Phytomedicine 23 (2016) 359–366

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed

Glabridin induces apoptosis and autophagy through JNK1/2 pathway in human hepatoma cells

Ming-Ju Hsieh[a,b,c], Mu-Kuan Chen[d], Chih-Jung Chen[e,f,g], Ming-Chang Hsieh[h,i], Yu-Sheng Lo[a], Yi-Ching Chuang[a], Hui-Ling Chiou[h,i,*], Shun-Fa Yang[c,j,*]

[a] Cancer Research Center, Changhua Christian Hospital, Changhua 50006, Taiwan
[b] School of Optometry, Chung Shan Medical University, Taichung 40201, Taiwan
[c] Institute of Medicine, Chung Shan Medical University, Taichung 40201, Taiwan
[d] Department of Otorhinolaryngology-Head and Neck Surgery, Changhua Christian Hospital, Changhua 500, Taiwan
[e] Department of Surgical Pathology, Changhua Christian Hospital, Changhua 500, Taiwan
[f] Department of Medical Technology, Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli, Taiwan
[g] School of Medicine, Chung Shan Medical University, Taichuang 40201, Taiwan
[h] School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taichung 40201, Taiwan
[i] Department of Clinical Laboratory, Chung Shan Medical University Hospital, Taichung 40201, Taiwan
[j] Department of Medical Research, Chung Shan Medical University Hospital, Taichung 40201, Taiwan

ARTICLE INFO

Article history:
Received 16 November 2015
Revised 18 January 2016
Accepted 26 January 2016

Keywords:
Glabridin
Apoptosis
Autophagy
JNK
Hepatoma

ABSTRACT

Background: Extensive research results support the use of herbal medicine or natural food to augment therapy for various cancers. Studies have associated glabridin with numerous biological activities, such as regulating energy metabolism and estrogenic, neuroprotective, antiosteoporotic, and skin-whitening activities.

Hypothesis/Purpose: However, how glabridin affects tumor cell autophagy has not been clearly determined.

Methods: Autophagy is a lysosomal degradation pathway essential for cell survival and tissue homeostasis. In this study, the roles of autophagy and related signaling pathways during glabridin-induced autophagy in human liver cancer cells were investigated. Additionally, the molecular mechanism of the anticancer effects of glabridin in human hepatoma cells was investigated.

Results: The results revealed that glabridin significantly inhibited cell proliferation in human hepatoma cells. Glabridin induced apoptosis dose-dependently in Huh7 cells through caspase-3, -8, and -9 activation and PARP cleavage. Furthermore, autophagy was detected as early as 12 h after exposure to a low dose of glabridin, as indicated by the up-regulated expression of LC3-II and beclin-1 proteins. The inhibition of JNK1/2 and p38 MAPK by specific inhibitors significantly reduced glabridin-induced activation of caspases-3, -8, and -9. Blocking autophagy sensitize the Huh7 cells to apoptosis.

Conclusion: This study demonstrated for the first time that autophagy occurs earlier than apoptosis does during glabridin-induced apoptosis in human liver cancer cell lines. Glabridin induces Huh7 cell death through apoptosis through the p38 MAPK and JNK1/2 pathways and is a potential chemopreventive agent against human hepatoma.

© 2016 Elsevier GmbH. All rights reserved.

Introduction

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver. HCC is now the third leading cause of cancer deaths worldwide, with over 500,000 people affected (Kessler et al. 2015). The incidence of hepatocellular carcinoma is highest in Asia and Africa. This type of cancer occurs more often in men than women. Patients with hepatitis B or C are at high risk of liver cancer, even

Abbreviations: hepatocellular carcinoma, HCC; dietary supplement, DS; focal adhesion kinase, FAK; transforming growth factor beta, TGF-β; nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB; activator protein 1, AP-1; 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide, MTT; bafilomycin A1, BafA1; 4'-6-diamidino-2-phenylindole, DAPI; Dulbecco's modified Eagle's medium, DMEM; fetal bovine serum, FBS; polyvinylidene difluoride, PVDF; acidic vesicular organelles, AVOs; poly (ADP-ribose) polymerase, PARP; microtubule-associated protein 1A/1B-light chain 3, LC3; mitogen-activated protein kinases, MAPK.

* Corresponding authors: Institute of Medicine, Chung Shan Medical University, 110, Section 1, Chien-Kuo N. Road, Taichung 402, Taiwan, ROC. Tel.: +886-4-24730022x34253; fax: +886-4-23248171.

http://dx.doi.org/10.1016/j.phymed.2016.01.005
0944-7113/© 2016 Elsevier GmbH. All rights reserved.

E-mail addresses: hlchiou@csmu.edu.tw (H.-L. Chiou), ysf@csmu.edu.tw (S.-F. Yang).

if they do not develop cirrhosis (Leake 2014). HCC is not the same as metastatic liver cancer, which starts in another organ (such as the breast or colon) and spreads to the liver. Chemoprevention, using phytochemicals with potent antioxidant and anti-inflammatory properties, represents a fascinating strategy, which has been a subject of intense investigation in the recent years (Panossian 2014). Increasing attention has been paid to the use of products derived from natural plants against malignant invasive progression in the late stage of neoplastic diseases (Ivanov et al. 2007) and as potent chemopreventive drugs (da Rocha et al. 2001), particularly for relatively chemorefractory tumors, such as HCC (Chua and Choo 2011). Increasing attention has been focused on providing a scientific basis for using these agents as a preventive strategy for people with a high risk of cancer.

Isoflavones are secondary vegetable substances, produced from a branch of the general phenylpropanoid pathway that produces all flavonoid compounds in higher plants, which can act as estrogens in the body and have protective functions. Isoflavones have been identified as dietary components having an important role in reducing the incidence of breast and prostate cancers (Sarkar and Li 2003). Glabridin is an isoflavone and, as a key chemical and biological marker of *G. glabra*, is critical in the food, dietary supplement (DS), and cosmetics industries. Glabridin is a part of a larger family of plant-derived molecules, the natural phenols. Previous study revealed that glabridin can inhibit lung and breast cancer metastasis by inhibiting the Focal Adhesion Kinase (FAK)/rho signaling pathway (Hsu et al. 2011; Tsai et al. 2011). Glabridin-mediated anti-inflammatory action and attenuates colonic inflammation in mice with dextran sulfate sodium-induced colitis (Kwon et al. 2008). Glabridin has exhibited positive effects in down-regulating iNOS expression and activity under high glucose stress and inflammation (Yehuda et al. 2015). Glabridin inhibits the CSC-like properties of HCC cells through the miR-148a-mediated inhibition of the Transforming growth factor beta (TGF-β)/SMAD2 signal pathway (Jiang et al. 2014). Glabridin also inhibits migration and invasion by transcriptionally inhibiting matrix metalloproteinase 9 through the modulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1) activity in human liver cancer cells (Hsieh et al. 2014a). Glabridin attenuates the migratory and invasive capacity of breast cancer cells by activating microRNA-200c (Ye et al. 2014). Furthermore, glabridin mediates caspase activation and induces apoptosis in human promyelocytic leukemia cells (Huang et al. 2014). However, the effects of glabridin on human hepatoma autophagy have yet to be evaluated.

