**Hesperitin derivative-11 suppress hepatic stellate cell activation and proliferation
by targeting PTEN/AKT pathway**

Wan-Xia Li^{a,b,c,d}, Xin Chen^{a,b,c,d}, Yang Yang^{a,b,c,d}, Hui-min Huang^{a,b,c,d}, Hai-di Li^{a,b,c,d},
Cheng Huang^{a,b,c,d}, Xiao-Ming Meng^{a,b,c,d}, Jun Li^{a,b,c,d*}

Abbreviations: Hesperidin derivative, HD-11; phosphatase and tension homologue deleted on chromosome ten, PTEN; Hepatic stellate cells, HSCs; Transforming growth factor- β 1, TGF- β 1; alpha-Smooth muscle actin, α -SMA; Potassium bisperoxo oxovanadate, bpv; Alanine aminotransferase, ALT; Aspartate aminotransferase, AST.

Abstract

Hesperitin derivative (HD-11) is a monomeric compound derived from Hesperidin, which is a naturally occurring flavanone glycoside that exerts extensive clinical effects such as anti-inflammatory, anti-oxidant and anti-angiogenic. However, the role and fundamental mechanism of HD-11 in hepatic fibrosis is still unrevealed. In this study, HD-11 not only alleviates ECM deposition in rats with liver fibrosis, but also reduces the expression of α -SMA and colla1 in TGF- β 1-induced HSC-T6 cells. Moreover, it was demonstrated that HD-11 significantly promoted the expression of PTEN *in vivo* and *in vitro*. In order to evaluate the involvement of HD-11 in TGF- β 1-induced HSC-T6 activation, a specific blocking agent of PTEN (bpv) and PTEN small interfering (si)-RNA-mediated silencing were used. Interestingly, HD-11 treatment couldn't inhibit α -SMA and colla1 expression on the basis of PTEN knockdown. On the contrary, over-expression of PTEN had an opposite effect on the expression of α -SMA and colla1 in TGF- β 1-induced HSC-T6 cells after treatment of HD-11. In addition, HD-11 remarkably inhibited the expression of p-AKT *in vivo* and *in vitro*. Taken together, all the above results indicate that HD-11 may play the part of an effective modulator of PTEN/AKT signaling pathway.

Keywords: Hesperidin derivative-11; PTEN; AKT; liver fibrosis

1. Introduction

Hepatic fibrosis is the consequence of a persistent chronic liver injury and a continuous wound-healing process mainly owing to chronic viral infection, alcohol

45 consumption, parasitic disease, genetic abnormalities, toxic damage, *etc* (Bataller and
 46 Brenner, 2005; Giannitrapani *et al.*, 2014). It is characterized by excessive deposition
 47 of extracellular matrix (ECM) components, especially for α -SMA and collagen I
 48 (Brenner *et al.*, 2000; Son *et al.*, 2013). Progressive scarring in response to a
 49 persisting liver injury eventually leads to cirrhosis or even hepatocellular carcinoma
 50 (HCC) which is one of the leading causes of death worldwide (Ismail and Pinzani,
 51 2009). Numerous studies indicate that hepatic stellate cells (HSCs), which are located
 52 in the perisinusoidal space of disse, are considered as the main producer of ECM
 53 during liver fibrosis (Friedman, 2008b). It is well accepted that quiescent HSCs
 54 (qHSCs) activation is the defining event in the development and maintenance of liver
 55 fibrosis (Hellerbrand, 2013). Upon chronic liver injury, the qHSCs are
 56 transdifferentiated to myofibroblast-like cells characterized by remarkable
 57 up-regulation of α -smooth muscle actin (α -SMA) and type I collagen (coll1a1), which
 58 refers to as the activated HSCs (Mallat and Lotersztajn, 2013). Therefore, the
 59 preferred therapeutic target for the treatment of hepatic fibrosis is inhibition of the
 60 activation and function of HSCs.

61 Phosphatase and tension homolog deleted on chromosome 10 (PTEN) is a
 62 phosphatase that de-phosphorylates phosphatidylinositol-3, 4, 5-trisphosphate (PIP₃)
 63 produced by phosphatidylinositol-3-kinase (PI3K) to influence cell growth signaling
 64 (Maehama and Dixon, 1998). PI3K/AKT signaling characterized in regulating
 65 apoptosis and cellular proliferation is the only pathway targeted by PTEN through its
 66 lipid phosphatase activity (Pitha-Rowe *et al.*, 2009). Therefore, the loss of PTEN may

cause an accumulation of PIP₃, which in turn increases AKT activity, leading to decreased cellular apoptosis and/or increased cell proliferation (Bhaskara *et al.*, 2006; Liu *et al.*, 2010). This suggests that promotion of PTEN may limit HSC activation in liver fibrosis.

To date, the application of traditional Chinese medicine to treat liver fibrosis has gained increasing attention. Hesperitin derivative (HD-11), a flavanone found abundantly in fruit peels of the genus citrus, has been reported to exert biological effects such as free-radical scavenging and inhibition of cellular proliferation, as well as antiallergic, anti-oxidative stress and anti-inflammatory (Ross and Kasum, 2002; Zielinska-Przyjemska and Ignatowicz, 2008). However, its role in the activation and proliferation of HSCs is still unrevealed. Furthermore, the potential role of HD-11, as shown in **Fig.1**, during hepatic fibrogenesis in vivo also has not been explored. Thus, we investigated the protective effect of HD-11 and the potential mechanisms on rat with liver fibrosis and TGF-β1-induced HSC-T6 cells.

