

## Research Article

# Compound *Phyllanthus urinaria* L Inhibits HBV-Related HCC through HBx-SHH Pathway Axis Inactivation

Yun Li, Mingjie Jiang, Mingshun Li, Yingjie Chen , Chunshan Wei, Lisheng Peng, Xinliang Liu, Zhen Liu, Guangdong Tong , Daqiao Zhou , and Jinsong He 

Shenzhen Traditional Chinese Medicine Hospital,  
The Fourth Clinical Medical College of Guangzhou University of Chinese Medicine, China

Correspondence should be addressed to Jinsong He; [hjsprayforyou@sina.com](mailto:hjsprayforyou@sina.com)

Received 20 October 2018; Accepted 24 February 2019; Published 24 March 2019

Academic Editor: Yuewen Gong

Copyright © 2019 Yun Li et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

*Compound Phyllanthus urinaria* L (CP) is a traditional formula widely used in clinical practice for hepatocellular carcinoma (HCC), especially HBV-related HCC. HBx, HBV X gene encoded X protein, has positive correlation with the abnormal SHH pathway in HBV-related HCC. So, we predicted that CP has the capability of anti-HBV-related HCC maybe via inactivating the HBx-Hedgehog pathway axis. HepG2-HBx cells, HBx overexpression, were treated with CP (70 µg/ml and 35 µg/ml, respectively) for 48 hours and the mice which received the HepG2-HBx cells were treated with CP (625 mg/kg and 300 mg/kg, respectively) for 17 days to evaluate the effect of CP on HBV-related HCC. HBx could accelerate HepG2 cells proliferation, clone formation, and migration in vitro and also could strengthen tumor growth in mice. However, CP could significantly decrease HepG2-HBx cells proliferation, clone formation, and migration in vitro and also could inhibit tumors growth in mice in a dose-dependent manner. Mechanism studies suggested that HBx upregulated the mRNA and proteins expression of Sonic hedgehog (SHH), transmembrane receptor patched (PTCH-1), smoothened (SMO), oncogene homolog transcription factors-1 (GLI-1), and oncogene homolog transcription factors-2 (GLI-2), which are compositions of the SHH pathway. CP could inhibit the mRNA and proteins expression of SHH, PTCH-1, GLI-1, and HBx. It may be one of the underlying mechanisms of CP to delay the HBV-related HCC development through the HBx-SHH pathway axis inactivation.

## 1. Introduction

Hepatocellular carcinoma (HCC) is one of the common cancers with high mortality and morbidity rate, as well as recurrence rate in spectrum of diseases. Chronic Hepatitis B virus (HBV) infection is a main risk factor for HCC development [1]. There were estimated nearly two hundred million people with HBV, which led to nearly one million people death all over the world in 2015 [2].

HBV X gene encode X protein (HBx) is the core protein in HBV replication progression, strongly associated with tumorigenesis [3]. Growing evidence suggests that HBx protein promotes cell cycle progression, inactivates the expression of tumor suppressor gene, such as p53, and inhibits negative growth regulator [4]. However, the whole spectrum of HBx-modulating HBV-related HCC remains not fully uncovered.

The Hedgehog pathway plays a critical role in tissue repair and regeneration, as well as involved in tumorigenesis [5]. The Hedgehog pathway includes three types of Hh ligands, SHH, Indian Hedgehog (IHH), Desert Hedgehog (DHH), and one receptor patched (Ptc), which in human is PTCH-1. In the absence of ligand, GLI is inactivated and that maintains normal homeostasis. Once ligand binds to Ptc, GLI is activated and migrates to cell nucleus; a wide tissue-specific target genes are activated, such as cyclins and bcl-2, as well as its compositions GLI-1 and GLI-2 [6, 7]. Accumulating evidence from in vitro and in vivo indicated that abnormal SHH pathway activation has been found in breast cancer, prostatic cancer, and ovarian cancer as well as liver cancer [8–10]. The SHH pathway is becoming a potential target for treating cancers. In our previous study, we found that SHH, PTCH-1, and GLI-2 were overexpression in the paracarcinoma tissue of HBV-related HCC patients,

and they have positive correlation with HBx (data not show), which revealed that maybe HBx activate the SHH pathway, contributing to HBV-related HCC development.

*Compound Phyllanthus urinaria L* (CP) [*Phyllanthus urinaria L*, *Astragalus membranaceus* (Fisch.) Bunge, *Scutellaria barbata* D. Don, *Curcuma zedoaria* (Christm.) Rosc, and *Cremastra appendiculata* (D. Don) Makino] is a formula widely used to treat HCC, especially HBV-related HCC in clinical practice [11]. CP has various pharmacological functions, such as antiviral and antifibrosis, and also can stimulate immune response. Except for HCC, CP also can be used to treat liver injury, hepatic fibrosis, and cirrhosis [12, 13]. However, the molecular mechanism of CP anti-HBV-related HCC is uncertain. In our previous studies, we have proved that CP has a capability of reducing the expression of HBx [14]; moreover, we also found that HBx has a positive correlation with the SHH pathway activation in HBV-related HCC patients' liver samples (data not shown). Therefore, we predicted that HBx might activate the SHH pathway to promote HBV-related HCC development, and the capability of CP anti-HBV-related HCC might be based on HBx-SHH pathway axis inactivation.

## 2. Materials and Methods

**2.1. Preparation and HPLC Analysis of CP.** CP [*Phyllanthus urinaria L* (30g), *Astragalus membranaceus* (Fisch) Bunge (15g), *Scutellaria barbata* D. Don (10g), *Curcuma zedoaria* (Christm) Rosc (10g), and *Cremastra appendiculata* (D. Don) Makino (10g)] purchased from the first affiliated hospital of Guangzhou university of Chinese medicine (Guangzhou, China), was soaked in 1L water for 40 min at room temperature and incubated at 100°C for 1 h, and then, the extract was concentrated at rates of 50rpm, 50°C by Rotary Evaporator (RE-2000, Yarong), and filtrated with 0.22 $\mu$ m filter in Clean Bence (Healforce, China).

The main ingredients of CP were analyzed using an Agilent HPLC system (1260 series, Agilent Technology, Santa Clara, CA, USA) with a phenomenon Luna 5u C18(2) 100A column (250mm $\times$ 4.6mm, 5 $\mu$ m). Signals were detected at 270 nm using a UV monitor (1260 series, Photo-Diode Array UV/V, Agilent Technology). The gallic acid, quercetin, and luteolin were quantitated using a calibration curve established by each standard (500 $\mu$ g/ml, 502.17 $\mu$ g/ml, and 505 $\mu$ g/ml, respectively) in to HPLC system. Gallic acid, quercetin, and luteolin for HPLC analysis were purchased from Yuanye Phytochemicals Ltd. (Shanghai, China).