Autophagy is a major intracellular degradation mechanism that operates under stress conditions to promote survival during starvation or cause type II programmed cell death under specific conditions, such as the inhibition of apoptosis (Gozuacik and Kimchi 2007; Liu and Lenardo 2007; Yu et al. 2004). The autophagy process is initiated by the engulfing of large sections of a cytoplasm by a crescent-shaped phagophore that elongates into an autophagosome, which subsequently fuses with a lysosome, causing its contents to be degraded by lysosomal hydrolases (Chang et al. 2007; Kanzawa et al. 2003). Because autophagy is vital in regulating growth and maintaining homeostasis in multicellular organisms, defective autophagy contributes to the pathogenesis of several diseases, including myopathies, neurodegenerative diseases, and some cancers (Kelekar 2005). The study characterized the effects of glabridin and the underlying molecular mechanism of autophagy and apoptosis in glabridin-induced cytotoxicity. We investigated the cytotoxic effects of glabridin on hepatoma and its underlying mechanisms *in vitro*.

Materials and methods

Chemicals

Glabridin, ≥98% (HPLC), powder was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solution of Glabridin was made at 25, 50 and 100 mM concentration in DMSO and stored at −20 °C. The final concentration of DMSO for all treatments was consistently less than 0.1%. 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), bafilomycin A1 (BafA1), 4'-6-Diamidino-2-phenylindole (DAPI), wortmannin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). General caspase inhibitor Z-VAD-FMK was purchased from Promega (Madison, WI, USA). Specific inhibitors for caspase-3 (Z-DEVE-FMK), caspase-8 (Z-IETD-FMK) or caspase-9 (Z-LEHO-FMK) were purchased from BioVision (Mountain View, CA). Specific inhibitors for ERK1/2 (U0126), JNK1/2 (SP600125) or p38 (SB202190) were purchased from Calbiochem (San Diego, CA). Antibodies were purchased from Cell Signaling.

Cell culture

Huh7, HepG2 and Sk-Hep-1, human hepatoma cell line, obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan), were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 1 mM glutamine, 1% penicillin/streptomycin, 1.5 g/l sodium bicarbonate, and 1 mM sodium pyruvate (Sigma, St. Louis, Mo, USA).

In vitro *cytotoxicity assay*

The effect of glabridin on cell growth was assayed by the MTT method, as previously described (Yang et al. 2010). Briefly, cells were cultured in 24-well plates and stimulated with different concentrations of glabridin. After 24, 48 or 72 h of glabridin stimulation, MTT was added to each well (0.5 mg/ml final concentration) with a further incubation for 4 h. The viable cell number was directly proportional to the production of formazan following the solubilization with isopropanol. The color intensity was measured at 595 nm.

DAPI (2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride) staining

As described in detail previously (Hsieh et al. 2013) after being subjected to indicate treatment, cells were fixed with 4% paraformaldehyde for 20 min. Extensive PBS washing was conducted between each reaction to remove any residual solvent. Cells were subjected to DAPI staining for 10 min and then observed under fluorescence microscopy equipped with filters for UV.

Cell cycle analysis

Cells were first cultured in serum-free medium for starvation at 18 h and then exposed to glabridin for 24 h. Cells were fixed with 70% ethanol and incubated for 30 min in the dark at room temperature with propidium iodide (PI) buffer. The cell cycle distribution was analyzed for 3000 collected cells by a FACS Vantage flow cytometer that uses the CellQuest acquisition and analysis program (Becton Dickinson FACSCalibur).

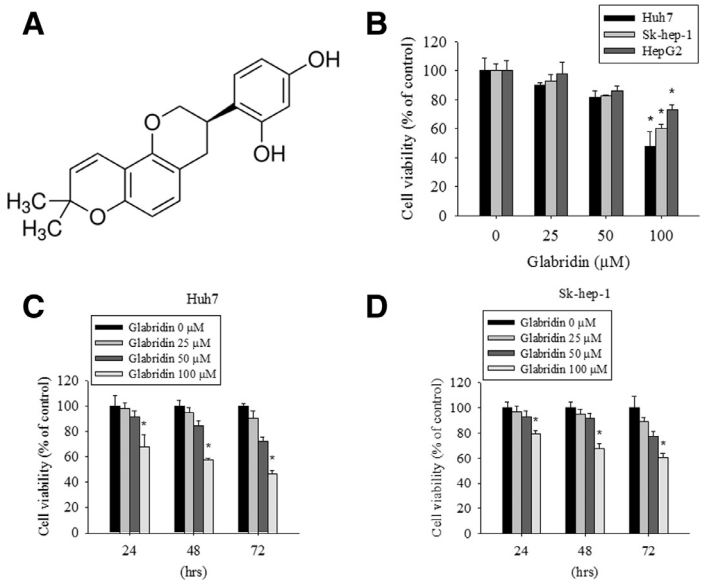

Fig. 1. Cytotoxic effect of glabridin in HCC cells. (A) Structure of glabridin. (B) Cell viability analysis of human hepatoma cells (Huh7, Sk-hep-1 and HepG2) (C) Huh7 cells cultured in presence of glabridin for 24, 48 and 72 h by MTT assay. (D) Sk-hep-1 cells cultured in presence of glabridin for 24, 48 and 72 h by MTT assay. Data represent mean of 3 determinations per condition repeated 3 times. Results are shown as mean ± SE. (*) $p < 0.05$ compared to the vehicle control groups.

Annexin V/PI double staining

As previously described (Ko et al. 2015) a Muse Annexin V & Dead Cell Assay Kit (Millipore) was used to quantify cell number in different stages of cell death. Briefly, 1×10^5 cells were resuspended in 100 μl PBS (2% BSA). Add 100 μl of Muse™ Annexin V & Dead Cell Reagent to each tube, the cell suspension was incubated for 20 min at room temperature in the dark. Analyze by Muse Cell Analyzer flow cytometry and analysis data by the Muse® Cell Analyzer Assays (Millipore).

Quantification of acidic vesicular organelle (AVO) formation

The occurrence of AVOs was assessed by a previously described method (Hsieh et al. 2013). Briefly, cells were washed with PBS, followed by staining with 1 μg/ml acridine orange for 15 min. Afterwards, cells were washed with PBS. For quantification of AVOs, acridine orange-stained cells were harvested, washed twice with PBS, resuspended in PBS containing 5% FBS and then analyzed by flow cytometry.

Western blot analysis

Cell lysates were separated in a 10% or 15% polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. The blot was subsequently incubated with 0.1% BSA in TBST for 1 h to block non-specific binding, and probed with a corresponding antibody against a specific protein for overnight at 4 °C, and then with an appropriate peroxidase conjugated secondary antibody for 1 h. After the final washing, signal was developed by ECL detection system and relative photographic density was quantitated by a gel documentation and analysis (AlphaImager 2000, Alpha Innotech Corporation, San Lean 189 dro, CA, USA).

