Cordyceps militaris fraction inhibits the invasion and metastasis of lung cancer cells through the protein kinase B/glycogen synthase kinase 3β/β-catenin signaling pathway

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Abstract. *Cordyceps militaris* is widely used as a traditional Chinese medicine health supplement, and is also used in the development of anticancer agents. In our previous studies, it was revealed that *C. militaris* fraction (CMF) possessed an antitumor effect against K562 cells *in vitro*, induced apoptosis and caused cell cycle arrest in the S phase. The published results also demonstrated that CMF-induced apoptosis was involved in mitochondrial dysfunction. The aim of the present study was to investigate the anti-invasion and anti-metastasis effects of CMF in NCI-H1299 and Lewis lung cancer (LLC) cell lines, which have high metastatic potential. MTT and clone formation assays were initially used to investigate the inhibitory effect of CMF on the viability of NCI-H1299 and LLC cells. The results of cell adhesion, wound healing, migration and Matrigel invasion assays *in vitro* indicated that NCI-H1299 cells (treated with 1, 3, 10 or 30 μg/ml CMF) and LLC cells (treated with 0.1, 0.3, 1 or 3 μg/ml CMF) demonstrated a concentration-dependent reduction in cell migration and invasion compared with the control. *In vivo* experiments demonstrated that the oral administration of CMF (65, 130 or 260 mg/kg) decreased the tumor growth and decreased the lung and liver metastasis in an LLC xenograft model, compared with untreated mice. Furthermore, western blot analysis was used to investigate the mechanism of the effect of CMF on the migration of NCI-H1299 cells and metastasis in the xenograft model. The results revealed that CMF may promote glycogen synthase kinase 3β (GSK-3β)-mediated degradation of β-catenin inhibited the phosphorylation of upstream protein kinase B (Akt), which resulted in the attenuation of the expression of matrix metalloproteinase (MMP)-2 and MMP-9. These results suggested that CMF may possess potential for the treatment of lung cancer metastasis via the Akt/GSK-3β/β-catenin pathway.

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Introduction

Lung cancer continues to be the leading cause of cancer-associated mortality globally and its rates of incidence and mortality have increased rapidly in developing countries (1,2). Previous studies have primarily focused on advancing the discovery and understanding of the development of lung cancer and metastasis (3-5). Clinical applications of cancer immunotherapies, radio therapies, gene therapies or target-oriented therapies have been suggested as potential approaches to lung cancer treatment (6). However, due to the considerable toxicity and in efficiency of these treatments, the results of standard chemotherapy and radiotherapy for the treatment of patients with an advanced stage of or locally recurring lung cancer remain unsatisfactory (7). Metastasis is responsible for a substantial number of the associated mortalities and the poor prognosis of patients with all types of cancer (4). Tumor metastasis is an exceedingly complex process and prevailing models of metastasis reflect that metastasis is a late, acquired event in tumorigenesis (8). Increasingly, evidence supports the view that the matrix metalloproteinases (MMPs) are proteolytic enzymes that mediate a number of the changes in the microenvironment during tumor progression (9-11). Among the members of the MMP family, MMP-2 and MMP-9 are type IV collagenases primarily involved in the degradation of the extracellular matrix (ECM) (11). In patients clinically diagnosed with lung cancer metastasis, serum levels of MMP-2 and MMP-9 are significantly increased compared with patients with an absence of distant metastasis or in healthy volunteers (12,13). Additionally, the Wnt/β-catenin pathway has been identified as providing tumor cells with the capacity to become resistant to treatment, self-renewal and metastasis (14).

A study revealed that the invasion of hepatocellular carcinoma BEL-7402 cells would be suppressed by inhibiting the expression of phosphorylated-glycogen synthase kinase (p-GSK), β-catenin and its target proteins (15). A number of molecular predictors, including β-catenin, MMPs, disheveled segment polarity protein (Dvl) and protein kinase B (Akt) have been proposed and are usually over expressed in non-small-cell lung cancer (NSCLC) (16-18). Therefore, the specific inhibition of expression of these proteins may serve as an effective method to prolong the survival and improve the prognosis of patients with lung cancer.

Cordyceps militaris has been extensively used as in the formula of nutraceuticals and as a tonic supplement for sub-healthy patients who are generally not completely healthy, particularly in China and Korea (19,20). At present, cultured *C. militaris* has been well established and a variety of constituents extracted from *C. militaris* (21). In addition to functional foods and supplements, *C. militaris* also has various pharmacological activities, including antioxidation (22), anti-inflammation (23), anti-proliferation (24) and anti-metastasis (25) in numerous tumor types. Therefore, *C. militaris* has good development prospects not only for healthcare but also for cancer treatment.

In our previous study, *C. militaris* fraction (CMF) was demonstrated to inhibit the proliferation of K562 cells and to induce apoptosis in addition to cell cycle arrest in the S phase. The mechanism underlying CMF-induced apoptosis was involved in mitochondrial dysfunction (26). In the present study, the aim was to investigate the inhibitory effects of CMF on the migration and invasion of NCI-H1299 and Lewis lung cancer (LLC) cell lines, in addition to metastasis in a xenograft model.

Materials and methods

Fraction preparation and materials. Cultured *C. militaris* was purchased from Shaanxi Honghao BioTech Co., Ltd. (Jiangmen, China). CMF was prepared as previously described (26), dissolved in serum-free of RPMI-1640 or DMEM medium to make a 1 mg/ml stock solution, and stored at -20˚C in multiple aliquots. RPMI-1640 medium and Dulbecco's modified Eagle's medium (DMEM) were purchased from Thermo Fisher Scientific, Inc., (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Anti-MMP-2 (cat no. 4022), MMP-9 (cat no. 3852), Akt (cat no. 9272), p-Akt (cat no. 9271), GSK-3β (cat no. 9832), p-GSK-3β (cat no. 9323) and MYC proto-oncogene (c-Myc) (cat no. 9402) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-β-catenin (cat no. sc7963) and Dvl-2 (cat no. sc8026) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-β-actin (cat no. ab16039) and GAPDH (cat no. ab181602) were obtained from Abcam (Cambridge, UK). HRP, Rabbit Anti-Goat IgG (H+L) (cat no. E030130) and HRP, Mouse Anti-Goat IgG (H+L) (cat no. E030110-01) were obtained from Earth Ox Life Sciences (Millbrae, CA, USA).