**2.2. Establishment of Stable Cell Lines.** Lentiviral particles expressing HBx or control sequence (NC) were constructed by Wenqi Bioscience Inc. (Guangzhou, China). HepG2 cells (American Type Culture Collection) were infected with lentiviral particles expressing control sequence or HBx and then selected with 1.6 $\mu$ g/ml puromycin (MedChemExpress, China). After antibiotic election for around 7 days, remaining cells (HepG2-HBx; HepG2-NC) were collected and over-expression of HepG2-HBx cells was used for next studies.

**2.3. MTT Assays.** HepG2-HBx cells were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA). HepG2-HBx cells were treated with CP at different concentrations for 24 h and 48 h; the OD<sub>490</sub> and cell viability were measured with the MTT (Beyotime, Guangzhou) using Microplate reader (Thermo. USA) [15]. IC<sub>50</sub> of CP was analyzed by Graphpad prism 5 and the dosages of 1/2 and 1/4 IC<sub>50</sub> were used for the next studies in vitro.

Then, the HepG2-HBx cells were treated with CP at the concentration of 1/2 IC<sub>50</sub> and 1/4 IC<sub>50</sub> to observe the effect of CP on HepG2-HBx cells proliferation. Cyclopamine (70 $\mu$ M), SHH pathway inhibitor, was used in our studies.

**2.4. Migration Assay.** HepG2-HBx cells could be scratched at interval 0.5cm in 6-well plate and were treated with CP (1/2 IC<sub>50</sub>; 1/4 IC<sub>50</sub>, respectively) and cyclopamine (70 $\mu$ M) for 48 h, respectively. HepG2-NC cells were treated without any medicine. The cells could be pictured by Fluorescence Inversion microscope system (OLYMPUS, USA).

**2.5. Clone Formation.** 3  $\times$  10<sup>2</sup> HepG2-HBx cells and HepG2-NC cells were cultured in 6-well plate for 2 days. On 3 day, the HepG2-HBx cells were treated with cyclopamine (70 $\mu$ M) and CP (1/2 IC<sub>50</sub>; 1/4 IC<sub>50</sub>, respectively). After 14 days, the cells were fixed with 4% paraformaldehyde and stained with Giemsa (Leagene, Beijing). The pictures were performed by Single Lens Reflex (Canon, Japan).

**2.6. Real-Time PCR.** Total RNA was isolated by using TRIzol reagent (Takara, Japan). The first-strand cDNA in a 10 $\mu$ L volume was carried out with conditions comprised 15 min at 30°C, 42°C for 60 min, and 72°C for 10 min. Real-time PCR was carried out using the SYBR Premix Ex Taq (Takara, Japan) according to the manufacturer's protocol with ABI-7500 (ABI, USA). Briefly, each PCR was measured in duplicate in a 20 $\mu$ L volume cycling conditions comprised 2 min at 50°C, followed by 40 cycles at 95°C for 10 min, 95°C for 15s, 60°C for 1 min, 95°C for 30s, and 60°C for 15 min.  $\beta$ -actin was used as the internal control. The mRNA relative expression of HBx, SHH, PTCH-1, SMO, GLI-1, and GLI-2 was quantified using 2<sup>- $\Delta\Delta$ CT</sup> method. Primer sequences are shown in Table 1.

**2.7. Western Blot Analysis.** Total protein was extracted from the cells into the RIPA buffer (Beyotime, China) and supplemented with protease inhibitor PMSF (Beyotime, China). Western blot were performed by SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membranes were probed with primary antibodies: HBx, SHH, PTCH-1, SMO, GLI-1, GLI2, and GAPDH (Abcam, USA). The results were visualized with the C-DiGit imager (Li-Cor, USA), and the levels of proteins expression were normalized by GAPDH loading control.

**2.8. Immunohistochemistry.** The tumors were fixed with 4% paraformaldehyde and embedded in paraffin. The sections (5 $\mu$ m) were incubated with antibodies: HBx, SHH, PTCH-1, SMO, and GLI-1 (Abcam, USA). The pictures were captured

TABLE 1: Primer sequences used in RT-PCR.

Genes		Primer sequence
GLI-1	Forward	CTGGATCGGATAGGTGGTCT
	Reverse	CAGAGGTTGGGAGGTAAGGA
GLI-2	Forward	GCCCTTCCTGAAAAGAAGAC
	Reverse	CATTGGAGAAACAGGATTGG
SHH	Forward	AAAGCTGACCCCTTTAGCCTA
	Reverse	TTCGGAGTTTCTTGTGATCTTCC
PTCH-1	Forward	CCGTTCAGCTCCGCACAGA
	Reverse	CTCACTCGGGTGGTCCCATAAA
SMO	Forward	GAGCGTAGCTTCCGGGACTA
	Reverse	CTGGGCCGATTCTTGATCTCA
HBx	Forward	TTTTCAGAGGCCAGTCAACGA
	Reverse	GAGCCCTGTCAGGTCCACAA
$\beta$ -ACTIN	Forward	ATCGTGCGTGACATTAAGGAGAAG
	Reverse	AGGAAGGAAGGCTGGAAGAGTG

by Fluorescence Inversion microscope system (Olympus, USA)

**2.9. Immunofluorescence Staining.**  $6 \times 10^4$  cells were cultured in laser confocal dishes (NEST, China). After treated for 48 h with cyclopamine and CP (1/2  $IC_{50}$ ; 1/4  $IC_{50}$ , respectively), the cells were fixed by 4% paraformaldehyde and then permeabilized followed by probed with GLI-1 and DAPI. The pictures were captured by Confocal Laser Scanning Microscope (Leica, Germany).

**2.10. Animals and Experimental Design.** All BABL/c nude mice (n=30) were purchased from Medical Experiment Center of Guangdong Province (Guangzhou, China) and were received humane care and used in accordance with the Guangzhou University Chinese Medical Animal Care and Welfare Committee. After one week of acclimatization, a total of  $1 \times 10^7$  HepG2-HBx cells were suspended in PBS and injected subcutaneously into the mice to build inducing HepG2-HBx cells transplant tumor models. The mice which received HepG2-HBx cells were divided into four groups (n=6 in each group): HepG2-HBx group [PBS, intragastrically (i.g.)], cyclopamine group 30 mg/kg, i.g., and CP groups (625mg/kg and 300 mg/kg, i.g., respectively). The HepG2-NC cells were injected subcutaneously into the mice in the group of HepG2-NC to build inducing HepG2-NC cells transplant tumor models (n=6). After two weeks, the medicines were given to the mice receiving the HepG2-HBx cells when the tumors volume is  $3 \times 3\text{mm}^2$ . Tumors size and mice weight were measured over a three-day interval. After 17 days, the mice were sacrificed, and the tumors were dissected.