Statistical analysis

Values represent the means ± standard deviation and the experiments were repeated three times ($n = 3$). Statistical analyses were performed using the one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used when more than three groups were analyzed. Data comparisons were performed with 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

Glabridin inhibit cell viability on human hepatoma cells

The chemical structure of glabridin is shown in Fig. 1a. To determine the cytotoxicity and effect on cell proliferation of glabridin on human liver cell lines (Huh7, HepG2, and Sk-hep-1), cells were treated with different concentrations of glabridin for 24, 48, and 72 h. The results of MTT assay testing for cell viability revealed that glabridin inhibited growth in a dose-dependent and time-dependent manner (Fig. 1b–d). Significant cell death was observed 24 h after glabridin (100 μM) treatment. Thus, all subsequent experiments used this glabridin concentration range (0–100 μM).

Glabridin-induced cell apoptosis in Huh7 cells

To determine whether the inhibitory effect of the cell viability of glabridin is associated with cell apoptosis induction, Huh7 cells were treated with different concentrations (0–100 μM) of glabridin for 24 h. A DAPI staining assay was conducted to reveal the presence of nuclei condensing and apoptotic bodies in glabridin-treated Huh7 cells in a dose-dependent manner (Fig. 2a). The accumulation of cell population in the sub-G1 and G0/G1 phases increased dose-dependently after 24 h of treatment with 100 μM glabridin (Fig. 2b). Meanwhile, Annexin-V and PI double-staining revealed an increased proportion of apoptotic cells after 24 h of treatment with glabridin (Fig. 2c), respectively.

Glabridin induced activation of caspase-3, -8 and -9 in Huh7 cells

To further confirm the involvement of caspase activation in glabridin-induced apoptosis, caspase-3, -8, and -9 activation and PARP cleavage were detected. Fig. 3a demonstrates that the exposure of Huh7 cells to glabridin (0–100 μM) for 24 h caused a concentration-dependent increase of the cleaved fragments of

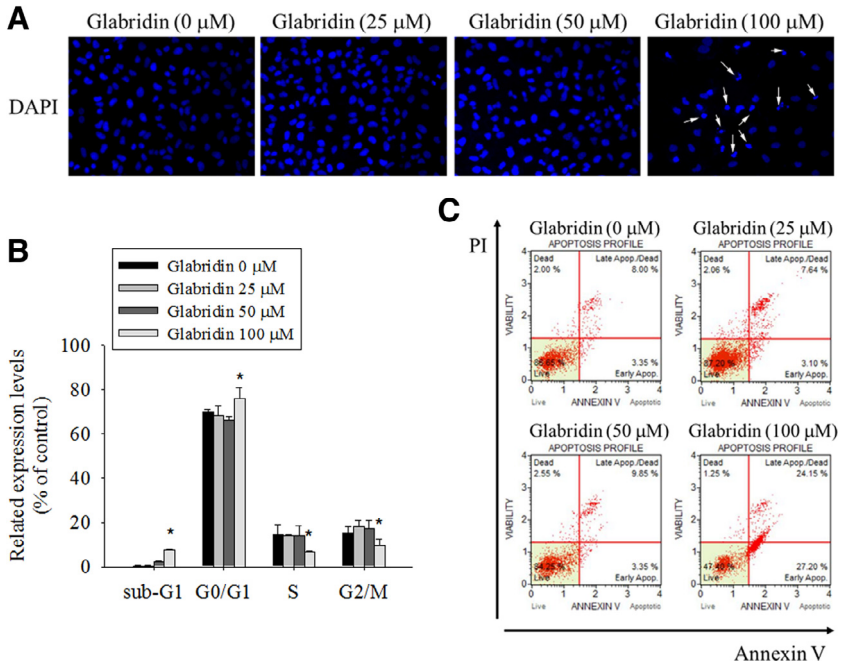

Fig. 2. Glabridin induced cell apoptosis in Huh7 cells. (A) Cells were stained with DAPI and observed under a UV-light microscope to examine the nuclear morphology of the Huh7 cells. The arrows show the areas with intense fluorescence staining and condensed nuclei (at a magnification of 200x). (B) Huh7 cells were incubated for 18 h in the absence of serum and then treated with indicated concentrations of glabridin for 24 h, after which the cells were stained with PI, and analyzed for DNA content by flow cytometry. (∗) $p < 0.05$ compared to the vehicle control groups. (C) After being treated with different concentration of glabridin for 24 h, cells were harvested and then subjected to quantitative analysis of cell apoptosis by Annexin-V and PI double-stained flow cytometry. Data represent mean of 3 determinations per condition repeated 3 times.

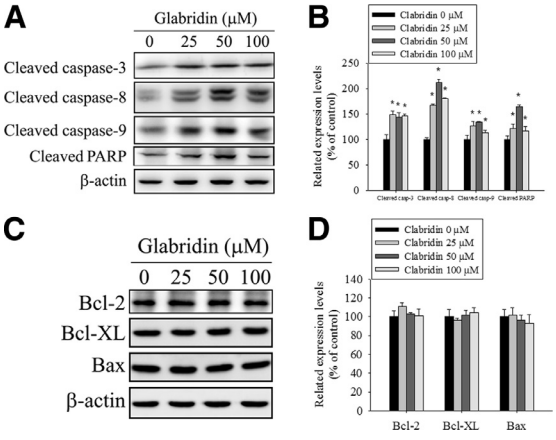

Fig. 3. Activation of caspase 3, -8, -9 and PARP were increased in Glabridin-treated Huh7 cells. (A) Huh7 cells were treated with 25, 50 and 100 μM glabridin for 24 h and subjected to western blotting with an antibody against PARP or caspase-3, -8 and -9 antibody. (B) The values under each lane indicate relative density of the band normalized to β-actin using a densitometer. Values represent the mean ± SE of three independent experiments. (∗) $p < 0.05$ compared to the vehicle control groups. (C) Huh7 cells were treated with 25, 50 and 100 μM glabridin for 24 h and subjected to western blotting with an antibody against bcl-2, bcl-xL and bax antibody. Data represent mean of 3 determinations per condition repeated 3 times. (D) The values under each lane indicate relative density of the band normalized to β-actin using a densitometer. Data represent mean of 3 determinations per condition repeated 3 times. Values represent the mean ± SE of three independent experiments. (∗) $p < 0.05$ compared to the vehicle control groups.

caspases-3, -8, and -9. Furthermore, cleaved PARP was significantly increased in glabridin-treated Huh7 cells. Glabridin treatment at 50 μM for 24 h increased the expression levels of cleaved caspases-3, -8, and -9 and poly (ADP-ribose) polymerase (PARP) by 49.1, 127, 41, and 53%, respectively, compared with the control (Fig. 3b). The Bcl-2 protein family determines the commitment of cells to apoptosis. The anti-apoptotic members of this family, such as Bcl-2 and Bcl-XL, prevent apoptosis either by sequestering proforms of death-driving cysteine proteases or by preventing the release of mitochondrial apoptogenic factors into the cytoplasm. In Fig. 3c and d, the results show that the expression of Bcl-2, Bcl-XL and Bax were no influence on Huh7 cells exposure to glabridin (0–100 μM) for 24 h.

