



# Hyperoside suppresses BMP-7-dependent PI3K/AKT pathway in human hepatocellular carcinoma cells

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**Background:** New therapeutics for hepatocellular carcinoma (HCC) are urgently needed and searching for new anti-cancer compounds in plant medicines may represent a promising approach. The present study was conducted to clarify the role of hyperoside (HP) and its underlying molecular mechanism in a cancer cell.

**Methods:** Bone morphogenetic protein 7 (BMP-7) protein expression was measured in Human HCC tissue. *In vitro* experiments, HP effects on cell proliferation and the mechanism were investigated deeply.

**Results:** The result showed a higher expression of BMP-7 in human HCC compared to adjacent noncancerous counterparts, and that silencing of BMP-7 suppressed HepG2 cell proliferation, suggesting BMP-7 plays an anti-cancer role in HCC. Furthermore, we found that HP could induce cell cycle arrest in proliferating HepG2 cells at the G1 phase by decreasing BMP-7 expression and that the phosphorylation of AKT and expression of PI3K were significantly down-regulated upon treatment of HP or BMP-7 knockdown. In addition, silencing of BMP-7 abrogated the difference of AKT phosphorylation between cells with and without HP treatment.

**Conclusions:** Our results indicated that HP suppressed cell proliferation by inhibiting the BMP-7-dependent PI3K/AKT signaling pathway in HepG2 HCC cells, and either HP supplement or targeting BMP-7 might be a promising treatment against HCC.

**Keywords:** Hepatocellular carcinoma (HCC); hyperoside (HP); bone morphogenetic protein 7 (BMP-7); cell proliferation; AKT

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## Introduction

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer-related death worldwide, accounting for approximately 8.3% of all cancers (1). Despite therapeutic approaches for patients with HCC having progressed rapidly over the past decades, the mortality rate remains high (2). One of the main reasons for the high mortality among

HCC patients is the lack of effective treatments to deal with the complicated molecular pathogenesis of HCC (3). Thus, it is urgent to discover new molecular targets and develop new therapeutics for the disease.

Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily which regulate cell

proliferation, apoptosis, and differentiation (4,5). In recent years, increasing studies have suggested that the aberrant expression of BMPs is positively correlated with disease progression and survival in several cancers (6-12). However, the role of BMP-7 in cancers is not well characterized and varies when the environment changes (13,14). For example, while BMP-7 can promote and also inhibit cell growth in breast cancer cells in suitable environments (15) and it has been reported as a prognostic biomarker for HCC (16), its role in HCC development remains elusive.

Plant species of the genus *Hypericum* are famous for their use in traditional medicine because of their therapeutic efficacy (17). Hyperoside (HP) is one of the flavonoid components of Celastraceae, Ericaceae and Guttifera, and has been reported to exert anti-cancer effects (18,19). Nevertheless, studies about their effect on HCC and its underlying mechanisms remain elusive. In the current study, we hypothesized that HP inhibits HCC cell proliferation, which may be associated with the down-regulation of BMP-7 expression and blockage of the PI3K/AKT signaling pathway. Our findings support a novel role of HP, as an effective modulator of BMP7 expression and finally a potential therapeutic choice in anti-cancer activity. We present the following article in accordance with the MDAR reporting checklist (available at <https://dx.doi.org/10.21037/atm-21-2980>).

## Methods

### *Reagents and antibodies*

HP was purchased from Selleck (S5453, Houston, TX, USA) and dimethyl sulfoxide (DMSO, D4540) and thiazolyl blue tetrazolium bromide (MTT, M5655) were purchased from Sigma (St. Louis, MO, USA). Mouse monoclonal antibodies  $\beta$ -actin (BS6007M) was purchased from Bioworld Technology (Bloomington, MN, USA), and rabbit anti-AKT (9272), rabbit anti-PI3K (4249), and rabbit anti-phosphorylated AKT (P-AKT, 4060) polyclonal antibody from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-BMP-7 (sc-53917) was purchased from Santa Cruz (Dallas, TX, USA) and rabbit anti-Cyclin D1 (ab226977) polyclonal antibody and rabbit anti-c-Myc (ab9106) polyclonal antibody from Abcam (Cambridge, UK).

### *Tissue samples*

Human HCC tissue samples were acquired from three

HCC patients who underwent liver resection surgery at the Department of Surgery, The First Affiliated Hospital, College of Medicine, Zhejiang University (Hangzhou, China). The patient's adjacent noncancerous tissues (>2 cm away from the tumor) served as the control group in this study. All procedures performed in this study were in accordance with the Declaration of Helsinki (as revised in 2013). Clinical Research Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University approved the design of the study (IIT20210), and the patient sample study protocol. All patients provided signed informed consent.