**2.11. Statistical Analysis.** All parameters in our studies were expressed with mean  $\pm$  standard deviation. Statistical analysis was using version 18.0 (SPSS, USA). Groups differences were performed by the Analysis of variance (ANOVA) followed by a least significant difference test (LSD).  $P < 0.05$  was considered to be significant. Graphs were plotted by Graphpad prism version 5 (GraphPad Software, San Diego, CA, USA).

### 3. Results

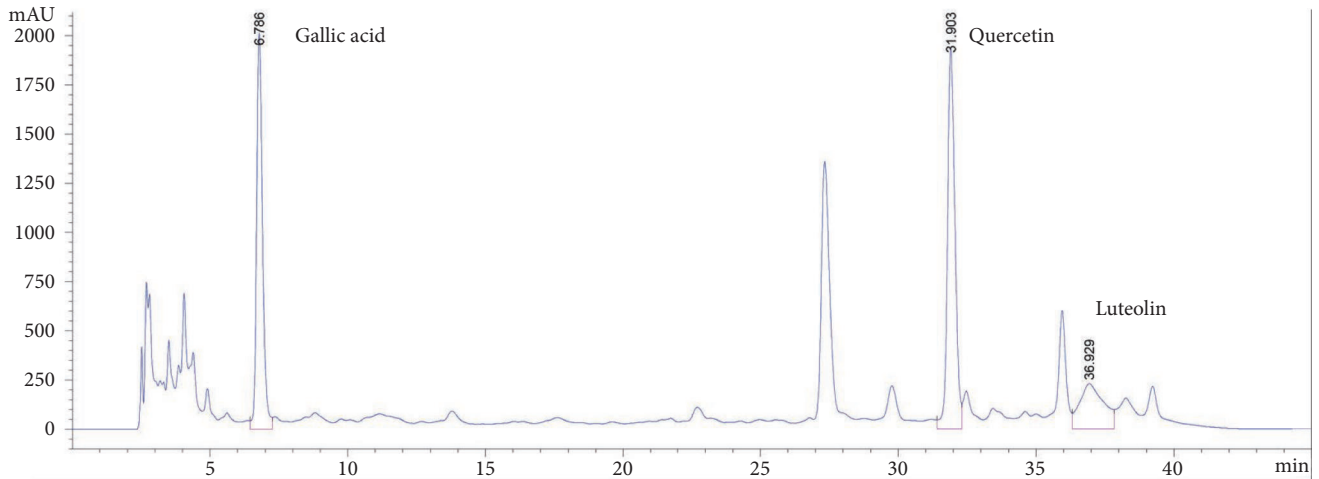
**3.1. HPLC Analysis of CP.** The ingredients of CP and standard compounds were analyzed by HPLC at 270 nm. Gallic acid, quercetin, and luteolin were detected in CP (Figure 1(a)), with their total peak area percentages were 4.2%, 8.9%, and 11.7%, respectively. The standard compounds were measured by HPLC (Figure 1(b)). The structures of gallic acid, quercetin, and luteolin were represented (Figures 1(c), 1(d), and 1(e), respectively).

**3.2. CP Could Inhibit Proliferation of HepG2-HBx Cells.** To evaluate the effect of CP on the HepG2-HBx cells, we analyzed the cells proliferation using MTT. Being treated for 24 h, only the CP dosages of 140  $\mu\text{g/ml}$  could inhibit HepG2-HBx cells proliferation (Figures 2(a) and 2(b)). However, CP could obviously inhibit the proliferation when HepG2-HBx cells were treated for 48 h (Figures 2(c) and 2(d)). We calculated the  $IC_{50}$  of CP which is 144.2  $\mu\text{g/ml}$  using the Graphpad prism soft when HepG2-HBx cells were treated for 48 h (Figure 2(e)). The CP dosages of 70  $\mu\text{g/ml}$  and 35  $\mu\text{g/ml}$  could be used for the next studies in vitro.

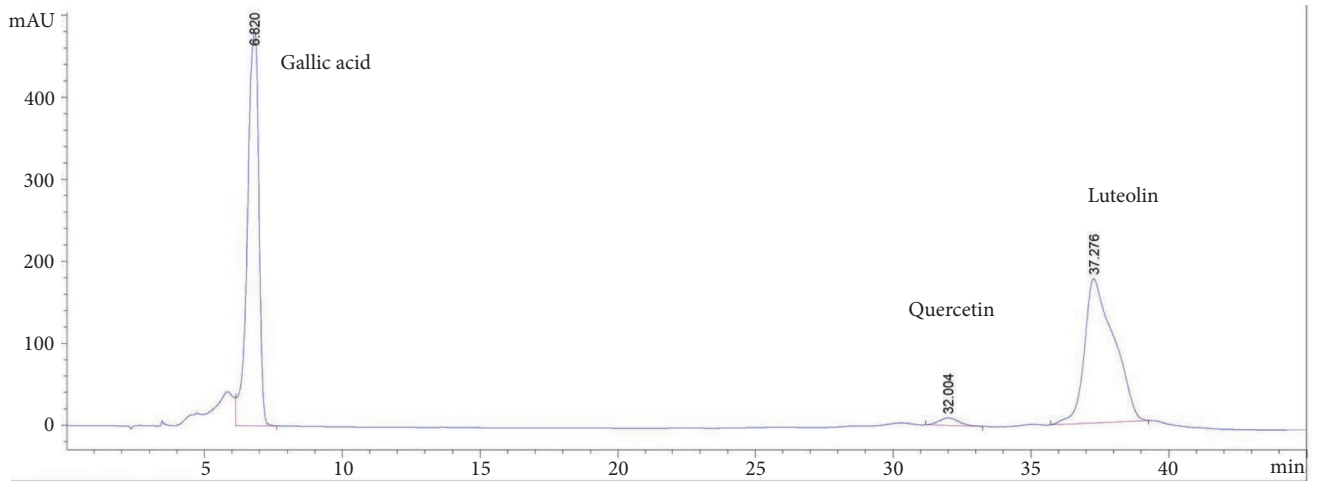
We found that HBx obviously promoted the proliferation of HepG2 cells, compared with the group of HepG2-NC. At the same time, cyclopamine (70  $\mu\text{M}$ ), a SHH pathway inhibitor, could suppress the HepG2-HBx cells proliferation compared with the group of HepG2-HBx. We also found that it could obviously decrease the proliferation of HepG2-HBx cells when HepG2-HBx cells are treated with CP (70  $\mu\text{g/ml}$ ; 35  $\mu\text{g/ml}$ , respectively) for 48 h, compared with the group of HepG2-HBx (Figure 2(f)).

