Liuwei Dihuang pill suppresses metastasis by regulating the wnt pathway and disrupting β-catenin/ T cell factor interactions in a murine model of triple-negative breast cancer


METHODS: Ninety-nine TNBC bearing-mice were distributed randomly to five groups: control (Con), paclitaxel (PTX), low-dose LWDHP (LLP, 2.3 g · kg⁻¹ · d⁻¹), middle-dose LWDHP (MLP, 4.6 g · kg⁻¹ · d⁻¹) and high-dose LWDHP (HLP, 9.2 g · kg⁻¹ · d⁻¹). The LWDHP were administered (p.o.) to the agonal stage. The morphology of BC cells was observed by hematoxylin & eosin staining. Expression of axin-2, β-catenin, T cell factor (TCF), cyclin-D1 and vascular endothelial growth factor (VEGF) was detected by western blotting or immunofluorescence. β-catenin/TCF-1 interaction was measured using a co-immunoprecipitation assay.

RESULTS: After LWDHP treatment, metastasis of BC cells to the lungs and liver was inhibited, expression of axin-2 was increased, expression of TCF-1, β-catenin, cyclin-D1 and VEGF was decreased, and β-catenin/TCF-1 interaction was disrupted.

CONCLUSION: The LWDHP could inhibit metastasis of BC cells to the liver and lungs. The molecular mechanism underlying this action may be regulation of protein expression and β-catenin/TCF-1 interactions in the Wnt pathway.

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Keywords: Triple negative breast neoplasms; Neoplasm metastasis; Beta catenin; T cell transcription factor 1; Wnt signaling pathway; Liuwei Dihuang pill

INTRODUCTION

Triple-negative breast cancer (TNBC) is one of the...
The LWDHPs used in the present study were manufactured by Beijing Tongrentang Pharmaceuticals (Beijing, China). Each LWDHP comprised six herbs: Shudihuang (Radix Rehmanniae Praeparata) 160 g, Shanzuyu (Fructus Corni) 80 g, Shanyao (Rhizoma Dioscoreae Oppositae) 80 g, Mudanpi (Cortex Moutan Radicis) 60 g, Zexie (Rhizoma Alismatis) 60 g and Fuling (Poria) 60 g.\(^{3,11}\) Paclitaxel was purchased from Bristol-Myers Squibb (Princeton, NJ, USA).

**Dose consideration of the LWDHP in model groups**

Following tumor development, all-bearing mice were distributed to five groups of 20 using a random-number table: control (Con), paclitaxel (PTX), low-dose LWDHP (LLP; 2.3 g·kg\(^{-1}\)·d\(^{-1}\)), medium-dose LWDHP (MLP; 4.6 g·kg\(^{-1}\)·d\(^{-1}\)) and high-dose LWDHP (HLP; 9.2 g·kg\(^{-1}\)·d\(^{-1}\)). The stock concentration of LWDHP was 24 g/100 mL, and was administered via the intragastric route. PTX-group mice were administered paclitaxel (25 mg/kg, i.p.) at cycles of 3–4 weeks. Chemotherapy was initiated 14–21 d when subcutaneous tumors of the mammary glands were palpated.\(^{11}\) Con-group mice received only an equal volume of physiologic (0.9%) saline. All mice were given agents to the agonal stage. The final diagnosis of BC was determined by histopathology. The weight and diameter of each tumor from each mouse were measured. Tumor dimensions were calculated using the following formula:

\[
\text{Weight} = \left(\frac{a \times b}{2}\right)
\]

where a is the largest diameter and b is the shortest dimension perpendicular to a. Cancer inhibition was calculated as:

\[
\text{Cancer inhibition} = \left(\frac{\text{weight}_{\text{con}} - \text{weight}_{\text{LWDHP}}}{\text{weight}_{\text{con}}}\right)
\]

**Histology**

In a proportion of cancer-bearing mice, tumor, liver, lung and normal mammary-gland (Nor) tissues were fixed in neutral formalin and processed by conventional means. Then, they were embedded in paraffin wax and sectioned at 5 μm, and sections were stained with hematoxylin and eosin for viewing under light microscopy. Serial sections of liver and lung tissues were prepared, sectioned and stained to detect microscopic evidence of metastatic spread.