### *Histopathology*

The liver tissues were fixed in 4% paraformaldehyde for at least 24 hours then processed and embedded in paraffin. Five  $\mu$ m thick sections were then stained with hematoxylin and eosin (H&E).

### *Cell culture*

Human HepG2 cells were obtained from the Cell Bank of Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS, Gibco) at 37 °C humidified incubator under 5% CO<sub>2</sub>. Cells were cultured by adding different concentrations of HP for 24 hours.

### *RNA extraction and quantitative real-time polymerase chain reaction*

Total RNA was extracted from cells using TRIzol (Invitrogen) according to the manufacturer's instruction, and reverse transcribed to cDNA using a cDNA synthesis kit (TaKaRa). The cDNA was subjected for the quantification of gene expression according to the manufacturer's instruction using a SYBR Green PCR Master kit (QIAGEN) and the primers used were listed as follows: BMP-7 (forward: 5'-TCAACCTCGTGGAACATGACA-3'; reverse: 5'-CTTGAAAGATCAAACCGGAACT-3'),  $\beta$ -actin (forward: 5'-CCCACACTGTGCCCATCTACG-3'; reverse: 5'-GCCATCTCTTGCTCGAAGTCC-3').  $\beta$ -actin served as the internal control.

### *Western blot*

Cells were lysed in protein extraction solution (Beyotime,

China) and the protein concentration of each sample was determined by a NanoDrop 2000 Spectrophotometer (Thermo Scientific). Equal amounts of protein were then electrophoresed using SDS-PAGE and blotted onto PVDF membranes (Millipore). After blockade of nonspecific protein binding, membranes were incubated with primary antibodies at 4 °C overnight, and the primary antibodies recognizing BMP-7, AKT, P-AKT, PI3K, Cyclin D1, c-Myc, and  $\beta$ -actin were used by a dilution 1:200, 1:500, 1:500, 1:500, 1:500, 1:500, and 1:3,000, respectively. The membranes were then washed and incubated with HRP-conjugated secondary antibodies. Finally, proteins were visualized with an ECL-chemiluminescent kit (Thermo Scientific).

### ***Cell cycle analysis***

A Cell Cycle Analysis Kit (Beyotime, China) was used according to the manufacturer's instructions. Analysis was performed on Cytomics FC 500 MCL (Beckman Coulter) and cell populations in the G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M phases were quantified by the Multicycle data analysis software package (Verity Software House).

### ***Immunohistochemistry***

Tissue sections were dehydrated, and antigen retrieval was performed in a microwave oven with inculcation in citrate buffer (pH =6.0) for 25 min. Sections were then blocked and incubated with primary antibody against BMP-7 (1:200) at 4 °C overnight. The sections were then washed and incubated with HRP-conjugated secondary antibody for 1 hour at room temperature and the expression of BMP-7 was visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining.

### ***Cell immunofluorescent staining***

Cells in eight-well culture slides were fixed with acetone for 10 min then blocked and incubated with primary antibody against BMP-7 (1:500) at 4 °C overnight. Following incubation with FITC-conjugated secondary antibody, the culture slides were counterstained with DAPI and observed using a Leica confocal microscope (Germany).

### ***Statistical analysis***

Results are presented as mean  $\pm$  SEM. The result analysis was performed using one-way analysis of variance (ANOVA)

or two-tailed unpaired Student's *t*-tests, and  $P < 0.05$  was considered statistically significant. At least three independent experiments are performed in every figure.

## **Results**

### ***Effect of BMP-7 on cell proliferation in human HCC***

BMP-7 has been implicated to be involved in regulating cancer cell proliferation, and to examine whether BMP-7 is involved in HCC, we collected three HCC tissues and their adjacent noncancerous counterparts (referred to as "controls"). H&E staining showed that human HCC tissues exhibited severe cellular edema and inflammatory infiltration compared to the controls (*Figure 1A*), and western blot analysis showed that the expression of BMP-7 was higher in HCC tissues compared to the control tissues (*Figure 1B*). In addition, immunohistochemistry results showed HCC tissues had more BMP-7 positive staining than their controls (*Figure 1C*). These data suggest that up-regulation of BMP-7 may be involved in the development and progression of HCC.