**3.3. CP Could Inhibit Clone Formation and Migration of HepG2-HBx Cells.** Cell migration is a physiological process of normal growth and involves in embryo development, angiogenesis, wound healing, immune response, inflammation, and cancer metastasis. At the same time, migration also indicates the capability of invasive and metastasis. Oncogenic HBx promoted HepG2 cell migration, whereas the migration in HepG2-HBx cell administration CP (70  $\mu\text{g/ml}$ ; 35  $\mu\text{g/ml}$ , respectively) was reduced compared with the group of HepG2-HBx (Figure 3(a)).

Clone formation is an indicator of cells proliferation rate and a capability of adaptability to living environment. HBx could accelerate the clone formation of HepG2 cells compared with HepG2-NC cells. CP could inhibit the clone formation in HepG2-HBx cells depending on dose-effect relationship (Figure 3(b)). And we also measured the clone numbers  $\geq 50$  cells. As expected, HBx significantly increased the clone numbers  $\geq 50$  cells compared with the HepG2-NC group; the clone numbers  $\geq 50$  cells were decreased in HepG2-HBx cells treatment with CP (70  $\mu\text{g/ml}$ ; 35  $\mu\text{g/ml}$ , respectively) (Figure 3(c)). Collectively, all of the data we presented indicated that HBx could robustly increase the proliferation, migration, and clone formation, whereas CP could significantly inhibit the proliferation, migration, and clone formation of HepG2-HBx cells in vitro.



(a)



(b)

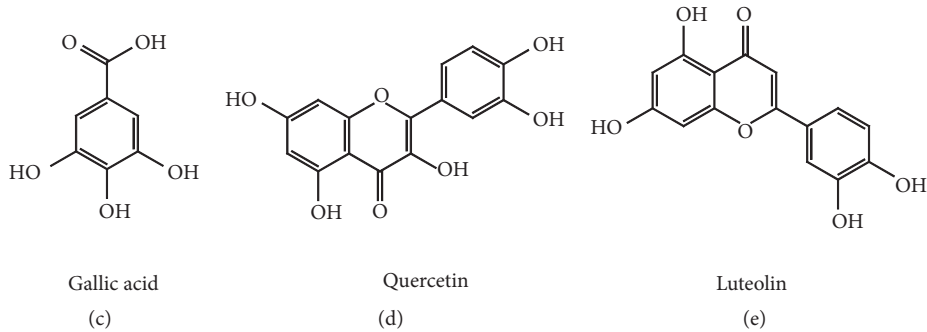


FIGURE 1: Representative high-performance liquid chromatography profile of CP. Gallic acid, quercetin, and luteolin were detected at 270 nm. (a) and (b) represented HPLC analysis of CP and standard compounds, respectively; (c), (d), and (e) presented the structures of gallic acid, quercetin, and luteolin, respectively.

**3.4. CP Decreased the mRNA Expressions of HBx, SHH, PTCH-1, and GLI In Vitro.** Based on our previous studies, we predicted that CP which inhibited HBV-related HCC might be through HBx-SHH pathway axis inactivation. To validate our prediction, we measured the mRNA expressions of HBx, SHH, PTCH-1, SMO, GLI-1, and GLI-2 in vitro. Our data revealed that mRNA expression levels of SHH, PTCH-1,

SMO, GLI-1, and GLI-2 were robustly elevated in HepG2-HBx cells compared with the HepG2-NC cells, which showed that HBx could activate the SHH pathway in HepG2 cells. As we expected, the mRNA expressions of SHH, PTCH-1, and GLI-1 were significantly decreased in HepG2-HBx cells treatment with CP (70  $\mu\text{g/ml}$ ; 35  $\mu\text{g/ml}$ , respectively). What is more, CP not only represses the above factors, but also

