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Evaluation of the combined use of metronomic zoledronic acid and *Coriolus versicolor* in intratibial breast cancer mouse model

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ABSTRACT

Ethnopharmacological relevance: *Coriolus versicolor* (CV) is a mushroom traditionally used for strengthening the immune system and nowadays used as immunomodulatory adjuvant in anticancer therapy. Breast cancer usually metastasizes to the skeleton, interrupts the normal bone remodeling process and causes osteolytic bone lesions. The aims of the present study were to evaluate its herb-drug interaction with metronomic zoledronate in preventing cancer propagation, metastasis and bone destruction.

Materials and methods: Mice inoculated with human breast cancer cells tagged with a luciferase (MDA-MB-231-TXSA) in tibia were treated with CV aqueous extract, mZOL, or the combination of both for 4 weeks. Alteration of the luciferase signals in tibia, liver and lung were quantified using the IVIS imaging system. The skeletal response was evaluated using micro-computed tomography (micro-CT). *In vitro* experiments were carried out to confirm the *in vivo* findings.

Results: Results showed that combination of CV and mZOL diminished tumor growth without increasing the incidence of lung and liver metastasis in intratibial breast tumor model. The combination therapy also reserved the integrity of bones. *In vitro* studies demonstrated that combined use of CV and mZOL inhibited cancer cell proliferation and osteoclastogenesis.

Conclusions: These findings suggested that combination treatment of CV and mZOL attenuated breast tumor propagation, protected against osteolytic bone lesion without significant metastases. This study provides scientific evidences on the beneficial outcome of using CV together with mZOL in the management of breast cancer and metastasis, which may lead to the development of CV as adjuvant health supplement for the control of breast cancer.

1. Introduction

Breast cancer in women usually associated with distant spread into the bone causing significant morbidity including pain, fracture and spinal cord compression (Coleman, 2001). Breast cancer-induced bone metastases activate osteoclastogenesis to break down existing bone. The transforming growth factor-β arising from cancer cells would increase parathyroid hormone-related protein secretion, and in turns producing osteolytic lesion and tumor expansion (Mundy, 2002). Meanwhile, zoledronic acid (ZOL) is the only bisphosphonate which is effective in reducing bone destruction and the numbers of skeletal related adverse events in patients with breast cancer-induced bone metastases (Coleman et al., 2011). It was demonstrated that treatment with ZOL alone or in combination with doxorubicin could protect the bone and reduced tumor growth in breast cancer metastasis mice model (Buijs et al., 2009; Ottewell et al., 2009; Syddall et al., 2010). In addition, ZOL has been shown to exhibit direct and indirect

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anti-cancer and anti-metastasis effects (Gnant and Clezardin, 2012). However, the optimal dose schedule for breast cancer patients is not yet established (Mahtani and Jahanzeb, 2010). One of the strategies to enhance the anti-tumor effects of chemotherapy agents is to divide a large single dose to repeated low doses (metronomic) (Zhao et al., 2010). A clinical study compared the biomarker changes between a weekly low dose and a conventional dosage of ZOL in breast cancer patients demonstrated that metronomic ZOL (mZOL) was better in reducing circulating VEGF levels (Zhao et al., 2010). In addition, mZOL had greater anti-tumor effects as compared with conventional single dose in breast tumor-bearing nude mice (with equivalent total administered dose) (Daubine et al., 2007). This report was in line to our recent findings that mZOL (0.0125 mg/kg i.p. injected twice a week for 4 weeks) was more effective than the conventional regimen (0.1 mg/kg i.p. injected once only) in reducing tumor burden and decreasing lung and liver metastasis in both primary and metastatic breast tumor mouse models (Luo et al., 2013).

Chinese medicines have been widely used as health-promoting food ingredients and supplements for the treatment of cancer. Patients with cancer frequently use herbal medicines along with the conventional medical treatment so as to produce more desirable result than either taken alone. It was reported that over half (53.9%) of cancer patients in Hong Kong took Chinese herbal medicines together with chemotherapeutic agents (Lam et al., 2009). Among these Chinese herbal medicines, a meta-analysis has provided strong evidence that the Chinese medicinal mushroom, *Coriolus versicolor* (CV) would improve the survival and quality of life in cancer patients, particularly in carcinoma of breast, gastric and colorectal, in combination with conventional chemotherapy (Eliza et al., 2012). CV is widely used as health supplement for the prevention and treatment of cancers in China. Medicinal mushroom CV [Scientific name: *Trametes versicolor* (L.) Lloyd (1920)], known as “Yun Zhi” in China; “Kawaratake” in Japan has been used for centuries in China. It is traditionally used to (i) invigorate the spleen to damp elimination; (ii) relieve cough and asthma and (iii) strengthen one's immune system and vital energy to live longer and healthier. In regard to its traditional use in immune modulation, in the past decade, it has been demonstrated that aqueous extract obtained from CV display different biological activities, including immune modulation and inhibition of cancer growth (Chu et al., 2002). CV extract was also effective *in vitro* for activating immune function in terms of promoting the production of antibodies and various cytokines such as interleukin (IL)-2 and IL-6, interferons, and tumor necrosis factors (TNF) (Ho et al., 2006). For *in vivo* studies, a significant reduction of tumor size after prolonged administration of CV extract was shown in mice inoculated with leukemia cells (Dong et al., 1996) and liver cancer cells (Chu et al., 2002). These observations were similar to our recent findings that aqueous CV extract inhibited the tumor growth in 4T1 xenograft breast tumor-bearing mouse model (Luo et al., 2014).