**Western blotting**

The protein concentration of homogenized lysates was measured using a Bradford assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts (50 μm) of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10%-15% polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, CA, USA). After blockade in T-TBS containing 5% skimmed milk for 1 h, immunoblotting was done by incubation overnight at 4 °C with primary antibodies corresponding to β-catenin (R & D Systems, Minneapolis, MN, USA), axin (Millipore, Billerica, MA, USA),

**MATERIALS AND METHODS**

**Ethical approval of the study protocol**

The Animal Ethics Committee of the National Research Institute for Family Planning (Beijing, China) approved the study protocol. Animal experiments were conducted according to the guidelines for the care and use of laboratory animals established by the Chinese Council on Animal Care (Beijing, China).

**Creation of the animal model**

Four-hundred and forty female Kunming mice (11.5 months; mean weight, 22 g) were provided by the animal center of Jiangxi TCM (production license number, SCSK-2015-0001). Trained technicians palpated the mammary glands of mice every 3 d, and noted the location and size of all nodules using standard methods. During 11-18 months, 20%-30% of mice developed a tumor in the mammary glands. The time of appearance of local tumors in mammary tumors was monitored by palpation and confirmed by histopathology.\(^{9,12}\)

**Preparation of the LWDHP and paclitaxel**

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TCF-1 (Abcam, Cambridge, UK), cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and vascular endothelial growth factor (VEGF; Santa Cruz Biotechnology) diluted 1:1000-1:2000 (v/v) in 5% skimmed milk. Immunoblotting was followed by incubation with secondary antibodies conjugated to horse-radish peroxidase (HRP) for 3 h at room temperature or overnight at 4 °C. After washing, the bands of interest were analyzed using a luminescent image analyzer FluorChem™ M (Proteinsimple, San Jose, CA, USA).

**Immunofluorescence**

Tumor tissue was cut to sections of size 8 μm using a freezing microtome, and fixed in 10% acetone for 8-10 min. Sections were air-dried and then washed thrice with phosphate-buffered saline (PBS). Sections were blocked with 2% goat serum for 20 min at 37 °C, and then incubated overnight at 4 °C with primary antibodies (1:1000 dilution). After rinsing with PBS, sections were incubated for 30 min at 37 °C with cyanine 3 or cyanine 5 (1:400 dilution; Abcam). 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA) was used to stain cell nuclei. The stained and mounted sections were captured with a charge-coupled device camera (SPOT Imaging, Sterling Heights, MI, USA).

**Co-immunoprecipitation assay**

Prepared cancer-tissue lysates that had undergone LWDHP treatment were incubated with anti-β-catenin or anti-TCF-1 primary antibodies overnight at 4 °C. Then, the immune complex was precipitated at 4 °C for 2 h by pre-washed Protein A-Sepharose beads (Santa Cruz Biotechnology). The proteins bound to β-catenin or TCF-1 primary antibodies and Protein A-Sepharose beads were washed, separated using 10% polyacrylamide gels, transferred to polyvinylidene-difluoride membranes, and then incubated with anti-TCF-1 or β-catenin primary antibody and HRP-conjugated secondary antibody. The immune-reactive bands were analyzed as Western blots.

**Statistical analyses**

SPSS v11.5 (IBM, Armonk, NY, USA) was employed for all analyses. Data were expressed as mean ± standard error of the mean (SEM). Comparisons between two groups were made using the least-square-difference test and Hochberg method. P < 0.05 was considered significant.

**RESULTS**

**Volume and weight of cancer tissue and mouse survival after LWDH treatment**

The tumor-surface vasculum was reduced and tumor volumes were smaller in LWDHP groups than those in the Con group, but the tumor volumes in MLP and HLP groups were greater compared with those in the PTX group (Figure 1A). Tumor weights were lower in LLP and MLP groups compared with that in the Con group, whereas the weight in LWDHP groups was higher compared with that in the PTX group (Figure 1B). Moreover, cancer inhibition in LWDHP groups was lower than that in the PTX group (Figure 1C). After treatment with LWDHP, survival of TNBC-bearing mice was prolonged compared with that in Con and PTX groups (Figure 1D).