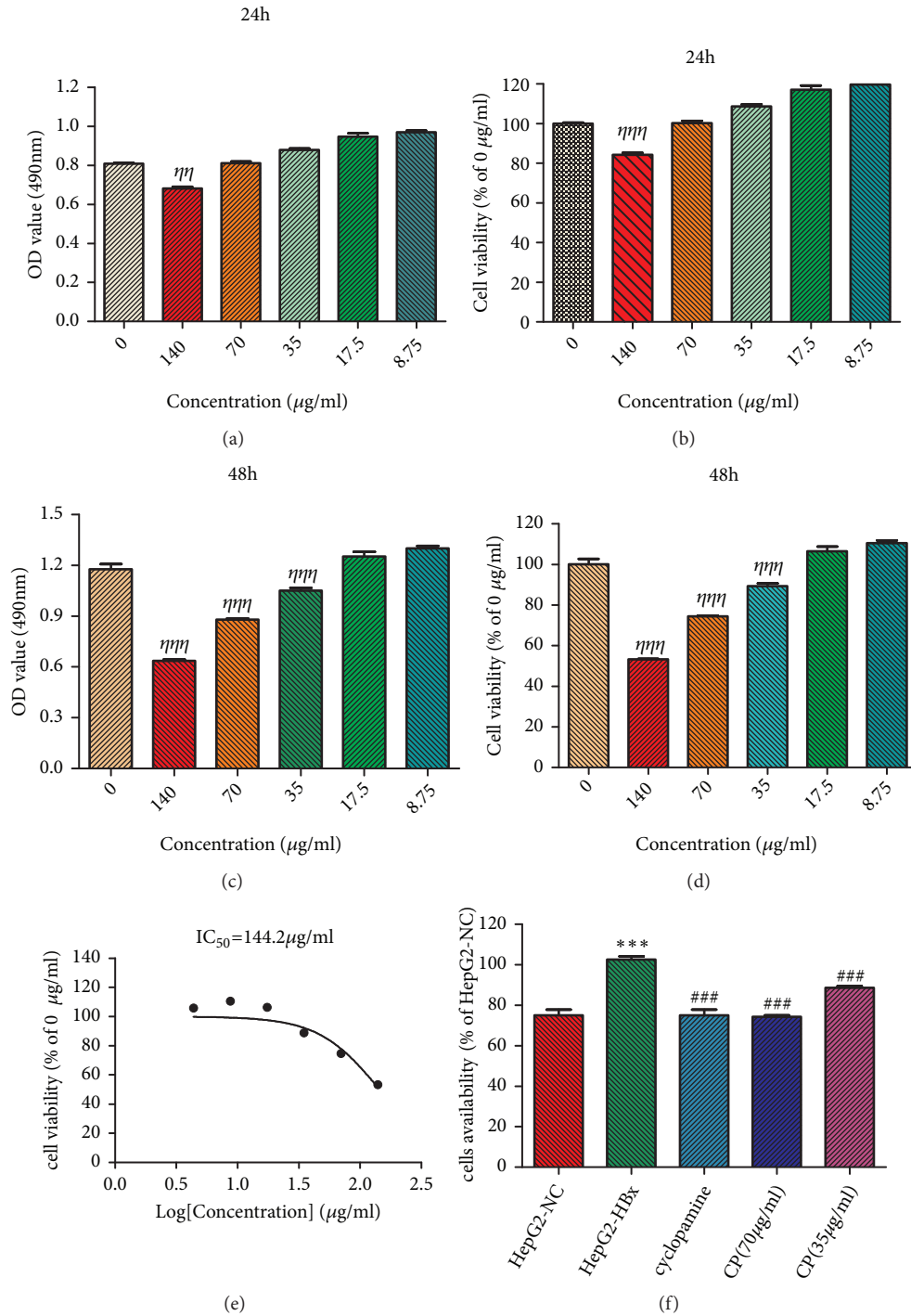


FIGURE 2: CP could inhibit proliferation of HepG2-HBx cells. Being treated for 24 h (a, b) and 48 h (c, d), the proliferation was measured by MTT. IC<sub>50</sub> of the CP was calculated using the Graphpad prism soft (e). Then we measured the proliferation of HepG2-HBx cells administrated with CP or cyclopamine (70 μM) for 48 h (f). <sup>\*\*\*</sup>P < 0.001, compared with HepG2-HBx cells administration with 0 μg/ml. <sup>\*\*\*</sup>P < 0.001, compared with the group of HepG2-NC. <sup>###</sup>P < 0.001, compared with the group of HepG2-HBx.

could inhibit HBx in a dose-effect relationship compared with the group of HepG2-HBx. However, CP (70 μg/ml; 35 μg/ml, respectively) has no inhibition on SMO and GLI-2 (Figure 4).

3.5. CP Inhibited the Proteins Expression of HBx, SHH, PTCH-1, and GLI-1 In Vitro. To further elucidate the speculation,

we measured the proteins expression in HepG2-HBx cells treatment with CP. In accordance with the results of mRNA, HBx could increase proteins expression of SHH, PTCH-1, SMO, GLI-1, and GLI-2 in HepG2-HBx cells compared with the HepG2-NC cells, which implied that HBx could activate the SHH pathway in HepG2 cells. The proteins expression of

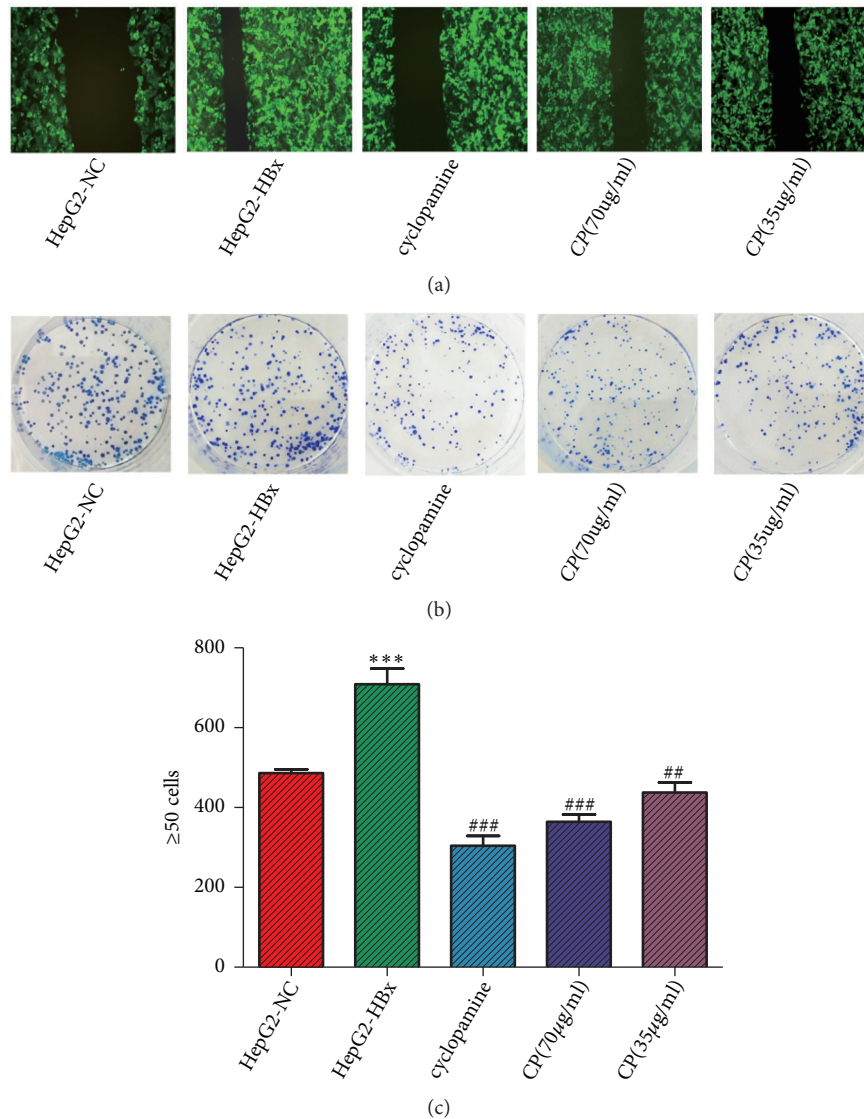


FIGURE 3: CP could inhibit migration and clone formation of HepG2-HBx cells. Migration (a), clone formation (b), and the clone numbers  $\geq 50$  cells (c) were measured when HepG2-HBx cells were treated with CP (70  $\mu\text{g}/\text{ml}$ ; 35  $\mu\text{g}/\text{ml}$ , respectively) and cyclopamine (70  $\mu\text{M}$ ) for 48 h. The pictures were captured by the Fluorescence Inversion microscope system ( $\times 10$ ) or Single Lens Reflex. \*\*\*  $P < 0.001$ , compared with the group of HepG2-NC; #  $P < 0.01$  and ###  $P < 0.001$ , compared with the group of HepG2-HBx.

SHH, PTCH-1, and GLI-1 was downregulated in HepG2-HBx cells treatment with CP (70  $\mu\text{g}/\text{ml}$ ; 35  $\mu\text{g}/\text{ml}$ , respectively) compared with HepG2-HBx cells. CP not only inhibited the proteins expression of SHH, PTCH-1, and GLI-1, but also could reduce the protein expression of HBx, compared with the HepG2-HBx cells. However, CP (70  $\mu\text{g}/\text{ml}$ ; 35  $\mu\text{g}/\text{ml}$ , respectively) could not reduce the proteins expression of SMO or GLI-2 (Figure 5). Taken together, all the results of mRNA and proteins implied that HBx activated the SHH pathway in HepG2 cells, and CP could inactivate HBx, SHH, PTCH-1, and GLI-1 in HepG2-HBx cells.

