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Antrodia cinnamomea sensitizes radio-/chemo-therapy of cancer stem-like cells by modulating microRNA expression

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ABSTRACT

Ethnopharmacological relevance: The discovery of many tissue-specific cancer stem cells (CSCs) continues to attract scientific attention. These CSCs are considered to be associated with chemo- and radio-resistance, and consequently, failure of conventional anticancer therapies. The recent demonstration of several microRNAs as enhancers of tumorigenicity via modulation of epithelial-mesenchymal transition and cancer stemness, makes them putative novel therapeutic target in oncology. *Antrodia cinnamomea* is a Chinese traditional medicine with several biological functions including anti-inflammation, antioxidant, and cancer prevention. However, the anti-CSC capability of *A. Cinnamomea* is not clear yet.

Aim of the study: To investigate the inhibitory effect of *A. cinnamomea* mycelium and extract on CSCs derived from various human cancer cell lines using our in-house therapeutics and human genome-wide miRNA screening panels.

Materials and methods: A broad range of human cancer cell lines, including the acute monocytic leukemia (THP-1), glioblastoma multiforme (GBM 8401), lung carcinoma (A549), breast adenocarcinoma (MDA-MB-231), hepatoblastoma (HepG2), colorectal adenocarcinoma (SW620), and foreskin fibroblast (HS68), were exposed to *A. cinnamomea* in this study. $CD133^+$ CSCs generated from the cell lines were characterized and isolated by flow cytometry, effect of chemo- and radiotherapy was assessed using the MTT assay, while the RT-PCR and human genome wide qRT-PCR determined the differential gene expression patterns. A comparative analysis of the anticancer effect of *A. cinnamomea* and Cisplatin, Taxol, or irradiation was also performed.

Results: Our results indicated that *A. cinnamomea* mycelium and its ethyl acetate extracts showed anti-proliferation effects against all types of CSCs, especially the lung, breast, and head and neck squamous cell carcinoma CSCs. Furthermore, CSCs treatment with *A. cinnamomea* combined with irradiation or chemotherapeutics demonstrated significant anti-cancer effect. We also established an association between the CSC-inhibitory effect of *A. cinnamomea* and significant downregulation of several microRNAs and cancer stemness expression levels in brain and breast CSCs. More importantly, higher CD133 expression is associated with poor prognosis in glioblastoma and breast cancer patients.

Conclusion: Herein, we demonstrate the putative role of *A. cinnamomea* as an effective ethnopharmacologic therapeutic agent for cancer treatment.

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1. Introduction

Conventional cancer treatment is mainly to inhibit the growth of the fast proliferating cancer cells and to induce their apoptosis (Gerl and Vaux, 2005). However, because of the heterogeneity of cancer cells, a highly malignant sub-population of cancer cells do escape detection by the host immune system and survive, leading to frequent cancer recurrence after initial response to chemotherapy and radiation therapy (Mimeault et al., 2007); this is supported by the proposed CSCs theory, which attempts to provide a new explanation for the therapeutic intractability of many cancers (Donnenberg and Donnenberg, 2005). Some studies have indicated that only a small subset of tumor cells are oncogenic, self-renewing, with ability to differentiate into a variety of cell lineages in the tumorous tissue (Scopelliti et al., 2009). Our team and others have shown that these few cells in different cancer tissues, including blood, breast, brain, ovarian, prostate, colorectal and oral, are more resistant to radiation or anticancer drugs than other cancer cells. This small population of cancer cells with stem cell-like characteristics are called cancer stem-like cells (CSCs) (Neuzil et al., 2007).

While CSCs can be isolated from patients' tumor tissues, they have also been shown to possess the ability to initiate tumor formation in the host's body (Liu et al., 2013). The modulation of the activities of these CSCs is closely related to tumor recurrence, remote invasion and patient survival rate (Kong et al., 2014). Similar to the normal stem cells, CSCs possess the ability to self-renew and differentiate. They grow continuously and differentiate into functionally and morphologically different tumor cells (Mukherjee et al., 2014). However, unlike normal stem cells, the self-renewal mechanisms of CSCs are modulated by dysregulated and aberrant signal cascades (Winquist et al., 2014). For instance, the surface antigen CD133, a glycoprotein with five transmembrane domains, first identified from $CD34^+$ precursor cells isolated from the blood and bone marrow of adult, as well as fetal liver cells was regarded as a marker of normal hematopoietic stem cells (Gotze et al., 2007). However, in the last decade, CD133 has been implicated as a surface marker for CSCs in blood, brain, retinoblastoma, kidney, pancreatic, prostate and colon cancers (Cheng et al., 2009; Horst et al., 2009). Recent reports also show that there are $CD133^+$ CSCs in medulloblastoma and glioma, with better proliferating and self-renewal abilities than the neighboring non-CSC tumor cells (Gu et al., 2011).