Glabridin induct autophagy in Huh7 cells

Autophagy is a major intracellular degradation mechanism operating under stress conditions to promote survival during starvation or under specific conditions such as the inhibition of apoptosis (Hsieh et al. 2012). However, the effects of glabridin on human hepatoma autophagy have yet to be evaluated. To prove that glabridin induces autophagy, Huh7 cells were treated with different concentrations (0–100 μM) of glabridin for 24 h. As shown in Fig. 4a, cytoplasmic microtubule-associated protein 1A/1B-light chain 3 (LC3) formations were observed in glabridin-treated Huh7 cells, which indicated the formation of autophagosomes. According to a previous study, autophagy is characterized by the formation of numerous acidic vesicular organelles (AVOs) (Hsieh et al. 2014b). For quantification, cells with AVOs exhibited enhanced red fluorescence when analyzed using flow cytometry, which significantly increased in a dose-dependent manner after glabridin treatment (Fig. 4b). In addition, increased LC3-II and beclin-1 protein expression was observed in glabridin-treated Huh7 cells in a dose- and time-dependent manner (Fig. 4c, d).

The apoptosis and autophagy induction by glabridin is dependent on the regulation of JNK and P38 signaling pathways in Huh7 cells

The Mitogen-activated protein kinases (MAPK) signaling pathway plays a critical role in how chemotherapeutic drugs regulate apoptosis (Chen and Wong 2008). In further investigating the

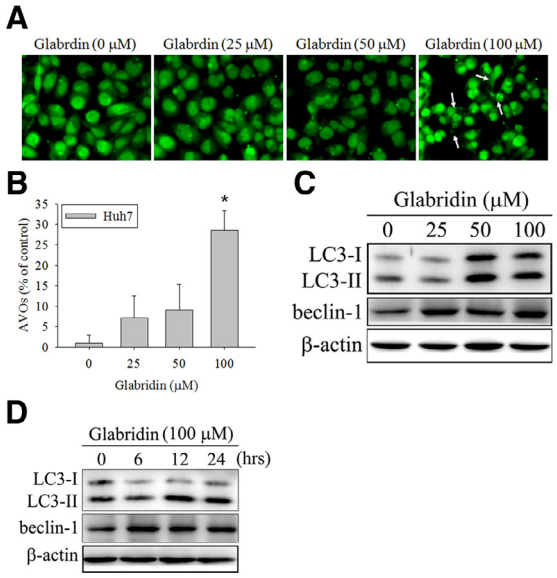

Fig. 4. Glabridin induces autophagy in Huh7 cells. (A) Huh7 cells were treated with glabridin for 24 h, followed by immunostaining and an observation of LC-3 under fluorescence microscopy. The arrows show the areas with intense fluorescence staining and condensed nuclei (at a magnification of 200x). (B) Huh7 cells were treated with glabridin (0–100 μM) for 24 h and detect AVOs. The percentage of cells with the formation of AVOs was detected by flow cytometry. (C) Cells were treated with glabridin for 24 h and Western blot analysis was performed to evaluate autophagy-related protein, LC3-I, LC3-II and beclin-1. β-actin was used as an internal control. (D) Huh7 cells were treated with glabridin (100 μM) for 0, 6, 12, 24 h and Western blot analysis was performed to evaluate autophagy-related protein, LC3-I, LC3-II and beclin-1. β-actin was used as an internal control.

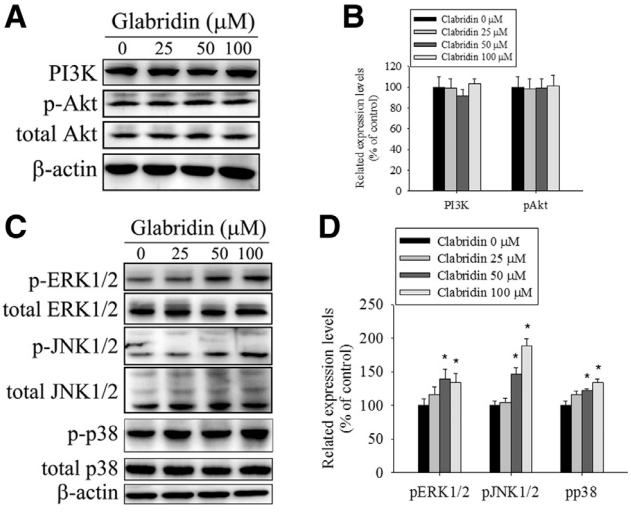

Fig. 5. Glabridin activates the phosphorylation of ERK1/2, p38 MAPK and JNK1/2 in Huh7 cells. (A and C) Cells were treated with different concentrations of glabridin (0–100 μM) for 24 h and then subjected to western blotting with an antibody against PI3K-Akt, ERK1/2, JNK1/2, and p38 MAPK. (B and D) Quantitative results of PI3K, p-Akt, p-ERK1/2, p-p38, and p-JNK1/2 protein levels, which were adjusted with the total Akt, ERK1/2, p38, and JNK1/2 protein levels and expressed as multiples of induction beyond each respective control. Values represent the mean ± SE of three independent experiments. (*) $p < 0.05$ compared to the vehicle control group.

underlying molecular mechanisms of this relationship, we used a Western blot analysis to determine whether MAPKs were activated in glabridin-treated Huh7 cells. The results revealed that the phosphorylation of ERK1/2, JNK1/2, and p38 was increased in cells treated with glabridin in a dose-dependent manner (Fig. 5a–d). Next, we investigated the relationships among the glabridin-induced activation of caspases, autophagy, and MAPKs. Huh7 cells were pretreated with 20 μM U0126 (an ERK inhibitor), SP600125 (a JNK inhibitor), or SB203580 (a p38 inhibitor) for 1 h, treated with 100 μM glabridin for another 24 h, and then analyzed using Western blotting. As Fig. 6a–d illustrates, treatment SP600125 and SB203580 significantly attenuated glabridin-induced caspase-3, -8, and -9 activation. Moreover, treatment SP600125 significantly attenuated glabridin-induced LC3-II and AVO expression. Conversely, treatment SB203580 had no significant effect on glabridin-induced LC3-II and AVO expression (Fig. 6e, f). These findings suggest that JNK1/2 activation might play a critical upstream role in glabridin-mediated caspase activation and the autophagy process in Huh7 cells.