Cells and culture. NCI-H1299 and Lewis lung carcinoma (LLC) cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). NCI-H1299 cells were cultured in RPMI-1640 medium with 10% FBS and LLC cells in DMEM with 10% FBS, each of which was supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37˚C.

MTT assay. A total of $3x10^3$ NCI-H1299 and LLC cells per well were seeded onto 96-well plates (cat no. 3599; Corning Incorporated, Corning, NY, USA) and treated with 100 μl of CMF at 0.3, 1, 3, 10, 30, 90 μ1/ml in NCI-H1299 cells and 0.03, 0.1, 0.3, 0.9 μg/ml in LLC cells. In a pre-screening experiment (data not shown), the effect of CMF was stronger against LLC cells than against NCI-H1299 cells. Therefore, the two cell lines were treated with different concentrations of CMF to obtain the IC_{50} values. The same volume (100 μl) of corresponding complete medium was used as a negative control. Following incubation for 24, 48, 72 h at 37˚C, 5% CO_2, 20 μl MTT solution (5 mg/ml) was added into each well. Then 200 μl DMSO was used to dissolve the formazan crystals and optical density (OD) absorbance was measured at 570 nm using a 96-well microplate reader. The results were presented as cell viability (%)=$OD_{treatment}/OD_{nc}$x100% and 50% inhibitory concentration (IC_{50}) was calculated by linear-regression analysis. All experiments were performed in triplicate.

Colony formation assay. NCI-H1299 and LLC cells were plated onto 6-well plates (200 cells/well; cat no. 3516; Corning Incorporated) and allowed to adhere overnight. The cells were then treated with 0.01, 0.03, 0.1, 0.3, 1 μg/ml of CMF or left untreated and cultured in corresponding complete medium at 37˚C, 5% CO_2 in air for up to 14 days. After 14 days, the cloned cells were fixed with absolute methanol for 30 min and stained with 0.1% crystal violet solution at room temperature for 20 min. Colonies of each well were photographed and counted with the naked eye.

Cell adhesion assay. CI-H1299 and LLC cells ($1x10^5$) were plated in 6-well culture dishes with or without CMF and allowed to adhere for 1.5 h. Subsequently, corresponding medium with non-adhered cells was discarded and cells were gently washed twice with PBS in order to remove any loosely attached cells. Adhered cells were then counted using a 0.1% crystal violet staining solution at room temperature for 20 min subsequent to being fixed with methanol for 30 min at room temperature. Data are expressed as a percentage in adhered cells treated with CMF relative to the control cells.

Wound healing assay. Cell migration was analyzed using a wound healing assay. NCI-H1299 and LLC cells ($2x10^5$) were seeded onto 6-well plates until they reached confluence. A scratch wound in confluent monolayer was made using a 10 μl sterile pipette tip. Subsequent to washing away all detached cells with PBS, the remaining cells were treated with or without CMF (NCI-H1299 cells were treated at 1, 3, 10, 30 μg/ml at 1 and LLC cells at 0.1, 0.3, 1, 3 μg/ml) in serum-free RPIM-1640 or DMEM medium. Photographs were taken at 0 and 24 h after treatment.

Transwell migration and invasion assay. A Transwell assay was used to test the migration ability of the cells. In order to test the invasion ability of cells, 6.5-mm Transwell inserts with

a 8.0-μm pore membrane (cat no. 3422; Corning Incorporated) coated with 50 μl of a 1:4 diluted Matrigel (BD Biosciences, San Jose, CA, USA) in cold RPIM-1640 or DMEM medium to form a thin continuous film on the top of the filter were used. The procedure was performed as previously described (22). A total of $4x10^4$ NCI-H1299 and LLC cells per well were seeded onto an insert with 200 μl serum-free RPIM-1640 or DMEM medium with or without CMF, and 600 μl corresponding medium containing 20% FBS was added into the bottom wells. Following 24 h incubation at 37˚C, 5% CO_2 in air, non-migrating cells were removed from the upper surface by wiping with a cotton swab. The bottom cells of the filter were fixed with absolute methanol for 30 min at room temperature and stained with 0.1% crystal violet for 20 min at room temperature. Subsequently, cells in 4 randomly selected fields were counted by a Digital Sight Inverted Light Microscope at x100 magnification (Nikon Corporation, Tokyo, Japan).

Western blot analysis. Whole cell lysate preparation and western blot analysis were performed as previously described (27). NCI-H1299 and LLC cells treated with or without CMF were collected and lysed in radio immunoprecipitation assay buffer (cat no. 66016413; Biosharp, Hefei, China) with 1% phenylmethylsulfonyl fluoride and 1% phosphatase inhibitor after 24 h cultivation. Cleared total cell lysate was quantified by BCA assay kit (Thermo Fisher Scientific, Inc.) and denatured by boiling for 10 min and loaded onto 10% SDS-PAGE with 40 μg per lane. Following electrophoretic separation, proteins were transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked for 2 h at room temperature in blocking buffer (5% skimmed milk in Tris-buffered saline with Tween-20 buffer) and incubated with the primary antibodies (AKT, p-AKT, GSK-3β, p-GSK-3β, c-MYC, MMP-2, MMP-9 were diluted to 1:1,000, DVL2 and β-catenin to 1:500, GAPDH and β-actin to 1:4,000) overnight at 4˚C. After washed with TBST three times, membranes which were incubated with AKT, p-AKT, p-GSK-3β, c-MYC, MMP-2, MMP-9, GAPDH and β-actin antibodies were then incubated with goat anti-rabbit IgG secondary antibody (1:4,000 dilutions), while membranes which were incubated with GSK-3β, DVL2 and β-catenin were then incubated with goat anti-mouse IgG HRP secondary antibodies (1:4,000 dilutions) for 2 h at room temperature. Finally, the membranes were developed by electrochemiluminescence substrates (Tanon Science and Technology Co., Ltd., Shanghai, China) and exposed onto X-ray films in a dark room. Results were analyzed using Image Lab Software (Version 5.1; Bio-Rad Laboratories, Inc., Hercules, CA, USA). GAPDH and β-actin were used as controls.