2. Materials and Methods

2.1. Reagents

Hesperidin derivative (HD-11) (HPLC≥98%) was synthesized by Wang Qian qian et al in our chemical laboratory. TGF-β1 was obtained from Pepro Tech Inc (Rocky Hill, USA). Anddimethyl sulfoxide (DMSO), bpv (PTEN) and methyl thiazolyl tetrazolium (MTT) were purchased from Sigma Chemical (St.Louis, MO, USA). Rat

monoclonal antibodies β -actin was purchased from Santa Cruz (California, USA) and Rabbit α -SMA, colla1 polyclonal antibody were purchased from Bioss (Beijin, China). Rabbit PTEN, c-myc, cyclinD1 polyclonal antibody were purchased from abcam (Cambridge, UK). Rabbit AKT, p-AKT polyclonal antibody were purchased from Cell Signaling Teconology (MA, USA). ALT and AST activity assay kit were purchased from Jiancheng (Nanjing, China).

94

2.2. Animal treatment

Six-week-old male Sprague-Dawley (SD) rats (n=40), weighting about 180 to 200 g each, were obtained from the Experimental Animal Center of Anhui Medical University. The animal experimental protocol was reviewed and approved by the University Animal Care and Use Committee. Eight rats were subjected subcutaneous injections of olive oil (2ml/kg weight) for 2 times per week up to 12 weeks, and served as the normal controls. The other 32 rats were injected by CCl₄ subcutaneously (40% CCl₄, diluted in olive oil, 2ml/kg) twice a week for 12 weeks. After 8-week CCl₄ administration, the rats were randomly divided into 4 groups (8 rats in each group). One group was just treated with CCl₄, while the other 3 groups were treated CCl₄ and 3 doses (1g/kg, 2g/kg, 4g/kg) of HD-11 dissolved in 5% sodium carboxymethylcellulose every day for 4 weeks, respectively.

107

2.3. ALT/AST activity assay

The levels of ALT and AST in serum from rats in model group and treatment group

were assayed using ALT and AST activity assay kits according to the manufacturer's instructions. The absorbance at 510 nm was obtained with a Multiskan MK3 (Biotek , USA).

2.4. Histopathology

The biggest lobe of the liver of each rat was excised and then perfused in 4% paraformaldehyde for at least 24 h. After fixation, the tissues were embedded in paraffin, and 5µm thick sections were then stained with hematoxylin and eosin (H&E) according to a standard procedure to observe the pathological changes by light microscopy.

2.5. Isolation of primary hepatic stellate cells

Primary HSCs were isolated from the livers of rats as previously described (Ramm, 1998). Briefly, the liver was perfused after opening the abdominal cavity in situ with PB, a kind of buffer solution containing NaCl, KCl, Hepes and NaOH, to purge the liver of blood. After digestion of the liver with collagenase IV (Sigma, St. Louis, MO, USA) and Pronase (Sigma, St. Louis, MO, USA), the liver was dissociated and disrupted in 1% BSA solution. The dispersed cell suspensions were layered by Nycodenz (Sigma, St. Louis, MO, USA) density gradient centrifugation according to manufacture protocol. The resulting upper layer contained the freshly isolated HSCs.

2.6. Cell Culture

The HSC-T6 cell line was obtained from Shanghai Fumeng Gene Biological Corporation (Shanghai, China). The HSC-T6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, USA) supplemented with 5% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine and incubated at 37 °C in an atmosphere of 5% CO₂.

2.7. Cell proliferation assays

Cell proliferation assay was determined by standard 3-(4, 5-dimethylthiazol-2-yl)-2, 4-diphenyl-tetrazolium bromide (MTT) assay. Briefly, the cells were dissolved at a density of 5×10^4 cells/ml and then 100 µl of the obtained cell suspension was added to each well of 96-well culture plates and incubated overnight to allow for cell attachment at 37 °C and 5% CO₂ atmosphere. After which they were treated with TGF-β1 and different concentrations of HD-11 (0, 0.15625, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40 µM) as described above. After 48 h, 20 µL of PBS containing 2.5 mg/mL of MTT (Sigma-Aldrich, USA) was added to each well. After 4 h, the medium was replaced by 150 µL DMSO to dissolve the purple formazan crystals produced. The absorbance at 490 nm of each well was measured by using a Thermomax microplate reader (BioTek, Winooski, VT, USA). All experiments were performed in triplicate and repeated at least three times.

2.8. Cell cycle and apoptosis analysis

For cell cycle, detection was performed by using Cell Cycle Analysis Kit

(Beyotime, China) according to the manufacturer's instructions. HSC-T6 cells were seeded in a 6-well plate. The cells were washed for three times by cold PBS, and then cells were fixed in 70% ethanol in PBS at -20°C for 24 h. After fixation, cells were washed with cold PBS and stained with 0.5 ml of propidium iodide (PI) staining buffer at 37 °C for 30 min in the dark. Flow cytometric analysis (FACS) was performed on a BDLSR (BD Biosciences), and data fitting was made with the Flow data analysis software package (TreeStar, USA). All experiments were performed in triplicate and repeated at least three times.

For apoptosis analysis, quantification of apoptotic cells was performed with Annexin-V-FITC Apoptosis Detection Kit (Bestbio, China). Apoptotic cells were defined as Annexin-Vpositive, PI-positive cells. Analyses were performed on BD FACSVerse (BD Biosciences, USA). Apoptosis was analyzed by using FlowJo data analysis software package (TreeStar, USA). All experiments were repeated three times.

2.9. Immunofluorescence staining

Cultured HSC-T6 cells were treated by TGF-β1 and different concentrations of HD-11. Immunofluorescence staining was performed with rabbit anti-PTEN (Abcam, Cambridge, UK). Alexa Fluor 594-conjugated anti-rabbit was used as secondary antibody. Counterstaining of nuclei was performed with 4', 6-diamidino-2-phenylindole (DAPI; Biyuntian biological technology, Shanghai, China).

