BJP British Journal of Pharmacology

DOI:10.1111/bph.12626
www.brjpharmacol.org

RESEARCH PAPER

Glabridin inhibits migration and invasion by transcriptional inhibition of matrix metalloproteinase 9 through modulation of NF-κB and AP-1 activity in human liver cancer cells

Correspondence
Hui-Ling Chiou or Mu-Kuan Chen, School of Medical Laboratory and Biotechnology, Chung Shan Medical University, 110, Section 1, Chien-Kuo N. Road, Taichung 40201, Taiwan.
E-mail: hlchiou@csmu.edu.tw; 53780@cch.org.tw

Keywords
glabridin; migration; invasion; matrix metalloproteinase 9; hepatoma

Received
6 September 2013
Revised
27 January 2014
Accepted
2 February 2014

Ming-Ju Hsieh[1,2,3], Chiao-Wen Lin[4,5], Shun-Fa Yang[3,6], Mu-Kuan Chen[7] and Hui-Ling Chiou[8,9]

[1]*Cancer Research Center, Changhua Christian Hospital, Changhua, Taiwan,* [2]*School of Optometry, Chung Shan Medical University, Taichung, Taiwan,* [3]*Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan,* [4]*Institute of Oral Sciences, Chung Shan Medical University, Taichung, Taiwan,* [5]*Department of Dentistry, Chung Shan Medical University Hospital, Taichung, Taiwan,* [6]*Department of Medical Research, Chung Shan Medical University Hospital, Taichung, Taiwan,* [7]*Department of Otorhinolaryngology-Head and Neck Surgery, Changhua Christian Hospital, Changhua, Taiwan,* [8]*School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taichung, Taiwan, and* [9]*Department of Clinical Laboratory, Chung Shan Medical University Hospital, Taichung, Taiwan*

BACKGROUND AND PURPOSE

High mortality and morbidity rates for hepatocellular carcinoma in Taiwan primarily result from uncontrolled tumour metastasis. Glabridin, a prenylated isoflavonoid of licorice (*Glycyrrhiza glabra*) roots, is associated with a wide range of biological properties, such as regulation of energy metabolism, oestrogenic, neuroprotective, anti-osteoporotic and skin whitening. However, the effect of glabridin on the metastasis of tumour cells has not been clarified.

EXPERIMENTAL APPROACH

A wound healing model and Boyden chamber assays *in vitro* were used to determine the effects of glabridin on the migration and invasion of human hepatocellular carcinoma (HHC) cells. Western blot analysis, gelatin zymography, real-time PCR and promoter assays were used to evaluate the inhibitory effects of glabridin on matrix metalloproteinase 9 (MMP9) expression in these cells.

KEY RESULTS

Glabridin significantly inhibited migration/invasion capacities of HCC cells, Huh7 and Sk-Hep-1, cell lines that have low cytotoxicity *in vitro*, even at high concentrations. Western blot analysis and gelatin zymography showed that glabridin inhibited the expression, activities and protein levels of MMP9 and the phosphorylation of ERK1/2 and JNK1/2. These inhibitory effects were associated with an up-regulation of tissue inhibitor of metalloproteinase-1 and a down-regulation of the transcription factors NF-κB and activator protein 1 signalling pathways. Finally, the administration of glabridin effectively suppressed the tumour formation in the hepatoma xenograft model *in vivo*.

CONCLUSION AND IMPLICATIONS
Glabridin inhibited the invasion of human HCC cells and may have potential as a chemopreventive agent against liver cancer metastasis.

Abbreviations
ChIP, chromatin immunoprecipitation assay; ECM, extracellularmatrix; HCC, hepatocellular carcinoma; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinases-1

Introduction

Hepatocellular carcinoma (HCC) is a common malignant neoplasm and major cause of cancer-related deaths in Asian countries (Yeh *et al.*, 2012). The metastasis of cancer cells typically involves multiple processes, including the invasion of surrounding tissue, penetration into blood or lymphatic vessels and the formation of new tumours (Chen *et al.*, 2011). Cancer cell metastasis involves multiple processes and various cytophysiological changes, including changes in the adhesive properties between cells and the extracellularmatrix (ECM). This involves proteolytic degradation and impaired intracellular interactions. Thus, degradation of the ECM and components of the basement membrane caused by a concerted action of proteinases, such as matrix metalloproteinases (MMPs, see Alexander *et al.*, 2013), cathepsins and plasminogen activator, play a critical role in tumour invasion and metastasis (Westermarck and Kahari, 1999; Yoon *et al.*, 2003). MMP9 is the protease most critically involved in the degradation of the basement membrane. A number of pathological states, including cancer, inflammation and vascular diseases, are associated with increased proteinase activities (Weng *et al.*, 2010; Yang *et al.*, 2010). The expression of MMPs is regulated by various factors, such as growth factors, cytokines and proteinase inhibitors. The endogenous tissue inhibitors of metalloproteinases-1 (TIMP-1) are the specific inhibitors of MMP9. An imbalance between the MMPs and TIMPs might, therefore, contribute to degradation or deposition of the ECM (Yang *et al.*, 2010; Yeh *et al.*, 2012). The inhibition of migration or invasion mediated by MMP2, MMP9 or u- plasminogen activator could, therefore, putatively provide a preventive measure against cancer metastasis (Bjorklund and Koivunen, 2005).

In recent years, naturally occurring plant products have gained increasing attention for their ability to inhibit malignant invasive progression in late stage neoplastic diseases (Shankar *et al.*, 2008; Ravindranath *et al.*, 2009). There is increasing focus on providing a scientific basis for use of these agents as a preventive strategy for people with high risk of cancers. Glabridin, a key chemical and biological component of licorice (*Glycyrrhiza glabra*) is present in many foods and dietary supplements as well as cosmetics. Glabridin is an isoflavane, a type of isoflavonoid, and is part of a larger family of plant-derived molecules, the natural phenols. It has been shown to have positive effects on the oxidation of low-density lipoprotein, to have anti-obesity properties and could potentially provide benefits to human health (Belinky *et al.*, 1998; Carmeli and Fogelman, 2009; Ahn *et al.*, 2013). In a previous study we showed that glabridin can inhibit lung and breast cancer metastasis (Hsu *et al.*, 2011; Tsai *et al.*, 2011). However, the effects of glabridin on HCC invasion and metastasis, and the underlying mechanisms of its antimetastatic effects, have yet to be evaluated. Hence, in the present study, we investigated the potential inhibitory effects of glabridin on HCC invasiveness and the possible mechanisms of glabridin-induced antimetastatic effects.

Methods

Cell lines

Huh7 and Sk-Hep-1, human hepatoma cell lines, obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan), were cultured in DMEM (Gibco BRL, Grand Island, NY, USA) supplemented with 10% FCS, 1 mM glutamine, 1% penicillin/streptomycin, 1.5 g L^{-1} sodium bicarbonate and 1 mM sodium pyruvate (Sigma Chemical Co., St. Louis, MO, USA).

Antibodies and other reagents

Glabridin, ≥98% (HPLC), powder was purchased from Sigma Chemical Co. and a stock solution of glabridin was made at 10, 20 and 40 μM concentration in DMSO and stored at –20°C. The final concentration of DMSO for all treatments was consistently less than 0.1%. Other chemicals, including 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), paraformaldehyde, Triton X-100, were obtained from Sigma Chemical Co. All antibodies were obtained from Cell Signaling (Cell Signaling Technology, Danvers, MA, USA).

The determination of cell viability (MTT assay)

The effect of glabridin on cell viability was assayed by the MTT method, as described previously (Yang *et al.*, 2010). Briefly, 2×10^5 cells per well were cultured in 6-well plates and stimulated with different concentrations of glabridin (0, 10, 20, 40 μM). After 24, 48 and 72 h, MTT was added to each well (at a final concentration of 0.5 mg mL^{-1}) and incubated for a further 4 h. The number of viable cells was directly proportional to the production of formazan, reflected by the colour intensity measured at 595 nm, following solubilization with isopropanol. Each condition was performed in three replicate wells and data were obtained from at least three separate experiments.

Colony formation assays

Huh7 and Sk-Hep-1 cell lines were seeded at a concentration of 1×10^4 cells per well in 6-well cell culture plates in appro-

priate media. After 24 h of incubation, the media were replaced with fresh media containing either glabridin at 10, 20 and 40 μM, or DMSO. Both treated and untreated cells were incubated and the media with added compounds was changed every 3 days. Colonies were allowed to form for 2 weeks and then stained with 0.3% crystal violet solution.

In vitro *wound closure*

Cells (1×10^5 cells per well) were plated in 6-well plates for 24 h, wounded by scratching with a pipette tip, then incubated with DMEM medium containing 0.5% FCS and treated with or without glabridin (0, 10, 20, 40 μM) for 0 and 24 h. Cells were photographed using a phase-contrast microscope (6100; Olympus, MA, USA).