Extracts or isolated fractions of CV are very popular in Chinese communities as they are regarded as a miracle cure for cancer. Cancer patients most often consumed CV extracts as part of their adjuvant cancer therapy. Given the increasing popularity and commercial development of different forms of CV extracts and related health supplements, there is an urgent need to study their interactions with mZOL for determining the safety and efficacy of these products when consumed together by those cancer patients at risk of multisite metastasis. Hence, in our present study design, more frequent but low doses of ZOL (metronomic ZOL), in combination with aqueous CV extract, were administered to mice bearing MDA-MB-231-TXSA breast tumor intratibial xenografts and their anti-cancer, anti-osteolytic and anti-metastatic efficacies were examined.

2. Materials and methods

2.1. Preparation of Coriolus versicolor aqueous extract

The raw herbal material of *Coriolus versicolor* (CV) was purchased from renowned supplier in Hong Kong. Morphological authentication was performed in accordance to Chinese Pharmacopoeia 2010 and further validated by botanical expert from Kunming Institute of Botany, China. Authenticated voucher specimen was deposited in the museum of Institute of Chinese Medicine, The Chinese University of Hong Kong, (voucher specimen number 20103291). For aqueous extract preparation, 1 kg of CV was added to 4 l of water and refluxed at 100 °C for 1 h, and repeated twice. The mixture was lyophilized into dried powder. As the major components of the CV aqueous extract are expected to be polysaccharides and triterpenoids, the standardization of the extract was performed using phenol-sulfuric acid method (Luo et al., 2014) and vanillin-perchloric acid method (Fan and He, 2006) for total polysaccharides and total triterpenoids, respectively.

2.2. Cells and reagents

The human breast cancer cells MDA-MB-231-TXSA tagged with the triple modality reporter construct (SFG-NES-TGL) was kindly provided by our co-investigator, Prof. Evdokiou and maintained in Dulbecco's modified Eagle's medium (DMEM), containing 10%v/v heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and those cells in the exponential growth phase were used for experiments. Zoledronic acid (ZOL) was purchased from Novartis Pharma AG (Orange Park, USA).

2.3. Intratibial bioluminescence breast tumor model

The experiments were approved by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong (Ref no.: 12/042/MIS). Female BALB/c nu/nu nude mice of 4 weeks old were implanted with human breast cancer cells tagged with the triple modality reporter construct in tibia. The inhibitory effects of CV and mZOL on tumor progression and bone destruction were investigated. The tumor burden and cancer-induced bone destruction of mice were evaluated by live animal bioluminescence imaging and micro-CT, respectively. In brief, mice were anesthetized under intraperitoneal anaesthesia with a cocktail of ketamine and xylazine. MDA-MB-231-TXSA cells (1×10^6) were transplanted into the marrow cavity of left tibia of mice using a Hamilton syringe. The right tibia was used as non-tumor bearing control. Two days after cancer cells implantation, mice were divided randomly into four groups (n=10): untreated control group (orally fed with distilled water daily), CV group (1 g/kg CV extract, orally fed daily), mZOL group (0.0125 mg/kg ZOL, i.p. injected twice a week), CV+mZOL group (1 g/kg CV, orally fed daily +0.0125 mg/kg ZOL, i.p. injected twice a week). Since CV aqueous extract was found to be effective previously at 0.2 g/kg in pilot dose finding experiment (data not shown), thus we used this dosage for combination studies. Naive mice without tumor and treatment were set as normal control. During treatment, body weight and bioluminescence measurement were performed once a week. After 4-week treatment, mice were sacrificed, lungs and livers were removed for bioluminescence imaging and quantification of tumor burden. Both tibias of each animal were removed for X-ray and μ-CT analysis.

2.4. In vivo bioluminescence imaging

Non-invasive, live animal whole-body imaging to monitor luciferase-expressing MDA-MB-231-TXSA cells in mice was performed weekly using the IVIS 200 Imaging system (Xenogen; Alameda, USA). Mice were injected with 100 μL of D-luciferin (Xenogen; Alameda, USA) i.p. at 250 mg/kg body weight and then gas anaesthetized with isoflurane. Images were acquired for 0.5–10 s and the photon emission transmitted from mice were quantified using Xenogen Living Image (Igor Pro version 3.2 software), and graphed according to the average radiance (photons/s/cm^2/sr).