Biomedicine & Pharmacotherapy 102 (2018) 18–25

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Medicinal mushroom *Phellinus igniarius* induced cell apoptosis in gastric cancer SGC-7901 through a mitochondria-dependent pathway

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ARTICLE INFO

Keywords:
Phellinus igniarius
Antitumor activity
Gastric cancer
SGC-7901
Apoptosis
Mitochondria-dependent pathway

ABSTRACT

Gastric cancer (GC) is the fourth most common diagnosed cancer and the second leading cause of cancer death worldwide. *Phellinus igniarius* is a traditional medicinal mushroom used in China and other countries of East Asia for the treatment of various diseases including cancer. The aim of this study is to evaluate the antitumor activity of *P. igniarius* and elucidate the possible mechanism. MTT assay displayed that the total ethanol extract of *P. igniarius* (TPI) had antitumor activities against five human tumor cell lines of HepG-2, AGS, SGC-7901, Hela and A-549. TPI was found the most cytotoxity against gastric cancer SGC-7901 *in vitro*, and strongly inhibited the tumor growth in xenograft nude mice *in vivo*. Typical morphological changes due to cell apoptosis including chromatin condensation, and nuclear fragmentation with the formation of apoptotic bodies were observed in the SGC-7901 cells after TPI treatment. TPI blocked SGC-7901 cell cycle at G_0/G_1 phase and caused apoptosis by down-regulated the expression of cyclin D1. TPI caused a remarkable collapse of mitochondrial membrane potential (MMP, $\triangle\psi m$) in SGC-7901 cells and induced the mitochondrial-dependent apoptosis by triggered the caspase-9, -3 activation and PARP cleavage. Moreover, TPI increased the ratio of Bax/Bcl-2 in SGC-7901 both *in vitro* and *in vivo*. These findings suggested that *P. igniarius* could be a potential natural derived therapeutic agent for the prevention and treatment of gastric cancer, as it could induce the cancer cell apoptosis through a mitochondria-dependent pathway.

1. Introduction

Gastric cancer (GC) is the fourth most common diagnosed cancer and the second leading cause of cancer death worldwide, and almost two-thirds of GC cases and deaths occur in underdeveloped regions [1]. In China, GC currently is the third among most common cancers, and will remain a serious burden in the next decades [2]. Current primary therapies for GC, including surgery, chemotherapy, and radiotherapy, are limited due to various diverse effects. The 5-year relative survival rate of GC is less than 25%, despite some new technical advances have been applied in last decades [3]. Now, natural herb-derived medicines in GC treatment are gaining more attention because of their advantages of efficiency, safety, and few side effects [4–6].

Macrofungi are mainly represented by mushrooms widely distributed in nature. Some mushrooms are edible and some possess valuable medicinal properties [7,8]. *Phellinus igniarius* (L.:Fr.) Quél. (Hemenochaetaceae, Agaricomycetes), a perennial wood-decaying mushroom with orange to yellow color, hosted on the trunks of mulberry, poplar, willow, and other broad-leaved trees, mainly distributed in China, Japan, Mongolia, Korea and some other Asian countries. As a precious medicinal fungus, *P. igniarius* has been widely used as health supplement and ancient herbal medicine in East Asian countries, especially in China, Korea and Japan. The fruiting body of *P. igniarius* is commonly recorded as “Sanghuang” in Traditional Chinese Medicine for the treatment of bloody gonorrhea, festering wounds, diarrhea, and menoxenia [9].

As a well-known Oriental medicinal fungus, *P. igniarius* was reported to have a variety of biological activities [10–14], especially its potential in cancer prevention and treatment. Yang et al. reported that *P. igniarius* extract significantly prolonged the survival of Sarcoma180 tumor-bearing mice [15]. The extracts of *P. igniarius* were reported to possess antiproliferative, antimetastatic, and antimutagenic effects *in vitro* [16,17]. Polysaccharides from *P. Igniarius* were found to have antitumor effect [18]. In addition, a series of yellow polyphenols known as styrylpyrones have been found in this fungus in past decades [19], of which, hispolon is the most important bioactive compound and showed significant effects against various cancers including gastric cancer [20], leukemia [21], breast and bladder cancer [22], liver cancer [23],

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https://doi.org/10.1016/j.biopha.2018.03.038
Received 14 December 2017; Received in revised form 9 March 2018; Accepted 9 March 2018

cervical cancer [24] and lung cancer [25].

To provide additional scientific evidence, especially the mechanistic insights to support the medicinal use of this mushroom, the present study mainly attempts to explore the antitumor activity of *P. igniarius* on gastric cancer SGC-7901 as well as the related molecular mechanism.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) and 3-(4, 5-dimethylthiazolyl-2)- 2, 5- diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). The antibodies against Bcl-2, Bax, caspase-9, caspase-3, PARP and Cyclin D1 proteins were obtained from Cell Signaling Technology (Beverly, MA).

2.2. Mushroom material and preparation of extract

The fruiting bodies of *P. igniarius* were collected at Tiantangzhai National Nature Reserve, Jinzhai, Anhui Province, People's Republic of China, in July 2014, and were identified by Prof. Kai-Jin Wang from the School of Life Sciences, Anhui University, where a voucher specimen (No. 2014011) was deposited. The dried fruiting bodies of *P. igniarius* (2.0 kg) were cut into small pieces and extracted three times with 90% ethanol (10 L × 3) under reflux for 3h at 80 °C. The combined solution was filtrated and then evaporated *in vacuo* at 50 °C to afford the total extract of *P. igniarius* (TPI, 160 g) and stored in a refrigerator until used.