**Effect of LWDHP treatment upon metastasis of BC cells to the lungs or liver**

Treatment with LWDHP or PTX could inhibit meta-

![Diagram](https://example.com/diagram.png)

Figure 1 LWDHP effects on cancer volumes, weight, inhibition and mouse survival

A: cancer volumes; B: cancer weight; C: cancer inhibition; D: mouse survival. Con: control group, received only an equal volume of physiologic (0.9%) saline; PTX: paclitaxel, administered paclitaxel (25 mg/kg, i.p.) at cycles of 3-4 weeks; LLP: low-dose LWDHP (2.3 g · kg⁻¹ · d⁻¹); MLP: medium-dose LWDHP (4.6 g · kg⁻¹ · d⁻¹); HLP: high-dose LWDHP (9.2 g · kg⁻¹ · d⁻¹). Each bar represents the mean ± standard error of mean of 19 mice. LWDHP: Liuwei Dihuang pill. Asterisks or triangles denote significance levels. *P* < 0.01 and *P* < 0.05 compared with control, *P* < 0.05 compared with PTX.
tasis of BC cells to lung/liver tissue compared with that in the Con group (Figure 2A, 2B). Histopathology revealed that the LWDHP reduced the number and size of nodules metastasizing to the liver compared with that in Con and PTX groups. Typically, liver nodules were adenocarcinomas in PTX and LWDHP groups (Figure 2B). However, in LWDHP groups, the liver-tumor nodules were smaller and fewer than those in Con and PTX groups (Figure 2B).

**The LWDHP regulates protein expression in the Wnt signaling pathway**

Compared with the Con group, axin-2 expression was increased significantly in PTX and HLP groups ($P < 0.05$ for both) whereas, compared with normal mammary-gland tissue, Axin-2 expression in Con, LLP and MLP groups was lower (Figure 3A1, A2). β-catenin expression was significantly higher in Con and MLP groups compared with that in normal mammary-gland tissue whereas, in PTX, LLP and HLP groups, it was compared with that in the Con group ($P < 0.05$) (Figure 3B1, B2). Compared with normal mammary-gland tissue, TCF-1 expression was higher in LWDHP, PTX and Con groups, but TCF-1 expression in LWDHP and PTX groups was significantly lower compared with that in the Con group ($P < 0.05$) (Figure 3C1, C2). β-catenin accumulation was decreased simultaneously in the nucleus and cytosol, and TCF-1 fluorescence intensity was reduced significantly after LWDHP treatment ($P < 0.05$) (Figure 4A1-A6, B1-B6).

**LWDHP treatment disrupts β-catenin/TCF complexes in nuclei**

The co-immunoprecipitation assay showed that MLP treatment resulted in inhibited expression of β-catenin upon immunoprecipitation with TCF antibodies (Figure 5A). Conversely, MLP treatment decreased TCF levels when immunoprecipitated with β-catenin antibodies (Figure 5A, IP), suggesting that MLP treatment disrupted association of β-catenin/TCF complexes in nuclei (Figure 4A, IP). Simultaneously, compared with the Con group, expression of cyclin-D1 and VEGF decreased in tumor bearing-mice treated with the LWDHP ($P < 0.05$) (Figure 5B, C, D, E).

**DISCUSSION**

Dissemination of BC cells and eventual metastatic growth to distant organs (predominantly the liver and lungs) is a major clinical problem because metastatic disease is the primary cause of death for the vast majority of TNBC patients. TCM compounds can improve the symptoms of BC patients in multifactorial and multitargeted ways. When the LWDHP has been employed to treat cancer, therapeutic regimens have been able to amplify the therapeutic efficacies or reduce the adverse effects of each of the six agents within the LWDHP, leading to maximal therapeutic efficacy with minimal adverse effects. Our findings suggest that the LWDHP decreased the prevalence of liver/lung metastasis compared with the Con group, and also reduced the number and size of metastatic liver nodules compared with Con and PTX.
groups (Figure 2). However, the best effect for inhibiting liver metastases was use of MLP.