Cell invasion and migration assays

Cell invasion and migration were assayed according to the methods described by Yang *et al.* (2010). After being treated with glabridin (0, 10, 20, 40 μM) for 24 h, surviving cells were harvested and seeded on a Boyden chamber (Neuro Probe, Cabin John, MD, USA) at 10^4 cells per well in a serum-free medium and then incubated for 24 h at 37°C. For the invasion assay, 10 mL Matrigel (25 mg 50 mL^{-1}; BD Biosciences, MA, USA) was applied to 8 mm (pore size) polycarbonate membrane filters; the bottom chamber contained standard medium. Filters were then air-dried for 5 h in a laminar flow hood. The invaded cells were fixed with 100% methanol and stained with 5% Giemsa. Cell numbers were counted under a light microscope. The migration assay was carried out as described in the invasion assay with no coating of matrigel (Yang *et al.*, 2010).

Gelatin zymography

The activity of MMP9 in a conditioned medium was measured using a gelatin zymography protease assay, as described previously (Yang *et al.*, 2008). Aliquots of the media, of an appropriate volume, were subjected to 0.1% gelatin–8% SDS-PAGE electrophoresis. After electrophoresis, gels were washed with 2.5% Triton X-100 and incubated in reaction buffer (40 mM Tris–HCl, pH 8.0; 10 mM $CaCl_2$ and 0.01% NaN_3) for 24 h at 37°C. The gel was, then, stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA, USA).

RNA interference

Small interfering RNAs (siRNA) targeting human MMP9 siRNA (sc-29400) or control siRNA-A (sc-37007) were from Santa Cruz Biotechnology. Cells were transfected by siRNA Transfection Reagent: sc-29528 according to the manufacturer's recommendations (Santa Cruz Biotechnology, CA, USA). The silencer negative control siRNA-A (sc-37007), a nonsense siRNA duplex, was used as a control siRNA.

RNA preparation and SYBR green quantitative real-time PCR

Total RNA was isolated from HCC cells using Trizol (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Quantitative real-time PCR analysis was carried out using SYBR Green One-step PCR Master Mix (Applied Biosystems, Life Technologies, Waltham, MA, USA). A total of 100 ng of cDNA was added to 25 mL reaction medium with MMP9 or GAPDH primers. Quantitative real-time PCR assays were carried out in triplicate on a StepOnePlus sequence detection system. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected.

Western blot analysis

Cells were lysed in cell lysis buffer (RIPA buffer) supplemented with protease inhibitor (Roche, Hoffmann-La Roche, Nutley, NJ, USA). Protein concentrations of the extracts were measured with BCA assay (Thermo Fisher Scientific Inc., Waltham, MA, USA) and equalized with the extraction reagent. Equal amounts of the extracts or concentrated cell culture supernatants were loaded and separated on a 10 or 15% polyacrylamide gel and transferred onto a PVDF membrane (Millipore Corporation, Billerica, MA, USA). The blot was subsequently incubated with 3% non-fat milk in PBS for 1 h to block non-specific binding, and probed with a corresponding antibody [antibodies for MMP9, TIMP-1, NF-κB (p65), phosphor-I, were from Cell Signaling; antibodies for C23, GAPDH, c-Jun and c-Fos were from Santa Cruz Biotechnology; antibodies for PI3K, Akt, phospho-Akt, p38, JNK1/2 and β-actin were from BD Biosciences; antibodies for ERK1/2, phospho-ERK1/2, phospho-p38 and phospho-JNK were from Millipore Corporation] against a specific protein for 37°C at 2 h or overnight at 4°C, and then with an appropriate peroxidase conjugated anti-rabbit or anti-mouse IgG secondary antibody for 1 h. Extensive washing with wash buffer was conducted between incubations and after the final washing, the signal was developed and identified using an ECL detection system. The relative photographic density was quantified by a gel documentation and analysis system (Alpha Imager 2000, Alpha Innotech Corporation, San Leandro, CA, USA). The quantitative results for the protein of interest levels are expressed as relative to an internal housekeeping control such as β-actin, C23 or GAPDH.

Transfection and MMP9 promoter-driven luciferase assays

Cells were seeded at a concentration of 5×10^4 cells per well in 6-well cell culture plates. After 24 h of incubation, pGL3-basic (vector) and MMP9 promoter plasmid were cotransfected with a β-galactosidase expression vector (pCH110) into cells using Turbofect (Fermentas, Carlsbad, CA, USA). After 12 h of transfection, cells were treated with vehicle or glabridin (0, 10, 20, 40 μM) for 24 h. The cell lysates were harvested, and luciferase activity was determined using a luciferase assay kit. The value of the luciferase activity was normalized to transfection efficiency and monitored by β-galactosidase expression.

Chromatin immunoprecipitation analysis (ChIP)

ChIP was performed as described previously (Yeh *et al.*, 2012). DNA immunoprecipitated with anti-NF-κB (p65) and anti-activator protein 1 (AP-1) was purified and extracted using phenol-chloroform. Immunoprecipitated DNA was analysed by PCR or quantitative PCR by using specific primers.

Preparation of cell nuclear and cytosolic extracts

Nuclear extracts and cytosolic extracts were prepared essentially as described previously (Hsieh *et al.*, 2012). Briefly, cells were rinsed with cold PBS twice and then harvested with trypsin-EDTA and then centrifuged at 500× *g* for 5 min. After removal of the supernatant, cold reagents were added to the cell pellet with a volume ratio of CER I : CER II : NER at 200:11:100. All of the reagents contained protease inhibitors. After the addition of cold CER I, the samples were vortexed for 15 s, incubated on ice for 10 min and then cold CER II was added. After a 5-min centrifugation at the maximum speed (12 000× *g*), the resultant supernatant (cytoplasmic extract) was stored at –80°C. Meanwhile, the insoluble (pellet) fraction was resuspended in cold NER. This vortex procedure was repeated four times with a 10-min incubation on ice between each vortex. After centrifugation at the maximum speed (12 000× *g*), the resultant supernatant (nuclear extract fraction) was stored at –80°C.

Measurement of xenograft tumour growth

These experiments were carried out in 5–6-week-old male *BALB/c* nude mice (18–22 g) (National Taiwan University Animal Center, Taipei, Taiwan, ROC). All animal care and experimental procedures were according to the guidelines of the Institutional Animal Care and Use Committee of Chung Shan Medical University (IACUC, CSMC) for the care and use of laboratory animals. SK-Hep-1 cells (3×10^6 per mouse) were resuspended in 200 μL of sterile PBS and injected s.c. into the right flank of the mouse. Mice were randomized into two groups (5 mice per group). All animals were housed with a regular 12-h light/12-h dark cycle and water and food, standard rodent chow diet (LaboratoryRodent Diet 5001, LabDiet, St. Louis, MO, USA), available *ad libitum*. They were kept in a pathogen-free environment at the Laboratory Animal Unit, temperature 22°C, humidity 30~70% and 5 mice per cage. Seven days after tumour cell injection, the treatment group received glabridin (10 mg kg^{-1}, i.p., three times per week). The control group received an equal volume of DMSO vehicle. Tumour volumes were determined from caliper measurements obtained every 7 days. At the end of the experiment, mice were killed and the tumours were excised and weighed. The tumour volume was calculated by the following formula: $0.5 \times \text{length} \times \text{width}^2$. Mean weight of mice at the beginning and end of the study did not differ significantly between the groups. All studies involving animals are reported in accordance with ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Statistical analysis

Values represent the means ± SD and the experiments were repeated three times (n = 3). Statistical analyses were performed using the one-way ANOVA followed by Tukey's *post hoc* test when more than three groups were analysed. Data comparisons were performed by use of Student's *t*-test (Sigma-Stat 2.0, Jandel Scientific, San Rafael, CA, USA) when two groups were compared. A P value < 0.05 was considered to be statistically significant.

Results

Effects of glabridin on the cell cytotoxicity of Huh7 and Sk-Hep-1 cells

The chemical structure of glabridin is shown in Figure 1A. To assess the effects of glabridin on cell viability, Huh7 and Sk-Hep-1 cells were treated with glabridin at various concentrations (0–40 μM) for 24, 48 and 72 h and then analysed by the MTT assay. As shown in Figure 1B and C, glabridin had no effect on the cell viability of Huh7 and Sk-Hep-1 cells, as compared with that of untreated cells. Also treatment with glabridin (0–40 μM) resulted in no significant change in colony formation of the Huh7 and SK-Hep-1 cells (Figure 1D). Thus, all subsequent experiments used glabridin in this concentration range.

Effects of glabridin on wound closure, invasion and migration in Huh7 and Sk-Hep-1 cells in vitro

The results shown in Figure 2 display the findings from the wound closure assay used to determine the effects of glabridin on the migration of Huh7 and Sk-Hep-1 cells. Figure 2A shows representative photographs of Huh7 and Sk-Hep-1 cells migrating into scratch wounds. The number of cells migrating into the wound was decreased by glabridin in a concentration-dependent manner (Figure 2B). At 40 μM, glabridin decreased the number of migrated cells number to 55% for Huh7 and 14% for Sk-Hep-1 at 24 h respectively. Figure 2C and E show the cell invasion and migration of Huh7 and Sk-Hep-1 cells treated with glabridin for 24 h (cell invasion) and 16 h (cell migration). At 40 μM, glabridin decreased, the cell invasion to 58% for Huh7 and 53% for Sk-Hep-1 (Figure 2D) respectively. In addition, glabridin decreased the cell migration to 41% for Huh7 and 45% for Sk-Hep-1 (Figure 2F) respectively. Hence, glabridin markedly reduced the invasion and migration abilities of Huh7 and Sk-Hep-1 cells in a dose-dependent manner.

