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A supercritical-CO2 extract of Ganoderma lucidum spores inhibits cholangiocarcinoma cell migration by reversing the epithelial–mesenchymal transition

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ABSTRACT

Background: *Ganoderma lucidum (G. lucidum)* is an oriental medical mushroom that has been widely used in Asian countries for centuries to prevent and treat different diseases, including cancer.
Hypothesis/Purpose: The objective of this study was to investigate the effect of A supercritical-CO2 extract of *G. lucidum* spores on the transforming growth factor beta 1 (TGF-β1)-induced epithelial–mesenchymal transition (EMT) of cholangiocarcinoma cells.
Study design: This was an *in vitro* study with human cholangiocarcinoma TFK-1 cells treated with varying concentrations of *G. lucidum*.
Methods: A supercritical-CO2 extract of G. lucidum spores (GLE) was obtained from completely sporoderm-broken germinating G. lucidum spores by supercritical fluid carbon dioxide (SCF-CO2) extraction. GLE pre-incubated with human cholangiocarcinoma TFK-1 cells prior to TGF-β1 treatment (2 ng/ml) for 48 h. Changes in EMT markers were analyzed by western blotting and immunofluorescence. The formation of F-actin stress fibers was assessed via immunostaining with phalloidin and examined using confocal microscopy. Additionally, the effect of the GLE on TGF-β1-induced migration was investigated by a Boyden chamber assay.
Results: TGF-β1-induced reduction in E-cadherin expression was associated with a loss of epithelial morphology and cell–cell contact. Concomitant increases in N-cadherin and Fibronectin were evident in predominantly elongated fibroblast-like cells. The GLE suppressed the TGF-β1-induced morphological changes and the changes in cadherin expression, and also inhibited the formation of F-actin stress fibers, which are a hallmark of EMT. The GLE also inhibited TGF-β1-induced migration of TFK-1 cells.
Conclusion: Our findings provide new evidence that GLE suppress cholangiocarcinoma migration *in vitro* through inhibition of TGF-β1-induced EMT. The GLE may be clinically applied in the prevention and/or treatment of cancer metastasis.

Introduction

Cholangiocarcinoma (CCA) is the second most common primary malignancy of the liver (Carriaga and Henson, 1995) and the incidence and mortality rate has been steadily growing worldwide (von Hahn et al., 2011). Despite advances in surgical and medical therapy, treatment options for CCA remain unsatisfactory (Blechacz and Gores, 2008). The primary reason for the poor prognosis is metastasis, which precludes curative surgical resection. Therefore, screening suitable compounds targeting tumor metastasis is an effective way to treat this highly invasive cancer.

Cancer metastasis refers to the spread of cancer cells from the primary neoplasm and the growth of secondary tumors at sites distant from the primary tumor. Metastasis occurs through a complex multistep process consisting of invasion into the circulation from a primary tumor, immigration to distant organs, adhesion to endothelial cells and infiltration into the tissue. The process of epithelial–mesenchymal transition (EMT) is characterized by the epithelial cells converting into the elongated, motile and invasive mesenchymal phenotype (Thiery et al., 2009), which enhances cellular motility. It is considered an early event of metastasis and a critical step in the dissemination of tumor cells

Abbreviations: G. lucidum, *Ganoderma lucidum*; GLE, supercritical-CO2 extract of *G. lucidum* spores; CAA, cholangiocarcinoma; TGF-β1, transforming growth factor beta 1; EMT, epithelial–mesenchymal transition.

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(Yilmaz and Christofori, 2010). Increasing evidence suggests that EMT is important during the progression and recurrence of CCA (Ryu et al., 2012). Therefore, the inhibition of EMT could be therapeutically important for the inhibition of invasion and metastasis in CCA.

Ganoderma lucidum (G. lucidum) has been used as a dietary therapeutic in traditional Chinese medicine for several millennia. The fruit bodies, cultured mycelia and the spores of *G. lucidum* have been reported to be effective in the treatment of various types of diseases, including cancer (Chen and Zhong, 2011; Liu and Zhong, 2011; Seto et al., 2009). Although the fruiting body of *G. lucidum* has been used as a traditional herbal medicine since ancient times, the spores only came to be utilized in the late 20th century. It is known that the anticancer effects of *G. lucidum* may be derived from the triterpenoids, polysaccharides or immunomodulatory protein components (Zhou et al., 2007). A supercritical-CO2 extract that we have obtained from *G. lucidum* spores (GLE) has high bioactive triterpenoid contents. More recently, we have reported GLE exert antitumor activities by direct tumoricidal effects or indirectly by activating monocytes/macrophages (Zhang et al., 2009).

However, to date, the ability of GLE to antagonize transforming growth factor beta 1 (TGF-β1)-mediated changes associated with EMT in CCA cells has not been explored. In the present study, we evaluated the effect of GLE on the TGF-β1-induced EMT of CCA cells. Our findings suggest that GLE inhibits TGF-β1-induced EMT development, thus suppressing EMT-activated CCA cell migration.

