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Original Article

Bi-directional solid fermentation products of *Trametes robiniophila* Murr with Radix Isatidis inhibit proliferation and metastasis of breast cancer cells

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Abstract

Background: The bi-directional solid fermentation product extract of *Trametes robiniophila* Murr (Huaier) with Radix Isatidis (TIF) has been shown to have good anti-tumor activity. However, the mechanisms of this activity are still unknown. In the present study, we aimed to investigate its inhibitory effect on both SK-BR-3 and MDA-MB-231 cells, and explore the possible mechanisms of its anti-cancer effect *in vitro*.
Methods: The experiment comprised a control group, Radix Isatidis group, Huaier group, and TIF group. The cell viability was measured by MTT and the distribution of cell cycle and apoptosis levels were analyzed by flow cytometry. Cell scratch, Transwell, and adhesion assays were used to measure the effects of the test compounds on the migration, invasion, and adhesion capability of SK-BR-3 and MDA-MB-231 cells. The effects of TIF on the mRNA and protein expression related to apoptosis and migration were measured by using semi-quantitative RT-PCR and western blotting.
Results: TIF strongly inhibited the cell proliferation of the SK-BR-3 and MDA-MB-231 cells in a time-dependent manner and induced G2/M arrest and apoptosis. Furthermore, TIF significantly inhibited the proliferation, migration, invasion, and adhesion capabilities of SK-BR-3 and MDA-MB-231 cells. Compared with other treatments, the anticancer effect of TIF were stronger in MDA-MB-231 cells. Semi-quantitative RT-PCR suggested that TIF may upregulate the expression of p53 and caspase-3 to inhibit cell proliferation, and downregulate the expression of MMP-9/Snail and MMP-9/MMP-2 to inhibit the migration, invasion, and adhesion capabilities of SK-BR-3 and MDA-MB-231 cells. Western blotting results showed that TIF increased the expression of p53 protein and decreased the expression of MMP-9 protein in SK-BR-3 and MDA-MB-231 cells.
Conclusion: The results indicated that the bi-directional solid fermentation may enhance the efficacy of Huaier in MDA-MB-231 cells and that TIF may be an effective complementary medicine for cancer treatment.

Keywords: Apoptosis; Breast cancer; Fermentation; Huaier

1. Introduction

In many countries, breast cancer is the most frequent type of malignancy among women,[1,2] and it is believed to be the second leading cause of cancer death.[3] The incidence of breast cancer in women is reported to be more than 100 times higher than in men.[4] Despite significant advances in the treatment of breast cancer, such as hormonal therapy, chemotherapy,

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radiotherapy, and surgery, the incidence and mortality rates of the disease continue to rise, owing to drug resistance and metastases.[5,6] Unfortunately, breast cancer is highly resistant to chemotherapy; therefore, there is an urgent need to seek new therapeutic strategies and develop novel drugs with low toxicity for the prevention and treatment of breast cancer. From this perspective, the study of natural products extracted from traditional Chinese Medicine (TCM) remains one of the most challenging fields in cancer research; in China, over the past few decades, compounds used in TCM have been mined for the discovery of potential anticancer agents.[7–9]

Trametes robiniophila Murr, commonly called "Huaier" in Chinese, was recorded for the first time in Zhou Hou Fang 1500 years ago. It is a type of officinal fungus and has been used as a TCM to treat a wide variety of diseases.[10] In recent decades, Huaier extract has been reported to be effective in the treatment of cancers, including hepatocellular carcinoma and breast cancer,[11,12] and displays various biological activities, such as apoptosis, anti-angiogenesis, drug resistance reversal, anti-metastasis, and system immune activation.

