

Diosmetin inhibits the metastasis of hepatocellular carcinoma cells by downregulating the expression levels of MMP-2 and MMP-9

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Abstract. Hepatocellular carcinoma (HCC) is one of the most malignant types of tumor worldwide with a high rate of mortality. Diosmetin (DIOS) exhibits various activities, including anti-cancer activities. However, the role of DIOS in the metastasis of HCC, and its underlying molecular mechanism, remain to be fully elucidated. In the present study, the antimetastatic effects of DIOS were investigated in SK-HEP-1 and MHcc97H HCC cell lines. Cell proliferation, wound healing, motility, invasion and adhesion capacities were examined to evaluate the inhibitory effect of DIOS on the metastasis of HCC cells. Cell viability was detected using an MTT assay in order to verify the inhibitory effect of DIOS on the proliferation of HCC cells. Cell migration was assessed using wound healing and motility assays in order to verify the inhibitory effect of DIOS on the migration of HCC cells. Cell invasion and adhesion assays were performed in order to verify the inhibitory effect of DIOS on the invasion and adhesion of HCC cells. Matrix metalloproteinase (MMP)-2/9, proteins of the mitogen-activated protein kinase (MAPK) pathway (c-Jun N-terminal kinase, extracellular signal-regulated kinase and p38 MAPK) and protein kinase C- δ were detected in order to verify the potential molecular mechanisms of DIOS in the inhibition of the metastasis of HCC cells. DIOS was observed to inhibit the metastasis of SK-HEP-1 and MHcc97H cells by downregulating the expression of MMP-2/9 via the PKC/MAPK/MMP pathways. DIOS also inhibited the migration and invasion of the HCC cells, and may serve as a potential candidate agent for the prevention of HCC metastasis.

Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant types of tumor worldwide, and is the third most common cause of cancer-associated mortality (1,2). Due to the limitations of surgery and liver transplantation, including inoperable tumors and tissue matching, chemotherapy remains the major treatment method for HCC (3). Previous studies have shown that the metastasis of cancer cells involves complex processes, in which the cancer cells invade the surrounding tissue, enter the bloodstream or lymph circulation, and form new tumors (4,5). The degradation of the extracellular matrix (ECM) is crucial in cancer cell migration and invasion, and a series of proteinases are involved in this process, including matrix metalloproteinases (MMPs) (6).

Diosmetin (3',5,7-trihydroxy-4'-methoxyflavone (C₁₆H₁₂O₆; DIOS; Fig. 1) is found in the legume, *Acacia farnesiana*, and in the leaves of *Olea europaea* L., and is the aglycone of the flavonoid glycoside, diosmin (7). It has been confirmed that DIOS has several medicinal properties, including antibacterial (8), antimicrobial (9), anti-inflammatory (10) and antioxidant (11) activities. It has also been confirmed that DIOS exerts cytostatic effects in MDA-MB 468 cells, a breast cancer cell line, by inducing cell cycle arrest (12). However, the effect of DIOS on the invasion and metastasis of HCC cells, and the antimetastatic mechanisms of DIOS remain to be fully elucidated. The aim of the present study was to investigate the anti-metastatic effect of DIOS on HCC cells and the underlying mechanisms.

Materials and methods

Reagents and antibodies. Diosmetin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO; MP Biomedicals, Santa Ana, CA, USA) at a stock solution concentration of 5 mg/ml, and was diluted as a working fluid for cell culture medium prior to use. Concentrations of DIOS used in the MTT assay were 0, 2, 5, 10, 20, 30, 40, 50 and 100 μ g/ml; whereas 0, 10, 20 and 40 μ g/ml DIOS was used in the other assays. The 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. Matrigel was purchased from BD Biosciences (Franklin Lakes, NJ, USA). SYBR Premix Ex Taq™ II kits were purchased from Takara Bio, Inc. (Shiga, Japan). Antibodies against GAPDH, MMP-2, MMP-9, c-Jun N-terminal kinase (JNK), phosphorylated

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(p)-JNK, extracellular signal-regulated kinase (ERK)1/2, p-ERK1/2 and protein kinase C (PKC)- δ were purchased from Cell Signal Technology, Inc. (Boston, MA, USA). Horseradish peroxidase-(HRP) conjugated goat anti-rabbit immunoglobulin G secondary antibody was purchased from EarthOx Life Sciences (Millbrae, CA, USA).

Cell culture. The MHcc97H and SK-HEP-1 HCC cell lines were purchased from the Shanghai Cell Bank of Chinese Academy of Science (Shanghai, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), and were cultured in a 37°C, 5% CO₂ incubator. The cells were passaged at 90% confluence.