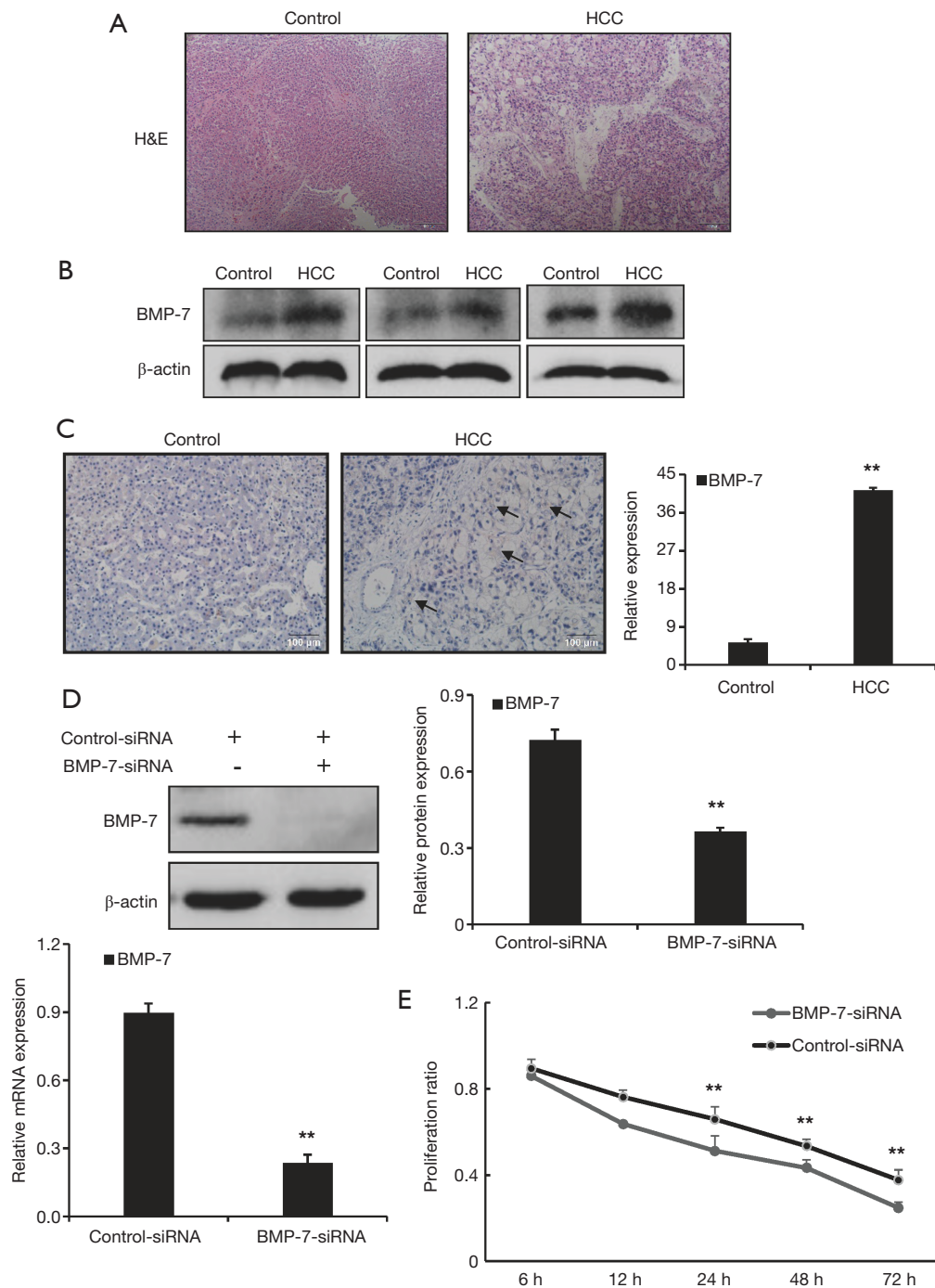
To investigate the role of BMP-7 in cancer cells, we generated BMP-7 knockdown HepG2 cells using siRNA and found that at 24 hours after transfection, BMP-7 was efficiently silenced in BMP-7-siRNA HepG2 cells (*Figure 1D*). Notably, BMP-7-siRNA HepG2 cells showed suppressed proliferation in MTT assay (*Figure 1E*). In summary, our results indicated that BMP-7 played a significant role in the proliferation of HepG2 cells, and that it may be a potential target for human HCC.