Glabridin-induced cell apoptosis was increased by the treatment of autophagy inhibitors

To clarify the interaction between glabridin-induced apoptosis and autophagy, specific caspase inhibitors were used with the following experiment. Huh7 cells were pretreated with caspase inhibitors (Z-DEVE-FMK, Z-IETD-FMK, and Z-LEDH-FMK) for 1 h and then glabridin for 24 h, and then subjected to an MTT assay. The results (Fig. 7a) demonstrate that caspase inhibitors pretreatment reduced the proportion of glabridin-induced cell death. Wortmannin, a cell-permeable, fungal metabolite that acts as a potent, selective, and irreversible inhibitor of PI3K has been used to inhibit autophagic sequestration (Arcaro and Wymann 1993; Blommaart et al. 1997). Cells were pretreated with wortmannin for 1 h and then glabridin for 24 h. The proportion of cell viability decreased in cells pretreated with wortmannin (Fig. 7b). To determine the effects of BafA1, an inhibitor of vacuolar ATPase used to prevent the fusion of lysosomes and autophagosomes, cells were pretreated with BafA1 for 1 h and then glabridin for 24 h. A similar result was obtained using an MTT assay (Fig. 7b). Furthermore, a cotreatment of glabridin and Z-VAD-FMK, a broad-spectrum caspase inhibitor, was conducted and revealed that the proportion of annexin V-positivity was reduced by Z-VAD-FMK; however, a cotreatment of glabridin and autophagy inhibitors (wortmannin and BafA1) was increased (Fig. 7c). Clearly, autophagy inhibition increased glabridin-induced cell apoptosis and inhibition of apoptosis prolong autophagy activity (Fig. 7d). These results indicate that glabridin-induced Huh7 cell apoptosis, however, autophagy induction plays a protective effect.

Discussion

Hepatocellular carcinoma is the most common type of liver cancer. Most HCC cases are secondary to a viral hepatitis infection or cirrhosis (Alter 2007; Wang et al. 2002). The treatment options for and prognosis of HCC depend on numerous factors, particularly tumor size and stage. High-grade tumors have a poor prognosis, whereas low-grade tumors may go unnoticed for many years, as is the case in cancers affecting many other organs. Hepatocellular carcinoma is a relatively uncommon cancer in the United States. In countries where hepatitis is uncommon, most liver cancers are not primary HCC but metastasis (Cheng et al. 1992). The use of natural herbal products in developing novel chemotherapeutics for various cancers appears promising (Casciola-Rosen et al. 1996; Jia et al. 2003; Lee et al. 2008). Various societies worldwide have traditionally used herbal products to prevent or treat several chronic diseases (Li and Wang 2005; Wang et al. 2013; Zhou et al. 2013). Glabridin, a polyphenolic flavonoid, is a main constituent in the roots of *Glycyrrhiza glabra* and has been reported as possessing various biological activities such as protecting against oxidation and obesity and inhibiting lung and breast cancer metastasis (Hsu et al. 2011; Kim et al. 2013; Wu et al. 2013). Being a natural product known to be safe for use by humans, glabridin is an appropriate candidate for drug development (Aoki et al. 2007). The present

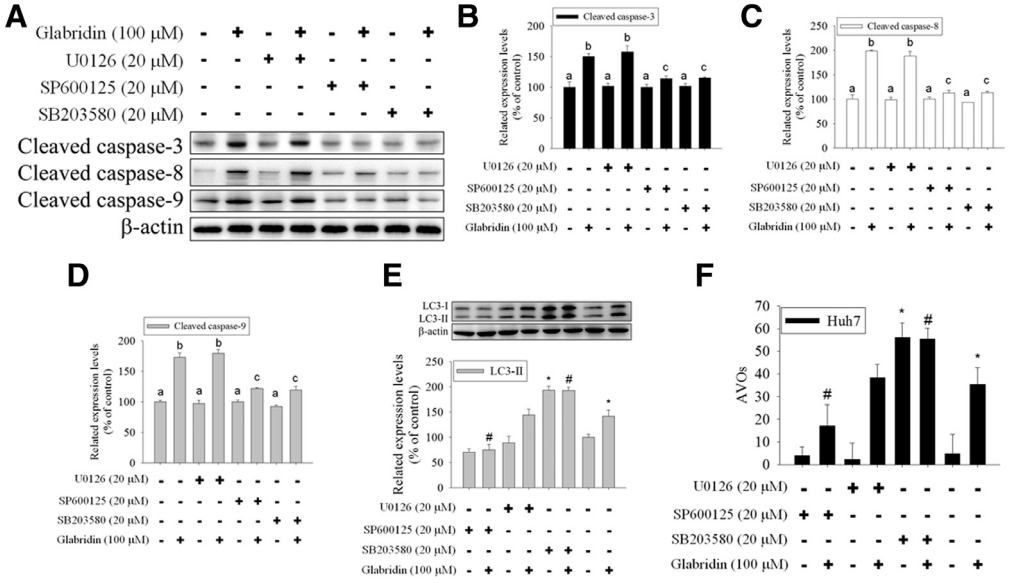

Fig. 6. JNK1/2 and p38 MAPK are essential for caspase activation and autophagy induced by glabridin. (A) Huh7 cells were treated for 24 h with 100 μM glabridin with or without a 1 h pretreatment of 20 μM U0126, SP600125, or SB202190. The expression of cleaved caspase-3, -8 and -9 were detected by western blotting. (B–D) Quantitative results of cleaved caspase-3, -8, and -9 protein levels, which were adjusted to the β-actin protein level and expressed as multiples of induction beyond each respective control. Values represent the mean ± SE of three independent experiments. Data were analyzed using a one-way ANOVA with Tukey's post hoc tests at 95% confidence intervals; different letters represent different levels of significance. (E, upper panel) Huh7 cells were treated for 24 h with 100 μM glabridin with or without a 1 h pretreatment of 20 μM U0126, SP600125, or SB202190. The expression of LC3-I and LC3-II were detected by western blotting. (E, lower panel) Quantitative results of LC3-II protein levels, which were adjusted to the β-actin protein level and expressed as multiples of induction beyond each respective control. Values represent the mean ± SE of three independent experiments. (*) $p < 0.05$ compared to the vehicle control group. (#) $p < 0.05$ compared to the control treatment with glabridin group. (F) Huh7 cells were treated for 24 h with 100 μM glabridin with or without a 1 h pretreatment of 20 μM U0126, SP600125, or SB202190 and detect AVOs. The percentage of cells with the formation of AVOs was detected by flow cytometry. Values represent the mean ± SE of three independent experiments. (*) $p < 0.05$ compared to the vehicle control group. (#) $p < 0.05$ compared to the control treatment with glabridin group.

study demonstrates, for the first time, that glabridin induces hepatoma cell apoptosis and autophagy.