The conventional cancer treatment modalities are radiation and neoadjuvant or adjuvant chemotherapy to inhibit the cancer growth and induce apoptosis. However, the highly malignant $CD133^+$ cells often survive chemotherapy and/or radiation therapy, as well as escape detection by the host's immune system, subsequently leading to recurrent malignancies after initial response to treatment and short remission period (Kim et al., 2009). Recently, therapeutic or molecular targeting of the $CD133^+$ CSCs has been proposed as a clinically adaptable strategy for dealing with 'difficult-to-cure', 'easy-to-relapse' and metastatic malignancies (Bruttel et al., 2014; Zhang et al., 2015). In the last decade, it has been demonstrated that oncogenic microRNAs (miRNAs), characterized by aberrant expression pattern, contribute to tumorigenesis and cancer progression, and are putative molecular or therapeutic targets in anticancer therapy (Gambari et al., 2016). Furthermore, there is cumulative evidence supporting a non-conventional mechanism of cancer progression by the self renewal of cancer stem cells modulated by a regulatory network of miRNAs and signaling pathways such as the Wnt/β-catenin, Notch, and Hedgehog pathways (Prokopi et al., 2014). Thus, targeting CSCs-associated oncomiRNAs remains a promising effective strategy for cancer eradication.

Antrodia cinnamomea, also called 'Niu Chang-Zhi' or 'Niu Chang-Gu' in Chinese, is a Taiwan-endemic perennial red camphor mushroom species of the *Polyporaceae* family and *Aphyllophorales* order, commonly found growing on the inner wall of the *Cinnamomum kanehirae* Hay tree. In traditional Taiwanese medicine, *Antrodia cinnamomea* is commonly used as an antidote, liver protective and anti-cancer drug (Chang et al., 2015; Tsai et al., 2016). *Antrodia cinnamomea*, is an edible medicinal mushroom rich in nutrients including triterpenoids, polysaccharides and adenosine, which are bioactive compounds with documented immunomodulatory, anti-allergy, anti-bacterial, blood pressure lowering, hypoglycemic, hypocholesterolemic, and anti-tumor effects (Huang et al., 2013).

The aim of the present study was to investigate the anticancer potential of *A. cinnamomea* extract on CSCs isolated from various solid tumor cell lines and probe the possibility and degree of therapeutic synergism when combined with radiation or chemotherapeutic treatment. To the best of our knowledge, this is the first study demonstrating the tissue-independent inhibitory effect of *Antrodia cinnamomea* on CSCs from several human cancer cell lines.

2. Materials and methods

2.1. Materials and chemicals

The mycelium, concentrate, and lyophilized powder of *A. cinnamomea* were obtained from New Bellus Enterprises Co., Ltd. (Tainan, Taiwan). Anhydrous ethyl acetate (> 99.8% purity) and ethanol (> 99.8% purity) were purchased from Sigma Aldrich (St. Louis, MO, USA). The bioactivity, chemical, and metabolite profile of *A. cinnamomea* is well documented (Chen et al., 2016; Wu et al., 2007).

2.2. Preparation of A. cinnamomea extracts

A. cinnamomea was incubated to produce *A. cinnamomea* fermented concentrate and a total of five *A. cinnamomea* extract were further prepared: *A. cinnamomea* concentrate (D1); ethyl acetate extract of *A. cinnamomea* concentrate (D2); lyophilized powder of *A. cinnamomea* concentrate (D3); ethyl acetate extract of lyophilized powder of *A. cinnamomea* concentrate (D4); and ethyl acetate extract of *A. cinnamomea* mycelium (D5).

A. cinnamomea was incubated to generate *A. cinnamomea* fermentation broth and then was concentrated by filtering through the membrane at 10 °C to generate *A. cinnamomea* concentrate (D1), which was further freeze-dried to generate the lyophilized powder of *A. cinnamomea* concentrate (D3).

The ethyl acetate extract of *A. cinnamomea* concentrate was prepared by adding 100 ml of *A. cinnamomea* concentrate to 100 ml of ethyl acetate to partition and repeated 3 times. The ethyl acetate layer was then collected, concentrated and dried to generate the ethyl acetate extract (D2).

The ethyl acetate extract of lyophilized powder of *A. cinnamomea* concentrate was prepared from 10 g of lyophilized powder of *A. cinnamomea* concentrate (D3) by adding 100 ml of 95% ethanol for reflux extraction at room temperature for 3 h. After concentrating and drying, 100 ml of ddH_2O and 100 ml of ethyl acetate were added to partition 3 times. The ethyl acetate layers was then collected, concentrated and dried to generate the ethyl acetate extract of lyophilized powder of *A. cinnamomea* concentrate (D4).

The ethyl acetate extract of *A. cinnamomea* mycelium (D5) was prepared by adding 10 g of *A. cinnamomea* mycelium to 100 ml of 95% ethanol for reflux extraction at room temperature for 3 h. After concentration and drying, 100 ml of ddH_2O and 100 ml of ethyl acetate were added to partition 3 times successively. The ethyl acetate layer was then collected, concentrated and dried to generate the ethyl acetate extract of *A. cinnamomea* mycelium (D5).

2.3. Cell culture and preparation of cancer stem cells

Human acute monocytic leukemia (THP-1, BCRC60430), brain glioblastoma multiforme (GBM 8401, BCRC60163), lung carcinoma (A549, BCRC60074), breast adenocarcinoma (MDA-MB-231, 60425),