### ***HP reduces BMP-7 expression in HepG2 cells***

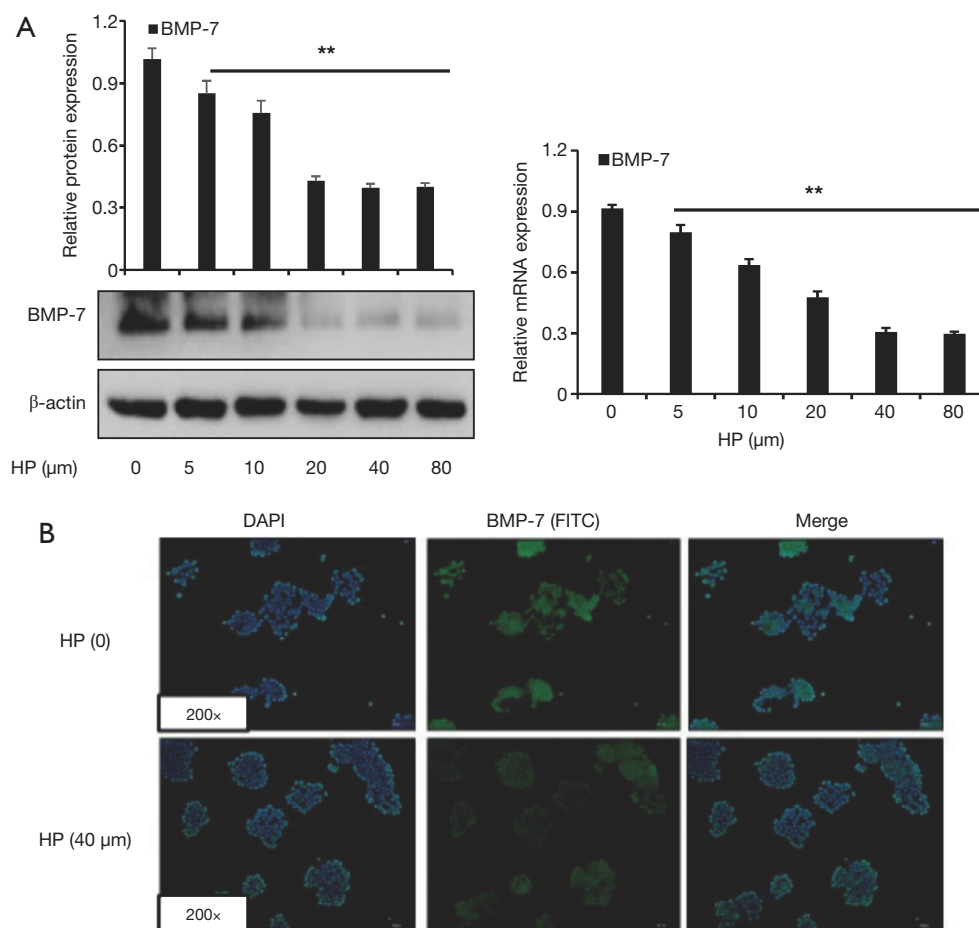
To investigate the effect of HP on the expression of BMP-7 in HepG2 cells, cells were treated with HP (5, 10, 20, 40, and 80  $\mu$ M) for 24 hours. The protein and mRNA levels of BMP-7 in the treatment group were significantly decreased compared with the control group in a dose-dependent manner (*Figure 2A*). Immunofluorescent staining of BMP-7 also indicated that BMP-7 was down-regulated in HP-treated HepG2 cells (*Figure 2B*).

### ***HP suppresses the proliferation and induces cell cycle arrest in HepG2 cells***

To determine the effect of HP on the proliferation of HepG2 cells, cells were treated with HP (5, 10, 20, 40, and



**Figure 1** BMP-7 expression and the effect of BMP-7 on cell proliferation in human HCC. (A) H&E staining was performed in HCC samples and their adjacent noncancerous counterparts (control) (100 $\times$  magnification). (B) Western blotting result shows the protein expression of BMP-7 in human HCC tissues and controls. (C) Immunohistochemistry of BMP-7 in HCC tissues and controls (200 $\times$  magnification). (D) BMP-7 knockdown was performed in HepG2 cells, and the mRNA and protein expression of BMP-7 was detected. (E) Effect of BMP-7 on cell proliferation in HepG2 cells. Cell viability was assessed by MTT assay. Data are expressed as mean  $\pm$  SEM. \*\*,  $P < 0.01$ ; and at least three independent experiments were performed in each panel. BMP-7, bone morphogenetic protein 7; HCC, hepatocellular carcinoma; H&E, hematoxylin and eosin; MTT, thiazolyl blue tetrazolium bromide.



**Figure 2** HP reduces BMP-7 expression in HepG2 cells. (A) Cells were treated with HP (5, 10, 20, 40, and 80 μM) for 24 hours, the protein and mRNA levels of BMP-7 in the cells were measured by western blot and qPCR, and β-actin was served as an internal control. (B) Immunofluorescent staining of BMP-7 was performed in HP-treated HepG2 cells and their controls (200× magnification). Data are expressed as mean ± SEM. \*\*, P<0.01; and at least three independent experiments were performed in each panel. HP, hyperoside; BMP-7, bone morphogenetic protein 7.

80 μM) for 24 hours, and cell viability was measured by MTT assay. The MTT result showed that HP inhibited cell proliferation by 6.87%±0.032%, 23.03%±0.043%, 47.27%±0.035%, 65.36%±0.020%, and 65.5%±0.011%, respectively (Figure 3A), suggesting that HP suppresses HepG2 cell proliferation in a dose-dependent manner.