The liver and lungs are the most susceptible organs to metastasis of BC cells, which can differ in terms of evolution, treatment, morbidity and mortality. Metastasis to the lungs and liver may be the final step in BC cell-related mortality. Conventional therapies such as chemotherapy and surgery can be successful, but metastasis-related BC mortality remains high. Thus, a novel approach to prevent metastasis of BC cells is needed, and the LWDHP may be a very important means for achieving this aim.

Deregulation of Wnt signaling is an important event in BC development. In TNBC, this pathway is constitutively active at a high level due to defective β-catenin genes, and plays a crucial part in progression of a subset of BC cells, so Wnt signaling could be an important target in the control of metastasis. The LWDHP could regulate higher expression of axin-2 and lower expression of β-catenin or TCF-1 in the Wnt/β-catenin signaling pathway according to western blotting and immunofluorescence (Figures 3, 4). Thus, altered expression of proteins could be related to inhibition of the growth and metastasis of BC cells, and improve metastasis.

Activated β-catenin/TCF-1 complexes in Wnt signaling lead to the transcription of downstream genes such as VEGF and cyclin-D1, and increases in cell proliferation. MLP treatment could destroy β-catenin/TCF complexes and decrease expression of VEGF and cyclin-D1 (Figure 5). These results also suggest that LWDHP treatment inhibited expression of β-catenin and TCF-1. This would result in formation of fewer β-catenin/TCF-1 complexes, leading to prevention of β-catenin stabilization and activation of the β-catenin/TCF-1-mediated transcriptional responsive genes VEGF and cyclin-D1, as well as reduction of metastases of BC cells.

In conclusion, the LWDHP is efficacious against TNBC, and can inhibit metastasis in TNBC-bearing mice. We identified that its anti-metastasis mechanism centers on the Wnt signaling pathway. Our findings suggest that a similar strategy could be beneficial for clinical treatment of TNBC.

REFERENCES


Figure 4 Immunofluorescence intensity of β-catenin and TCF-1 after LWDHP treatment
A1-A6: β-catenin; B1-B6, E1-E6: DAPI; C1-C6, F1-F6: merge. A1, B1, C1, D1, E1, F1: Nor: normal breast tissue. A2, B2, C2, D2, E2, F2: Con: control group, received only an equal volume of physiologic (0.9%) saline. A3, B3, C3, D3, E3, F3: PTX: paclitaxel, administered paclitaxel (25 mg/kg, i.p.) at cycles of 3–4 weeks. A4, B4, C4, D4, E4, F4: LLP: low-dose LWDHP (4.6 g · kg⁻¹ · d⁻¹); A6, B6, C6, D6, E6, F6: HLP: high-dose LWDHP (9.2 g · kg⁻¹ · d⁻¹). G: β-catenin immunofluorescence intensity; H: TCF-1 immunofluorescence intensity. LWDHP: Liuweidi-huang pill; TCF: T cell factor; DAPI: 2-(4-aminophenyl)-6-indolecarbamidine dihydrochloride. Scale bar = 50 μm. *P < 0.05 compared with the normal group; **P < 0.05 compared with control.


8. Wang J, Yao KW, Yang XC, et al. Chinese patent medicine Liu Wei Di Huang Wan combined with antihypertensive drugs, a new integrative medicine therapy, for the
Figure 5: Immunoprecipitation of lysates of breast-cancer tissue

A: β-actin (immunoprecipitation (IP)); B: VEGF (Western blotting); C: Cyclin-D1 (Western blotting); D: VEGF protein expression level; E: Cyclin-D1 protein expression level. Nor: normal breast tissue. Con: control group, received only an equal volume of physiologic (0.9%) saline. PTX: paclitaxel, administered paclitaxel (25 mg/kg, i.p.) at cycles of 3-4 weeks; LLP: low-dose LWDHP (2.3 g · kg⁻¹ · d⁻¹), MPL: medium-dose LWDHP (4.6 g · kg⁻¹ · d⁻¹) and HLP: high-dose LWDHP (9.2 g · kg⁻¹ · d⁻¹). LWDHP: Liuwei Dihuang pill; TCF: T cell factor; VEGF: vascular endothelial growth factor. *P < 0.05, compared with control; **P < 0.05, compared with the normal group.