176

2.10. RNA interference (RNAi)

RNAi was performed by using Lipofectamine™ 2000 (Invitrogen, California, USA) according to the manufacturer's protocol. Small interfering RNA (siRNA) oligonucleotides against PTEN gene was purchased from GenaPharma Corporation (Shanghai, China). A negative scrambled siRNA (GenePharma, Shanghai, China) was used in parallel, as shown in **Table 1**. HSC-T6 cells were cultured in serum-free DMEM for 12 h and then subjected to reverse transfection with siRNA-PTEN or the scrambled sequences in Opti-MEM (Gibco, USA). The culture medium was changed 6 h after transfection. Transfection was allowed to proceed for various times and cells were processed for different assays. The siRNA transfection efficiency of Lipofectamine™ 2000 in cells was determined by Real-time PCR test and Western blot analysis. All experiments were repeated three times.

2.11. Generation of recombinant PTEN plasmid

Plasmid construction of GV141-PTEN was obtained from Genechem (Shanghai, China). We acknowledge the principal investigators sincerely. HSC-T6 cells were activated by TGF-β1 (10 ng/ml, 48 h), and then were transfected with GV141-PTEN to induce the ectopic expression of PTEN, and empty GV141 vector (GV141-control) as a control.

2.12. Total RNA extraction and quantitative real-time PCR

Total RNA was extracted from primary HSC and HSC-T6 cells by using TRIzol (Invitrogen) according to the manufacture's instruction, and the first-strand cDNA was synthesized using a Thermoscript RT-PCR synthesis kit (Fermentas, Canada) according to the manufacturer's instructions.. Realtime quantitative PCR analyses for mRNA of PTEN, α -SMA, *colla1*, and β -actin were performed by using Thermoscript RT-qPCR kits (Fermentas, USA) in an ABI Prizm step-one plus real-time PCR system (Applied Biosystems, USA). The primers used were as shown in **Table 2**. PCR was performed 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and at 60°C about 1 minute by using Thermo Step One. Reactions were conducted three times, and threshold cycle values were normalized to GAPDH gene expression. The specificity of the products was determined by melting curve analysis. Relative mRNA expression of target genes were obtained by normalized to control group and the level of β -actin.

2.13. Western Blot

Primary HSC and HSC-T6 cells were lysed in protein extraction solution for Western (Beyotime, China). Protein concentration of the extract was determined by using NanoDrop 2000 Spectrophotometer (Thermo scientific, USA). Equal amounts of protein were electrophoresed on SDS-PAGE and blotted onto PVDF membranes (Millipore Corp, Billerica, MA, USA). After blockade of nonspecific protein binding, nitrocellulose blots were incubated for 6 h with primary antibodies diluted in primary antibody dilution buffer (Beyotime, China). The primary antibodies recognizing

PTEN, α -SMA, colla1, cyclinD1, c-myc, AKT, p-AKT and β -actin were used 1:1000, 1:200, 1:200, 1:1000, 1:1000, 1:500, 1:500 and 1:3000, respectively. The membranes were then incubated in TBST containing 5 % skim milk at 37°C for 3 h, and with specific primary antibodies at 4°C overnight. The next day, the membranes were washed 3 times with TBS/Tween20 (0.075%), followed by incubation with HRP-conjugated secondary antibodies(1:8000–1:10000) at 37°C for 1 h. After washing three times with TBST (Boster, China), proteins were visualized with ECL-chemiluminescent kit (ECL-plus, Thermo Scientific, USA).

2.14. Statistical Analysis

Statistical analysis was completed with the Statistical package for Social Sciences (SPSS Inc., Chicago, IL, USA, version 13.0). All data were presented as means $\pm$ SD and statistical significances were determined by one-way ANOVA with the post-hoc Dunnett's test. In all cases, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Hepatic protective effects of HD-11 *in vivo*

In order to investigate the effect of HD-11 on rats with liver fibrosis, histopathological study was performed. H&E staining and Masson Trichrome staining showed that CCl₄ (20%) injection caused remarkable liver injury including hepatocyte steatosis, necrosis and liver lobule structural failure as compared with the control

group (**Fig.2A**). Moreover, α -SMA immunostaining signal was increased in fibrotic liver tissue from CCl₄-treated rats compared to that from vehicle-treated groups (**Fig.2B**). Meanwhile, the serum levels of ALT and AST were markedly elevated in rats with liver fibrosis (**Fig.2C**). Furthermore, the result of western blot and q-PCR indicated that the expression of α -SMA and *colla1* were up-regulated in primary HSCs isolated from CCl₄-induced liver tissue compared with vehicle group (**Fig.2D**). These results suggest that the rat liver fibrosis model is successfully built.

Then, different concentrations of HD-11 had varying degrees of effect on hepatic protection as shown in H&E staining and Masson Trichrome staining compared with model group, respectively (**Fig.2A**). Meanwhile, α -SMA immunostaining signal was reduced by treatment with HD-11 compared with CCl₄-treated rats (**Fig.2B**). Furthermore, the levels of ALT and AST were reduced after treated with HD-11, especially in the concentration of 4g/kg compared with model group (**Fig.2C**). Moreover, western blot and q-PCR showed that the primary HSCs isolated from CCl₄-induced liver tissue treatment with HD-11 (4g/kg) had lower expressions of α -SMA and *colla1* compared with model group (**Fig.2D**). In conclusion, these results suggest that HD-11 have an obvious hepatic protective effect *in vivo*.