The antitumor activities of glabridin, including inducing oxidative stress, cell cycle arrest, and apoptosis, has been extensively studied (Cheema et al. 2014; Huang et al. 2014; Kim et al. 2013). Glabridin is a potent antioxidant that prevents oxidative DNA fragmentation and the activation of apoptosis-associated proteins in human keratinocytes (Veratti et al. 2011). Studies have suggested that glabridin may partially act through the initiator caspase-8 and then the executioner caspase-3 to increase the cleavage form of PARP, thereby inducing apoptosis in human promyelocytic leukemia cells (Huang et al. 2014). Moreover, many papers have reported that the ability of anticancer agents to induce tumor cell apoptosis is correlated with the ability to reduce Bcl-2 expression (Vermes et al. 1995). We found that the expression of the antiapoptotic proteins Bcl-2 and Bcl-xL in Huh7 cells was not affected by glabridin treatment for 24 h. Other Bcl-2 family members are not death inhibitors, but rather promote procaspase activation and cell death. Some of these apoptosis promoters, such as Bad, function by binding to and inactivating the death-inhibiting family members, whereas others, like Bax and Bak, stimulate the release of cytochrome *c* from mitochondria. Bax and Bak are themselves activated by other apoptosis-promoting Bcl-2 family members, such as Bid (Cotter 2009). In the present study, the protein expression levels of Bcl-2, Bcl-XL and Bax are no change. However, caspase-3, -8, or -9 activation occurred after treatment with glabridin, suggesting that glabridin may influence the other anti-apoptotic proteins or proapoptosis proteins to induce apoptosis in Huh7 cells. It may need more experiments to prove.

Recently, autophagy has become a promising target of research for drugs to treat various diseases and has been implicated in the pathogenesis of cancers and diseases (Kondo et al. 2005; Shintani and Klionsky 2004). Furthermore, many anticancer agents have been reported as inducing autophagy (Fang et al. 2014; Lin et al. 2011). Sulforaphane protected prion protein-mediated neurotoxicity and increased autophagy flux marker microtubule-associated protein 1 light chain 3-II protein levels (Lee et al. 2014). Autophagy is a type II programmed cell death and a lysosomal degradation pathway essential for homeostasis. When autophagy is induced, beclin-1 and LC3-II are distributed to the membrane of autophagosomes, thus determining the extent of autophagosome formation (Kelekar 2005). In this study, glabridin was revealed as inducing autophagy as early as 12 h after the addition of glabridin. Furthermore, LC3-II expression analysis indicated that autophagy induction was dose-dependent (Fig. 4).

Promoters of autophagy, which is genetically programmed, are clinically beneficial in cancer prevention. By using autophagy inhibitors, wortmannin and BafA1, the role of autophagy in glabridin-induced cell death was further investigated. Glabridin-induced cell death was increased by wortmannin and BafA1 (Fig. 7). The proportion of cell viability was reduced in cells pretreated with wortmannin and BafA1 (Fig. 7b). Furthermore, a cotreatment of glabridin and Z-VAD-FMK was conducted, revealing that the proportion of annexin V-positivity was abolished by Z-VAD-FMK, but that cotreatment with glabridin and autophagy inhibitors (wortmannin and BafA1) exerted more effect (Fig. 7c). Therefore, these results indicate that glabridin also induce Huh7 cell autophagy, in addition apoptosis. At early stage, cells cause endogenous autophagy response in order to survive in glabridin-treatment (Fig. 7d).

Studies have suggested that glabridin increases the phosphorylation of ERK1/2, p38 MAPK, and JNK1/2 in a dose- and time-dependent manner (Huang et al. 2014). The results showed that the phosphorylation of ERK1/2, JNK1/2, and p38 MAPK increased in glabridin-treatment Huh7 cells. However, treatment with a JNK- or p38 MAPK-specific inhibitor effectively inhibited

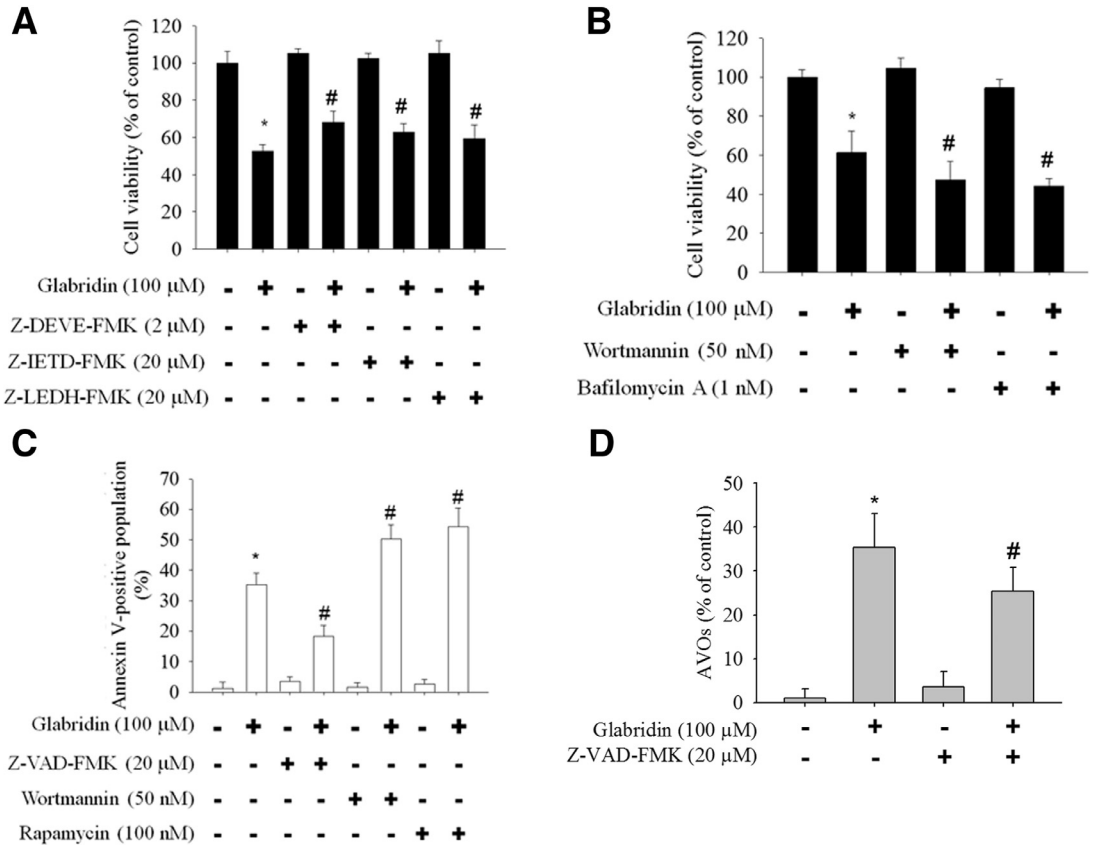

Fig. 7. Glabridin-induced Huh7 cell death causes apoptosis and autophagy induction. (A) Huh7 cells were treated for 24 h with 100 μM glabridin with or without a 1 h pretreatment of Z-DEVE-FMK (2 μM), Z-IETD-FMK (20 μM), or Z-LEDH-FMK (20 μM) and detect cell viability by MTT assay. (B) Huh7 cells were treated for 24 h with 100 μM glabridin with or without a 1 h pretreatment of wortmannin (50 nM) or bafilomycin A1 (1 nM) and detect cell viability by MTT assay. (C) Huh7 cells were treated for 24 h with 100 μM glabridin with or without a 1 h pretreatment of Z-VAD-FMK (20 μM), wortmannin (50 nM) or rapamycin (100 nM) and detect cell apoptosis by Annexin-V and PI double-stained flow cytometry. (D) Huh7 cells were treated for 24 h with 100 μM glabridin with or without a 1 h pretreatment of Z-VAD-FMK (20 μM) and quantification of AVOs formation by flow cytometry. Values represent the mean ± SE of three independent experiments. (∗) $p < 0.05$ compared to the vehicle control group. (#) $p < 0.05$ compared to the control treatment with glabridin group.

glabridin-induced caspase-3, -8, and -9 activation, whereas U0126 did not affect glabridin-induced caspase activation. Moreover, treatment SP600125 significantly attenuated glabridin-induced LC3-II and AVO expression. Conversely, treatment SB203580 no affected glabridin-induced LC3-II and AVO expression (Fig. 6e and f). These findings suggest that JNK1/2 activation might play a critical upstream role in glabridin-mediated caspase activation and the autophagy process in Huh7 cells.