Glabridin inhibits the expression of MMP9 and increases that of the endogenous inhibitor TIMP-1

MMP9 is the protease involved in the degradation of the basement membrane in tumour invasion and metastasis. Previous results have indicated that *Terminalia catappa* inhibits HCC cell metastasis through regulation of MMP9 (Yeh *et al.*, 2012). To further investigate the mechanism by which glabridin inhibits cell invasion and migration in Huh7 and Sk-Hep-1 cells, we analysed the expression levels of MMP9. Glabridin (40 μM) significantly inhibited MMP9 enzyme activity (Figure 3A and B) and protein expression (Figure 3C and D) in a dose-dependent manner. In contrast, the expression of the endogenous inhibitor of MMP9, TIMP-1, was increased in glabridin-treated Huh7 and Sk-Hep-1 cells. The expression levels of MMP9 were also down-regulated by the specific MMP9 siRNA (Figure 3E) and, similar to glabridin, the cell invasion and migration ability were also inhibited in both cells transfected with MMP9 siRNA. These results indicate that the inhibitory effects of MMP9 siRNA on cell invasion and migration ability were comparable to those of glabridin (Figure 3F and G).

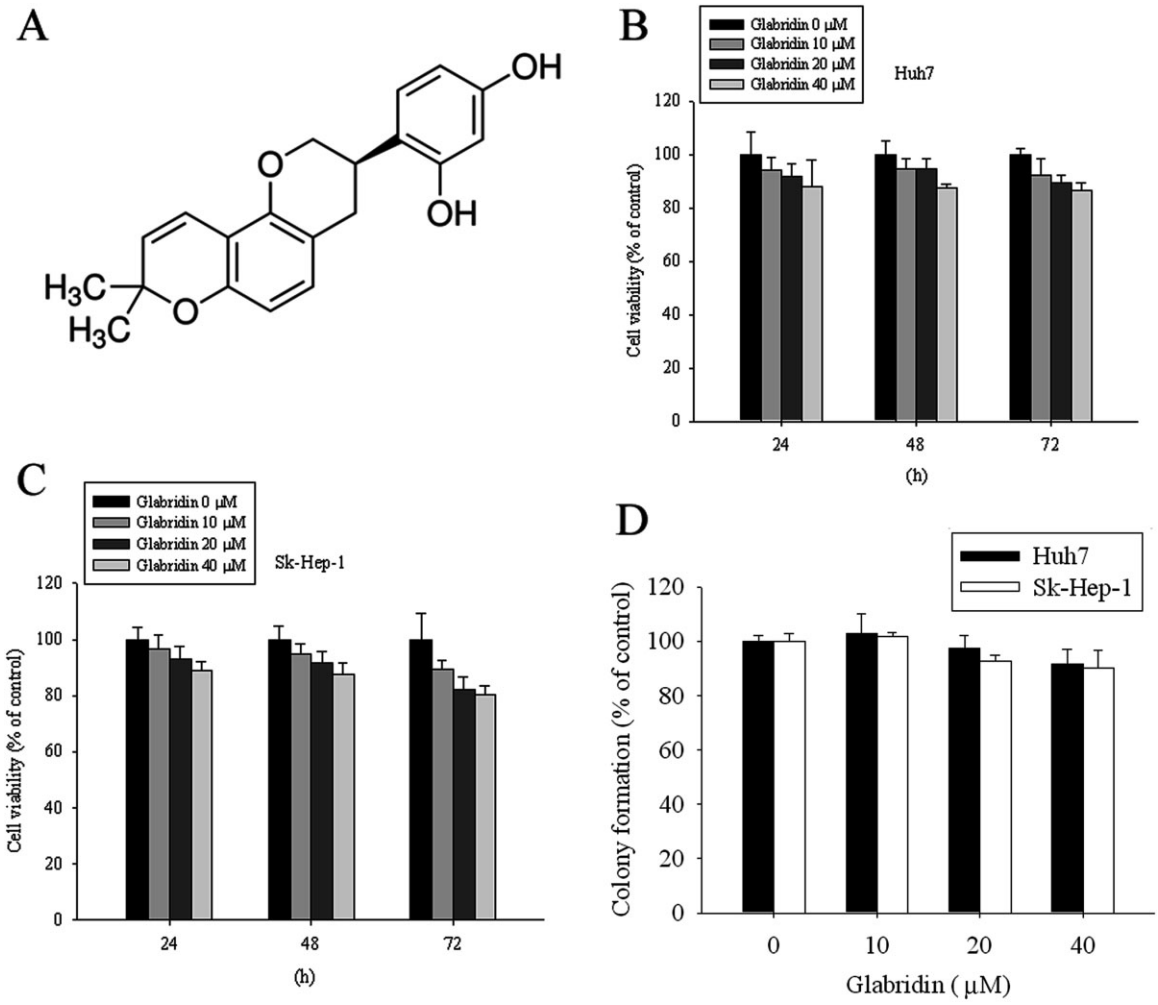

Figure 1

Effect of glabridin on cell viability in Huh7 and Sk-Hep-1 cell lines. (A) Structure of glabridin. Cell viabilities of Huh7 (B) and Sk-Hep-1 cells (C) cultured in absence or presence of glabridin (0–40 μM) for the indicated period were analysed by MTT assay. (D) Representative cultures of Huh7 and SK-Hep-1 cells in the colony-forming assay. Results are shown as mean ± SD from three separate experiments with three determinations in each experiment.

Glabridin suppresses MMP9 expression at the transcriptional level

To further investigate the inhibitory effects of glabridin on MMP9 expression in Huh7 and Sk-Hep-1 cells, cells were treated with 0, 10, 20 and 40 μM glabridin for 24 h. The mRNA levels of MMP9 were then analysed by reverse-transcriptase and real-time PCR and found to be significantly decreased in a dose-dependent manner by glabridin (Figure 4A and B). Promoter analysis using a luciferase assay kit also identified significant inhibition of the luciferase activities of MMP9 (Figure 4C). These results show that glabridin regulates the expression of MMP9 at a transcriptional level in Huh7 and Sk-Hep-1 cells.

Glabridin suppresses MMP9 expression by affecting the expression of NF-κB and AP-1 transcription factors

To determine whether certain transcription factors are involved in the effects of glabridin on MMP9 in Huh7 and Sk-Hep-1 cells, we evaluated the effect of glabridin on the nuclear translocation of NF-κB and AP-1. Treatment of Huh7 and Sk-Hep-1 cells with 40 μM glabridin reduced the nuclear translocation of NF-κB and AP-1. Using the ChIP assay, we then investigated the involvement of NF-κB and AP-1 transcription factors in the glabridin-induced transcriptional inhibition of MMP9 (Figure 5A). Results from the quantitative real-time PCR indicated that glabridin significantly reduced the binding of NF-κB and AP-1 to the MMP9 promoters (Figure 5B). To further investigate the involvement of NF-κB and AP-1 in the transcriptional regulation of MMP9 by glabridin in these cells, we analysed the expression of NF-κB, c-fos and c-Jun by Western blotting to confirm the effect of glabridin on nuclear translocation. Cells treated with 40 μM glabridin had reduced nuclear translocation of NF-κB (Figure 5C and D). In addition, glabridin also inhibited the expression of c-Fos, c-Jun and phosphorylation of IκB (Figure 5E and F). These findings indicate that glabridin inhibits the transcription of MMP9 in Huh7 and Sk-Hep-1