3.2. Hepatic protective effect of HD-11 in vitro

The cytotoxicity test of HD-11 was achieved by MTT assay. HSC-T6 cells were treated with TGF- β 1 in advance. As shown in **Fig.3A**, at low-concentration (0.15625, 0.3125, 0.625, 1.25, 2.5), HD-11 had no effect on HSC-T6 cell viability compared

with control group. However, higher dose (5 μ M and above) had significant inhibition of cell viability, so 5 μ M was chosen to be used in the following experiments. To study the effect of HD-11 on HSC-T6 cells, cells were treated by HD-11 (1.25, 2.5, 5, 10 μ M) for 48 h. Western blot analysis showed that HD-11 suppressed α -SMA and colla1 expression compared with TGF- β 1-treated group (**Fig.3B**). Similar findings were demonstrated by q-PCR analysis (**Fig.3B**). These data indicated that HD-11 could induce PTEN expression *in vitro*.

3.3. HD-11 promoted PTEN expression in primary HSCs isolated from liver tissue and TGF- β 1-induced HSC-T6 cell

Firstly, immunohistology was used to evaluate the level of PTEN in liver tissue with liver fibrosis. As shown in **Fig.4A**, the expression of PTEN was down-regulated in CCl₄-treated group but significantly up-regulated in HD-11-treated group. Moreover, result of western blot indicated that the expression of PTEN was down-regulated in primary HSCs isolated from CCl₄-induced liver tissue compared with vehicle group, while HD-11 (4g/kg) could up-regulated it (**Fig.4B**). Similar findings were also demonstrated by q-PCR, HD-11 could up-regulate the PTEN mRNA expression *in vitro* (**Fig.4B**). Then, HSC-T6 cells were treated with different concentrations of HD-11, then the result of western blot and q-PCR showed that HD-11 could induce PTEN protein and mRNA expression *in vitro* (**Fig.4C**). Furthermore, western blot detected the expression of PTEN in liver tissue and showed that HD-11 could up-regulate PTEN in a dose dependent manner (**Fig.4D**). In addition,

immunofluorescence staining also showed that PTEN expression was down-regulated in TGF- β 1-treated group compared with control group whereas HD-11 up-regulated PTEN expression compared with TGF- β 1-treated group (**Fig.4E**). All the data indicated that HD-11 could up-regulate PTEN expression.

3.4. HD-11 inhibited α -SMA and colla1 expression by targeting PTEN

To further examine that whether HD-11 can reduce α -SMA and colla1 expression by targeting PTEN in HSC-T6 cells. Firstly, cells were treated with HD-11 (5 μ M) after PTEN over-expression successfully. The result of western blot showed that HD-11 and GV141-PTEN remarkably raised the expression of PTEN, respectively. Meanwhile, both HD-11 and GV141-PTEN significantly inhibited the expression of α -SMA and colla1. However, there was no significant difference in PTEN expression between PTEN-overexpression cells and HD-11-treated cells (**Fig.5A, 5B**). Furthermore, western blot and q-PCR analysis showed that HD-11 significantly promoted the expression of PTEN after treated with TGF- β 1 in HSC-T6 cells, but HD-11 had no influence on PTEN expression while PTEN was silenced (**Fig.5C, 5D**). In addition, bpv, an insulin-mimetic small molecule as an inhibitor of PTEN, was used to knockdown PTEN. Similar findings were demonstrated by western blot analysis (**Fig.5E**).

3.5. HD-11 inhibited cell cycle in activated HSC-T6 cells but had no influence on cell apoptosis

In order to investigate that whether HD-11 could regulate TGF- β 1-induced HSC proliferation or apoptosis, we used Flow cytometric analyses to measure the effect of HD-11 on induction of cell cycle and apoptosis. HSC-T6 cells were treated with TGF- β 1 and HD-11 for 48 h. Cell cycle analysis showed that treatment with HD-11 resulted in increased percentage of the activated HSC-T6 cells in G0/G1 phase and decreased population of cells in G2 phase in comparison to the model group (**Fig.6A**). However, as illustrated in **Fig.6B**, HD-11 had no influence on the apoptosis of HSC-T6 cells. In a word, HD-11 could inhibit activated HSC-T6 cell proliferation induced by TGF- β 1 via promoting cell cycle arrest without promoting cell apoptosis.

3.6. HD-11 may protect liver through PTEN/AKT pathway

It has been reported that PI3K/AKT signaling pathway is involved in regulating HSCs proliferation, and AKT is the direct target of PTEN (Takashima *et al.*, 2009; Gupta and Dey, 2012). Hence, in order to illuminate that whether HD-11 had an influence on AKT pathway through PTEN, we isolated primary HSCs and tissue protein from liver tissue with liver fibrosis. Immunohistology was used to evaluate the expression of p-AKT in liver tissue and the result showed that it was obviously increased in the model group, yet it was decreased in HD-11 treated group (**Fig.7A**). As shown in **Fig.7B**, the results of western blot revealed that CCl₄-treatment increased the expression of p-AKT and cell cycle relevant proteins like cyclinD1 and c-myc. However, HD-11 reversed the change of these proteins especially in the high dose group. The similar finding was shown in **Fig.7C**. Secondly, we treated HSC-T6 cells

with TGF- β 1 and HD-11 and then protein was extracted, respectively. Western blot showed that TGF- β 1 up-regulated p-AKT, cyclinD1 and c-myc, nevertheless, HD-11 inhibited them in a dose-dependent manner (**Fig.7D**). Furthermore, the result of western blot demonstrated that transfection of GV141-PTEN plasmid significantly decreased the expression of p-AKT, cyclinD1 and c-myc in comparison with control plasmid as well as treatment with HD-11 (**Fig.7E**). However, there is no difference in plasmid group and plasmid plus HD-11 group. This indicated that HD-11 played a similar role to plasmid. Moreover, siRNA specific for rat PTEN (PTEN-siRNA) was used to knockdown the PTEN expression. The results of western blot revealed that activated HSC-T6 cells produced more p-AKT, cyclinD1 and c-myc after transfected with siRNA-PTEN, but HD-11 had no influence in their expression while PTEN was silenced (**Fig.7F**). In addition, bpv, an inhibitor of PTEN, was used to block PTEN, similar phenomenon was found (**Fig.7G**). All above these data indicated that HD-11 developed an inhibition of cell proliferation through PTEN/AKT pathway in TGF- β 1-induced HSC-T6 cells.