Cell proliferation assay. Cell proliferation rates were detected using an MTT assay. The cells were seeded into a 96-well plate at a density of 10⁴ per well in 100 μ l culture medium. Following 24 h adhesive culture at 37°C, the medium was removed and replaced with the same volume of medium containing either 2, 5, 10, 20, 30, 40, 50 and 100 μ g/ml DIOS, with cells cultured in normal medium as a control group. After 24 h incubation at 37°C, 20 μ l MTT stock solution, at a concentration of 5 mg/ml, was added to each well of the plate and, following 3 h incubation at 37°C, the medium was removed gently and 200 μ l DMSO was added per well. The absorbance was then detected using a microplate reader (PerkinElmer, Waltham, MA, USA) at a wavelength of 570 nm. These experiments were performed independently in triplicate.

Wound healing assay. Migration capacities of the HCC cell lines under DIOS treatment were detected using a wound healing assay. The cells were seeded in a 24-well plate in DMEM containing 10% FBS for 24 h, when the cells were at 100% confluence. A wound was then created in the cell layer using a pipette tip. Following washing twice with phosphate-buffered saline (PBS) to remove cellular debris, the cells were cultured in the absence or presence of 5, 10 or 20 μ g/ml DIOS in DMEM containing 1% FBS for 24 h at 37°C. The cells were observed under a microscope, and images of the cells were captured when the wound was created and at 24 h-post wounding. Migration rates were calculated using the following formula: Migration rate = [width of (0-24 h) / width of 24 h] x 100%. The experiments were performed in triplicate independently.

Cell motility assay. Cells were seeded in Transwell chambers, comprising porous polycarbonate membranes with a pore size of 8.0 μ m (Corning, Corning, NY, USA), at a concentration of 1x10⁵ cells/chamber in the absence or presence of 100 μ l 5, 10 or 20 μ g/ml DIOS in DMEM. Chambers were then fitted into the lower wells of the Transwell system in a 24-well plate (BD Biosciences), which contained 600 μ l DMEM containing 10% FBS. Following incubation for 24 h at 37°C, the cells that passed through the membrane were fixed in 70% ethanol, and then stained with 0.1% crystal violet (Amresco, Solon, OH, USA). The cells were observed under a Olympus IX70 microscope (Olympus Corporation, Tokyo,

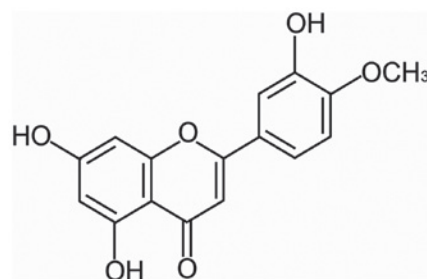


Figure 1. Chemical structure of Diosmetin.

Japan) and counted in the last four fields of each group. Three independent experiments were performed in triplicate.

Cell invasion assay. The methods used for the cell invasion assay were similar to those of the cell motility assay, with the exception that each Transwell chamber was pretreated with Matrigel (1:10 diluted in DMEM), of which 100 μ l per chamber was added. The chambers were placed into a 37°C incubator for 2 h prior to use.

Cell adhesion assay. A cell adhesion assay were performed, as previously reported (3). Briefly, each well of a 96-well plate was coated with 10 μ l fibronectin (R&D systems, Minneapolis, MN, USA), and the plates were placed in to a 37°C incubator for 2 days. The plates were washed twice with DMEM prior to use. The cells were pretreated with 5, 10 or 20 μ g/ml DIOS for 24 h at 37°C, following which the cells were harvested and seeded into the 96-well plate coated with fibronectin, at a density of 5x10⁵ cells/ml for 100 μ l. After 2 h, the medium and non-adhesive cells were removed, and the adhered cells were detected using an MTT assay.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The expression levels of the MMPs and tissue inhibitors of MMPs were detected using RT-qPCR. The total RNA of the cells were extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and reverse transcription to cDNA was performed using a PrimeScriptTM RT reagent kit with gDNA Eraser (Takara Bio, Inc.). qPCR reactions were performed using a Roche LightCycler 480 II (Roche Diagnostics, Basel, Switzerland), according to the instructions of the SYBR[®] Premix Ex TaqTM II, ROX plus (Takara Bio, Inc.). Specific primers for each gene were designed as follows: GAPDH, forward 5'-TGCACCACCAAC TGCTTAG-3' and reverse 5'-AGTAGAGGCAGGGATGAT GTTC-3' as internal control; MMP2, forward 5'-CCACAG GAGGAGAAGGCTGT-3' and reverse 5'-CTCCAGTTA AAGGCGGCATC-3'; and MMP9, forward 5'-ACGACGTCT TCCAGTACCGA-3' and reverse 5'-TTGGTCCACCTGGTT CAACT-3'. Thermal cycling conditions were 95°C for 30 sec, followed by 40 cycles of amplification at 95°C for 5 sec and 60°C for 30 sec. Data are representative of three independent assays and expression levels were calculated according to the $\Delta\Delta$ Cq method and expressed as 2^{- $\Delta\Delta$ Cq} (13).

