EXPERIMENTAL STUDY

Paeoniflorin inhibited the tumor invasion and metastasis in human hepatocellular carcinoma cells

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Abstract: **Objective:** Evidence suggests that paeoniflorin may be involved in anticancer activities. Here, we have investigated the effects of paeoniflorin and correlative mechanisms on anti-invasion and anti-metastasis in human hepatocellular carcinoma (HCC) cell lines.

**Materials and methods:** In the current study, we have applied 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to determine the proliferative effect of HepG2 and Bel-7402, two human hepatoma cell lines, and we have established a boyden chamber assay, a wound healing assay and cell adhesion assay to detect and quantify the invasion, metastasis and adhesion of both HepG2 and Bel-7402. In addition, we have analyzed the protein expression of matrix metalloproteinas (MMP)-9, E-cadherin (E-cad) and extracellular signal-regulated kinase (ERK) in both cell lines through western blot analysis.

**Results:** Paeoniflorin (6.25–200 μM) had inhibitory effect on the growth of HepG2 and Bel-7402 cell lines, and reduced significantly invasion, metastasis and adhesion of HCC cell lines. In addition, paeoniflorin decreased the expression of MMP-9 and ERK in HepG2 and Bel-7402 cells, and increased expression of E-cad in both cell lines.

**Conclusions:** Paeoniflorin is effective anti-metastatic and anti-invasive agent for suppressing HCC invasion and metastasis (Fig. 5, Ref. 30).

Key words: paeoniflorin, hepatocellular carcinoma, anti-invasion, anti-metastasis.


Hepatocellular carcinoma (HCC) is the most common malignant tumour, it is the third cause of cancer-related death worldwide due to its poor prognosis (1, 2). The prognosis of HCC patients is primarily determined by the incidence of recurrence after surgery and the occurrence of invading metastases into the remaining liver parenchyma. Invasion and metastasis are fundamental properties of malignant HCC (3–5), the formation of metastatic nodules of hepatocellular carcinoma involves an intricate multiprocessing cascade, including cell adhesion, migration and proteolysis of the extracellular matrix and so on (6). Therefore it is imperative to identify agents that are highly effective on HCC invasion and metastasis.

Paeoniflorin (structure shown in Figure 1), a characteristic monoterpene glucoside, is one of the main effective components of the total glucosides of paeony (TGP) from the root of Raidix Paeoniae Alba (PAR). PAR is known to have diverse pharmacologic activities including anti-inflammation, antioxidant, antiproliferatory, analgesia, immunoregulation (7–11) and anticancer (12, 13). PAR has been extensively used in China for liver diseases.

![Fig. 1. Chemical structure of paeoniflorin.](image-url)
for thousands of years. Our previous studies also indicated that extract of PAR possess protective effect on liver injury, hepatic fibrosis (14–16) and inhibit the proliferation of HCC cells. In this study, we studied the effect of paeoniflorin on the anti-metastasis and anti-invasion in Bel-7402 and HepG2 cell lines, two human hepatoma cell lines. Simultaneously we detected that effects of paeoniflorin on the protein expression of MMP-9, E-cad and ERK in Bel-7402 and HepG2 cells to investigate underlying signaling molecular mechanisms, by which paeoniflorin inhibits invasion and metastasis in HCC cells.

Materials and methods

Reagents

Dulbecco’s modified Eagle’s medium (DMEM) and 3-(4,5-di-methylthiazole-2-yl)-2, 5-biphenyl tetrazolium bromide (MTT) were obtained from Gibco Chemical Company (Gibco, USA). Anti-ERK, anti-E-cad and anti-MMP-9 antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and (Bio-world). All chemicals were purchased in the purest form available. Paeoniflorin was provided by phytochemistry lab of Institute of Clinical Pharmacology of Anhui Medical University, and dissolved in DMEM. The purity of paeoniflorin was determined by high-performance liquid chromatography (HPLC) as 98.9 %.

Cell line and culture conditions

Human HCC Bel-7402 and HepG2 cell lines, obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences, was grown in DMEM supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Gibico, USA), 100 μg/ml streptomycin and 100 U/ml penicillin in a humidified atmosphere containing 5 % CO₂ at 37 °C. Cells during exponential growth phase were used in the experiments. However, in the invasion and metastasis experiments, cells were cultured in a serum-free medium.