4. Discussion

Liver fibrosis has been acknowledged as a reversible wound-healing response of liver to many liver injuries, which is a common outcome of virtually all chronic liver diseases. Hepatic fibrosis is characterized by a distortion of hepatic tissue and extensive deposition of ECM proteins such as type I and III collagens (Du *et al.*, 1999; Ding *et al.*, 2005). Hepatic stellate cell activation acts as a critical event in liver

fibrosis because they become the primary source of extracellular matrix in liver upon injury (Hernandez-Gea and Friedman, 2011). During fibrosis, HSCs undergo transdifferentiation from a quiescent, vitamin A-storing cell to a myofibroblast-like cell which is highly proliferative and synthesize more ECM components including α -SMA and *colla1* (Friedman, 2008a; Lakner *et al.*, 2012). Therefore, reducing the activation and suppressing the proliferation of HSCs were the most efficient treatment strategies for liver fibrosis (Woodhoo *et al.*, 2012).

HD-11 is an monomeric compound derives from Hesperidin which was extracted from fruit peels of the genus citrus, has been described to exhibit various biological activities and pharmacological properties, especially anti-inflammation and anti-tumor. Hesperetin dramatically suppressed secretion of tumor necrosis factor (TNF)- α , interleukin (IL)-6, and IL-1 β through inhibiting NF- κ B and activating Nrf2/HO-1 (Ren *et al.*, 2016). In addition, hesperetin could induce esophageal cancer cells apoptosis via mitochondrial-mediated intrinsic pathway by accumulation of ROS (Wu *et al.*, 2016). However, there are only few correlational studies about the anti-fibrotic activity of HD. Thus, we investigate the anti-fibrotic effect of HD-11 in rat with liver fibrosis and TGF- β 1-induced HSC-T6 cells.

PTEN is known to act as a tumor suppressor protein which dephosphorylates the D3 position of phosphatidylinositol 3, 4, 5-trisphosphate (PIP3) thereby inhibiting the PI3K/AKT pathway, and influence cell processes including growth, proliferation and polarization (Song *et al.*, 2012; Worby and Dixon, 2014). Decreased or absence PTEN expression has been observed in many kinds of malignant tumors, such as the

brain, breast, endometrium, prostate, bladder, thyroid, lung and liver (Hao *et al.*, 2009). Furthermore, the study showed a loss of expression of PTEN during HSC activation and in the fibrotic liver tissue from CCl₄-treated rats (Bian *et al.*, 2012). Therefore, we focus on that the effect of HD-11 regulating PTEN *in vivo* and *in vitro*, respectively. In this study, our results showed that the anti-fibrotic effect of HD-11 seem to be dose-dependent because treatment of higher dose of HD-11 (400 mg/kg) was more effective compared with lower-dose (100 mg/kg). In addition, HD-11 inhibited the expressions of α -SMA and colla1 but promoted the expression of PTEN in primary HSCs isolated from rat with liver fibrosis and TGF- β 1-treated HSC-T6 cells. More interestingly, the expressions of α -SMA and colla1 were not too big change in HD-11 plus si-PTEN treated group compared with HD-11 treated group. However, HD-11 greatly inhibited α -SMA and colla1 expression in the presence of GV141-PTEN. These results showed that HD-11 may partly target PTEN to exert anti-fibrotic effect. All above results showed that HD-11 might act as an agonist of PTEN to activating PTEN to exert anti-fibrotic effects.

The AKT pathway, activated by growth factors, cytokines and integrin signaling, plays an important role in controlling cancer cell growth, cell cycle progression and survival (Fresno Vara *et al.*, 2004; Liu *et al.*, 2011). One of the silencing mechanisms underlying PTEN down-regulation is concerned with methylation in the *PTEN* gene promoter, which has frequently been found in diverse cancer types (Soria *et al.*, 2002; Furuta *et al.*, 2004; Mirmohammadsadegh *et al.*, 2006). It has been demonstrated that aberrant methylation and subsequent silencing of the *PTEN* gene causes the activation

of the PI3K/AKT and ERK pathways and consequently promotes cancer cell proliferation and ultimately contributes to the progression of tumor (Phuong *et al.*, 2011). It has also been documented that the activation of PI3K/AKT pathway promotes HSC activation and liver fibrogenesis (Liu *et al.*, 2011). In this study, we observed that HD-11 inhibited cell cycle in TGF- β 1-treated HSC-T6 cells but had no influence on apoptosis and the activity of AKT was enhanced in the primary HSCs isolated from rat with liver fibrosis and in activated HSC-T6 cells in vitro. In the present study, we found that blocking of PTEN by bpv or PTEN siRNA or over-expression plasmid of PTEN, PTEN regulating AKT expression lead to the change of c-myc and cyclinD1 expression to influence HSC-T6 cell proliferation could be proved. Taken together, these results suggested that PTEN subsequently inhibited TGF- β 1-induced AKT pathway activation in HSC-T6 cell.