Previous studies have shown that glabridin induced HL-60 cell apoptosis through p38 MAPK and JNK1/2 pathways (Huang et al. 2014) induces oxidative stress mediated apoptosis in malaria parasite plasmodium falciparum (Cheema et al. 2014). However, whether glabridin can exert similar effect on other tumor cells such as breast tumor cells, it may need more experiments to prove.

In conclusion, this study demonstrated that glabridin-induced apoptosis in Huh7 cells through p38 and JNK1/2 phosphorylation and subsequently stimulates caspase-3, -8, and -9 activation, which eventually causes PARP cleavage and inhibits proliferation. Glabridin cause autophagy at early stage to suppress glabridin-induced apoptosis in Huh7 cells through JNK1/2 phosphorylation. This is the first study to indicate that glabridin have autophagy induction processes ability in human hepatoma. Our findings suggest that glabridin may be a useful chemotherapeutic agent.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgments

This study was supported by grants from National Science Council, Taiwan (MOST 103-2314-B-371-007-MY2) (MOST 104-2314-B-371-008-MY2) and Changhua Christian Hospital (104-CCH-IRP-094) (104-CCH-IRP-061). The authors of the manuscript do not have a direct financial relation with the commercial identity mentioned in this paper.

References

Alter, M.J., 2007. Epidemiology of hepatitis C virus infection. World J. Gastroenterol.: WJG 13, 2436–2441.

Aoki, F., Nakagawa, K., Kitano, M., Ikematsu, H., Nakamura, K., Yokota, S., Tominaga, Y., Arai, N., Mae, T., 2007. Clinical safety of licorice flavonoid oil (LFO) and pharmacokinetics of glabridin in healthy humans. J. Am. Coll. Nutr. 26, 209–218.

Arcaro, A., Wymann, M.P., 1993. Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. Biochem. J. 296 (Pt 2), 297–301.

Blommaart, E.F., Krause, U., Schellens, J.P., Vreeling-Sindelarova, H., Meijer, A.J., 1997. The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. Eur. J. Biochem. / FEBS 243, 240–246.

Casciola-Rosen, L., Rosen, A., Petri, M., Schlissel, M., 1996. Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: implications for coagulation events and antigenic spread in systemic lupus erythematosus. Proc. Nat. Acad. Sci. U.S.A. 93, 1624–1629.

Chang, C.P., Yang, M.C., Liu, H.S., Lin, Y.S., Lei, H.Y., 2007. Concanavalin A induces autophagy in hepatoma cells and has a therapeutic effect in a murine in situ hepatoma model. Hepatology 45, 286–296.