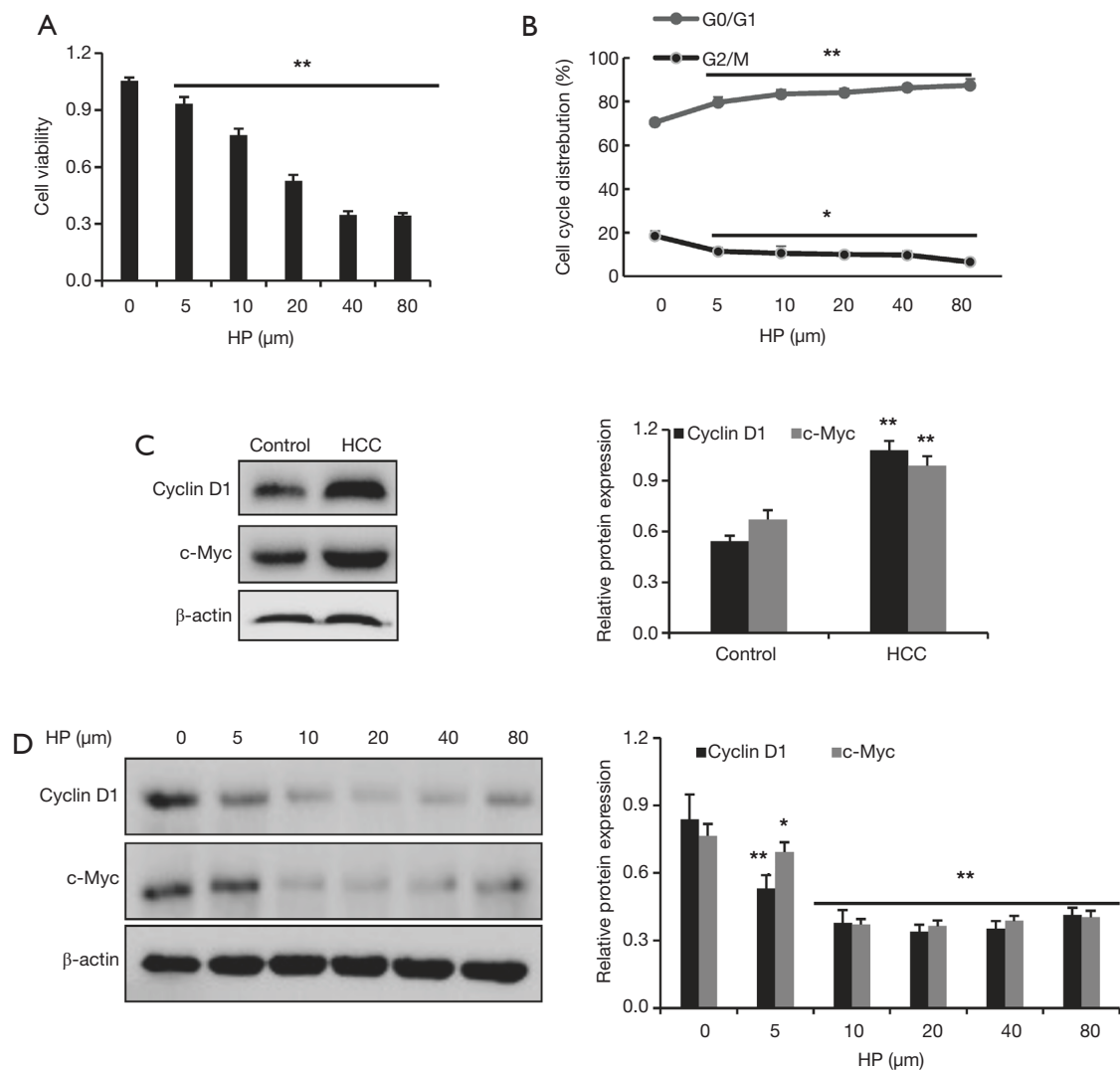
To investigate the potential mechanism by which HP suppresses the proliferation of HepG2 cells, the cell cycle was analyzed by flow cytometry after HP treatment. The results showed that HP remarkably enhanced the percentage of HepG2 cells in the G<sub>0</sub>/G<sub>1</sub> phase compared with that in the control group, and concurrently, cell numbers in the G<sub>2</sub>/M phase decreased in HP-treated cells (Figure 3B).

Cyclin D1 and c-Myc are involved in the progression

of the G<sub>1</sub>/S phase and were obviously enhanced in human HCC tissues (Figure 3C). However, the expression of Cyclin D1 and c-Myc were significantly reduced in cells under administration of HP (Figure 3D). Taken together, these results suggest that HP inhibits cell proliferation in HepG2 cells through cell cycle arrest.