In summary, our findings from the present study suggested that flavonoid compound HD-11 functioned potential therapeutic effect of liver fibrosis most likely through activating PTEN, which is known as a “fibrotic suppressor” gene. Administration of HD-11 significantly attenuated liver fibrosis and regulated the expression of PTEN in rat with liver fibrosis and decreased the expression of α -SMA and colla1 in activated HSC-T6 cell. In addition, inhibition of PTEN by bpv or PTEN siRNA, the anti-fibrotic effect of HD-11 was reversed, the results showed that HD-11 up-regulated the expression of PTEN in a dose dependent manner. Moreover, we have proven that HD-11 indeed inhibited AKT signaling pathway activation through up-regulating PTEN expression. Collectively, our findings may

support a novel role of HD-11, as an effective modulator of PTEN expressional activity and a potential therapeutic choice in treating liver fibrosis.

Acknowledgments

This project was supported by the National Science Foundation of China (Nos. 81273526, 81473268); the Anhui Provincial Higher Education Natural Science Foundation (KJ2016A364); and the Anhui Provincial Natural Science Foundation (21408085MKL31).

References

- Bataller, R., Brenner, D.A., 2005. Liver fibrosis. *J Clin Invest* 115, 209-218.
- Bhaskara, V.K., Sundaram, C., Babu, P.P., 2006. pERK, pAkt and pBad: a possible role in cell proliferation and sustained cellular survival during tumorigenesis and tumor progression in ENU induced transplacental glioma rat model. *Neurochem Res* 31, 1163-1170.

- 437 Bian, E.B., Huang, C., Ma, T.T., Tao, H., Zhang, H., Cheng, C., Lv, X.W., Li, J., 2012.
438 DNMT1-mediated PTEN hypermethylation confers hepatic stellate cell activation and liver
439 fibrogenesis in rats. *Toxicol Appl Pharmacol* 264, 13-22.
- 440 Brenner, D.A., Waterboer, T., Choi, S.K., Lindquist, J.N., Stefanovic, B., Burchardt, E., Yamauchi, M.,
441 Gillan, A., Rippe, R.A., 2000. New aspects of hepatic fibrosis. *J Hepatol* 32, 32-38.
- 442 Ding, J., Yu, J., Wang, C., Hu, W., Li, D., Luo, Y., Luo, H., Yu, H., 2005. Ginkgo biloba extract
443 alleviates liver fibrosis induced by CCl in rats. *Liver Int* 25, 1224-1232.
- 444 Du, W.D., Zhang, Y.E., Zhai, W.R., Zhou, X.M., 1999. Dynamic changes of type I,III and IV collagen
445 synthesis and distribution of collagen-producing cells in carbon tetrachloride-induced rat liver
446 fibrosis. *World J Gastroenterol* 5, 397-403.
- 447 Fresno Vara, J.A., Casado, E., de Castro, J., Cejas, P., Belda-Iniesta, C., Gonzalez-Baron, M., 2004.
448 PI3K/Akt signalling pathway and cancer. *Cancer Treat Rev* 30, 193-204.
- 449 Friedman, S.L., 2008a. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver.
450 *Physiol Rev* 88, 125-172.
- 451 Friedman, S.L., 2008b. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 134, 1655-1669.
- 452 Furuta, J., Umebayashi, Y., Miyamoto, K., Kikuchi, K., Otsuka, F., Sugimura, T., Ushijima, T., 2004.
453 Promoter methylation profiling of 30 genes in human malignant melanoma. *Cancer Sci* 95,
454 962-968.
- 455 Giannitrapani, L., Soresi, M., Bondi, M.L., Montalto, G., Cervello, M., 2014. Nanotechnology
456 applications for the therapy of liver fibrosis. *World J Gastroenterol* 20, 7242-7251.
- 457 Gupta, A., Dey, C.S., 2012. PTEN, a widely known negative regulator of insulin/PI3K signaling,
458 positively regulates neuronal insulin resistance. *Mol Biol Cell* 23, 3882-3898.
- 459 Hao, L.S., Zhang, X.L., An, J.Y., Karlin, J., Tian, X.P., Dun, Z.N., Xie, S.R., Chen, S., 2009. PTEN
460 expression is down-regulated in liver tissues of rats with hepatic fibrosis induced by biliary
461 stenosis. *APMIS* 117, 681-691.
- 462 Hellerbrand, C., 2013. Hepatic stellate cells--the pericytes in the liver. *Pflugers Arch* 465, 775-778.
- 463 Hernandez-Gea, V., Friedman, S.L., 2011. Pathogenesis of liver fibrosis. *Annu Rev Pathol* 6, 425-456.
- 464 Ismail, M.H., Pinzani, M., 2009. Reversal of liver fibrosis. *Saudi J Gastroenterol* 15, 72-79.
- 465 Lakner, A.M., Steuerwald, N.M., Walling, T.L., Ghosh, S., Li, T., McKillop, I.H., Russo, M.W.,
466 Bonkovsky, H.L., Schrum, L.W., 2012. Inhibitory effects of microRNA 19b in hepatic stellate
467 cell-mediated fibrogenesis. *Hepatology* 56, 300-310.