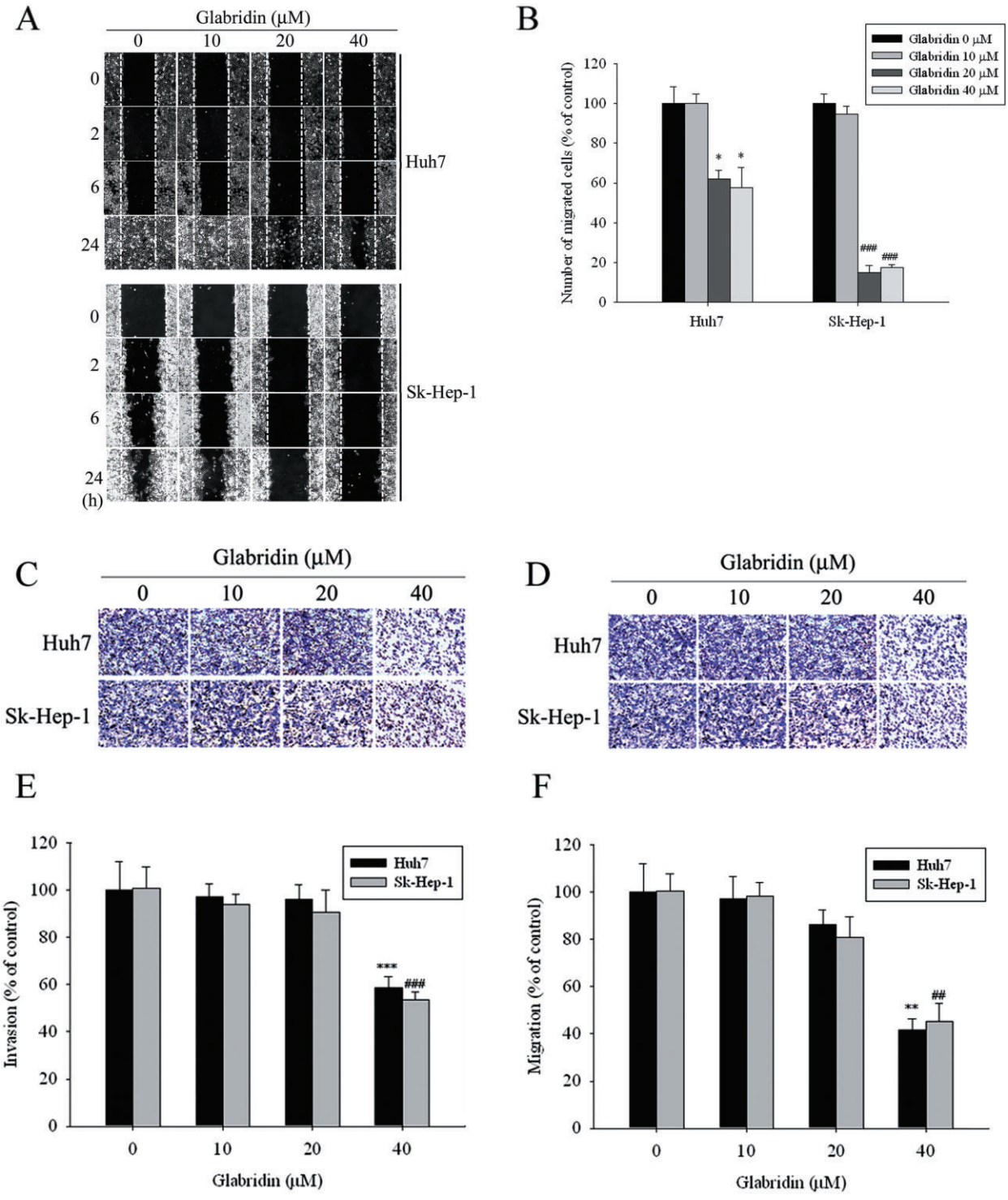

Figure 2

Glabridin inhibits wound closure, invasion and migration in Huh7 and Sk-Hep-1 cells. (A) Huh7 and Sk-Hep-1 cells were wounded and then treated with vehicle (DMSO) or glabridin (0–40 μM) for 24 h in 0.5% FBS-containing medium. At 0, 2, 6 and 24 h, phase-contrast pictures of the wounds at three different locations were taken. The cells migrating into the wound area were counted based on the dash line as time zero. (B) Quantitative assessment of the mean number of cells in the denuded zone at 24 h, expressed as means ± SD ($n = 3$). *$P < 0.05$ as compared with the untreated Huh7 cells. ###$P < 0.001$ as compared with the untreated Sk-Hep-1 cells. (C) Cell invasion was measured using Matrigel-coated transwell chambers for 24 h. (D) Cell migration was measured using a Boyden chamber for 16 h with polycarbonate filters. (E and F) The invasion and migration abilities of Huh7 and Sk-Hep-1 cells were quantified by counting the number of cells that invaded to the underside of the porous polycarbonate as described in the Methods section. The values represent the means ± SD from three determinations per condition repeated three times ($n = 3$). **$P < 0.01$, ***$P < 0.001$ as compared with the untreated Huh7 cells. ##$P < 0.01$, ###$P < 0.001$ as compared with the untreated Sk-Hep-1 cells.

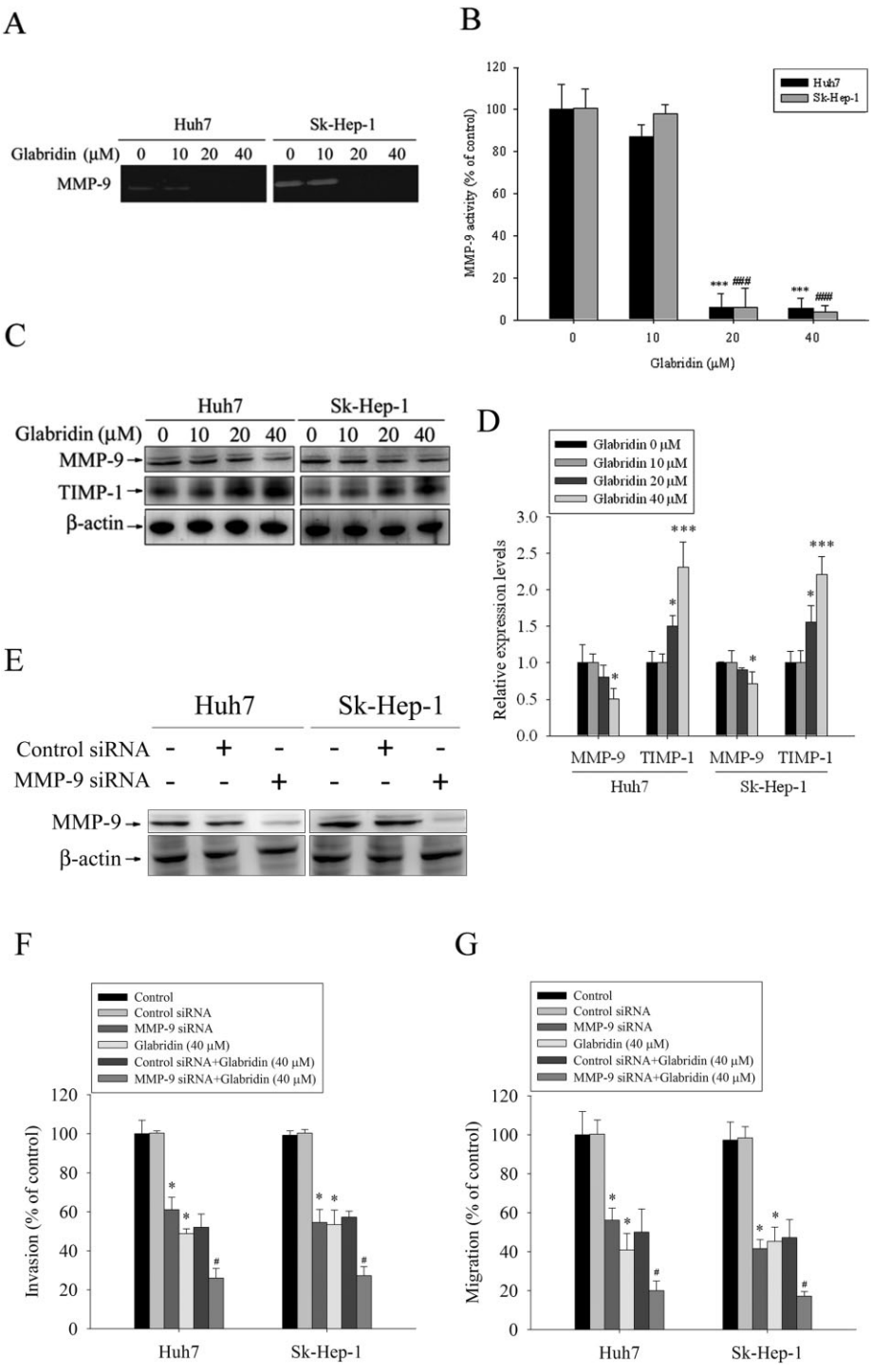

Figure 3

Glabridin inhibits MMP9 and increases TIMP-1 protein expression. (A), (B) Huh7 and Sk-Hep-1 cells were treated with glabridin (0–40 μM) for 24 h in serum-free medium and then subjected to gelatin zymography to analyse the activity of MMP9. ***$P < 0.001$ as compared with the untreated Huh7 cells. ###$P < 0.001$ as compared with the untreated Sk-Hep-1 cells. (C) Western blotting to analyse the protein levels of MMP9 and TIMP-1. (D) Quantitative results of MMP9 and TIMP-1 protein levels after being adjusted to the β-actin protein level. The values represent the means ± SD from three determinations per condition repeated three times. *$P < 0.05$, ***$P < 0.001$ as compared with that of untreated cells. (E) Huh7 and Sk-Hep-1 cells were transfected with the control or MMP9 siRNA at 24 h and then subjected to Western blotting to study the expression levels of MMP9. (F) Huh7 and Sk-Hep-1 cells were transfected with the control or MMP9 siRNA. After a successful transfection, Huh7 and Sk-Hep-1 cells were treated with glabridin 40 μM. Cell invasion was measured using Matrigel-coated transwell chambers for 24 h. (G) Cell migration was measured using a Boyden chamber for 16 h with polycarbonate filters. The values represent the means ± SD from three determinations per condition repeated three times ($n = 3$). *$P < 0.05$ as compared with the untreated cells, #$P < 0.05$ as compared with the cells treated with glabridin (40 μM).