Western blot analysis. The protein expression levels of MMP-2, MMP-9, JNK, p-JNK, ERK1/2, p-ERK1/2 and PKC- δ were

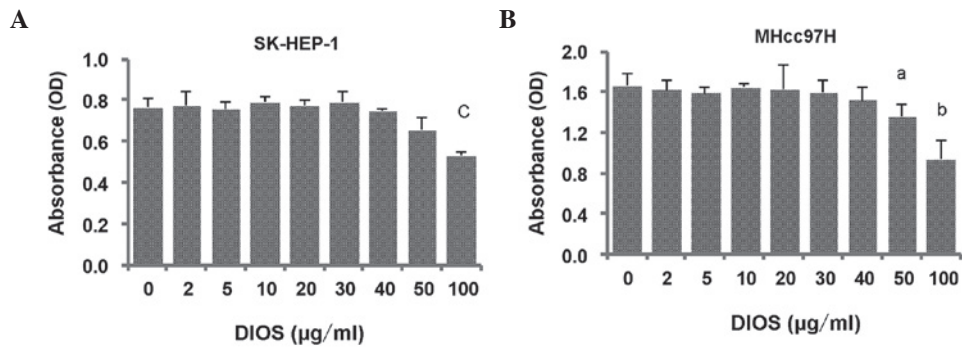


Figure 2. Concentrations of DIOS <math>< 50 \mu\text{g/ml}</math> has no inhibitory effect on the proliferation of SK-HEP-1 or MHcc97H cells. Cell viability following treatment with different concentrations of DIOS for 24 h was detected using a 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (A) Absorbance in the SK-HEP-1 cells; (B) Absorbance in the MHcc97H cells. Each experiment was performed ≥ 3 times and data are presented as the mean \pm standard error of the mean. ^a $P < 0.05$, ^b $P < 0.01$ and ^c $P < 0.001$, vs. control. Data were analyzed using Student's *t*-test. DIOS, diosmetin; OD, optical density.