- 468 Liu, H., Li, H., Guo, L., Li, M., Li, C., Wang, S., Jiang, W., Liu, X., McNutt, M.A., Li, G., 2010.
469 Mechanisms involved in phosphatidylinositol 3-kinase pathway mediated up-regulation of the
470 mu opioid receptor in lymphocytes. *Biochem Pharmacol* 79, 516-523.
- 471 Liu, Y., Wang, Z., Kwong, S.Q., Lui, E.L., Friedman, S.L., Li, F.R., Lam, R.W., Zhang, G.C., Zhang,
472 H., Ye, T., 2011. Inhibition of PDGF, TGF-beta, and Abl signaling and reduction of liver
473 fibrosis by the small molecule Bcr-Abl tyrosine kinase antagonist Nilotinib. *J Hepatol* 55,
474 612-625.
- 475 Maehama, T., Dixon, J.E., 1998. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid
476 second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 273, 13375-13378.
- 477 Mallat, A., Lotersztajn, S., 2013. Cellular mechanisms of tissue fibrosis. 5. Novel insights into liver
478 fibrosis. *Am J Physiol Cell Physiol* 305, C789-799.
- 479 Mirmohammadsadegh, A., Marini, A., Nambiar, S., Hassan, M., Tannapfel, A., Ruzicka, T., Hengge,
480 U.R., 2006. Epigenetic silencing of the PTEN gene in melanoma. *Cancer Res* 66, 6546-6552.
- 481 Phuong, N.T., Kim, S.K., Lim, S.C., Kim, H.S., Kim, T.H., Lee, K.Y., Ahn, S.G., Yoon, J.H., Kang,
482 K.W., 2011. Role of PTEN promoter methylation in tamoxifen-resistant breast cancer cells.
483 *Breast Cancer Res Treat* 130, 73-83.
- 484 Pitha-Rowe, I., Liby, K., Royce, D., Sporn, M., 2009. Synthetic triterpenoids attenuate cytotoxic retinal
485 injury: cross-talk between Nrf2 and PI3K/AKT signaling through inhibition of the lipid
486 phosphatase PTEN. *Invest Ophthalmol Vis Sci* 50, 5339-5347.
- 487 Ramm, G.A., 1998. Isolation and culture of rat hepatic stellate cells. *J Gastroenterol Hepatol* 13,
488 846-851.
- 489 Ren, H., Hao, J., Liu, T., Zhang, D., Lv, H., Song, E., Zhu, C., 2016. Hesperetin Suppresses
490 Inflammatory Responses in Lipopolysaccharide-Induced RAW 264.7 Cells via the Inhibition
491 of NF-kappaB and Activation of Nrf2/HO-1 Pathways. *Inflammation* 39, 964-973.
- 492 Ross, J.A., Kasum, C.M., 2002. Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu*
493 *Rev Nutr* 22, 19-34.
- 494 Son, M.K., Ryu, Y.L., Jung, K.H., Lee, H., Lee, H.S., Yan, H.H., Park, H.J., Ryu, J.K., Suh, J.K., Hong,
495 S., Hong, S.S., 2013. HS-173, a novel PI3K inhibitor, attenuates the activation of hepatic
496 stellate cells in liver fibrosis. *Sci Rep* 3, 3470.
- 497 Song, M.S., Salmena, L., Pandolfi, P.P., 2012. The functions and regulation of the PTEN tumour
498 suppressor. *Nat Rev Mol Cell Biol* 13, 283-296.
- 499 Soria, J.C., Lee, H.Y., Lee, J.I., Wang, L., Issa, J.P., Kemp, B.L., Liu, D.D., Kurie, J.M., Mao, L.,
500 Khuri, F.R., 2002. Lack of PTEN expression in non-small cell lung cancer could be related to
501 promoter methylation. *Clin Cancer Res* 8, 1178-1184.

Takashima, M., Parsons, C.J., Ikejima, K., Watanabe, S., White, E.S., Rippe, R.A., 2009. The tumor suppressor protein PTEN inhibits rat hepatic stellate cell activation. *J Gastroenterol* 44, 847-855.

Woodhoo, A., Iruarrizaga-Lejarreta, M., Beraza, N., Garcia-Rodriguez, J.L., Embade, N., Fernandez-Ramos, D., Martinez-Lopez, N., Gutierrez-De Juan, V., Arteta, B., Caballeria, J., Lu, S.C., Mato, J.M., Varela-Rey, M., Martinez-Chantar, M.L., 2012. Human antigen R contributes to hepatic stellate cell activation and liver fibrosis. *Hepatology* 56, 1870-1882.

Worby, C.A., Dixon, J.E., 2014. Pten. *Annu Rev Biochem* 83, 641-669.

Wu, D., Zhang, J., Wang, J., Li, J., Liao, F., Dong, W., 2016. Hesperetin induces apoptosis of esophageal cancer cells via mitochondrial pathway mediated by the increased intracellular reactive oxygen species. *Tumour Biol* 37, 3451-3459.

Zielinska-Przyjemska, M., Ignatowicz, E., 2008. Citrus fruit flavonoids influence on neutrophil apoptosis and oxidative metabolism. *Phytother Res* 22, 1557-1562.

Figure legends

Fig.1 The chemical structure of HD-11.

Fig.2 Detection of the protective effect of HD on hepatic fibrosis *in vivo*, n=6 per group. **A.** Vehicle, fibrotic and HD-treatment rat liver tissue sections were detected by hematoxylin eosin (H&E) staining and Masson staining. Representative views from each group are presented ($\times 100$). **B.** The expression of α -SMA was analyzed by immunohistochemistry in liver tissue of vehicle control, model group and treatment group. Representative views from each group are presented ($\times 100$). **C.** The serum level of ALT and AST was measured as described in Materials and methods. Each group consists of 6 rats. The results (mean $\pm$ SD) were calculated relative expression against control values. **D.** The expression of α -SMA and Colla1 in primary HSCs isolated from rat liver tissues with fibrosis was detected by western blot and qPCR. The results are expressed as relative expression against negative control expression. All the experiments were repeated thrice, and a representative result is shown. N = normal rats; M = CCl₄; L = HD-11 (1g/kg) + CCl₄; Mi = HD-11 (2g/kg) + CCl₄; H =

HD-11 (4g/kg), **P < 0.01 vs control, ##P < 0.01 vs model.

Fig.3 Detection of the protective effect of HD-11 in HSC-T6 cells in vitro. A. MTT assay was performed to assess the effects of different concentrations of HD on HSC-T6 proliferation. **B.** The expression of α -SMA and Coll α 1 in primary HSCs isolated from rat liver tissues with fibrosis was detected by western blot and qPCR. The results are expressed as relative expression against negative control expression. qPCR was performed to determine the mRNA expression of α -SMA and Coll α 1. The results were expressed as relative expression against control expression. All the experiments were repeated thrice, and a representative result is shown. All values were expressed as mean $\pm$ SEM. **P < 0.01 vs control, ##P < 0.01 vs model.