#### **BMP-7 regulates the PI3K/AKT signaling pathway in HepG2 cells**

The PI3K/AKT signaling pathway plays a pivotal role in fundamental cellular functions such as cancer cell differentiation, cell proliferation, and tissue polarity (20). To determine the effect of BMP-7 on the PI3K/AKT

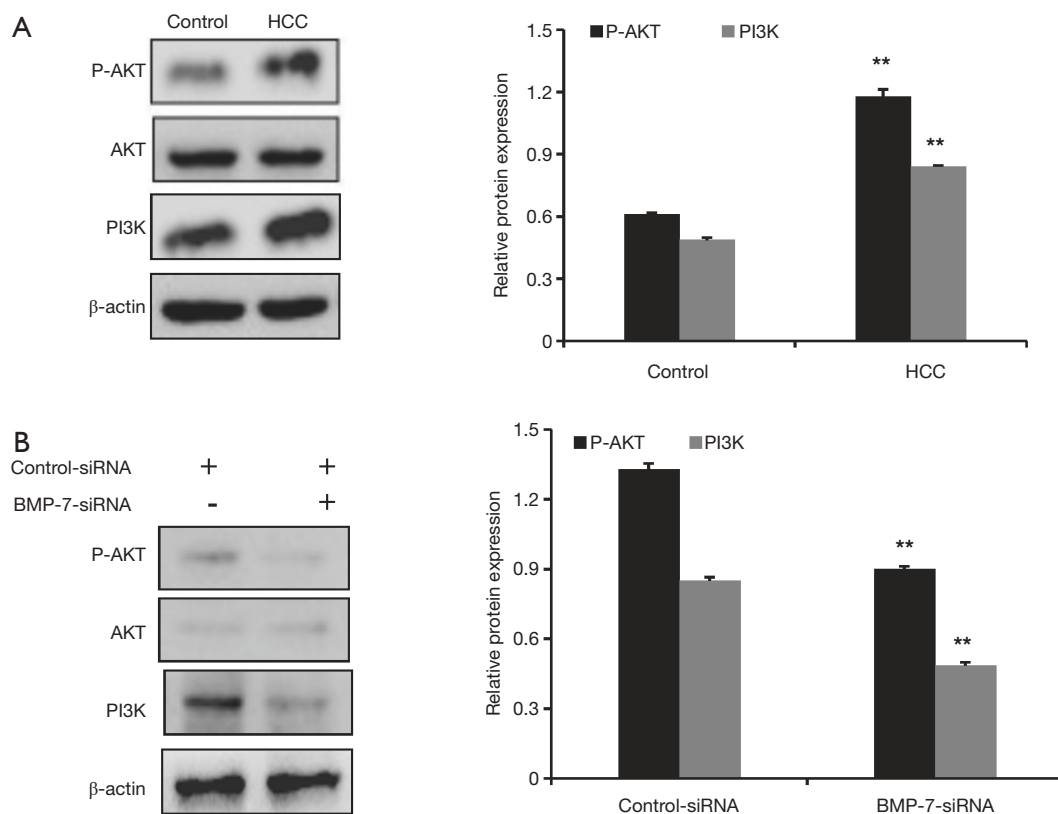


**Figure 3** HP suppresses cell proliferation and induces cell cycle arrest. (A) Cell viability was assessed by MTT assay after HP treatment for 24 hours. (B) Flow cytometry for cell cycle analysis was performed in cells treated with HP for 24 hours. (C) The expression of Cyclin D1 and c-Myc were analyzed by western blot in human HCC and controls. (D) The expression of Cyclin D1 and c-Myc were analyzed by western blot in HepG2 cells treated with HP for 24 hours. Data are expressed as mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; and at least three independent experiments were performed in each panel. HP, hyperoside; MTT, thiazolyl blue tetrazolium bromide; HCC, hepatocellular carcinoma.

signaling pathway in HepG2 cells, the levels of P-AKT, AKT, and PI3K were examined, and western blot analysis indicated that PI3K and P-AKT were obviously up-regulated in human HCC tissues (Figure 4A). Furthermore, the protein expression of P-AKT and PI3K was remarkably down-regulated in cells transfected with BMP-7-siRNA, compared with those in control-siRNA transfected cells (Figure 4B). These data suggest BMP-7 can regulate the PI3K/AKT signaling pathway in HepG2 cells.

#### *HP inhibits the PI3K/AKT signaling pathway through BMP7 regulation in HepG2 cells*

Since both HP and BMP-7 knockdown inhibit hepatocellular cancer cell proliferation and given that HP could attenuate BMP-7 expression in HepG2 cells, we hypothesized that HP inhibited the BMP-7-dependent PI3K/AKT pathway. HepG2 cells were treated with HP for 24 hours, following which the expression of P-AKT and