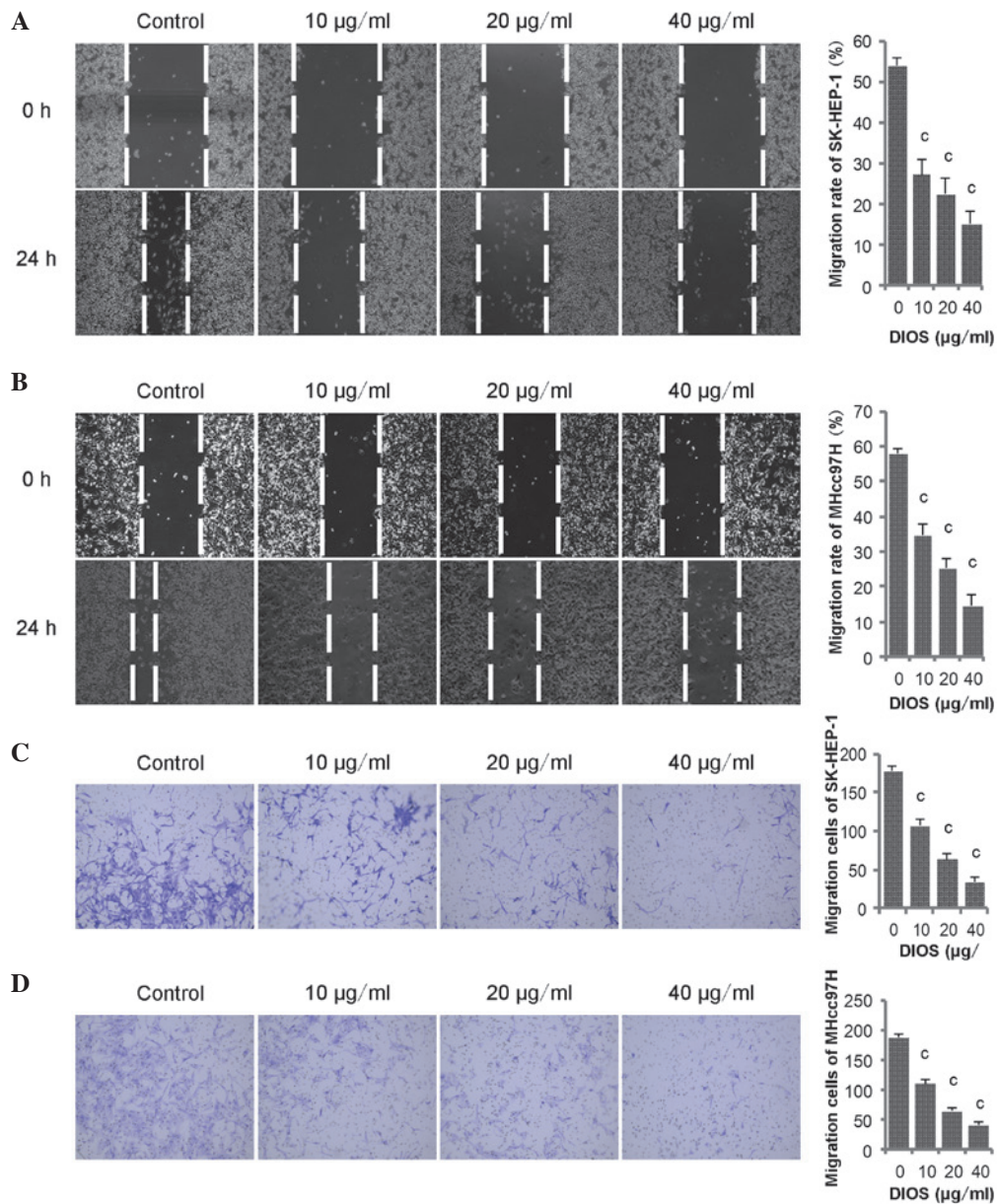


Figure 3. DIOS inhibits the migration of SK-HEP-1 and MHcc97H cells. (A) Results of the wound healing assay showed that DIOS treatment for 24 h inhibited the migration of SK-HEP-1 cells in a concentration-dependent manner. (B) Results of the wound healing assay of MHcc97H cells following treatment with different concentrations of DIOS for 24 h. (C) Results of the cell motility assay of SK-HEP-1 cells following treatment with different concentrations of DIOS for 24 h. (D) Results of the cell motility assay of MHcc97H cells following treatment with different concentrations of DIOS for 24 h. Data are presented as the mean \pm standard error of the mean. Magnification, 100x. ^c $P < 0.001$, vs. 0 $\mu\text{g/ml}$ (control). Data were analyzed using Student's *t*-test. DIOS, diosmetin.