**3.6. CP Could Inhibit the GLI-1 Expression in Cytoplasm.** GLI-1 is an indicator of the SHH pathway activation. From the data from the mRNA and protein, we found that the

HBx could increase the expression of GLI-1 in HepG2-HBx cells. In order to clearly observe the change of GLI-1 in HepG2-HBx cells treated with CP (70  $\mu\text{g}/\text{ml}$ ; 35  $\mu\text{g}/\text{ml}$ , respectively), we measured the level of GLI-1 in cytoplasm by immunofluorescence. As expected, HBx could augment fluorescence intensity of GLI-1 in cytoplasm compared with the HepG2-NC cells. And CP (70  $\mu\text{g}/\text{ml}$ ; 35  $\mu\text{g}/\text{ml}$ , respectively) could robustly attenuate the expression of GLI-1 in cytoplasm compared with the HepG2-HBx cells (Figure 6). Our data showed that CP could reduce the expression of GLI-1 in cytoplasm.

**3.7. CP Could Inhibit Tumors Growth in Mice.** To validate our results in vivo, we transplanted the HepG2-NC cells and HepG2-HBx cells into the Balb/c nude mice to observe

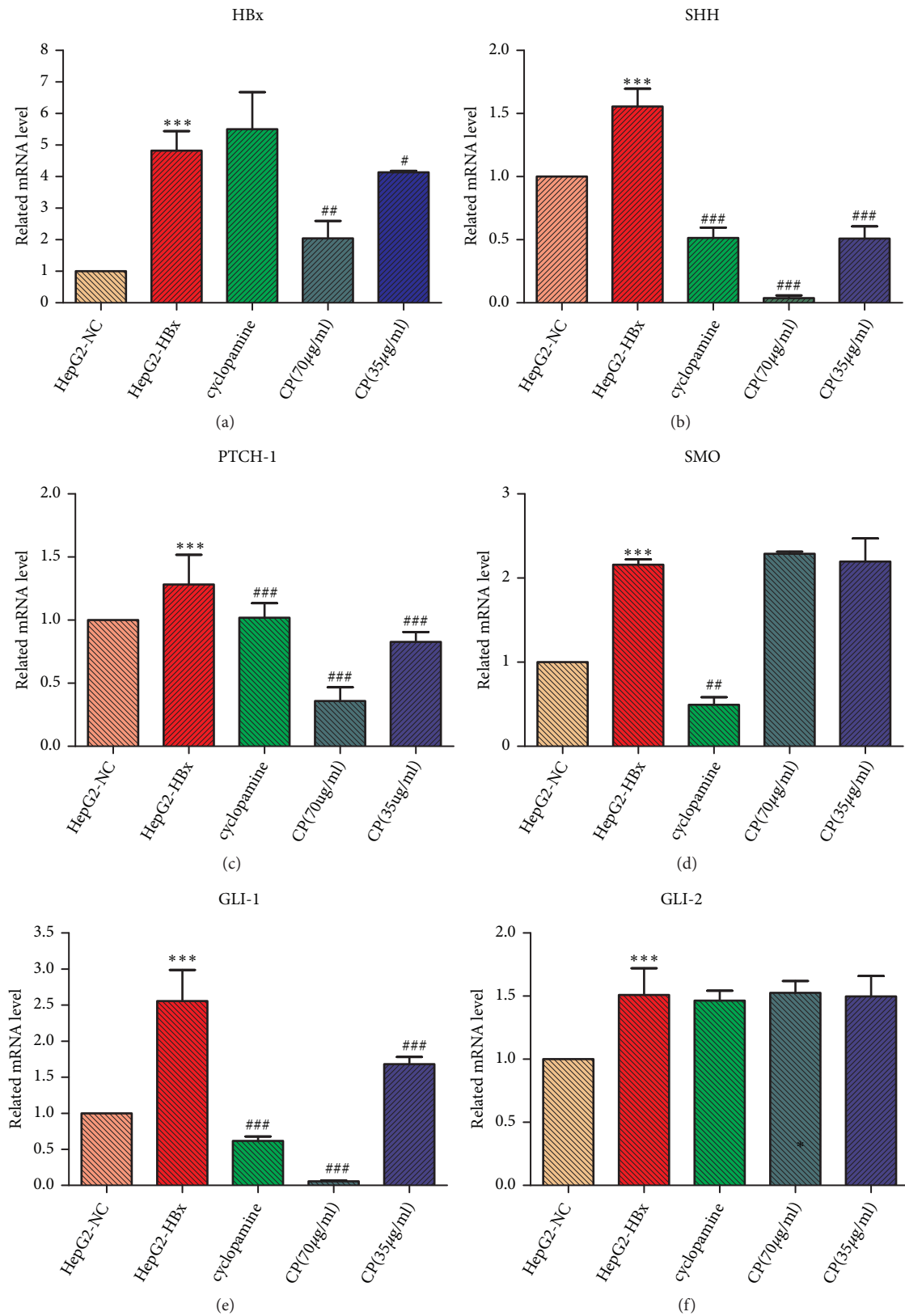


FIGURE 4: CP decreased the mRNA expressions of HBx, SHH, PTCH-1, and GLI-1 in HepG2-HBx cells. The mRNA was measured when HepG2-HBx cells were treated with CP (70μg/ml; 35 μg/ml, respectively) and cyclopamine (70μM) for 48 h using RT-PCR. \*\*\* $P < 0.001$ , compared with the group of HepG2-NC; # $P < 0.05$ , ## $P < 0.01$ , and ### $P < 0.001$ , compared with the group of HepG2-HBx.