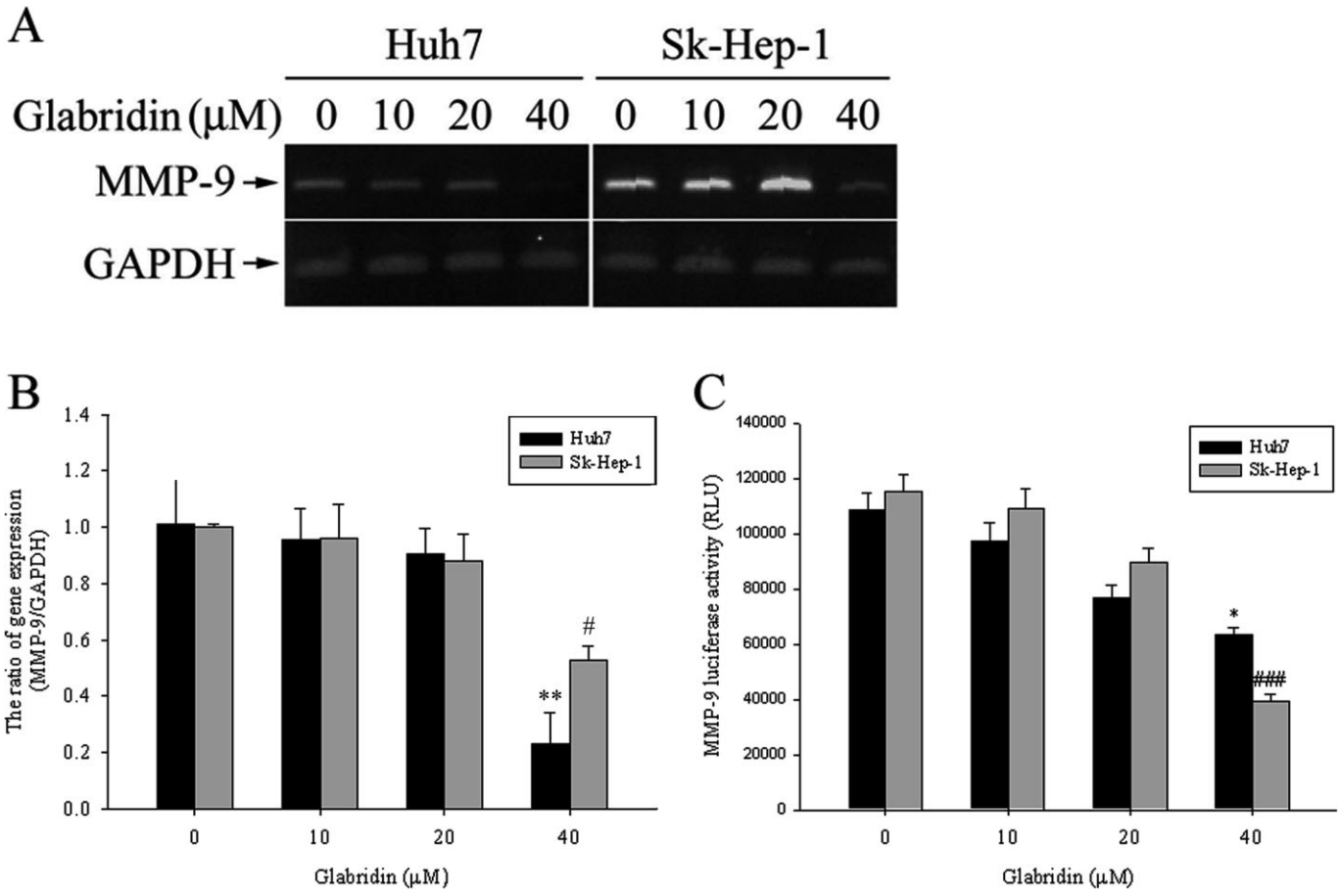

Figure 4

Glabridin inhibits MMP9 mRNA expression and promoter luciferase activities. (A) Huh7 and Sk-Hep-1 cells were treated with glabridin (0–40 μM) for 24 h and then subjected to semi-quantitative reverse-transcriptase PCR and (B) real-time PCR to analyse the expression of MMP9 mRNA. (C) MMP9 promoter reporter assay to analyse the promoter activity of MMP9. Luciferase activity, determined in triplicate, was normalized to β-galactosidase activity. The values represent the means ± SD from three determinations per condition repeated three times (n = 3). *P < 0.05, **P < 0.01 as compared with the untreated Huh7 cells. #P < 0.05, ###P < 0.001 as compared with the untreated Sk-Hep-1 cells.

cells by suppressing the nuclear translocation and MMP9 promoter binding activity of NF-κB and AP-1.

Glabridin suppresses cell invasion and migration by inhibiting the phosphorylation of ERK1/2 and JNK1/2

The effects of glabridin on the expressions of MAPK and PI3K/Akt pathways were investigated using Western blotting to elucidate further the underlying mechanisms for its inhibitory effect on cell migration/invasion. Glabridin reduced the phosphorylation of ERK1/2 and JNK 1/2 in Huh7 and Sk-Hep-1 cells (Figure 6A). From the densitometric analyses of blots, compared to the control, treatment of glabridin at 40 μM resulted in a reduction in the phosphorylation of ERK1/2 to 51% and reduction in the phosphorylation of JNK1/2 to 64%. However, the phosphorylation of the PI3K/Akt and p38 pathways remained unaffected (Figure 6B and C).

To further determine whether glabridin's inhibitory effects on cell invasion and migration were cause mainly by inhibition of the phosphorylation of ERK1/2 and JNK1/2, we investigated its effects on Huh7 and Sk-Hep-1 cells when combined with specific inhibitors of ERK1/2 (U0126) and JNK1/2 (SP600125). The inhibition of Huh7 and Sk-Hep-1 invasion and migration was further increased when glabridin was combined with these specific inhibitors (Figure 7). Therefore, the inhibition of the ERK1/2 and JNK1/2 signalling pathways may result in a reduced expression of tumour cell invasion and migration.

Glabridin inhibits tumour growth in SK-Hep-1 tumour xenografted nude mice model

The effects of glabridin on tumour growth *in vivo* were investigated in the xenograft nude mice model, BALB/c male mice injected in the right flank with SK-Hep-1 human HCC cells.). The control group of animals treated with DMSO (i.p.) showed a progressive increase in their tumour volumes, whereas, experimental animals treated with glabridin (10 mg kg^{-1}, i.p.) developed tumours of significantly smaller