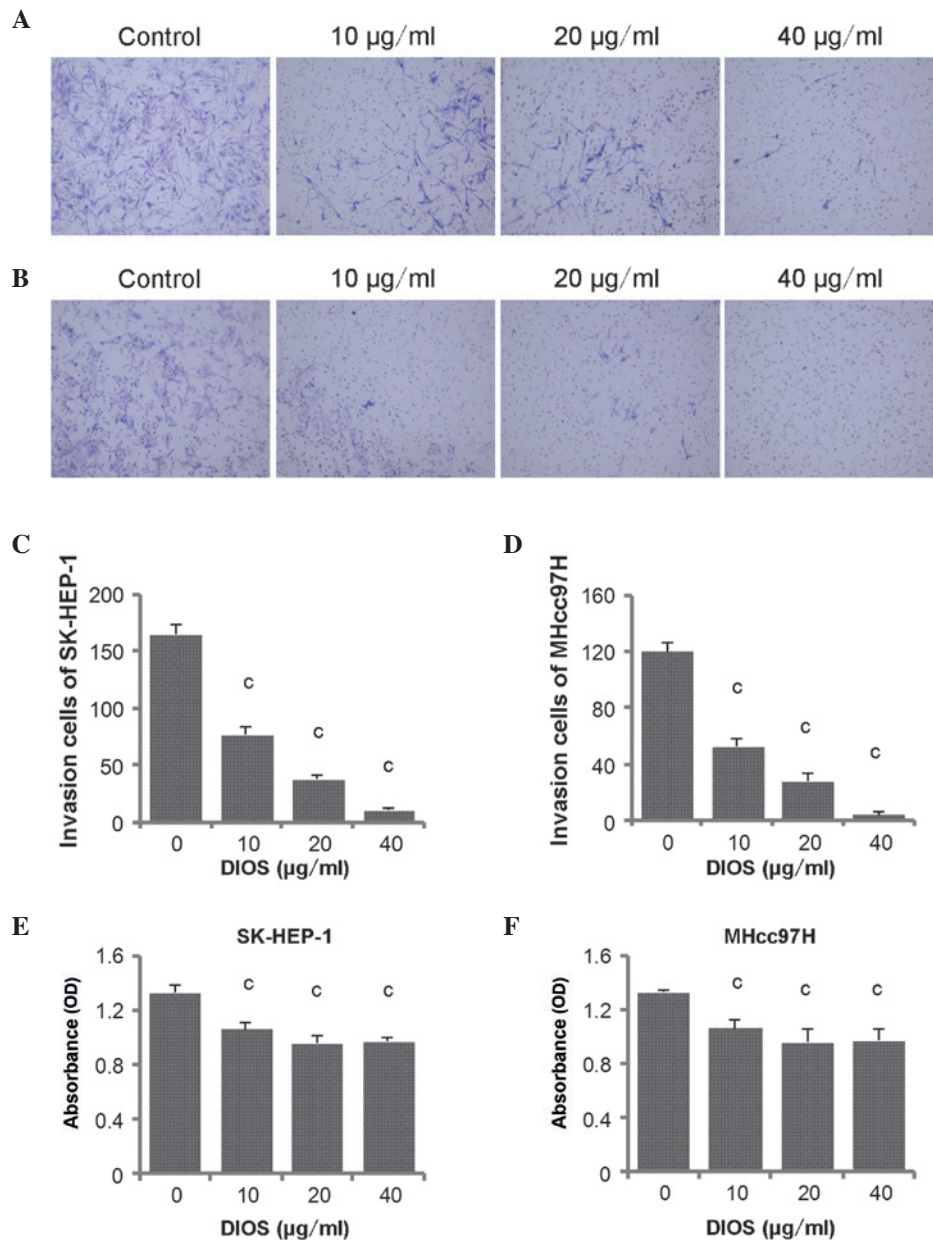


Figure 4. DIOS inhibits invasion and adhesion in SK-HEP-1 and MHcc97H cells. Results of invasion assays of the (A) SK-HEP-1 and (B) MHcc97H cells following treatment with different concentrations of DIOS for 24 h. Quantification of the numbers of invaded cell in the (C) SK-HEP-1 and (D) MHcc97H cells. Absorbance of the (E) SK-HEP-1 and (F) MHcc97H cells using an adhesion assay. ^cP<0.001, vs. 0 µg/ml (control). Data were analyzed using Student's t-test and are presented as the mean ± standard error of the mean. DIOS, diosmetin; OD, optical density.

detected using western blotting, the protocol of which was as reported previously (14). Briefly, cells were seeded in 100 mm culture dishes at a density of 10^5 cells/ml in 10 ml culture media and subsequently cultured at 37°C for 24 h in an atmosphere containing 5% CO₂. Subsequently, cells were exposed to various concentrations of DIOS (0, 10, 20 and 40 µg/ml) and were washed in PBS twice and suspended in lysis buffer for 30 min on ice. Lysates were then centrifuged at 13,000 x g at 4°C for 10 min, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were then blocked in Tris-buffered saline (Beyotime Institute of Biotechnology, Haimen, China) with 0.1% Tween 20 (Sangon Biotech Co., Ltd., Shanghai, China) (TBST), containing 5% bovine serum

albumin for 1 h. The membranes were then incubated with the following rabbit anti-human primary antibodies at 4°C overnight: Anti-MMP-2 monoclonal antibody (mAb; 13132), anti-MMP-9 mAb (13667), anti-JNK polyclonal antibody (pAb; 9258), anti-p-JNK mAb (4668), anti-ERK1/2 mAb (4695), anti-p-ERK1/2 mAb (8544) and anti-PKC-δ pAb (all 1:1,000; 2058). Following washing three times with TBST supplemented with 150 mM NaCl for 10 min, the membranes were incubated with HRP-conjugated goat anti-rabbit immunoglobulin G secondary antibody (1:1,000; E030120-02) for 2 h at room temperature. Membranes were washed three times with TBST for 10 min and the bands were exposed in a dark room and analyzed using Alpha-view gradation analyzing system (Alpha View SA 3.4.0, ProteinSimple, Santa Clara, CA, USA).