3-(4,5-Dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay

The antiproliferative effects of paeoniflorin with different concentrations on HCC cell lines were detected by MTT assay. Cells were seeded at a density of 5×10⁴ per well in 100 μl of DMEM with 10 % FBS in 96-well plates. The next day, the medium was changed into a serum-free DMEM with paeoniflorin (from 6.25 μM to 200 μM) for HepG2, and Bel-7402 cells. After incubation for 24 hours, each well was added 20 μl MTT (5 mg/l), and the plates were incubated for additional 4 hours. Then the medium was discarded, 150 μl of dimethyl sulfoxide was added and the absorbance (A) of the oxidized MTT solution was determined at 490 nm by a multiwell spectrophotometer (Bio-Tek, XL-808, USA). Inhibitory rate of cell was calculated as follows: Inhibitory rate (%) = (1- A of the treated wells / A of the control wells) × 100.

Boyden chamber invasion assay

The invasive ability of HepG2 and Bel-7402 cells was determined by using Boyden chamber assays as previously described (17). Briefly, 24-well transwell units with polycarbonate membranes (8 μm pore size, Costar, USA) were coated with 100 μl matrigel (25 μg in 100 μl PBS, Becton Dickinson), dried in a laminar hood overnight, and reconstituted in 100 μl PBS at 37 °C for 2 hours. HepG2 and Bel-7402 cells to be tested for invasion were resuspended in DMEM with 0.5 % BSA (5×10⁴ cells/200 μl) in the presence or absence of paeoniflorin, added to the upper side of the invasion chamber. DMEM (500 μl) with 2.5 % FBS was added to the lower chamber. After 24 hours of incubation, filter inserts were removed from the wells, the cells on the upper surface of the filter were wiped off using cotton swabs. The cells that penetrated to the lower surface were fixed with 4 % paraformaldehyde, stained with 0.1 % crystal violet in 20 % ethanol, than counted in five randomly selected fields under phase contrast microscope. The invading cells were monitored by photographing under x200 magnification with an Olympus Microscope. The assay was performed in triplicate.

Wound-healing assay

Cell migration was examined using the wound-healing assay. Briefly, HCC cells were cultured to about 80–90 % confluence in a 6-well plate at 37 °C and 5 % CO₂. A wound about 1 mm in width was created by scratching cells with a sterile 100 μl micro-pipette tip. Cells were washed with PBS (pH 6.8) three times to remove nonadherent cells. Then 1 ml of serum-free DMEM was added. A computer-based microscopy imaging system was used to determine wound healing at 0 hour with a microscope at 200X magnification. Then 1 ml of serum-free DMEM was added with different concentrations of paeoniflorin (6.25, 12.5 and 25 μM). After 24 hours, photos of the wound were taken under x200 magnification. Wound-healing was assessed by measuring the pixel of wound area by Photoshop 7.01 software. The experiments were performed in triplicate.

Cell adhesion assay

Briefly, 96-well tissue culture plates were coated with the matrigel (12.5 μg in well 50 μl PBS). Coated plates were incubated at 37 °C for 40 minutes, and 100 μl of the Bel-7402 cells (1×10⁶ cells/ml) suspended in DMEM with 10 % FBS was added to each well. The cells were incubated in the absence or presence of paeoniflorin for 24 hours, then were weaved at speed of 100 rpm for 1 hour, washed three times with PBS to remove the unattached cells. Each well was added 100 μl of same DMEM with MTT (5 mg/l), and cells were incubated for additional 4 hours. Then medium was discarded, 150 μl of dimethyl sulfoxide was added to each well. The absorbance (A) of the oxidized MTT was determined at 490 nm by a multiwell spectrophotometer (Bio-Tek, XL-808, USA). The percentage of cell inhibition was calculated as follows, increasing adhesion rate (%) = (A of the treated wells – A of the control wells) / A of the control wells × 100. The experiments were performed in triplicate.

Western blots

Cells were plated onto culture flasks at a density of 2×10⁵ cells/ml and cultured at 37 °C and 5 % CO₂. The next day, different concentrations of paeoniflorin (6.25, 12.5 and 25 μM) were
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added. After 24 hours, the levels of MMP-9, E-cad and ERK proteins were quantified through western blots. Proteins were extracted through the addition of 200 μl of lysis buffer (1 mM EDTA, 1.5 mM MgCl₂, 150 mM NaCl, 50 mM Hepes, 50 μM DTT, 1 mM phenylmethylsulfonyl fluoride and 10 mg/ml leupeptin pH 7.4) to each well. The cell lysates were incubated on ice for 30 minutes vortexing every 10 minutes, followed by centrifugation at 12,000 g for 30 minutes at 4 °C. 50 μg/μl protein of cell lysate was mixed equally with 2 X electrophoresis buffer (50 % glycerol, 25 % mercaptoethanol, 10 % SDS, 0.3M Tris (pH 6.8), 0.025 % bromphenol blue) and boiled for 10 minutes. The samples (50 μg of protein) of total cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore corporation) in transfer buffer containing 25 mM Tris, 150 mM glycine and 20 % methanol. The membranes were blocked using 5 % BSA (pH 7.4, 0.5 % Tween 20). The membrane was then incubated with primary antibodies,

![Graph showing inhibitory rate (%) vs concentration of proliferation (μM)](image)

**Fig. 2.** Effects of paeoniflorin on the proliferation of HepG₂ and Bel-7402 cells. Cells were treated with different concentrations of paeoniflorin for 24 hours. The absorbance (A) of the oxidized MTT solution was determined at 490 nm. Inhibitory rate of different concentrations paeoniflorin was calculated. The experiments were performed in triplicate.