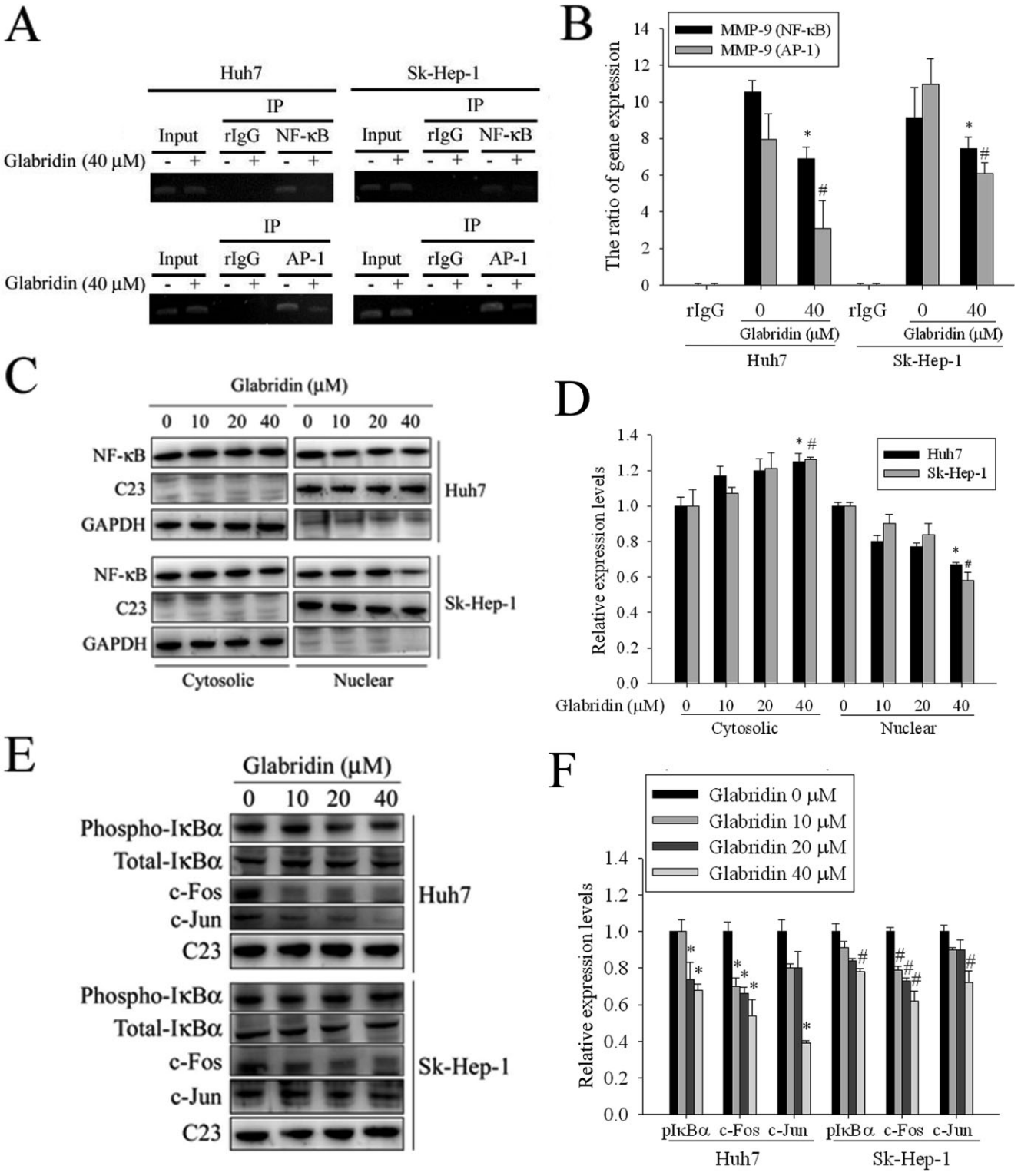

Figure 5

Critical role of NF-κB and AP-1 in glabridin-induced transcriptional inhibition of MMP9 in Huh7 and Sk-Hep-1 cells. (A) (B) Huh7 and Sk-Hep-1 cells were treated with glabridin (0–40 μM) for 24 h and then the nuclear fraction was prepared as described in the Methods section. ChIP analysis of the association of various transcription factors with the MMP9 promoter region in Huh7 and Sk-Hep-1 cells. (C and E) Representative results of NF-κB protein levels and phosphorylation of IkBα obtained by Western blot analysis. (D and F) Quantitative results of NF-κB, phosphorylation of IkBα, c-Fos and c-Jun protein levels, which were adjusted to the internal control C23 or GAPDH protein level. The values represent the means ± SD from three determinations per condition repeated three times ($n = 3$). *$P < 0.05$ as compared with the untreated Huh7 cells. #$P < 0.05$ as compared with the untreated Sk-Hep-1 cells.

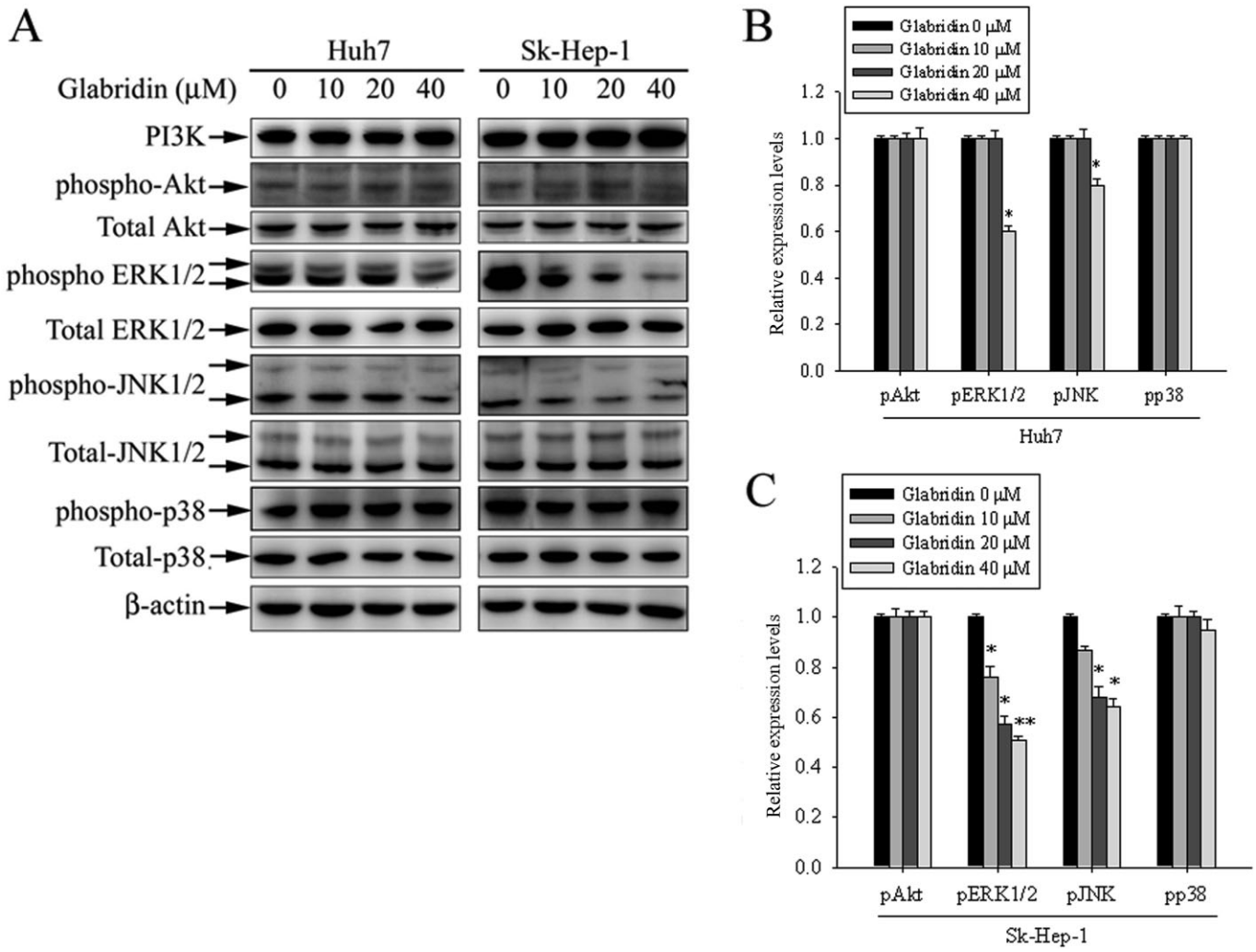

Figure 6

Effects of glabridin on the MAPKs pathway and PI3K/Akt signallings. (A) Huh7 and Sk-Hep-1 cells were treated with glabridin (0–40 μM) for 24 h and then the cell lysates were subjected to SDS-PAGE followed by Western blots with anti-ERK1/2, anti-JNK, anti-p38 and anti-PI3K and anti-Akt antibodies as described in Methods section. (B and C) Quantitative results of ERK1/2, JNK, p38, PI3K and Akt protein levels, which were adjusted to the β-actin protein level. The values represent the means ± SD from three determinations per condition repeated three times ($n = 3$). *$P < 0.05$, **$P < 0.01$ as compared with the untreated cells.

volume (Figure 8A). The results show that treatment with glabridin resulted in a dramatic suppression of tumour growth of SK-Hep-1 xenografts, whereas there were no statistically significant changes in body weight after drug exposure compared with the DMSO control (Figure 8B).

Discussion

HCC is a common malignant neoplasm and major cause of cancer-related deaths in Asian countries. Various ethnic societies worldwide have traditionally used herbal products in the prevention and/or treatment of several chronic diseases and their potential anticancer and antimetastatic effects are currently under investigation (Jia *et al.*, 2003; Li and Wang, 2005). Glabridin an isoflavane, a type of isoflavonoid, has been reported to protect against oxidation, have anti-obesity effects and inhibit lung and breast cancer metastasis (Hsu *et al.*, 2011). However, so far, no studies have investigated the antimetastasis (inhibition of migration and invasion) effects of glabridin on HCC cells. In the present study we demonstrated, for the first time, that glabridin inhibits the invasion and migration of Huh7 and Sk-Hep-1 liver cancer cells. Glabridin also inhibited the secretion and enzyme activity of MMP9 and increased TIMP-1 expression, and inhibited NF-κB and AP-1 nuclear translocation and their binding activity to the MMP9 promoter. The breakdown of the basement membrane is a critical step for invasion and metastasis of cancer cells and requires activation of MMPs (Liotta and Stetler-Stevenson, 1991; Coussens and Werb, 1996). Hence, MMPs play an important role in tumour angiogenesis and metastasis (Coussens and Werb, 1996). Inhibition of MMP expression or enzyme activity can provide early targets for prevention of cancer metastasis (Okada *et al.*, 2001; Waas