Cheema, H.S., Prakash, O., Pal, A., Khan, F., Bawankule, D.U., Darokar, M.P., 2014. Glabridin induces oxidative stress mediated apoptosis like cell death of malaria parasite Plasmodium falciparum. Parasitol. Int. 63, 349–358.
Chen, T., Wong, Y.S., 2008. Selenocystine induces S-phase arrest and apoptosis in human breast adenocarcinoma MCF-7 cells by modulating ERK and Akt phosphorylation. J. Agric. Food Chem. 56, 10574–10581.
Cheng, W.S., Govindarajan, S., Redeker, A.G., 1992. Hepatocellular carcinoma in a case of Wilson's disease. Liver 12, 42–45.
Chua, C.W., Choo, S.P., 2011. Targeted therapy in hepatocellular carcinoma. Int. J. Hepatol. 2011, 348297.
Cotter, T.G., 2009. Apoptosis and cancer: the genesis of a research field. Nat. Rev. Cancer 9, 501–507.
da Rocha, A.B., R.M., Lopes, Schwartsmann, G., 2001. Natural products in anticancer therapy. Curr. Opin. Pharmacol. 1, 364–369.
Fang, J., Bao, T., Zhou, H., Wang, N., Wang, Y., Huang, W., Wu, Y., 2014. Autophagy and apoptosis of HeLa cells induced by recombinant human endostatin combined with hypoxia. Chin. J. Cell. Mol. Immunol. 30, 1255–1257.
Gozuacik, D., Kimchi, A., 2007. Autophagy and cell death. Curr. Topics Dev. Biol. 78, 217–245.
Hsieh, M.J., Lin, C.W., Yang, S.F., Chen, M.K., Chiou, H.L., 2014. Glabridin inhibits migration and invasion by transcriptional inhibition of matrix metalloproteinase 9 through modulation of NF-kappaB and AP-1 activity in human liver cancer cells. Br. J. Pharmacol. 171, 3037–3050.
Hsieh, M.J., Lin, C.W., Yang, S.F., Sheu, G.T., Yu, Y.Y., Chen, M.K., Chiou, H.L., 2014. A combination of pterostilbene with autophagy inhibitors exerts efficient apoptotic characteristics in both chemosensitive and chemoresistant lung cancer cells. Toxicol. Sci. 137, 65–75.
Hsieh, M.J., Tsai, T.L., Hsieh, Y.S., Wang, C.J., Chiou, H.L., 2013. Dioscin-induced autophagy mitigates cell apoptosis through modulation of PI3K/Akt and ERK and JNK signaling pathways in human lung cancer cell lines. Arch. Toxicol. 87, 1927–1937.
Hsieh, M.J., Yang, S.F., Hsieh, Y.S., Chen, T.Y., Chiou, H.L., 2012. Autophagy inhibition enhances apoptosis induced by dioscin in huh7 cells. Evid. Based Complement. Altern. Med.: eCAM 2012, 134512.
Hsu, Y.L., Wu, L.Y., Hou, M.F., Tsai, E.M., Lee, J.N., Liang, H.L., Jong, Y.J., Hung, C.H., Kuo, P.L., 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.
Huang, H.L., Hsieh, M.J., Chien, M.H., Chen, H.Y., Yang, S.F., Hsiao, P.C., 2014. Glabridin mediate caspases activation and induces apoptosis through JNK1/2 and p38 MAPK pathway in human promyelocytic leukemia cells. PloS one 9, e98943.
Ivanov, V., Roomi, M.W., Kalinovsky, T., Niedzwiecki, A., Rath, M., 2007. Antiatherogenic effects of a mixture of ascorbic acid, lysine, proline, arginine, cysteine, and green tea phenolics in human aortic smooth muscle cells. J. Cardiovasc. Pharmacol. 49, 140–145.
Jia, W., Gao, W., Tang, L., 2003. Antidiabetic herbal drugs officially approved in China. Phytother. Res.: PTR 17, 1127–1134.
Jiang, F., Mu, J., Wang, X., Ye, X., Si, L., Ning, S., Li, Z., Li, Y., 2014. The repressive effect of miR-148a on TGF beta-SMADs signal pathway is involved in the glabridin-induced inhibition of the cancer stem cells-like properties in hepatocellular carcinoma cells. PloS one 9, e96698.
Kanzawa, T., Kondo, Y., Ito, H., Kondo, S., Germano, I., 2003. Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. Cancer Res. 63, 2103–2108.
Kelekar, A., 2005. Autophagy. Ann. New York Acad. Sci. 1066, 259–271.
Kessler, S.M., Barghash, A., Laggai, S., Helms, V., Kiemer, A.K., 2015. Hepatic hepcidin expression is decreased in cirrhosis and HCC. J. Hepatol. 62, 977–979.
Kim, H.S., Suh, K.S., Ko, A., Sul, D., Choi, D., Lee, S.K., Jung, W.W., 2013. The flavonoid glabridin attenuates 2-deoxy-D-ribose-induced oxidative damage and cellular dysfunction in MC3T3-E1 osteoblastic cells. Int. J. Mol. Med. 31, 243–251.
Ko, C.P., Lin, C.W., Chen, M.K., Yang, S.F., Chiou, H.L., Hsieh, M.J., 2015. Pterostilbene induce autophagy on human oral cancer cells through modulation of Akt and mitogen-activated protein kinase pathway. Oral Oncol. 51, 593–601.
Kondo, Y., Kanzawa, T., Sawaya, R., Kondo, S., 2005. The role of autophagy in cancer development and response to therapy. Nat. Rev. Cancer 5, 726–734.
Kwon, H.S., Oh, S.M., Kim, J.K., 2008. Glabridin, a functional compound of liquorice, attenuates colonic inflammation in mice with dextran sulphate sodium-induced colitis. Clin. Exp. Immunol. 151, 165–173.
Leake, I., 2014. Hepatocellular carcinoma. Treatment potential of targeting Toll-like receptors in HCC. Nat. Rev. Gastroenterol. Hepatol. 11, 518.
Lee, J.H., Jeong, J.K., Park, S.Y., 2014. Sulforaphane-induced autophagy flux prevents prion protein-mediated neurotoxicity through AMPK pathway. Neuroscience 278, 31–39.
Lee, Y.R., Lei, H.Y., Liu, M.T., Wang, J.R., Chen, S.H., Jiang-Shieh, Y.F., Lin, Y.S., Yeh, T.M., Liu, C.C., Liu, H.S., 2008. Autophagic machinery activated by dengue virus enhances virus replication. Virology 374, 240–248.
Li, X., Wang, H., 2005. Chinese herbal medicine in the treatment of chronic kidney disease. Adv. Chronic Kidney Dis. 12, 276–281.
Lin, J.F., Lin, Y.C., Lin, Y.H., Tsai, T.F., Chou, K.Y., Chen, H.E., Hwang, T.I., 2011. Zoledronic acid induces autophagic cell death in human prostate cancer cells. J. Urol. 185, 1490–1496.
Liu, Z., Lenardo, M.J., 2007. Reactive oxygen species regulate autophagy through redox-sensitive proteases. Dev. Cell 12, 484–485.
Panossian, A., 2014. 18 years with phytomedicine!. Phytomed. : Int. J. Phytother. Phytopharmacol. 21, 781–782.
Sarkar, F.H., Li, Y., 2003. Soy isoflavones and cancer prevention. Cancer Invest. 21, 744–757.
Shintani, T., Klionsky, D.J., 2004. Autophagy in health and disease: a double-edged sword. Science 306, 990–995.
Tsai, Y.M., Yang, C.J., Hsu, Y.L., Wu, L.Y., Tsai, Y.C., Hung, J.Y., Lien, C.T., Huang, M.S., Kuo, P.L., 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 Therapies 10, 341–349.
Veratti, E., Rossi, T., Giudice, S., Benassi, L., Bertazzoni, G., Morini, D., Azzoni, P., Bruni, E., Giannetti, A., Magnoni, C., 2011. 18beta-glycyrrhetinic acid and glabridin prevent oxidative DNA fragmentation in UVB-irradiated human keratinocyte cultures. Anticancer Res. 31, 2209–2215.
Vermes, I., Haanen, C., Steffens-Nakken, H., Reutelingsperger, C., 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J. Immunol. Methods 184, 39–51.
Wang, J., Xiong, X., Xing, Y., Liu, Z., Jiang, W., Huang, J., Feng, B., 2013. Chinese herbal medicine for acute mountain sickness: a systematic review of randomized controlled trials. Evid. Based Complement. Altern. Med.: eCAM 2013, 732562.
Wang, X.W., Hussain, S.P., Huo, T.I., Wu, C.G., Forgues, M., Hofseth, L.J., Brechot, C., Harris, C.C., 2002. Molecular pathogenesis of human hepatocellular carcinoma. Toxicology 181-182, 43–47.
Wu, F., Jin, Z., Jin, J., 2013. Hypoglycemic effects of glabridin, a polyphenolic flavonoid from licorice, in an animal model of diabetes mellitus. Mol. Med. Rep. 7, 1278–1282.
Yang, S.F., Chen, M.K., Hsieh, Y.S., Yang, J.S., Zavras, A.I., Hsieh, Y.H., Su, S.C., Kao, T.Y., Chen, P.N., Chu, S.C., 2010. Antimetastatic effects of Terminalia Catappa L. on oral cancer via a down-regulation of metastasis-associated proteases. Food Chem. Toxicol. 48, 1052–1058.
Ye, X., Jiang, F., Li, Y., Mu, J., Si, L., Wang, X., Ning, S., Li, Z., 2014. Glabridin attenuates the migratory and invasive capacity of breast cancer cells by activating microRNA-200c. Cancer Sci. 105, 875–882.
Yehuda, I., Madar, Z., Leikin-Frenkel, A., Tamir, S., 2015. Glabridin, An isoflavan from licorice root, down-regulates iNOS expression and activity under high glucose stress and inflammation. Mol. Nutr. Food Res.
Yu, L., Alva, A., Su, H., Dutt, P., Freundt, E., Welsh, S., Baehrecke, E.H., Lenardo, M.J., 2004. Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. Science 304, 1500–1502.
Zhou, F., Hao, Y.F., Chen, Y., Wang, T., 2013. Chinese herbal medicine in treatment of polyhydramnios:a meta-analysis and systematic review. Chin. Med. Sci. J. 28, 72–81.