Animal experiments. A total of 40 C57BL/6 mice (male, aged 6-8 weeks, weighted 16-18 g) were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). All animal experiments were carried out in compliance with the Animal Management Rules of the Ministry of Health of the People's Republic of China and approved by the Animal Care and Use Committee of Jinan University (Guangzhou, China). The mice were housed and maintained under sterile conditions at 23-27˚C, 40-60% relative humidity in a 12 h light/12 h dark cycle and *ad libitum* access to food and water. Mice were randomly divided into five groups (8 mice per group). A total of $6x10^6$ LLC cells were injected subcutaneously into the right armpit of the mice. After 24 h of inoculation, the mice were treated with different doses of CMF (65, 130 or 260 mg/kg) by oral administration once a day and the negative control group was administered the same volume of distilled water. The positive control group was administered cyclophosphamide (30 mg/kg) through intraperitoneal injection twice a week. During the treatment, the body weight of the mice was scaled and the volume of tumor was measured using a vernier caliper. Following a 4-week treatment course, mice were sacrificed, and tumors were removed and weighed. Half of each tumor was frozen in liquid nitrogen for western blot analysis (as previously described). The lung tissues were fixed in Bouin's solution (picranisic acid:formalin:glacial acetic acid=15:5:1) for 24 h at room temperature and then tumor nodules on the lung surface were counted. Hematoxylin and eosin staining of lung and liver tissues was performed at room temperature for 20 min to evaluate the morphology and then examined under a light microscope with x100 magnification (Nikon Corporation, Tokyo, Japan).

Statistical analysis. The data are presented as the mean ± the standard deviation. GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. The data for concentration and dosage effects were analyzed using a one-way analysis of variance followed by Tukey's multiple comparisons test. $P<0.05$ and $P<0.01$ were considered to indicate a statistically significant difference.

Results

CMF inhibits the viability of NCI-H1299 and LLC cells. The inhibitory effect of CMF on the viability of NCI-H1299 and LLC cells was investigated using an MTT assay at varying concentrations (0.3, 1.0, 3.0, 10.0, 30.0 or 90.0 μg/ml in NCI-H1299 cells; 0.03, 0.10, 0.30, 1.00, 3.00 or 9.00 μg/ml in LLC cells) and time periods (24, 48 or 72 h). As shown in Fig. 1A, CMF inhibited the viability of NCI-H1299 and LLC cells in a time- and concentration-dependent manner compared with the control. The half maximal inhibitory concentration (IC_{50}) values of CMF in NCI-H1299 cells were 16.58 and 7.95 μg/ml for 48 and 72 h, respectively. For the LLC cells, the IC_{50} values were only 1.63 μg/ml (48 h) and 0.58 μg/ml (72 h). Furthermore, a clone formation assay was used to evaluate the ability of single cell viability. As shown in Fig. 1B, that cell clone formation was significantly inhibited in a concentration-dependent manner in NCI-H1299 cells treated with ≥0.03 μg/ml CMF compared with the control ($P<0.05$) and in all CMF-treated LLC cells compared with the control ($P<0.01$). Subsequent to incubation with CMF at 1 μg/ml, the number of cells forming colonies in the two cell lines was decreased by almost 90% compared with that of control group. These results suggested that CMF might efficiently inhibit the viability of NCI-H1299 and LLC cells.

CMF suppresses the adhesion, migration and invasion of NCI-H1299 and LLC cells. Two-dimensional and physiological three-dimensional culture systems have been constructed to perform cell motility *in vitro*, which contributes to current