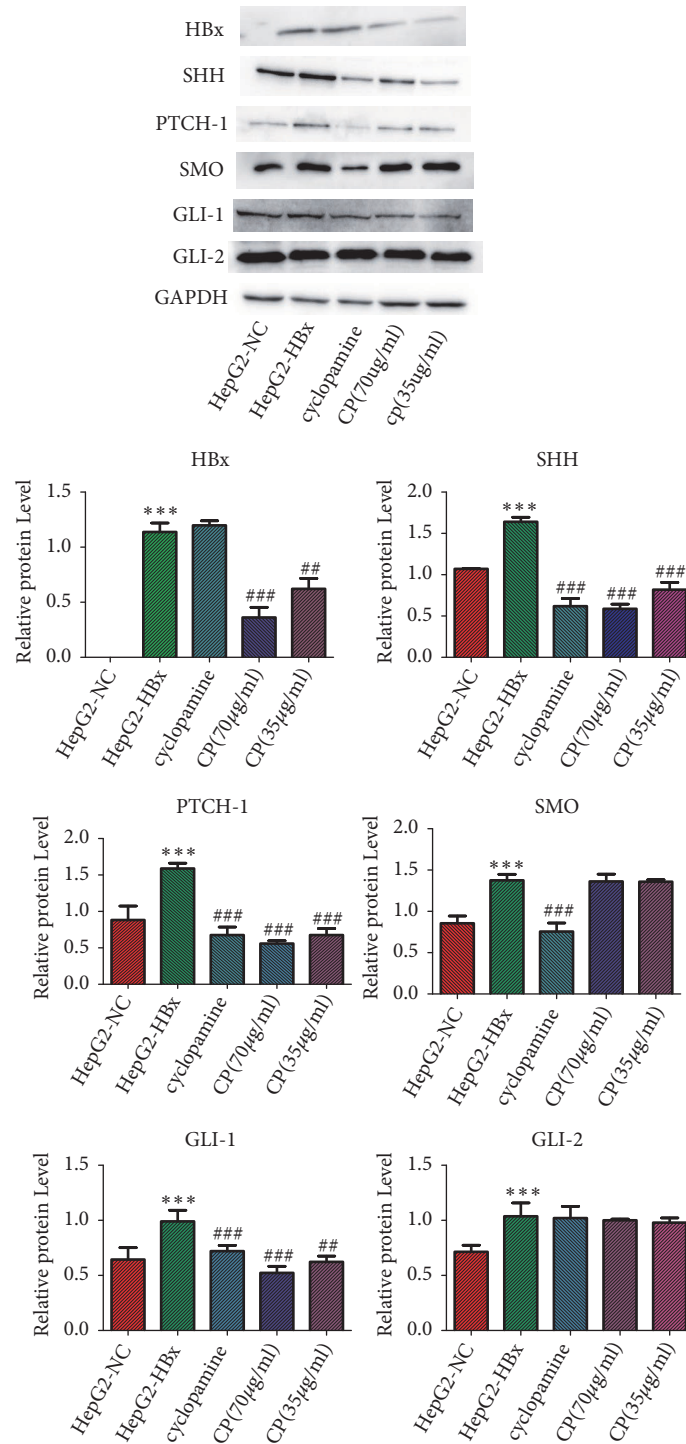


FIGURE 5: CP decreased the proteins expressions of HBx, SHH, PTCH-1, and GLI-1 in HepG2-HBx cells. HepG2-HBx cells were treated with CP (70µg/ml; 35 µg/ml, respectively) and cyclopamine (70µM) for 48 h. Then the proteins expressions were measured by Western blot. The related protein expressions were standard by GAPDH. \*\*\*  $P < 0.001$ , compared with the group of HepG2-NC; \*\*  $P < 0.01$  and ###  $P < 0.001$ , compared with the group of HepG2-HBx.

the effect of CP on HBV-related HCC. In our study, the mice weight of CP (625mg/kg) was heavier than the group of HepG2-NC and HepG2-HBx, but no statistic significance (Figure 7(a)). Oncogene HBx increased the tumor

volume and tumor/weight (%) in mice of HepG2-HBx group compared with that of HepG2-NC group, which showed that HBx could promote the tumor growth. The tumor volume and tumor/weight (%) were significantly decreased



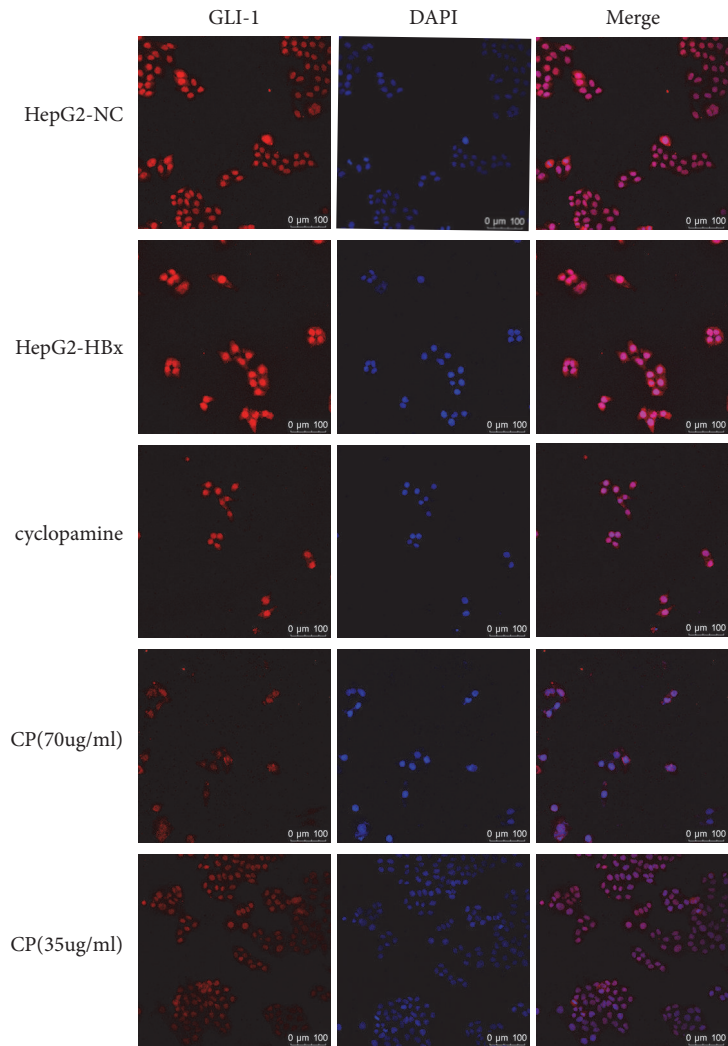


FIGURE 6: CP could inhibit the GLI-1 expression in cytoplasm. The expression of GLI-1 in HepG2-HBx cells treated with CP (70 $\mu$ g/ml; 35 $\mu$ g/ml, respectively) and cyclopamine (70 $\mu$ M) was measured by immunofluorescence. And the pictures were captured by Confocal Laser Scanning Microscope.

in CP (625mg/kg and 300 mg/kg) groups compared with the group of HepG2-HBx (Figures 7(b), 7(c), and 7(e)). HBx could decrease the tumor inhibition rate in HepG2 cells, which implied that HBx promoted the tumor growth. CP (625mg/kg; 300 mg/kg) could elevate the tumor inhibition rate (%) compared with HepG2-HBx group (Figure 7(d)). All of our data showed that HBx promoted tumor growth and CP (625mg/kg and 300 mg/kg) could inhibit growth of tumor in dose-effect relationship.