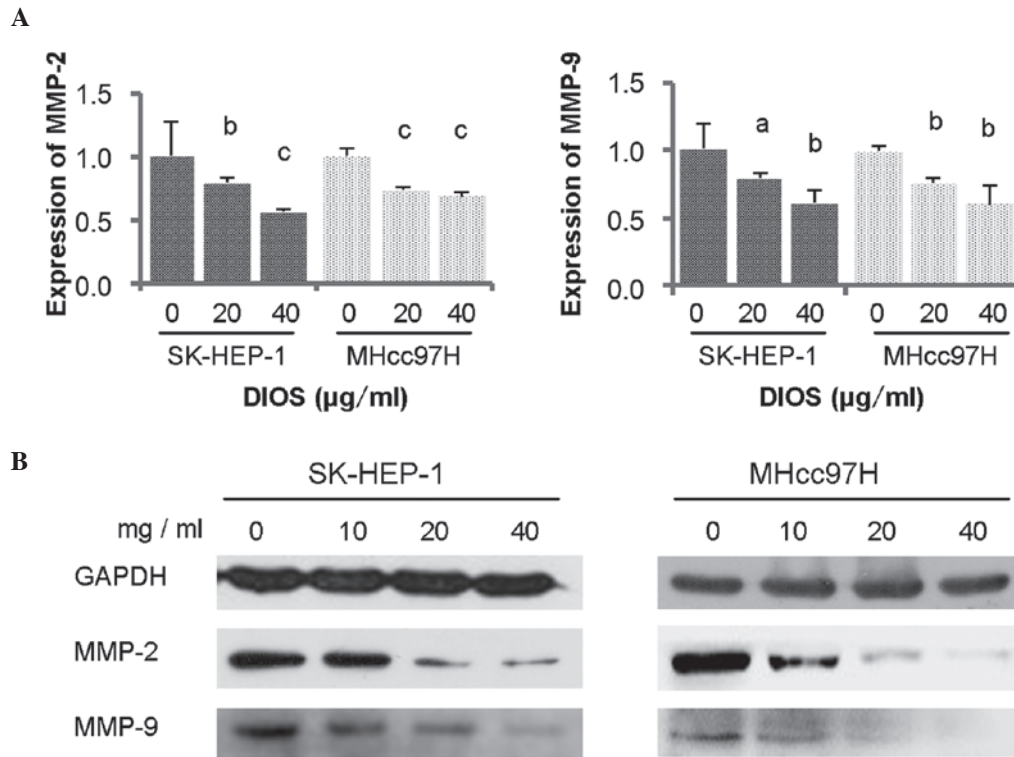


Figure 5. DIOS reduces the expression of MMP-2/9. (A) mRNA expression levels of MMP-2 and MMP-9 following treatment with different concentrations of DIOS for 24 h in SK-HEP-1 and MHcc97H cells. (B) Protein expression levels of MMP-2 and MMP-9 following treatment with different concentrations of DIOS for 24 h in SK-HEP-1 and MHcc97H cells. ^aP<0.05, ^bP<0.01 and ^cP<0.001, vs. 0 $\mu\text{g/ml}$ (control). Data were analyzed using Student's t-test and are presented as the mean \pm standard error of the mean. DIOS, diosmetin; MMP, matrix metalloproteinase.

Table I. Expression levels of related proteins following treatment with diosmetin (DIOS).

DIOS ($\mu\text{g/ml}$)	Expression levels of SK-HEP-1				Expression levels of MHcc97H			
	0	10	20	40	0	10	20	40
MMP-2	1.00	0.81	0.19	0.13	1.00	0.36	0.05	0.03
MMP-9	1.00	0.83	0.62	0.46	1.00	0.80	0.36	0.08
p38	1.00	1.02	1.14	0.81	1.00	0.88	0.70	0.78
p-p38	1.00	0.74	0.67	0.45	1.00	0.54	0.52	0.46
ERK 1/2	1.00	1.06	1.13	0.87	1.00	0.94	0.85	0.83
p-ERK 1/2 (p44)	1.00	1.13	0.56	0.70	1.00	0.62	0.57	0.24
p-ERK 1/2 (p42)	1.00	0.80	0.64	0.76	1.00	0.75	0.41	0.11
JNK (p54)	1.00	0.78	0.80	0.63	1.00	0.92	0.86	0.52
JNK (p46)	1.00	0.91	0.95	0.73	1.00	0.77	0.62	0.46
p-JNK (p54)	1.00	0.79	0.70	0.54	1.00	0.57	0.24	0.19
p-JNK (p46)	1.00	1.12	0.98	0.59	1.00	0.67	0.55	0.07
PKC- δ	1.00	0.81	0.75	0.57	1.00	0.97	0.29	0.43

MMP, matrix metalloproteinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PKC, protein kinase C.

Statistical analysis. Data were obtained from ≥ 3 independent experiments and all results are presented as the mean \pm standard error of the mean. Between-group differences were assessed via Student's t-test using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). Comparisons were relative to untreated controls. P<0.05 was considered to indicate a statistically significant difference.

Results

DIOS does not inhibit cell proliferation at a low concentration. MTT assays were performed to investigate the inhibitory ability of DIOS on the proliferation of SK-HEP-1 and MHcc97H cells. As shown in Fig. 2, the SK-HEP-1 and MHcc97H cells were treated with various concentrations of