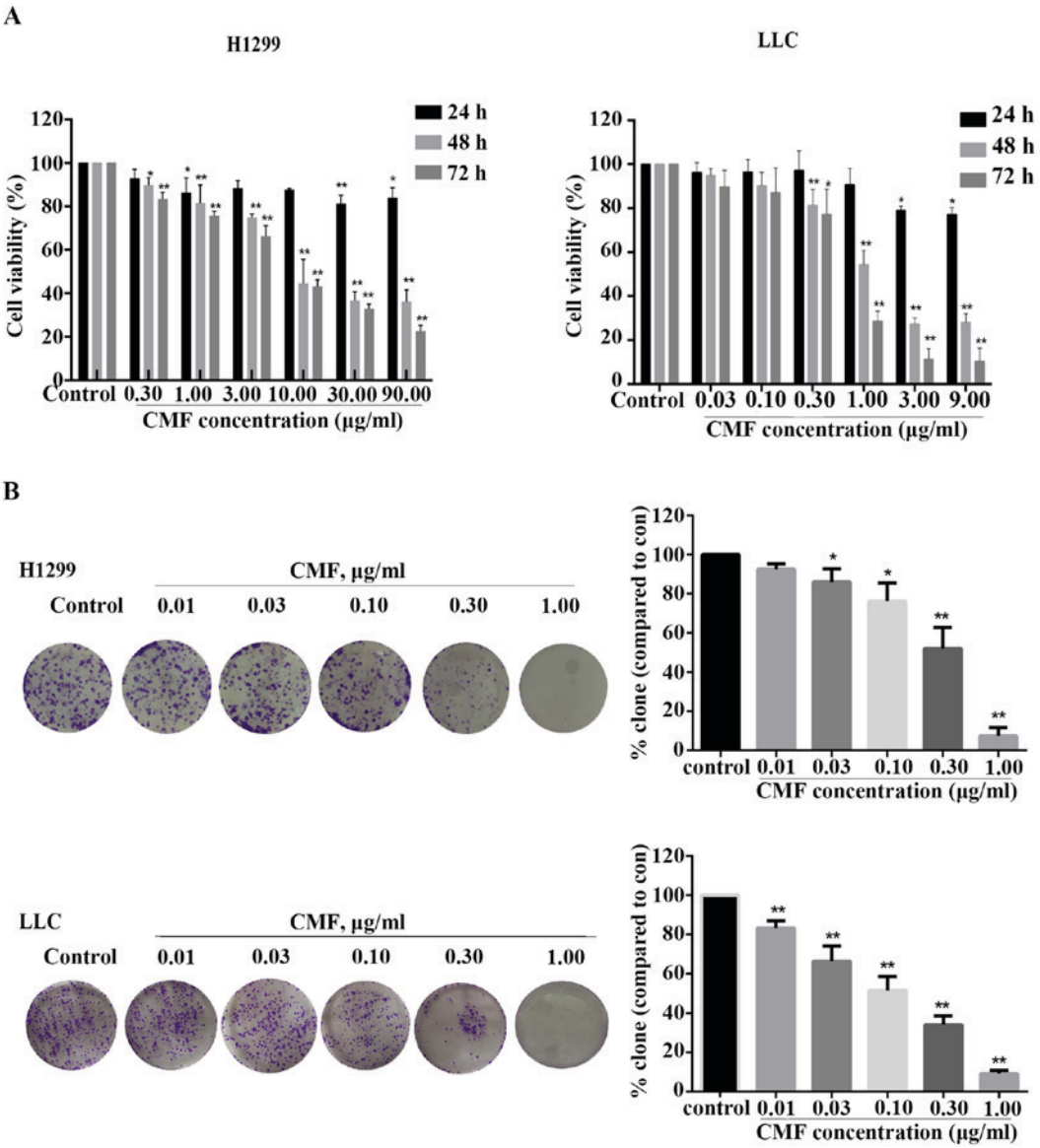

Figure 1. CMF inhibits the viability and colony formation of NCI-H1299 and LLC cells. (A) NCI-H1299 and LLC cells were treated with or without CMF at the indicated concentrations for 24, 48 or 72 h. Cell viability was determined using an MTT assay. *P<0.05 and **P<0.01 vs. the control. (B) Cell colonies of NCI-H1299 and LLC cells were counted visually and the percentage of colony formation was calculated based on the untreated cells, which were set as 100%. *P<0.05 and **P<0.01 vs. the control. Data are presented as the mean ± standard deviation of three independent experiments. CMF, *Cordycep smilitaris* fraction; LLC, Lewis lung cancer.

understanding of the mechanisms of cell migration (28). In the present study, a non-specific cell adhesion assay was used to investigate the effect of CMF on cell attachment. As shown in Fig. 2A, the number of NCI-H1299 and LLC cells that adhered was significantly decreased by 44 and 42.5%, respectively, compared with the control group at 3 μg/ml CMF (P<0.01). In addition, the migratory abilities of the two cell lines from one end of the wound to the other also significantly decreased following treatment with CMF for 24 h compared with the control (P<0.05; Fig. 2B). The results were further detected using a Transwell assay. As presented in Fig. 2C, CMF significantly reduced the numbers of NCI-H1299 cells (at all CMF concentrations) and LLC cells (CMF concentrations ≥0.3 μg/ml) migrating through the Transwell membrane to the lower chamber in a concentration-dependent manner, compared with the control (P<0.05), in which CMF at the concentration of 30 μg/ml decreased the number of migrating NCI-H1299 cells by >50%, and the number of LLC cells decreased 63.1% at the CMF concentration of 1 μg/ml, compared with the control. In a tumor micro-environment, an invaded cell must modify its shape and stiffness to interact with the surrounding tissue structures to migrate through a physical barrier of dense extracellular matrix (ECM) (29). A matrigel-coated chamber was used to simulate the ECM in order to study the effect of CMF on the invasive capacity of the two cell lines. As shown in Fig. 2D, the invasion of NCI-H1299 cells into the lower chamber was significantly decreased when treated with CMF compared with the control group (≥3 μg/ml; P<0.05). Furthermore, the number of LLC cells was also inhibited by ~37.2% at the CMF concentration of 1 μg/ml, demonstrating a significant difference compared with the control group (P<0.05). In the presence of the respective