Radix Isatidis (*Isatis Tinctoria*), named ''Banlangen'' in traditional Chinese medicine, is used to reduce inflammatory factors in the blood and to relieve convulsions. Many chemical compounds have been discovered in Radix Isatidis, including tryptanthrin B, indirubin, and organic acids. The functions of Radix Isatidis are widely reported to include antiviral, fever detoxification, and anti-inflammatory activities.[13] Indirubin and tryptanthrin B have been proven to be the main active components from Radix Isatidis from the perspective of their anticancer effects. Indirubin, an anticancer drug with an established clinical effect, has a clear effect on the treatment of chronic granulocytic leukemia.[14] Tryptanthrin B possessed antitumor activity to BEL-7402 human hepatocellular carcinoma cell and the A2780 human ovarian cancer cell, *in vitro*.[15]

Bi-directional solid fermentation, a new Chinese herbal fermentation technology with low toxicity, was able to enhance the properties of medicines and improve absorption.[16–18] The bi-directional solid fermentation product extract of *Trametes Robiniophila* Murr (Huaier) with Radix Isatidis (TIF) was developed based on the new bi-directional fermentation technology of modern Chinese medicine, established by Professor Yi Z in the 1980s.[19] Huaier was developed as a medicinal fungi, and the associated Huaiqi fungal substance has achieved a good clinical tests.[20] Radix Isatidis is rich in nutrients and bioactive composition, and has a wide range of clinical applications, including a significant anticancer effect. In this study, we acquired TIF and aimed to evaluate its effect on breast cancer cells and explore the underlying mechanisms.

2. Methods

2.1. Materials

The breast cancer cell lines SK-BR-3 and MDA-MB-231 were purchased from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Radix Isatidis (*Isatis Tinctoria*) was purchased from Tongrentang Chinese Medicine -Since 1669 (Beijing, China). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco-BRL (Rockville, IN, USA) and fetal bovine serum (FBS) and 0.25% trypsin−EDTA were supplied by Hyclone (Beijing, China). DMSO and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were supplied by Amresco (Beijing, China). A BCA protein assay kit, Annexin V-FITC Apoptosis Detection Kit, and Cell Cycle and Apoptosis Analysis Kit were purchased from Beyotime Biotechnology (Shanghai, China). Penicillin-Streptomycin Liquid was supplied by Solarbio Bioscience & Technology Co. (Shanghai, China). The RT-PCR Kit, 6 × Loading Buffer, 100 bp DNA Ladder, RNAiso Plus, and Regular Agarose were purchased from TaKaRa Bio Group (Daliang, China). BD Matrigel Basement Membrane Matrix was purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA) and Crystal Violet Staining Solution was supplied by Sigma−Aldrich (St Louis, MO, USA). Antibodies against p53, MMP-9, β-actin, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2. Preparation of TIF, Radix Isatidis, and Huaier extract

Samples of *T. robiniophila* Murr were obtained from The Northeast Food Medicine Institute of Fungi (Heilongjiang, China). The cultures were maintained on nutrient agar (NA) slants [20.0% (w/v) potato, 2.0% (w/v) glucose, and 2.0% (w/v) agar at 4 °C]. The activated cultures were then inoculated into fluid medium [2.5% (w/v) glucose, 0.05% (w/v) yeast powder, 2.5% (w/v) bran, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$] and incubated at 28 °C for 7 days. Then, bidirectional solid fermentation was used to acquire TIF. The cultures were used to inoculate Radix Isatidis prior to fermentation. For each sample, Radix Isatidis (10 g) was placed in a flask and then steamed at 121 °C for 30 min; subsequently, the samples were inoculated with fungus inoculum (10 mL) and incubated at 28 °C with a relative humidity of 80% for 40 days. A new type of mushroom was acquired, which was inoculated on the culture medium composed of Radix Isatidis. This was powdered and the obtained powder (50 g) was extracted with 70% ethanol (500 mL) by refluxing for 2 × 1 h periods. After merging and filtering, the supernatant was collected, concentrated under vacuum, and dried in a drying oven at 60 °C for 48 h. This obtained dry extract powder was TIF. The same method was used to prepare Radix Isatidis and ordinary Huaier, which was inoculated on agar medium. Three different extracts (0.5 g) were dissolved in complete medium (50 mL), sterilized by filtration through a 0.22 μm filter, and diluted to produce a 10 mg/mL stock solution suitable for long term storage at 4 °C.

2.3. Cell culture

Cells (SK-BR-3 and MDA-MB-231) were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in 5% CO_2 at 37 °C.

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