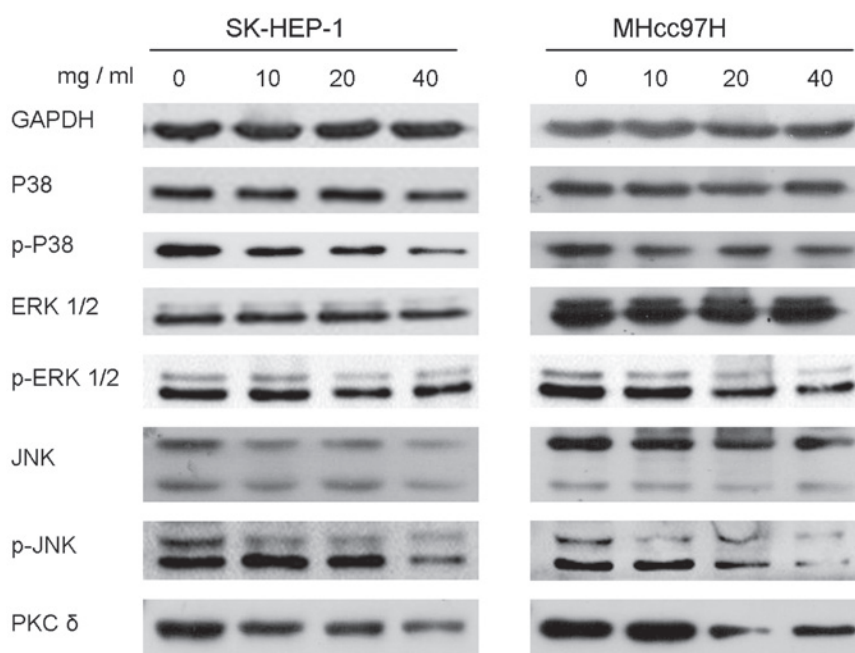


Figure 6. DIOS regulates the PKC- δ and mitogen-activated protein kinase pathways. DIOS, diosmetin.; p-, phosphorylated; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PKC, protein kinase C.

DIOS for 24 h, however, cell proliferation was not affected by DIOS until the concentration reached 50 $\mu\text{g}/\text{ml}$ for the MHcc97H cells ($P < 0.05$) and 100 $\mu\text{g}/\text{ml}$ for the SK-HEP-1 cells ($P < 0.001$).

DIOS inhibits the migration of SK-HEP-1 and MHcc97H cells. The role of DIOS in HCC metastasis was also investigated using a wound healing assay and cell motility assay. Initially, to identify whether the effect of DIOS on HCC occurred due the inhibition of cell proliferation or metastatic suppression, the SK-HEP-1 and MHcc97H cells were treated with 10, 20 and 40 mg/ml DIOS. As described above, the results of the previous MTT assay showed that these concentrations had no effect on cell proliferation. In the wound healing assay, the width of the wounds were measured at 0 h and at 24 h post-DIOS treatment and migration rates were calculated. The results are shown in Fig. 3, in which the migration rates of the SK-HEP-1 and MHcc97H cells were >50% higher, compared with those in the control groups 24 h post-wounding. However, the migration rate was significantly reduced in the groups treated with DIOS, which occurred in a dose-dependent manner (Fig. 3A and B; $P < 0.001$). In the cell motility assay, the cells which passed through the porous polycarbonate membranes were counted to assess whether DIOS affected the migration of the SK-HEP-1 and MHcc97H. The results showed that fewer cells passed through the membranes of the Transwell chambers following DIOS treatment (Fig. 3C and D; $P < 0.001$). The results of the wound healing and cell motility assays indicated that DIOS significantly inhibited the migration of the SK-HEP-1 and MHcc97H HCC cells.

DIOS inhibits the invasion of SK-HEP-1 and MHcc97H cells. In the present study, tumor aggressiveness was evaluated using a basement membrane invasion assay. The cells

observed to degrade the Matrigel and pass through the porous polycarbonate membranes were counted, and the results revealed that DIOS efficiently inhibited the invasion of the SK-HEP-1 and MHcc97H cells across the membranes pretreated with Matrigel. The numbers of cells on the lower surface of the membranes decreased in a dose-dependent manner following DIOS treatment (Fig. 4A and D; $P < 0.001$). These results indicated that DIOS significantly inhibited HCC cell invasion.

DIOS reduces the adherence abilities of SK-HEP-1 and MHcc97H cells. Cancer cell metastasis involves multiple processes, including migration, adhesion and invasion, and the adherence of cells to the ECM or the basement membrane is a crucial step during cancer invasiveness (3). In the present study, the adherence abilities of the SK-HEP-1 and MHcc97H cell lines were significantly reduced following DIOS pretreatment (Fig. 4E and F; $P < 0.001$). These results indicated that DIOS effectively inhibited the adherence ability of the SK-HEP-1 and MHcc97H cells.

DIOS downregulates the expression levels of MMP-2 and MMP-9 in SK-HEP-1 and MHcc97H cells. It is known that MMPs are key enzymes involved in degradation of the ECM, and that MMP-2 and MMP-9 are important in cancer invasion and metastasis (3). To determine the effect of DIOS on HCC cell metastasis, the expression levels of MMP-2/9 were detected using RT-qPCR and Western blot analyses. The results demonstrated that the expression levels of MMP-2/9 were significantly reduced following DIOS treatment (Fig. 5A and B; $P < 0.05$).