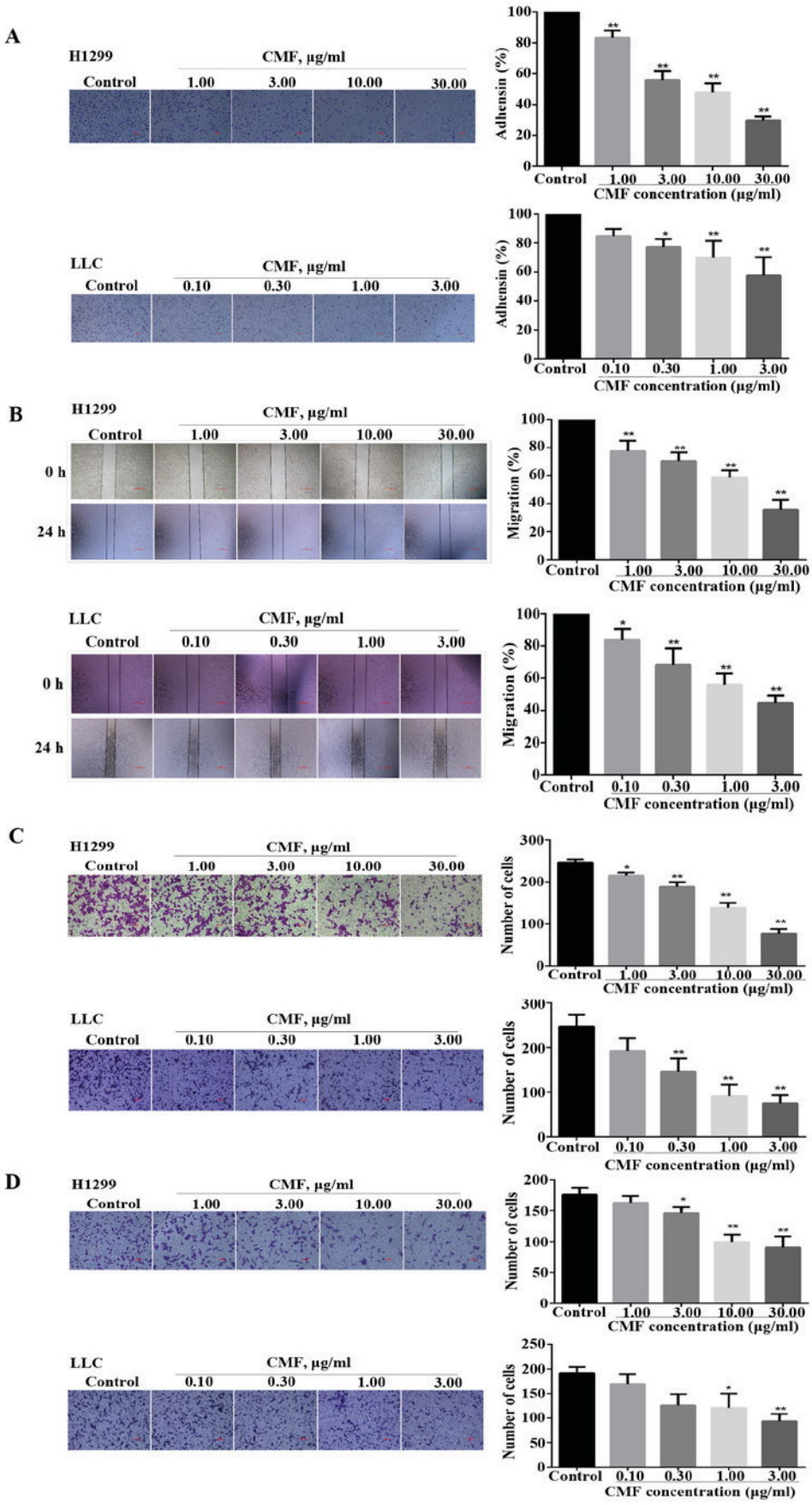

Figure 2. CMF suppresses the adhesion, migration and invasion of NCI-H1299 and LLC cells. (A) Images of the adhering cells were captured (magnification, x10) and the cells were counted. *P<0.05 and **P<0.01 vs. the control. (B) Representative images of wound healing at 0 and 24 h (magnification, x4) and the percentage of migrated cells were calculated based on the number of untreated cells, which were set as 100%. *P<0.05 and **P<0.01 vs. the control. Representative images (magnification, x10) and data statistics of cells that (C) migrated and (D) invaded through the Matrigel coated Transwell chamber. *P<0.05 and **P<0.01 vs. the control. Data are presented as the mean ± standard deviation of three independent experiments. CMF, *Cordyceps militaris* fraction; LLC, Lewis lung cancer.