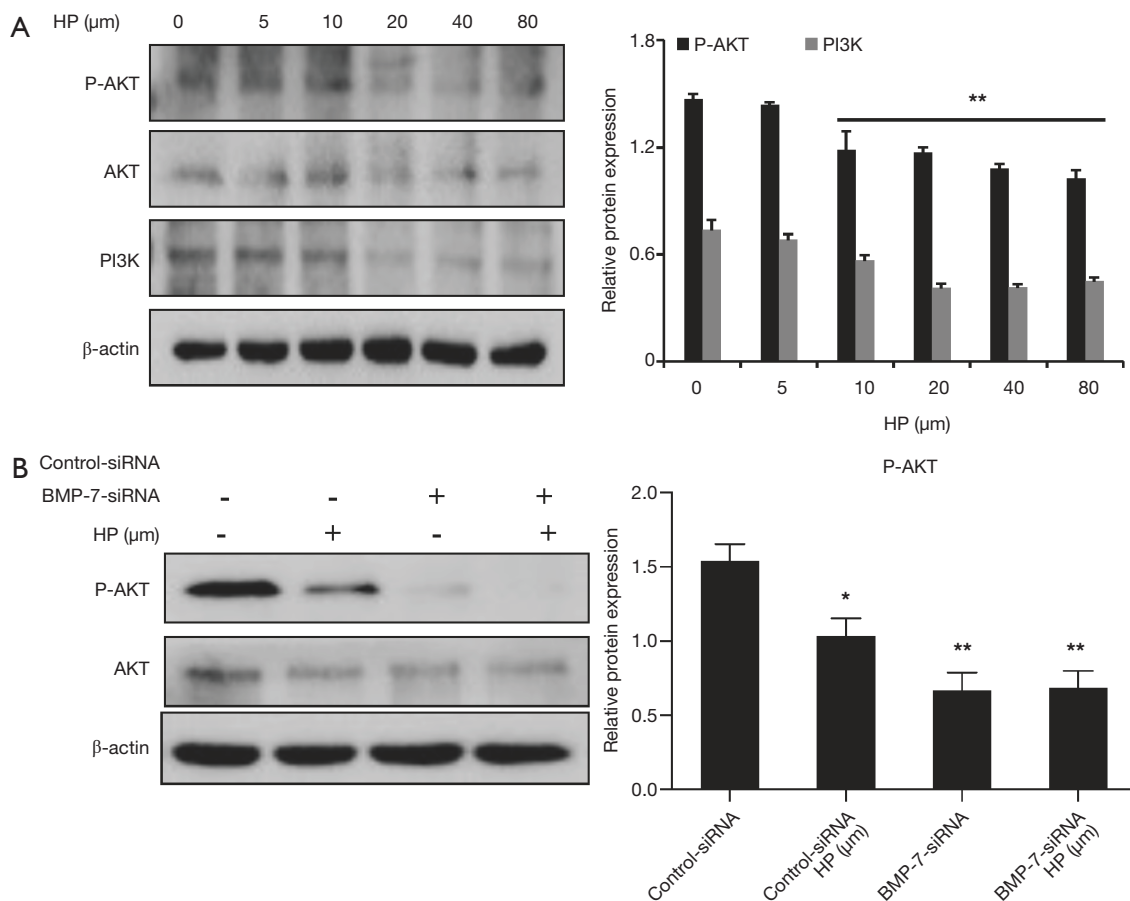
**Figure 4** Effect of BMP-7 on the PI3K/AKT signaling pathway in human HCC tissue and HepG2 cells. (A) The protein expression of PI3K and P-AKT were detected by western blot in human HCC tissues and their controls. (B) The protein expression of PI3K and P-AKT were detected by western blot in HepG2 cells with BMP-7-siRNA and control-siRNA. Data are expressed as mean  $\pm$  SEM. \*\*,  $P < 0.01$ ; and at least three independent experiments were performed in each panel. BMP-7, bone morphogenetic protein 7; HCC, hepatocellular carcinoma.

PI3K were detected by western blot. As shown in *Figure 5A*, the level of P-AKT and PI3K protein was significantly decreased under administration of HP in a dose-dependent manner, and BMP-7 siRNA could abrogate the difference of AKT phosphorylation between cells with and without HP treatment (*Figure 5B*). In conclusion, all of the above results show that HP inhibits cell proliferation by reducing the BMP-7-dependent PI3K/AKT signaling pathway in HCC cells.

## Discussion

In the current study, we found that HP inhibited the proliferation of HepG2 cells through cell cycle arrest and HP inhibited cell proliferation by down-regulating the BMP-7/PI3K/AKT signaling. Current study suggests a novel treatment using HP as a potential therapy against HCC.