![Images of control, paeoniflorin 6.25 μM, paeoniflorin 12.5 μM, paeoniflorin 25 μM](image)

**Fig. 3.** Paeoniflorin restricted invasion of HepG₂ and Bel-7402 cells in Boyden chamber invasion assay. HCC cells were added to the upper side of the invasion chamber, the cells on the upper surface of the filter were removed after 24 hours of incubation in the presence or absence of paeoniflorin. The cells on the lower surface were fixed, stained and monitored by photographing, then counted. (A) Representative photographs (x200 magnification) of HepG₂ cells from one independent experiment. (B) Representative photographs (x200 magnification) of Bel-7402 cells from one independent experiment. (C) The number of HepG₂ and Bel-7402 cells in the lower chamber, each bar represents the mean ± SD of three separate experiments (* p < 0.05, ** p < 0.01 compared with the control).
anti-MMP-9 (1:1000), anti-ERK (1:500), and anti-E-cad (1:500) for 16–18 hours at 4 °C. The membranes were subsequently probed with an anti-mouse or an anti-rabbit IgG antibody with the HRP-conjugated secondary antibody (1:5000) for 1 hour. Control blots were performed using anti-actin antibodies (1:500, Santa Cruz, CA). The membranes were washed in PBS for 30 minutes at room temperature, and detection was achieved by measuring the chemiluminescence of the blotting agent after exposure of the filters on X-omat films. The densities of the bands were quantified with a computerized densitometer (Image J Launcher, Broken Symmetry Software).

**Statistical analysis**

Statistical analyses were performed using the SPSS 11.0 software program (SPSS, USA). All data were presented as the mean ± standard deviation (S.D.). Statistical differences were determined by the Student’s t-test. Statistical significance of differences was accepted at $p < 0.05$. 

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Fig. 4. Paeoniflorin inhibited migration of HepG$_2$ and Bel-7402 cells. The cells in six-well plate were scratched with pipette tip, incubated at 37 °C, 5% CO$_2$, for 24 hours after being treated with paeoniflorin, then monitored by photographing for wounding. (A) Representative photographs (×200 magnification) of HepG$_2$ cells after wound was created. (B) Representative photographs (×200 magnification) of HepG$_2$ cells treated with and without paeoniflorin for 24 hours. (C) Representative photographs (×200 magnification) of Bel-7402 cells treated with and without paeoniflorin for 24 hours. (D) Values of wound-healing assessed by measuring the pixel of healing area. Bar represented the mean ± SD of three separate experiments (* $p < 0.05$, ** $p < 0.01$ compared with the control).
Results

**Effects of paeoniflorin on proliferation of HCC cells**

The cytotoxicity of paeoniflorin was evaluated by 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay. As illustrated in Figure 2, after HepG2 and Bel-7402 cells were treated with different concentrations of paeoniflorin for 24 hours, paeoniflorin can inhibit the proliferation of HepG2 and Bel-7402 cells in concentration-dependent manner, but paeoniflorin has a little cytotoxic effects on HepG2 and Bel-7402 cells at 6.25–25 μM (Fig. 2).

**Paeoniflorin inhibited invasion of HCC cells**

Paeoniflorin has no obvious inhibitory effect on proliferation of HCC cells at 6.25–25 μM for 24 hours, so we deployed these doses of paeoniflorin in boyden chamber invasion assay. The result indicated that paeoniflorin decreased significantly the numbers of the lower chamber HCC cells compared with the control in concentration-dependent manner. The maximum inhibitory rates for HepG2 and Bel-7402 cells were 83.35 % and 85.78 % respectively, as illustrated in Figure 3.

**Paeoniflorin inhibited migration of HCC cells**

In the wound-healing assay, we also deployed the doses of 6.25, 12.5 and 25 μM of paeoniflorin to detect its anti-migration effect on HepG2 and Bel-7402 cells. Results revealed that paeoniflorin delayed healing of wound, and notably inhibited migration of HepG2 and Bel-7402 cells by 48.51 % and 65.21 % respectively (Fig. 4).