Materials and methods

Reagents

Fetal bovine serum (FBS) was obtained from Hyclone, Thermo Fisher Scientific, Victoria, Australia, and Roswell Park Memorial Institute 1640 medium (RPMI 1640) was from Hyclone, Logan, UT, USA. TGF-β1 was from R&D Systems, Minneapolis, MN, USA, and 4'-6-diamidino-2-phenylindole (DAPI) and Alexa Fluor® 555 phalloidin were from Sigma–Aldrich, Seelze. Mouse monoclonal antibodies (mAb) against E-cadherin (610181), N-cadherin (610920) and Fibronectin (610077) were from BD Pharmingen, San Diego, CA, USA.

Plant and spores

G. lucidum was cultivated at a base in a high alpine forested area (1000 m above sea level) in Fujian Province, Southeast China, which was established by the Academy of Food and Health Engineering at Sun Yat-Sen University. The fungus was G. lucidum (Curtis: Fr.) Karst. (Polyporaceae), and voucher specimen of the sample was deposited in the Mycological Herbarium, Institute of Microbiology, Chinese Academy of Sciences. The spores of G. lucidum were collected and activated by placing the germination-induced spores in a well-ventilated culture box kept at constant temperature and humidity (relative humidity, 60% to 98%; temperature, 16 °C to 43 °C; activation period, 10 min to 24 h) to obtain the germination-activated spores. The germination rate of the spores was more than 95%. The sporoderm of germinating spores was then broken and the broken rate could reach 99.8%.

Sample preparation and determination of ganoderic acids content

The germination-activated, sporoderm-broken spores were placed in supercritical fluid carbon dioxide (SCF-CO2) extracting apparatus, the supercritical conditions included 5 M to 60 M Pa of pressure; 32°C to 85°C of temperature; and 5 kg/h to 80 kg/h of flow capacity rate. The total extraction time was between 0.5 to 6 h. 37 g extract was obtained from 100 g of completely sporoderm-broken germinating ganoderma spores (extraction yield = 37 g/100 g). The extract mainly consisted of triglycerides and *G. lucidum* triterpenoids (Liu et al., 2007; Yuan et al., 2006).

Ganoderic acids, the major bioactive triterpenoids components from *G. lucidum,* could be considered as the "marker compounds" for the chemical evaluation. Thus, the contents of the ganoderic acids in the GLE were determined following the previous method (Gao et al., 2004)**.** Briefly, GLE was dissolved in MeOH to prepare stock solution of 0.5 g/ml and filtered through a 0.45 μM membrane. Then, 20 μl of the supernatant diluted to appropriate concentration was injected into the HPLC System (Binary HPLC Pump 1525, Refractive Index Detector 2414, Photodiode Array Detector 2996; Waters, Milford, MA, USA) which included a Symmetrys C18 column (4.6 mm × 250 mm, 5 μm; Waters) and a Guard column (3.9 mm × 20 mm × 5 mm; Waters). The mobile phase was made by mixing solvent A (0.1% phosphoric acid aqueous solution) and solvent B (acetonitrile) using the following gradient program: 0 min, 20% B; 5 min, 30% B; 10 min, 36% B; 60 min, 40% B; 61 min, 20% B; 65 min, 20% B. The flow rate was set at 1.0 ml/min, and the detecting wavelength was set at 254 nm. The operating temperature was maintained at 30 °C. Chromatographic peak was identified by comparing the retention times and spectra against known standards. The method was validated for parameters such as linearity, precision, and accuracy following the previous method (Gao et al., 2004).

Cell culture and treatment

The human CCA TFK-1 cell line was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. Cells were maintained in RPMI 1640 medium with 10% FBS and 1% penicillin–streptomycin at 37°C in 5% CO_2 with constant humidity. The cells were provided with fresh medium every 2–3 days.

TFK-1 cells were pretreated with GLE at various concentrations (GLE at the concentrations less 400 μg/ml has no effect on the TGF-β1-induced EMT, data not shown) for 8 h, then incubated with 2 ng/ml TGF-β1 for 48 h. The GLE was dissolved in dimethyl sulfoxide (DMSO) so that the final concentration of DMSO was less than 0.1% (*v*/*v*).

Cell viability assay

TFK-1 cells were seeded at 2×10^4 cells/well in 96-well plates, and then cultured with GLE, TGF-β or medium alone for the indicated time. The viability of tumor cells was determined by Alamar Blue assay (AbD Serotec, Oxford, UK). The cell viability was calculated according to the following formula: Cell viability (%) = (1 – absorbance of experimental group/absorbance of control group) × 100%. Values are represented as means ± SEM of three independent experiments performed in triplicate.

Western blotting

Proteins were extracted as previously described and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), immunoblotted with an mAb against E-cadherin, N-cadherin, Fibronectin or actin (used as an internal control), and visualized with an ECL Kit (Pierce, Rockford, IL, USA).

Immunofluorescence

For immunofluorescence analysis, TFK-1 cells cultured on coverslips were fixed with ice-cold methanol and then stained with mouse anti-human E-cadherin, N-cadherin or Fibronectin, followed