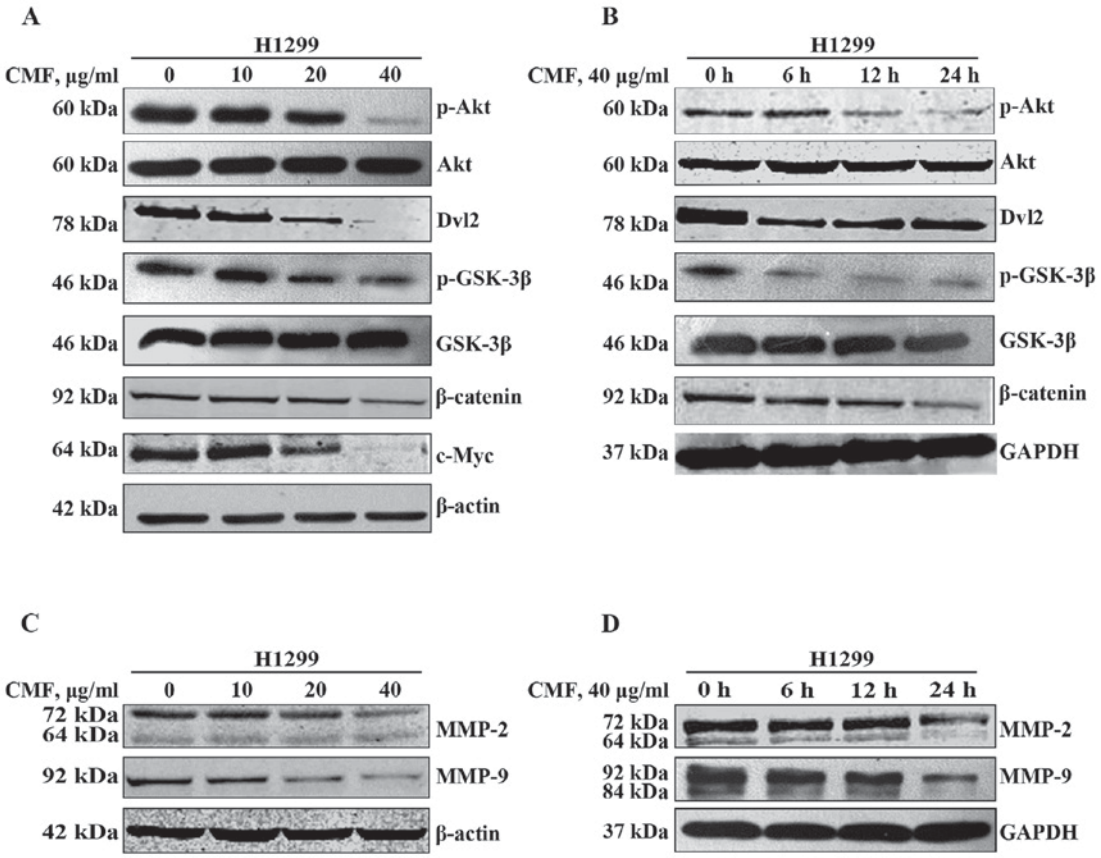

Figure 3. CMF suppresses Akt/GSK-3β/β-catenin signaling in addition to the expression of MMP-2 and MMP-9 in NCI-H1299 cells. (A) NCI-H1299 cells were treated with 10, 20 and 40 μg/ml CMF for 24 h. The protein expression of p-Akt, Akt, p-GSK-3β, GSK-3β, Dvl-2, β-catenin and c-Myc was determined in whole cell lysates by western blot analysis. (B) NCI-H1299 cells were treated with 40 μg/ml CMF for 6, 12 and 24 h. The protein expression of p-Akt, Akt, p-GSK-3β, GSK-3β, Dvl-2 and β-catenin were determined in whole cell lysates by western blot analysis. (C) NCI-H1299 cells were treated with 10, 20 or 40 μg/ml CMF for 24 h, and tested for MMP-2 and MMP-9 by western blot analysis. (D) NCI-H1299 cells were treated with 40 μg/ml CMF for 6, 12 or 24 h, and tested for MMP-2 and MMP-9 by western blot analysis. β-actin and GAPDH were used as loading controls. CMF, *Cordyceps militaris* fraction; Akt, protein kinase B; Dvl, Disheveled segment polarity protein; GSK-3β, glycogen synthase kinase 3β; c-Myc, MYC proto-oncogene, BHLH transcription factor; MMP, matrix metalloproteinase; p-, phosphorylated.

highest concentrations of CMF, NCI-H1299 and LLC cells barely invaded into the lower part of the insert. Taken together, these results demonstrated that CMF functions by acting directly on NCI-H1299 and LLC cells to inhibit the processes of adhesion, migration and invasion.

CMF inhibits the Akt/GSK-3β/β-catenin signaling pathway in NCI-H1299 cells. In order to investigate the mechanism of CMF on NCI-H1299 cells *in vitro*, western blot analysis was used to assay cell signaling transduction. CMF inhibited the phosphorylation of Akt at Ser473, which resulted in the down regulation of the expression of its target protein p-GSK-3β compared with the untreated cells (Fig. 3A). As GSK-3β also served an important role in the Wnt signaling pathway, its downstream protein β-catenin was investigated. Consistent with a previous study (30), the expression of β-catenin was attenuated when cells were treated with CMF at 40 μg/ml compared with untreated cells. It was reported that the overexpression of Dvl was evidence of the activation of the Wnt pathway in NSCLC (17). As a critical mediator of Wnt signaling, Dvl is hyperphosphorylated following linking to Wnt/Frizzled and prevents GSK-3β from phosphorylating β-catenin through its association with a cytoplasmic protein complex [Axin/APC, WNT signaling pathway regulator (APC)/GSK-3β complex] (31). The results of the present study revealed that CMF inhibited Dvl-2 expression at a concentration of ≥20 μg/ml, which indicated that Dvl protein also served a function in the regulation of GSK-3β activity. Additionally, these results were further confirmed by treatment with CMF at 40 μg/ml for different durations (0, 6, 12 or 24 h). The expression of p-Akt was decreased after 12 h incubation with CMF compared with cells treated for 0 h, while its downstream protein GSK-3β was activated even at 6 h, suggesting that Dvl-2 was also implicated in the regulation of GSK-3β (Fig. 3B). In addition, c-Myc, one of the β-catenin target proteins, decreased in expression with the increasing concentrations of CMF compared with the untreated cells. Taken together, these results illustrated that CMF may inhibit the migration and invasion ofNCI-H1299 cells by blocking the Akt/GSK-3β/β-catenin signaling pathway. Furthermore, Dvl-2 was partially involved in the regulation of GSK-3β.

CMF down regulates the expression of MMP-2 and MMP-9. MMPs have long been associated with cancer cell invasion and metastasis (32). Elevated levels of MMP-2 and MMP-9 have been demonstrated to promote metastasis and worsen the prognosis of patients with lung cancer (16,33). The results of the present study revealed that the expression of MMP-2 and MMP-9 was