**Paeoniflorin decreased adhesion of HCC cells**

Adhesion assays results displayed that paeoniflorin could significantly decrease the numbers of adhering HepG2 and Bel-7402 cells after they were treated with various concentrations of 6.25, 12.5 and 25 μM paeoniflorin compared to the control in concentration-dependent manner. The decreasing adhesive rates for HepG2 and Bel-7402 cells were by 89.21±15.4 %, 76.38±21.34 % respectively.

**Effects of paeoniflorin on the expression of MMP-9, ERK and E-cad protein in HCC cells**

To investigate underlying signaling molecular mechanisms, by which paeoniflorin inhibits invasion and metastasis in HCC cells, we have applied the Western blot analysis to detect the protein expression of MMP-9, E-cad and ERK in Bel-7402 cell and HepG2 lines. The results revealed that paeoniflorin dramatically inhibited the protein expression of MMP-9, and increased E-cad protein expression in both HepG2 and Bel-7402 cell lines in concentration-dependent manner, moreover paeoniflorin dra-
matically inhibited ERK protein expression in both cells (Fig. 5) after HCC cells were treated with different concentrations of paeoniflorin for 24 hours.

Discussion

HCC is one of the most common malignant tumors. Most patients with HCC die within one year after diagnosis largely because of frequent tumor recurrence and metastasis. Invasion and metastasis are fundamental properties of malignant HCC, which lead to recidivism rate of HCC larger after surgery therapeutic approaches (18, 19). The formation of metastatic nodules of HCC involves an intricate multiprocessing cascade, the adhesion and motility of tumor cells in the ECM are considered important steps in the invasive processes of metastatic tumor cells. When tumor metastasis occurs, tumor cells change many previous properties, including altered adhesiveness, motility, and invasive capacity, to complete the metastatic process. Therefore, we examined the effects of paeoniflorin on adhesion, metastasis and invasion in HCC cells.

First, we determined the effects of paeoniflorin on proliferation of HepG2 and Bel-7402 cells. The results indicated that paeoniflorin can inhibit the proliferation of HepG2 and Bel-7402 cells in concentration-dependent manner, however there is no obvious inhibitory effect of paeoniflorin on proliferation of HCC cell at 6.25–25 μM for 24 hours, so we deployed these doses of paeoniflorin in Boyden chamber invasion assay, wound healing assay and cell adhesion assay. We demonstrated that paeoniflorin decreased significantly the numbers of the lower chamber HepG2 and Bel-7402 cells in Boyden chamber invasion assay, decreased significantly area of wound-healing in wound healing assay, increase numbers of adhering HCC cells in cell adhesion assay. It indicated that paeoniflorin can significantly inhibit the invasion and metastasis of HCC cells and enhance the adhesion of HCC cells.

MMPs, which are a family of zinc-dependent endopeptidases, are deeply involved in the invasion and metastasis of various tumor cells (3–5). Tumor-secreted MMPs destroy extracellular matrix components in tissue surrounding a tumor, tumor cells enter and survive in the circulation, lymphatic or peritoneal spaces and arrest in a distant target organ. MMP-9 (gelatinase-B) are mostly associated with tumor migration, invasion and metastasis for various human cancers (20–23). Generally, MMP-9 can be stimulated by an inflammatory cytokine through activation of different intracellular-signaling pathways. The expression of MMP-9 is modulated by the activation of transcription factors including activator through mitogen-activated protein kinase (MAPK) -signaling pathway and phosphatidylinositol 3-kinase (PI3K) -signaling pathway (24–27). In the current study, we detected that effects of paeoniflorin on the protein expression of MMP-9 and ERK in HepG2 and Bel-7402 cells. We concluded that paeoniflorin could inhibit the expression of MMP-9 and ERK proteins in HepG2 and Bel-7402 cells. Meanwhile, we detected that effects of paeoniflorin on the protein expression of PI3K and Akt (not show), but paeoniflorin had no effects on expression of PI3K and Akt. So we deduced that paeoniflorin could inhibit HCC invasion and migration, its mechanisms might be related to decreasing the expression ERK, activating transcription factors, then inducing MAPK signaling pathway, at last inhibiting expression of MMP-9.

E-cad is a classical member of the cadherin families (28), E-cad-mediated cell-cell adhesions limits cell motility and establish apical-basal polarity, which is associated with tumor migration, invasion and metastasis (29, 30). In the current study, we also detected that effects of paeoniflorin on the protein expression E-cad in HepG2 and Bel-7402 cells. Paeoniflorin could increased the expression of E-cad, so we deduced that paeoniflorin could inhibit HCC invasion and migration, mechanisms also might be related to increasing the expression of E-cad via inducing MAPK signaling pathway.

Based on those results, we concluded that paeoniflorin is useful as a novel agent of controlling HCC invasion and metastasis, nevertheless, greatly understanding its anti-invasion and metastasis mechanisms is essential to further research.

References


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