Fig.4 Decreased expression of PTEN during HSC-T6 cells activation induced by TGF- β 1 and increased expression of PTEN after treated with different concentrations of HD. A. The level of PTEN was analyzed by immunohistochemistry in control, model and HD-treated livers. Representative views from each group are presented ($\times$ 100). **B.** The expression of PTEN in primary HSCs isolated from rat liver tissues with fibrosis was detected by western blot and qPCR. **C.** HSC-T6 cells were treated with different concentrations of HD (1.25, 2.5 5 10 μ M). Western blot analysis showed that PTEN expression up-regulated in a dose dependent manner. Similar findings were demonstrated by q-PCR analysis. The results were expressed as relative expression against control expression. All the experiments were repeated thrice, and a representative result is shown. All values were expressed as mean $\pm$ SEM. **P < 0.01 vs control, ##P < 0.01 vs model. **D.** The level of PTEN in the liver tissue was detected by western blot. **E.** Immunofluorescence staining for PTEN (green), was evaluated by inverted fluorescence microscope, 4,6-diamidino-2-phenylindole (DAPI) stained nuclei blue. Representative views from each group are presented ($\times$ 100).

Fig.5 Role of HD in PTEN-related HSC-T6 activation. HSC-T6 cells were

transfected with GV141-PTEN or GV141-control plasmid, and then they were activated by TGF- β 1 (10ng/ml, 48h). **A.** The protein level of PTEN, α -SMA and Coll α 1 were detected by western blot. **B.** The mRNA level of PTEN, α -SMA and Coll α 1 were assessed by q-PCR analysis. HSC-T6 cells were transfected with PTEN-siRNA or Scrambled-siRNA, then they were activated by TGF- β 1 (10ng/ml, 48h). **C.** Western blot was performed to assess the expression of PTEN, α -SMA and Coll α 1. **D.** Q-PCR was performed to examine the mRNA level of PTEN, α -SMA and Coll α 1. **E.** HSC-T6 cells were treated with TGF- β 1 (10ng/ml, 48h) and bpv (0.25 μ M, 48h). All the experiments were repeated thrice, and a representative result is shown. All values were expressed as mean $\pm$ SEM. **P < 0.01 vs control, ##P < 0.01 vs model, &&P < 0.01 vs model.

Fig.6 Effect of HD on cell proliferation, cell cycle and apoptosis in TGF- β 1-treated HSC-T6 cells. **A.** Effect of HD on cell cycle in TGF- β 1-treated HSC-T6 cells. Representative images of three independent experiments are shown. The results are expressed as relative expression against control group. **B.** Effect of HD on apoptosis in TGF- β 1-treated HSC-T6 cells. Representative images of three independent experiments are shown. The results are expressed as relative expression against control group.

Fig. 7 Role of HD in the activation of PTEN/AKT signaling pathway. **A.** The level of p-AKT was analyzed by immunohistochemistry in control, model and HD-treated group. **B.** Primary HSC cells were isolated from liver tissue with control, CCl $_4$ -treatment and CCl $_4$ plus HD treatment. Western blot was performed to examine the protein level of AKT, p-AKT, cyclinD1 and c-myc. Tissue protein was extracted from live tissue. Western blot was performed to examine the expressions of AKT and p-AKT. **D.** HSC-T6 cells were treated with TGF- β 1 (10ng/ml) and HD (0, 1.25, 2.5, 5, 10 μ M) for 48h. Western blot was performed to examine the protein level of AKT, p-AKT, cyclinD1 and c-myc. **E.** HSC-T6 cells were transfected with GV141-PTEN and then treated with TGF- β 1 (10ng/ml) and HD (10 μ M) for 48h. Western blot was

performed to examine the protein level of AKT, p-AKT, cyclinD1 and c-myc. **F.** HSC-T6 cells were transfected with PTEN-siRNA and then treated with TGF- β 1 (10ng/ml) and HD (10 μ M) for 48h. Western blot was performed to examine the protein level of AKT, p-AKT, cyclinD1 and c-myc. **G.** HSC-T6 cells were treated with TGF- β 1 (10ng/ml) and bpv (0.25 μ M) for 48h. Western blot was performed to examine the protein level of AKT, p-AKT, cyclinD1 and c-myc. All the experiments were repeated thrice, and a representative result is shown. All values were expressed as mean $\pm$ SEM. **P < 0.01 vs control, ##P < 0.01 vs model, &&P < 0.01 vs model.

Table 1 Sequences used for RNA interference

Gene	Forward Primer (5'→ 3')	Reverse Primer (5'→ 3')
Rat		
Si-PTEN	CCGAUACUUCUCUCCAAAUTT	AUUUGGAGAGAAGUAUCGGTT
Scrambled-siRNA	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Table 2 Primers used for real time-PCR.

Gene	Forward Primer (5'→ 3')	Reverse Primer (5'→ 3')
Rat		
β-actin	CCCATCTATGAGGGTTACGC	TTTAATGTCACGCACGATTTC
PTEN	CCATAACCCACCACAG	CAGTCCGTCCTTTCC
α-SMA	CGAAGCGCAGAGCAAGAGA	CATGTCGTCCCAGTTGGTGAT
Colla1	GATCCTGCCGATGTCGCTAT	TGTAGGCTACGCTGTTCTTGCA

Figure 1

Fig 1

Figure 2

Fig 3

Figure 4

Fig 5

Figure 6

Fig 6

Figure 7