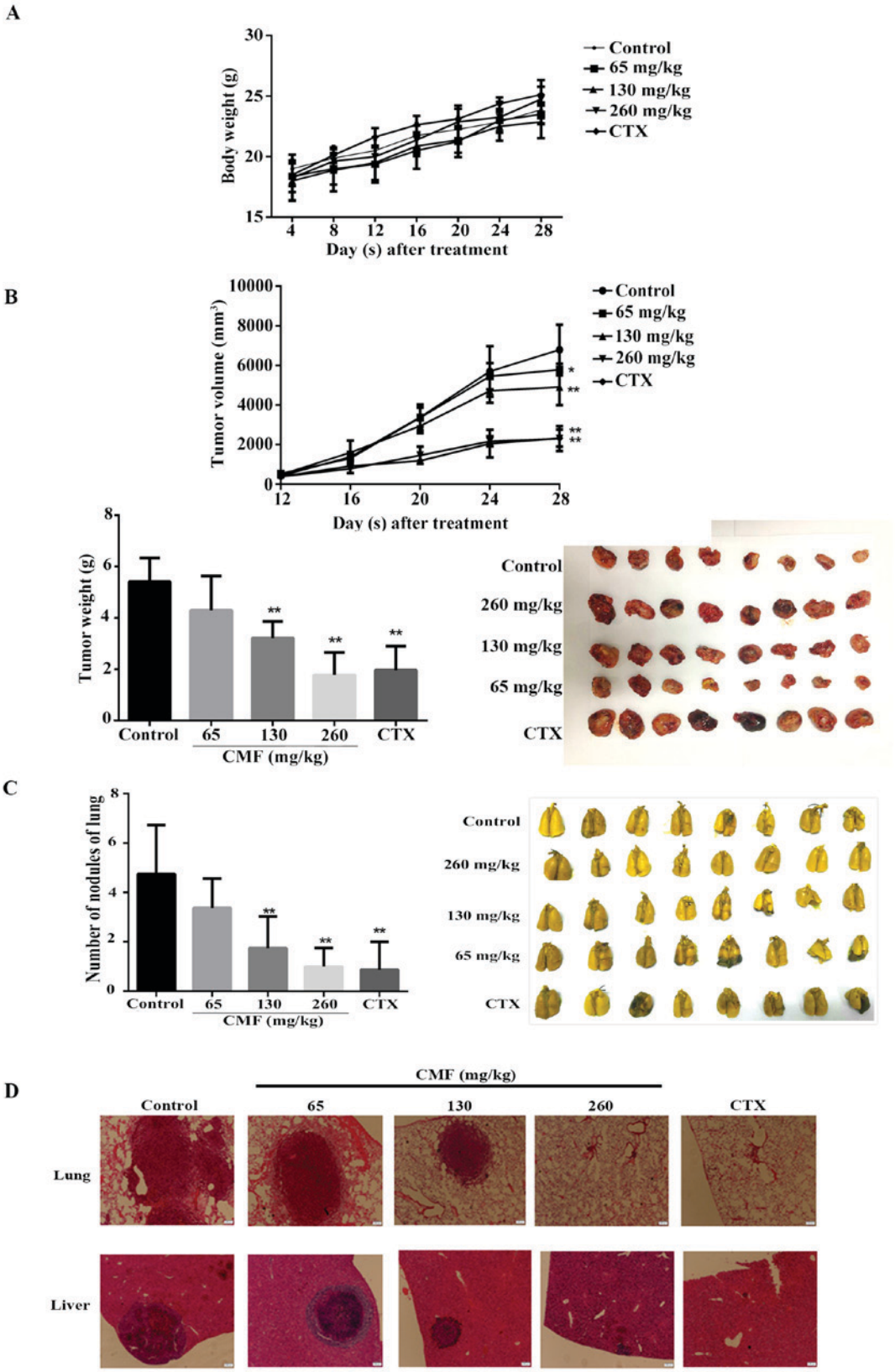

Figure 4. Oral administration of CMF inhibits tumor growth and metastasis in an LLC xenograft model. (A) Change in the body weight of the mice over a 4-week period (n=8). (B) Change in tumor size and tumor weight was scaled once the mice were sacrificed. *P<0.05 and **P<0.01 vs. the control. (C) The number of lung metastatic nodules on the surface of the lung tissue was counted. **P<0.01 vs. the control. (D) Hematoxylin and eosin staining was used to observe the metastatic clones of lung and liver tissues under images (magnification, x10). CMF, *Cordyceps militaris* fraction; CTX, cyclophosphamide; LLC, Lewis lung cancer.

inhibited subsequent to treatment with CMF, in a concentration- and time-dependent manner (Fig. 3C and D). This suggested that MMP-2 and MMP-9 were implicated in the effects of CMF on the migration and invasion of NCI-H1299 cells.

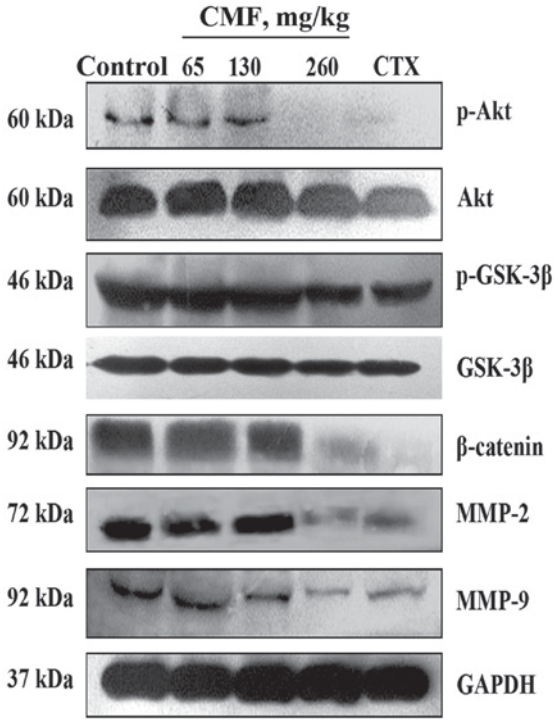

Figure 5. CMF induces the down regulation of MMP-2 and MMP-9 through Akt/GSK-3β/β-catenin signaling *in vivo*. Tumor tissue from the five groups of mice was randomly selected and the protein was obtained by lysis buffer. Protein expression of p-Akt, Akt, p-GSK-3β, GSK-3β, β-catenin, MMP-2 and MMP-9 was detected using a western blot analysis assay. GAPDH was used as a loading control. CMF, *Cordyceps militaris* fraction; Akt, protein kinase B; GSK-3β, glycogen synthase kinase 3β; MMP, matrix metalloproteinase; p-, phosphorylated; CTX, cyclophosphamide.