Downregulation of MMP-2/9 by DIOS is associated with the MAPK and PKC- δ pathways. The present study further investigated the mechanism underlying the inhibitory effect

of DIOS on HCC metastasis. MAPK and PKC- δ pathway proteins, including P38, ERK 1/2, JNK, PKC- δ and their phosphorylated forms, were measured using Western blotting. DIOS treatment for 24 h had no effect on the protein levels of P38 in the SK-HEP-1 or MHcc97H cell lines, however, the levels of the phosphorylated form, p-P38, were markedly decreased. DIOS treatment for 24 h markedly reduced the protein expression levels of ERK 1/2 and JNK, and markedly reduced the protein levels of p-ERK 1/2 and p-JNK. The results showed that DIOS also downregulated the protein levels of PKC- δ (Fig. 6).

Discussion

HCC is one of most malignant types of tumor and a major common cause of cancer-associated mortality. Several traditional Chinese medicines have been reported for their antitumor properties, including baicalein (15), dihydromyricetin (2,7,16) and resveratrol (17). As a flavonoids compound, DIOS has several medicinal properties, including antibacterial, antimicrobial anti-inflammatory and antioxidant effects (12). Although a previous study demonstrated that DIOS induces cell cycle arrest of HCC cells (18), the role of DIOS on the metastasis of HCC cells remains to be fully elucidated. The metastasis of cancer involves complex processes, including migration and invasion through the tumor stroma, intravasation, tumor cell dissemination, extravasation and cell growth at metastatic sites (19). The present study showed that DIOS inhibited the migration, invasion and adhesion of SK-HEP-1 and MHcc97H cells, which demonstrated that DIOS effectively suppressed the metastasis of SK-HEP-1 and MHcc97H cells.

MMPs are a family of proteolytic enzymes, which have a number of important physiological roles, including ECM modification, accelerating cell migration and cleaving cytokines (20). In the MMP family, MMP-2/9 are reported as substrate-specific gelatinases, which are critical in ECM degradation (12,21). Elevated levels of MMP-2/9 correlate with invasion, metastasis and poor prognosis in various types of cancer (22,23). Therefore, the suppression of MMP-2/9, is an important strategy to prevent cancer cell invasion (24). In the present study, MMP-2 and MMP-9 were downregulated following DIOS treatment for 24 h, in the SK-HEP-1 and MHcc97H cells. This result indicated that DIOS suppressed the metastasis of the SK-HEP-1 and MHcc97H cells through inhibition of the expression of MMP-2/9.

It has been observed previously that members of the MAPK family, including p38 MAPK, ERK 1/2 and JNK, are activated in several types of cancer (25,26). There is increasing evidence that the MAPK family is involved in the migration and invasion of cancer, and that all three of the proteins mentioned above regulate the expression of MMPs (23,27,28). To investigate whether the downregulation of MMP-2 and MMP-9 is associated with the MAPK family, the expression levels of p38, ERK 1/2 and JNK, and their phosphorylated forms, were detected using Western blotting. The data demonstrated no significant change in the the expression levels of p38 and ERK 1/2, however, the phosphorylations of p38 and ERK decreased significantly in the SK-HEP-1 and MHcc97H cells. The expression levels of JNK and p-JNK were downregulated significantly in the SK-HEP-1 and MHcc97H cells. These results suggested that the MAPK

family (p38 MAPK, ERK 1/2 and JNK) was important in the DIOS-mediated cell metastasis.

The upstream protein of ERK is PKC, which is a family of intracellular protein kinases that activate serine/threonine kinases, including MAPK, nuclear factor κ B and phosphatidylinositol-3-kinase by controlling the growth, migration and death of cells, and several PKCs are considered to be associated with tumor progression (23). It has been reported that PKC- δ is overexpressed in human ductal carcinoma (29). The inhibition of PKC- δ may suppress the migration of cells through the PKC/ERK/MMP-9 pathways (30-32). In the present study, PKC- δ was downregulated following DIOS treatment for 24 h in the SK-HEP-1 and MHcc97H cells, which indicated that DIOS inhibited the metastasis of the SK-HEP-1 and MHcc97H cell via the PKC/MAPK/MMPs pathways.

In conclusion, the results of the present study showed that DIOS inhibited the migration, invasion and adhesion of HCC cells by decreasing the gene and protein expression levels of MMP-2/9. Furthermore, the decreased expression of MMP-2/9 was regulated by the PKC- δ /MAPK/ MMPs pathways. These results suggested that DIOS has a potent antimetastatic effect on HCC cells.

Acknowledgements

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