supplementing Qi and blood, and balancing viscera function has achieved remarkable effects. In this experiment, LP and JP were used to treat spontaneous breast carcinoma. The results showed that they could inhibit cancer growth, with an inhibition rate of over 20%. Moreover, the low-dose group of both treatments had a significantly greater effect compared with the control group ($P < 0.05$). Both could prolong the survival time of tumor-bearing mice, and the survival of the LL mice was significantly longer compared with the control group ($P < 0.05$). Pathological analysis showed that both treatments could improve the pathological structure of cancer tissue, and promoted the recovery of breast ducts and cancer differentiation, with LL resulting in the best effect.

Both LP and JP could significantly inhibit VEGF, Cyclin D1 and ERK protein expression compared with the control ($P < 0.01$ and $P < 0.05$, respectively). VEGF, Cyclin D1 and ERK protein levels in the Liuweidihuang pill treated groups (LL or LH) were significantly lower compared with the JP treated groups ($P < 0.01$).

Yin-Yang is the general principle of syndrome differentiation, and various pathological changes in malignant tumors can be summarized as imbalances between Yin.
and Yang, Qi and blood. Timely correction of predomi-
nance and decline could improve the inner environ-
ment, causing our bodies to achieve dynamic balance
between Yin and Yang. LP nourishes kidney Yin, while
JP supplements kidney Yang, which enables tumor
mice to achieve a new balance between Yin and Yang
and results in inhibition of cancer growth, prolonging
the survival of tumor mice, and consequently improv-
ing the survival rate.23

LP prescriptions can achieve the effect of nourishing
three Yin together (kidney Yin, liver Yin and spleen
Yin) in terms of the prescription composition. For
example, Shudihuang (Radix Rehmanniae Praeparata) can
supplement kidney Yin, Shanzhuyu (Fructus Corni)
can supplement the kidney through supplementing the
liver, and Shanyao (Rhizoma Dioscoreae Opposite) can
nourish the kidney and supplement the whole body
through nourishing the spleen. Modern pharmacology
has demonstrated that all three medicines can improve
immune function, induce the production of interfer-
ons and antioxidants, and inhibit tumor growth.24 Mu-
danpi (Cortex Moutan Radix) regulates the immune
cells, while Poria is anti-inflammatory.25 Remedium car-
dinale consists of Fuzi (Radix Aconiti Lateralis Prepara-
ta) and Guizhi (Ramulus Cinnamomi); a small amount
in JP results in Tong Ren Tang, which means "junior
fire supporting vital Qi" and is intended to increase the
fire slightly to encourage kidney Yang, complementing
vital fire and letting the fire return to its origin. Radix
Rehmanniae supplement, for example, is included in
LP drugs to nourish Yin and produce Yin fluid, thus a
compatible prescription was based on the principle of
Yin-Yang interdependence and complementarity.22

A previous study reported that overexpression of inter-
leukin suppressed cancer cell growth in vitro, induced
cancer cell cycle arrest at the G1 phase, mediated ro-
 bust apoptosis induced by serum starvation, and stimu-
 lated TNF-α and IFN-γ through upregulation of Fas and
concurent downregulation correlates with tumor
progression. Aberrant expression of VEGF is a hall-
mark of malignant tumor development required for
the colonization of endothelial cells that allow tumor
nutrition.23 Thus, VEGF is a viable target for pharma-
co logical intervention in cancer.

This provides an interesting paradigm for VEGF rela-
tionships in signal transduction in cancer. Pharmacolo-
gical and genetic approaches following treatment
with Chinese medicine compound recipes were used to
dissect the signaling pathways involved in LH/IL-6 or
JH/JL-inhibition of VEGF protein expression. The
MAPK/ERK signaling pathway is generally involved in
the regulation of VEGF. MAPKs are very important
signaling components that convert extracellular stimuli
into cell proliferation. These results delineate for
the first time the mechanisms of Chinese medicine com-
 pound recipe regulation of VEGF and reinforce the
idea that the disruption of VEGF signaling could im-
 pact on breast cancer growth by inhibiting prolifera-
tion and angiogenesis.23

Together, the data suggest both LP and JP can inhibit
tumor growth and progression, promote the recovery
of breast ducts, stimulate the differentiation of tumor
cells, and inhibit the expression of VEGF, cyclin D1
and ERK in cancer tissues. However, the effect of LP
was obviously better than JP.

Cancer patients always exhibit symptoms of Yin defi-
ciency either before tumor development or during its
progression. LP nourishes three Yin (kidney Yin, liver
Yin and spleen Yin), while JP contains small amount
of Yang-supplementing components as well as a large
amount of Yin-nourishing components, thus, LP has
a better effect than JP in inhibiting tumor progres-
sion because excessive Yang-supplementing medicine
will result in the impairment of Yin due to the heat
it introduces.

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