Previous studies have reported that BMPs family display significantly higher expression in tumors, which have been used as new biomarkers for the prognosis of cancer patients. In HCC, the member of BMPs (BMP-2, -4, -6, -7, -8, -9, -10, -11, -13, and -15) revealed enhanced expression levels (21). In non-small cell lung cancer, BMP-2 level was increased in patients' serum, and was significantly correlated with poor prognosis. It can be regarded as an independent negative predictor for the prognosis of patients (6). Serum BMP-4 up-expression is closely associated with shorter patients' overall and disease-free survival, which serves as a pivotal marker for predicting the recurrence and prognosis of HCC patients after surgery (22). Recent studies have implied BMP-7 is present in cancers, including prostate, breast, and colon cancers, in which it is involved in regulating cancer cell proliferation (9,10,23). Our current study further verified the increased expression of BMP-7 in



**Figure 5** Effect of HP on the PI3K/AKT signaling pathway in HepG2 cells. (A) Cells were treated with HP for 24 hours, and the protein expression of PI3K and P-AKT were detected by western blot. (B) Cells were treated with or without HP (40  $\mu\text{m}$ ) and with control-siRNA or BMP-7-siRNA for 24 hours, and the protein expression of P-AKT were detected by western blot. Data are expressed as mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; and at least three independent experiments were performed in each panel. HP, hyperoside; BMP-7, bone morphogenetic protein 7.

human HCC tissues, and the silencing of BMP-7 restrained HepG2 cell proliferation *in vitro*. These data show that BMP-7 might be a promising target against human HCC.

BMP-7 is a secreted multifunctional protein, which has various biological activities. It participates in regulating cell proliferation, differentiation, and apoptosis, and plays a critical role in the formation of tissues and organs, the development of embryos, and the repair of injured tissues (24-26). Moreover, BMP-7, which belongs to the superfamily of TGF- $\beta$ , has been implied to play a role in fibrogenesis (27). Chronic fibrotic livers eventually develop liver cirrhosis, which causes HCC, and further investigations are warranted to determine whether targeting BMP-7 in fibrotic or cirrhotic livers can prevent HCC.

HP is a bioactive component isolated from Ericaceae, Celastraceae and Guttifera. Previous studies have revealed that HP has a variety of pharmacological and biological effects including anti-oxidant, anti-viral, and anti-coagulant, especially anti-inflammatory activities (28-30). It has been reported to have remarkable anti-cancer effects (19,31). Although HP has been implied to play a role in HCC cancer cells (32,33), its direct effect has never been determined. We found HP inhibited HCC cancer cell proliferation in a dose-dependent manner, and administration of HP also led to a decline in BMP-7 expression in a dose-dependent manner. Since BMP-7 knockdown also plays an inhibitory role in HepG2 cell proliferation, this suggests that HP suppresses cell proliferation by targeting BMP-7 in HepG2



cells.

The significance of the PI3K/AKT pathway and its potential as a therapeutic target in human cancers has been reported in several preclinical studies, including lung cancer (34), renal cancer (35), glioblastoma (36), breast cancer (37), and neuroblastoma (38). It has been reported that the PI3K/AKT signaling pathway plays a vital role in HCC and is activated in 30–55% of HCC cases (39). In our study, the expression of PI3K and P-AKT was markedly up-regulated in human HCC, which indicated that the mechanisms of BMP-7 involved activation of the PI3K/AKT signaling pathway in human HCC. We also discovered that HP exerted its effect through repressing the PI3K/AKT signaling pathway in HepG2 cells, and BMP-7 knockdown could not further inhibit AKT phosphorylation in HP-treated cells. This indicated that HP suppresses the PI3K/AKT pathway through down-regulating BMP-7.

Previous studies have indicated HP inhibits prostate cancer cell growth by regulation of microRNA-21 and microRNA-27a (17,18). Yet the results of the present study benefit us to offer a novel insight into the mechanism of the inhibitory effect of HP on cell proliferation in human HCC and other cancers. Moreover, Huang *et al.* have previously reported that HP attenuates the inflammatory response by activating PPAR- $\gamma$  in lipopolysaccharide (LPS)-treated macrophages (40). This indicated that HP plays an important role not only in cancer cells, but also in the microenvironment. Thus, further studies should pay close attention to the role of HP in cancer immunity in HCC.

In conclusion, HP, a flavonoid compound derived from a traditional Chinese medicine, suppressed the proliferation of HCC cancer cells by targeting BMP-7, accompanied by cell cycle arrest, and this anti-proliferating effect was mediated by the PI3K/AKT signaling pathway. Our study also highlights the role of BMP-7 in the proliferation of HCC cancer cells and suggests that BMP-7 is a new target against HCC. Our findings support the possibility of the application of flavonoid compounds in anti-HCC therapy, which might lead to new therapeutics for the treatment of HCC. In addition, it would be promising to develop the derivatives of HP according to its chemical structure and achieve the development of lead compounds with better anti-cancer activity.

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### Footnote

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*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/atm-21-2980>). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study were in accordance with the Declaration of Helsinki (as revised in 2013). Clinical Research Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University approved the design of the study (IIT20210), and the patient sample study protocol. All patients provided signed informed consent.

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