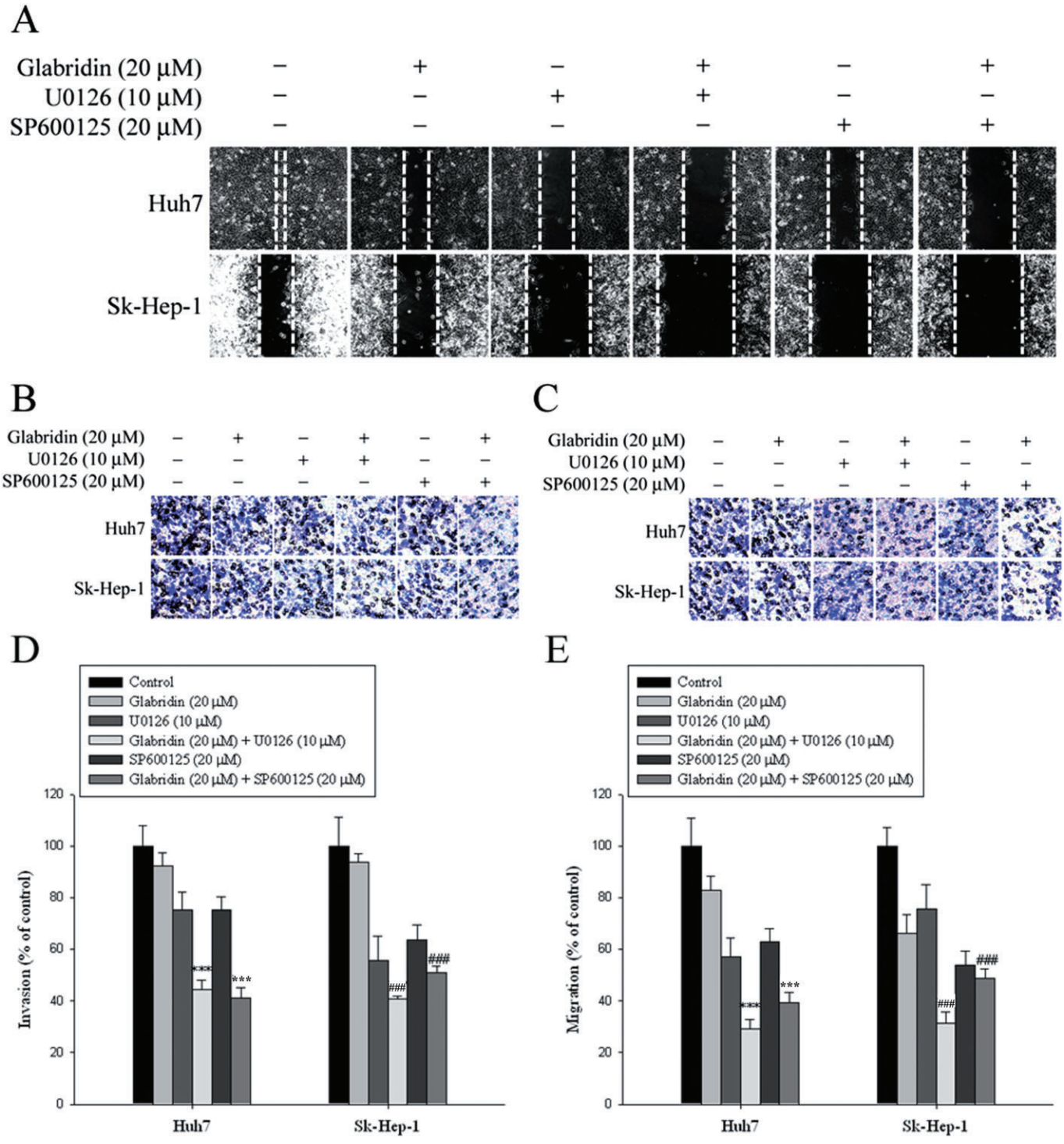

Figure 7

Effect of glabridin, ERK1/2 and JNK1/2 inhibitor on *in vitro* wound closure, cell migration and invasion in Huh7 and Sk-Hep-1 cells. (A–E) Huh7 and Sk-Hep-1 cells were pretreated with U0126 or SP600125 for 1 h and then incubated in the presence or absence of glabridin (20 μM) for 24 h and the cells were then subjected to *in vitro* wound closure, cell migration and invasion assay. The migration and invasion abilities of Huh7 and Sk-Hep-1 cells were quantified by counting the number of cells that invaded to the underside of the porous polycarbonate as described in the Methods section. The values represent the means ± SD from three determinations per condition repeated three times ($n = 3$). ***$P < 0.001$ as compared with the Huh7 cells only treated with glabridin (20 μM). ###$P < 0.001$ as compared with the Sk-Hep-1 cells only treated with glabridin (20 μM).

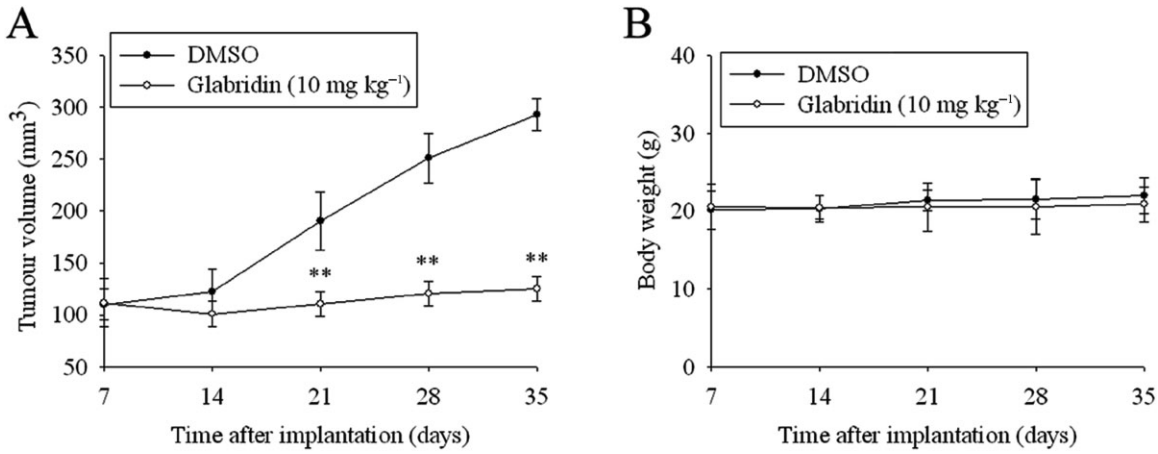

Figure 8

Glabridin suppressed tumour growth of SK-Hep-1 cells *in vivo*. SK-Hep-1 cells were injected into the tail veins of 6-week-old male *BALB/c* nude mice. After injection of SK-Hep-1 cells, nude mice were treated with either DMSO or glabridin (10 mg kg^{-1}) for 28 days. (A) Mice were killed and then analysed for the weight of tumours. The growth of the xenograft tumours was referred to the measurement of the long and short dimensions of the tumours, and the calculation of the tumour size were described in the Methods section. (B) Body weight changes of the mice during 35 days of treatment. $^{**}P < 0.01$, compared to the DMSO.

et al., 2003; Guruvayoorappan and Kuttan, 2008). Previous research has shown that MMP2 and MMP9 are associated with the invasive metastatic potential of tumour cells (Zhang *et al.*, 2004; Hwang and Lee, 2008). In the present study, the zymography data indicated that the secreted protein level of MMP-2 from Huh7 and Sk-Hep-1 cells was quite low (data not shown) whereas MMP9 expression was high. Furthermore, glabridin significantly inhibited MMP9 enzyme activity and protein expression in Huh7 and Sk-Hep-1 cells (Figure 3) and also inhibited promoter activity and MMP9 mRNA expression (Figure 4), indicating that glabridin regulates the expression of MMP9, at least partially, at the transcriptional level.

The promoter regions of inducible MMP genes show remarkable conservation of regulatory elements, including AP-1, NF-κB and SP-1 (Vincenti *et al.*, 1996; Benbow and Brinckerhoff, 1997; Shapiro, 1998). Transcription of the MMP9 gene is regulated by upstream sequences, including motifs corresponding to NF-κB or AP-1 binding sites (Takada *et al.*, 2004; Chien *et al.*, 2012). Inhibition of NF-κB and AP-1 activities has been shown to be effective at preventing and treating cancer (Aggarwal, 2004). The transcription nuclear factors NF-κB and AP-1 can promote tumourigenesis and are linked to invasion and metastasis. In the present study, glabridin inhibited the binding of NF-κB and AP-1 to the MMP9 promoter in Huh7 and Sk-Hep-1 cells (Figure 5). NF-κB, when activated, translocates from the cytosol to the nucleus to regulate gene expression at a transcriptional level. Activation of NF-κB occurs through the phosphorylation of IκBα. AP-1 is a transcription factor, which is a heterodimeric protein composed of proteins belonging to the c-Fos, c-Jun, activating transcription factor and Jun dimerization partners families. In the present study, glabridin treatment resulted in inhibition of NF-κB and AP-1 DNA binding activity, accompanied by inhibition of IκBα phosphorylation and decreased expression of c-Jun and c-Fos, leading to the down-regulation of MMP9. It is noteworthy that the expression levels of MMP9 mRNA and protein were not significantly changed by 20 μM glabridin in Huh7 cells (Figures 3C, 3D, 4A, 4B). Furthermore, cell invasion and migration ability of Huh7 cells were not different from control in the presence of 20 μM glabridin (Figures 2C, 2D, 7B, 7C), a significant inhibitory effect on MMP9 activity and wound healing was observed in these cells treated with glabridin 20 μM (Figures 2A, 2B, 3A, 3B). This suggests that MMP9 may not be the only target whereby glabridin inhibits Huh7 cell invasion and migration.

MAPKs are a family of serine/threonine kinases, activation of MAPKs is followed by phosphorylation of various cytosolic substrates and is involved in numerous cellular programmes, such as their proliferation, differentiation, invasion, migration and death (Reddy *et al.*, 2003; Yang *et al.*, 2010). Furthermore, glabridin inhibits the FAK/Src complex and blocks Akt and ERK1/2 activation in MDA-MB-231 cells (Hsu *et al.*, 2011). Glabridin also inhibits the migration, invasion and angiogenesis of A549 lung cancer cells by blocking Akt activation (Tsai *et al.*, 2011). Kim *et al.* (2010) showed that glabridin inhibits the degradation of IκBα/β, nuclear translocation of NF-κB p65/p50 and phosphorylation of ERK, JNK and p38 MAPKs. In the present study, we showed that glabridin inhibits the phosphorylation of ERK1/2 and JNK1/2 in HCC cell lines (Figure 6). In addition, when glabridin was combined with specific inhibitors of the JNK 1/2 pathway (SP600125) and ERK1/2 pathway (U0126) it further reduced the migration and invasion of these cells (Figure 7). This shows that glabridin inhibits the phosphorylation of ERK1/2 and JNK1/2, leading to the down-regulation of cell invasion and migration abilities in Huh7 and Sk-Hep-1 cells. Finally, glabridin was found to effectively suppress the tumour formation in the hepatoma xenograft model *in vivo*. The remarkable effect on xenograft growth (Figure 8A) is somewhat at odds with the *in vitro* data (Figure 1). This interesting result may be associated with the inhibitory effects of glabridin on mechanisms related to tumour growth, such as angiogenesis;

further research is needed to confirm this hypothesis. This is the first time that the antimetastasis effect of glabridin on HCC cells has been verified.