Oral administration of CMF inhibits tumor growth and metastasis in an LLC xenograft model. To determine the effect of CMF on tumor growth and metastasis *in vivo*, LLC cells were injected into the right armpit of C57BL/6 mice to establish an animal lung cancer model. Subsequent to intragastric administration of CMF once a day for 4 weeks, the volume and weight of the tumor were significantly inhibited in a dose-dependent manner compared with those of control group with no significant difference in body weight ($P<0.05$; Fig. 4A). In the group treated with 260 mg/kg CMF, tumor volumes were inhibited after 12 days of being injected with the cells, resulting in the final average volume being significantly smaller than that of the untreated group ($P<0.01$). Similarly, tumor weights were decreased to 57.5% of the control group when administered with 130 mg/kg (Fig. 4B).

Certain organs, including the lung (34) and liver (35) are prone to be sites for the formation of metastatic colonization. To investigate the effect of CMF on metastasis *in vivo*, whole lung tissue was collected once the mice were sacrificed and the nodules on the surface were counted (Fig. 4C). The results revealed that CMF significantly decreased the number of nodules in mice at dosages of 130 and 260 mg/kg compared with the control ($P<0.01$). Furthermore, CMF treatment also substantially decreased the size and number of metastatic clones in the lung and liver tissue (Fig. 4D). Western blot analysis results revealed that the expression of MMP-2 and MMP-9 was substantially down regulated in the tumor tissue of mice treated with CMF at a dose of 260 mg/kg (Fig. 5). Additionally, consistent with the *in vitro* results, the expression of p-Akt, p-GSK-3β and β-catenin was also decreased compared with that in control mice. Taken together, the results from the *in vitro* and *in vivo* assays revealed that CMF may inhibit the invasion and metastasis of lung carcinoma cells through the Akt/GSK-3β/β-catenin signaling pathway.

Discussion

Lung cancer develops more commonly in humans and usually has a poor clinical outcome and low survival rate due to high rates of metastasis at a late stage of the tumor development (36). Therefore, novel approaches are urgently required for the treatment of this disease and the prevention of its metastasis. Over previous years, natural compounds that are extracted and purified from herbal plants have widely attracted attention due to their preventative and treatment effects on cancer without serious side effects (37). The results of the present study demonstrated that CMF served an important functional role in suppressing the invasion and metastasis of lung cancer cells.

Cancer metastasis is a complicated multistep process involving the dissociation of malignant cells at the primary sites, invasion through the extracellular matrix, intravasation of invading cells into the vasculature or lymphatic systems, survival and finally proliferation at a distant organ (38,39). In the *in vitro* experiments in the present study, it was revealed that the cell adhesion, migration, invasion and clone formation in NCI-H1299 and LLC cells decreased in a concentration-dependent manner when treated with CMF (Fig. 2).

MMPs have been associated with cancer risk, clinical prognosis, metastasis and recurrence (10). A previous study in genetic mouse models of cancer has suggested that an MMP deficiency may result in decreased or increased tumor progression, incidence, size and metastasis (10). In the results of the present study, the expression of MMP-2 and MMP-9 was reduced by CMF in NCI-H1299 cells *in vitro* and LLC cells in C57BL/6 mice, suggesting that the two MMPs were associated with the metastasis of the lung cancer cells. Akt is a serine/threonine kinase that may phosphorylate and inactivate GSK-3β which may be involved in the regulation of the Wnt/β-catenin pathways by facilitating phosphorylation within other proteins (including APC and Axin) and promoting the degradation of β-catenin (15,40). It has been reported that the Akt/GSK-3β/β-catenin pathway is required for the epithelial-mesenchymal transition process induced by soluble interleukin-15 receptor α (41). It was revealed in the present study that CMF may inhibit the phosphorylation of the Akt protein at the Ser473 site which promoted the activation of its downstream protein GSK-3β *in vitro* and *in vivo*. Dvl proteins combine with Axin and Frat1, WNT signaling pathway regulator to form a complex to prevent β-catenin from degradation mediated by GSK-3β (42). The results demonstrated that Dvl-2 was also inhibited and the expression of β-catenin and its target protein c-Myc decreased following GSK-3β protein activation (Fig. 3), which were consistent with results previously reported (43). However, the association between Akt and Dvl-2, which regulate the activity of GSK-3β by CMF, remains unknown. Furthermore, the anti-metastatic effect of CMF *in vivo* was investigated using an LLC cell line xenograft model of C57BL/6 mice. Tumor sizes in addition

to lung and liver metastases were reduced following the oral administration of CMF.

To the best of our knowledge, the present study was the first to demonstrate that CMF may inhibit the viability, invasion and metastasis of lung carcinoma cells *in vitro* and *in vivo*. Furthermore, it was revealed that the inhibitory effect of CMF was primarily associated with the suppression of the phosphorylation of upstream Akt and an increase in the activity of GSK-3β, which promoted the degradation of downstream protein β-catenin. These results suggested that CMF may possess great potential for the treatment of lung cancer metastasis partially through Akt/GSK-3β/β-catenin signaling.

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Availability of data and materials

The datasets used and analyzed during the present study are available from the corresponding author on request.

Authors' contributions

QZ conducted the majority of experiments and drafted the manuscript. CH, QC and SB also conducted the experiments. ZZ and XH provided support during the experiments and technical assistance. RY and LS defined and guided the experiments, wrote and revised the manuscript.

Ethics approval and consent to participate

All animal experiments were carried out in compliance with the Animal Management Rules of the Ministry of Health of the People's Republic of China and approved by the Animal Care and Use Committee of Jinan University.

Competing interests

The authors declare that there are no conflicts of interest.

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