2.3. Cell culture

Five human cancer cell lines, liver cancer HepG-2, gastric cancer AGS and SGC-7901, cervical cancer Hela, and lung cancer A549 cells were obtained from Nanjing Kaiji Biotech Company (Nanjing, Jiangsu, China). All the cancer cells were cultured in DMEM medium, supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were incubated at 37 °C in a 95% humidity atmosphere containing 5% CO_2. Culture medium was changed every two days. TPI was dissolved in DMSO and diluted with 1640 medium before used.

2.4. In vitro cytotoxicity assay (MTT assay)

The inhibition of TPI against cancer cell was assayed by MTT method as previously described [26]. Briefly, cells were seeded in 96-well plates at 2×10^4 cells per well and allowed to adhere for 24 h. Cells were treated by TPI with final concentrations at 40, 80, 160, 320 and 640 μg/mL. After incubation, 20 μL of MTT (5 mg/mL) was added to each well, and the incubation continued for 4 h at 37 °C. Then, 150 μL of DMSO was added to each well to dissolve the formazan crystals of the viable cells. The plates were read at a wavelength of 570 nm using a Wallac 1420 ARVOsx microplate reader (Perkin-Elmer Life and Analytical Sciences, Inc., Boston, MA, USA). 5-Fluorouracil was used as the positive control. The 50% inhibitory concentration value (IC_{50}) was calculated to evaluate the cytotoxic activity.

2.5. DAPI staining assay

Nuclear fragmentation was examined by DAPI assay. Briefly, SGC-7901 cells (2×10^5 cells/mL) were seeded on glass slides and placed in 6-well plates for 48 h. Cells treated with a series concentrations of TPI (80, 160, 240, 320 and 400 μg/mL) for 48 h. After treatment, cells were washed and stained with DAPI (10 μg/mL) for 20 min at 37 °C. Slides were viewed using a fluorescence microscope (BX53, Olympus).

2.6. Electron microscopy

SGC-7901 cells were treated with vehicle control (DMSO, 3 μL) or propidium iodide (PI) at the dose of 15 μM for indicated time on 60 mm plates. Then the cells were collected and fixed in phosphate buffer (PH 7.4) containing 2.5% glutaraldehyde overnight at 4 °C.The cells were postfixed in 1% OsO4 at room temperature for 60 min, stained with 1% uranylacetate, dehydrated through graded acetone solutions, and embedded in epon. Areas containing cells were block mounted and cut into 70 nm sections and examined with the electron microscope (H-7500, Hitachi, Ibaraki, Japan).

2.7. Cell apoptosis via flow cytometry

The SGC-7901 cells were seeded at a density of 2×10^5 cells/well in 6-well plates for 12 h, and then treated with TPI (80, 160, 240, 320 and 400 μg/mL) for 24 h. The cells were harvested with trypsin, washed twice with ice-cold PBS, and resuspended in FITC Annexin V and PI (5 μL each) and incubated for 15 min at room temperature in the dark, and analyzed by a FACSCalibur flow cytometer (BD Biosciences, CA) at the excitation wavelength of 480 nm.

2.8. Cell cycle analysis

Cells were placed in 6-well plates for 12 h, and then treated with TPI (80, 160, 240, 320 μg/mL) for 48 h. Cells were collected, fixed in 70% ethanol for at least 24 h at −20 °C, and stained with 20 μg/mL PI containing 10 μg/mL RNase A for 30 min at room temperature. Cell cycle analysis was performed with a FACSCalibur (BD Bioscience, Franklin Lakes, USA) and analyzed by ModFiT LT software.

2.9. Evaluation of mitochondrial membrane potential (MMP, Δψm)

The effects of TPI on the cell mitochondrial membrane potential (Δψm) were examined by fluorescence microscope using JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′- tetraethyl-benzimidazolylcarbocyanine chloride) kit (Beyotime Biotech, Haimen, China). Briefly, after 48 h of exposure to TPI, the cells were trypsinized and collected, then washed twice with PBS and incubated with JC-1 working solution for 15 min at 37 °C and 5% CO_2 in the dark. The cells were washed twice with PBS and resuspended in 200 μL PBS. The stained cells were analyzed by flow cytometry to determine the change in the florescence from red to green.

2.10. Western blot analysis

Cells or tumor tissues were homogenized in protein lysis buffer, and debris was removed by centrifugation for 10 min at 4 °C. Protein concentrations in all samples were determined by using Bradford protein assay (Bio-Rad, Hercules, CA, USA). Protein samples were separated using 6–12% sodium dodecyl sulfate-polyacrylamide gels and transferred to PVDF membranes. Five percent nonfat milk was used to block nonspecific binding for 1 h at room temperature. Blots were then probed with specific primary antibodies. Horseradish peroxidase-conjugated secondary antibodies and ECL kit (Bio-Rad, Hercules, CA, USA) were used for detection.

2.11. In vivo xenograft model

All animal experiments complied with the Medical University's Policy on the Care and Use of Laboratory Animals. Protocols for animal studies were approved by the Medical Animal Policy and Welfare Committee. Four-week-old, athymic BALB/c nu/nu mice (18–20 g) were purchased from Vital River Laboratory (Beijing, China). Mice were housed at a constant room temperature with a 12 h:12 h light/dark cycle and fed a standard rodent diet and given water *ad lib*. SGC-7901 cells (5×10^6) were suspended in 0.2 mL PBS and injected