In conclusion, we demonstrated that glabridin inhibits the phosphorylation of IκB and the expressions of c-Jun and c-Fos and subsequently interferes with the binding activities of NF-κB and AP-1 DNA, which eventually results in the down-regulation of MMP9 expression and the inhibition of metastasis. Glabridin also inhibited cell invasion and migration by up-regulating TIMP-1 expression and inhibiting the phosphorylation of ERK1/2 and JNK1/2. By targeting the signal transduction mediators and transcriptional factors, it might be possible to develop specific mediators that are involved in the glabridin-induced antimetastatic effects in HCC cells. Glabridin might have potential for the development of preventive and treatment agents for cancer metastasis.

Acknowledgements

This study was supported by grants from National Science Council, Taiwan (NSC 102–2320-B-040-014). The authors of the manuscript do not have a direct financial relation with the commercial identity mentioned in this paper.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

Aggarwal BB (2004). Nuclear factor-kappaB: the enemy within. Cancer Cell 6: 203–208.

Ahn J, Lee H, Jang J, Kim S, Ha T (2013). Anti-obesity effects of glabridin-rich supercritical carbon dioxide extract of licorice in high-fat-fed obese mice. Food Chem Toxicol 51: 439–445.

Alexander S PH, Benson H E, Faccenda E, Pawson A J, Sharman J L, Spedding M, Peters J A, Harmar A J and CGTP Collaborators (2013). The Concise Guide to PHARMACOLOGY 2013/14: Enzymes. Br J Pharmacol 170: 1797–1867.

Belinky PA, Aviram M, Mahmood S, Vaya J (1998). Structural aspects of the inhibitory effect of glabridin on LDL oxidation. Free Radic Biol Med 24: 1419–1429.

Benbow U, Brinckerhoff CE (1997). The AP-1 site and MMP gene regulation: what is all the fuss about? Matrix Biol 15: 519–526.

Bjorklund M, Koivunen E (2005). Gelatinase-mediated migration and invasion of cancer cells. Biochim Biophys Acta 1755: 37–69.

Carmeli E, Fogelman Y (2009). Antioxidant effect of polyphenolic glabridin on LDL oxidation. Toxicol Ind Health 25: 321–324.

Chen TY, Li YC, Liu YF, Tsai CM, Hsieh YH, Lin CW *et al.* (2011). Role of MMP14 gene polymorphisms in susceptibility and pathological development to hepatocellular carcinoma. Ann Surg Oncol 18: 2348–2356.

Chien MH, Ying TH, Hsieh YS, Chang YC, Yeh CM, Ko JL *et al.* (2012). Dioscorea nipponica Makino inhibits migration and invasion of human oral cancer HSC-3 cells by transcriptional inhibition of matrix metalloproteinase-2 through modulation of CREB and AP-1 activity. Food Chem Toxicol 50: 558–566.

Coussens LM, Werb Z (1996). Matrix metalloproteinases and the development of cancer. Chem Biol 3: 895–904.

Guruvayoorappan C, Kuttan G (2008). Amentoflavone inhibits experimental tumor metastasis through a regulatory mechanism involving MMP-2, MMP-9, prolyl hydroxylase, lysyl oxidase, VEGF, ERK-1, ERK-2, STAT-1, NM23 and cytokines in lung tissues of C57BL/6 mice. Immunopharmacol Immunotoxicol 30: 711–727.

Hsieh MJ, Yang SF, Hsieh YS, Chen TY, Chiou HL (2012). Autophagy inhibition enhances apoptosis induced by dioscin in huh7 cells. Evid Based Complement Alternat Med 2012: 134512.

Hsu YL, Wu LY, Hou MF, Tsai EM, Lee JN, Liang HL *et al.* (2011). Glabridin, an isoflavan from licorice root, inhibits migration, invasion and angiogenesis of MDA-MB-231 human breast adenocarcinoma cells by inhibiting focal adhesion kinase/Rho signaling pathway. Mol Nutr Food Res 55: 318–327.

Hwang ES, Lee HJ (2008). Benzyl isothiocyanate inhibits metalloproteinase-2/-9 expression by suppressing the mitogen-activated protein kinase in SK-Hep1 human hepatoma cells. Food Chem Toxicol 46: 2358–2364.

Jia W, Gao W, Tang L (2003). Antidiabetic herbal drugs officially approved in China. Phytother Res 17: 1127–1134.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: Reporting *in vivo* experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Kim JY, Kang JS, Kim HM, Ryu HS, Kim HS, Lee HK *et al.* (2010). Inhibition of bone marrow-derived dendritic cell maturation by glabridin. Int Immunopharmacol 10: 1185–1193.

Li X, Wang H (2005). Chinese herbal medicine in the treatment of chronic kidney disease. Adv Chronic Kidney Dis 12: 276–281.

Liotta LA, Stetler-Stevenson WG (1991). Tumor invasion and metastasis: an imbalance of positive and negative regulation. Cancer Res 51: 5054s–5059s.

McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Okada N, Ishida H, Murata N, Hashimoto D, Seyama Y, Kubota S (2001). Matrix metalloproteinase-2 and -9 in bile as a marker of liver metastasis in colorectal cancer. Biochem Biophys Res Commun 288: 212–216.

Ravindranath MH, Ramasamy V, Moon S, Ruiz C, Muthugounder S (2009). Differential growth suppression of human melanoma cells by tea (Camellia sinensis) epicatechins (ECG, EGC and EGCG). Evid Based Complement Altern Med 6: 523–530.

Reddy KB, Nabha SM, Atanaskova N (2003). Role of MAP kinase in tumor progression and invasion. Cancer Metastasis Rev 22: 395–403.

Shankar S, Ganapathy S, Hingorani SR, Srivastava RK (2008). EGCG inhibits growth, invasion, angiogenesis and metastasis of pancreatic cancer. Front Biosci 13: 440–452.

Shapiro SD (1998). Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr Opin Cell Biol 10: 602–608.

Takada Y, Singh S, Aggarwal BB (2004). Identification of a p65 peptide that selectively inhibits NF-kappa B activation induced by various inflammatory stimuli and its role in down-regulation of NF-kappaB-mediated gene expression and up-regulation of apoptosis. J Biol Chem 279: 15096–15104.

Tsai YM, Yang CJ, Hsu YL, Wu LY, Tsai YC, Hung JY *et al*. (2011). Glabridin inhibits migration, invasion, and angiogenesis of human non-small cell lung cancer A549 cells by inhibiting the FAK/rho signaling pathway. Integr Cancer Ther 10: 341–349.

Vincenti MP, White LA, Schroen DJ, Benbow U, Brinckerhoff CE (1996). Regulating expression of the gene formatrix metalloproteinase-1 (collagenase): mechanisms that control enzyme activity, transcription, and mRNA stability. Crit Rev Eukaryot Gene Expr 6: 391–411.

Waas ET, Wobbes T, Lomme RM, DeGroot J, Ruers T, Hendriks T (2003). Matrix metalloproteinase 2 and 9 activity in patients with colorectal cancer liver metastasis. Br J Surg 90: 1556–1564.

Weng CJ, Tsai CM, Chen YC, Hsieh YH, Lin CW, Liu YF *et al*. (2010). Evaluation of the association of urokinase plasminogen activator system gene polymorphisms with susceptibility and pathological development of hepatocellular carcinoma. Ann Surg Oncol 17: 3394–3401.

Westermarck J, Kahari VM (1999). Regulation of matrix metalloproteinase expression in tumor invasion. FASEB J 13: 781–792.

Yang SF, Yang WE, Kuo WH, Chang HR, Chu SC, Hsieh YS (2008). Antimetastatic potentials of flavones on oral cancer cell via an inhibition of matrix-degrading proteases. Arch Oral Biol 53: 287–294.

Yang SF, Chen MK, Hsieh YS, Yang JS, Zavras AI, Hsieh YH *et al*. (2010). Antimetastatic effects of Terminalia catappa L. on oral cancer via a down-regulation of metastasis-associated proteases. Food Chem Toxicol 48: 1052–1058.

Yeh CB, Hsieh MJ, Hsieh YH, Chien MH, Chiou HL, Yang SF (2012). Antimetastatic effects of norcantharidin on hepatocellular carcinoma by transcriptional inhibition of MMP-9 through modulation of NF-kB activity. PLoS ONE 7: e31055.

Yoon SO, Park SJ, Yun CH, Chung AS (2003). Roles of matrix metalloproteinases in tumor metastasis and angiogenesis. J Biochem Mol Biol 36: 128–137.

Zhang L, Shi J, Feng J, Klocker H, Lee C, Zhang J (2004). Type IV collagenase (matrix metalloproteinase-2 and –9) in prostate cancer. Prostate Cancer Prostatic Dis 7: 327–332.