**3.8. CP Could Inhibit the mRNA Expression of HBx, SHH, PTCH-1, and GLI-1 In Vivo.** We had verified that CP could inactivate the HBx, SHH, PTCH-1, and GLI-1 in vitro. The data from in vivo also demonstrated that CP could inhibit the tumors growth. In order to further confirm our assumption, we observed the expressions of mRNA in tumors. As same as the data from in vitro, CP (625mg/kg; 300 mg/kg) could reduce the mRNA expressions of HBx, SHH, PTCH-1, and GLI-1 in dose-effect relationship compared with the group

of HepG2-HBx. However, CP could not inhibit the mRNA expressions of SMO and GLI-2 (Figure 8).

**3.9. CP Could Repress the Proteins Expression of HBx, SHH, PTCH-1, and GLI-1 In Vivo.** We observed the proteins expression of HBx, SHH, PTCH-1, SMO, GLI-1, and GLI-2 in vivo. Being consistent with the mRNA, the proteins expression of SHH, PTCH-1, SMO, GLI-1, and GLI-2 was significantly increased in the group of HepG2-HBx compared with the group of HepG2-NC. CP with two dosages (625mg/kg and 300 mg/kg, respectively) could reduce proteins levels of HBx, SHH, PTCH-1, and GLI-1 in a dose-dependent manner. However, CP could not inhibit the proteins expression of SMO and GLI-2 (Figure 9).

**3.10. CP Could Inactivate the SHH Pathway in Tumors.** We observed the expression of HBx, SHH, PTCH-1, and GLI-1 in tumors with Immunohistochemistry. The results were

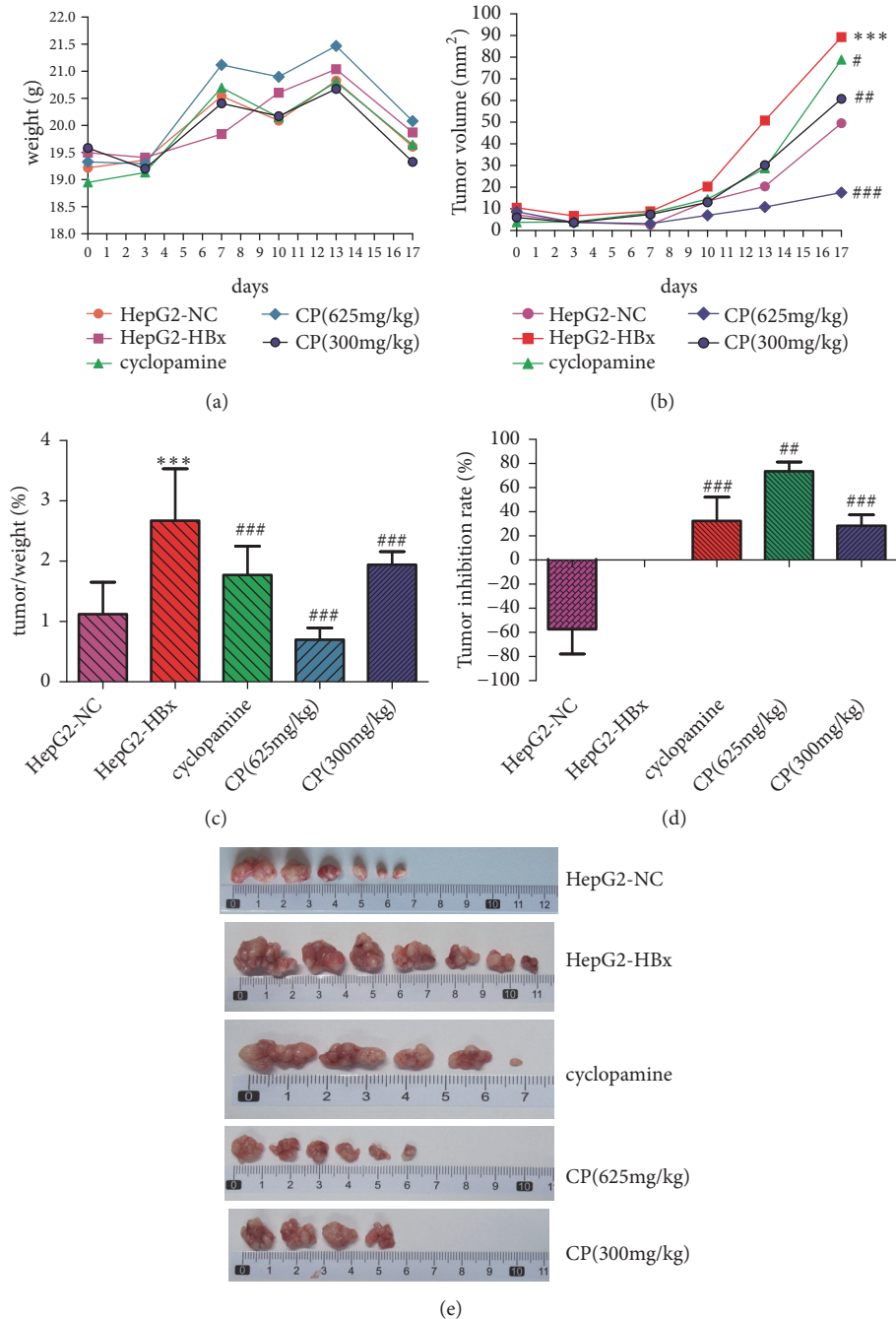


FIGURE 7: CP could inhibit tumors growth in vivo. We transplanted the HepG2-NC cells and HepG2-HBx cells into Balb/c nude mice, respectively. Body weights (a) and tumor volume (b, e) were measured at the indicated times. We measured the tumor/weight (%) (c) and tumor inhibition rate (%) (d) at the end of experiment. \*\*\*  $P < 0.001$ , compared with the group of HepG2-NC; #  $P < 0.05$ , ##  $P < 0.01$ , and ###  $P < 0.001$ , compared with the group of HepG2-HBx.

consistent with the expressions of mRNA and protein. HBx could increase the expression of SHH, PTCH-1, SMO, and GLI-1 in the group of HepG2-HBx compared with the group of HepG2-NC. The mice treatment with CP (625mg/kg and 300 mg/kg) could decrease the expression of HBx, SHH, PTCH-1, and GLI-1 in a dose-dependent manner. However, CP could not inhibit the protein expression of SMO in tumors (Figure 10).

#### 4. Discussion

Here, we investigated the effects of CP on HBV-related HCC in vitro and in vivo. All of the data we presented revealed that HBx activate the SHH pathway to promote the HBV-related HCC development. And CP has significant anti-HBV-related HCC activity that might be mediated by inactivation of HBx, SHH, PTCH-1, and GLI-1.

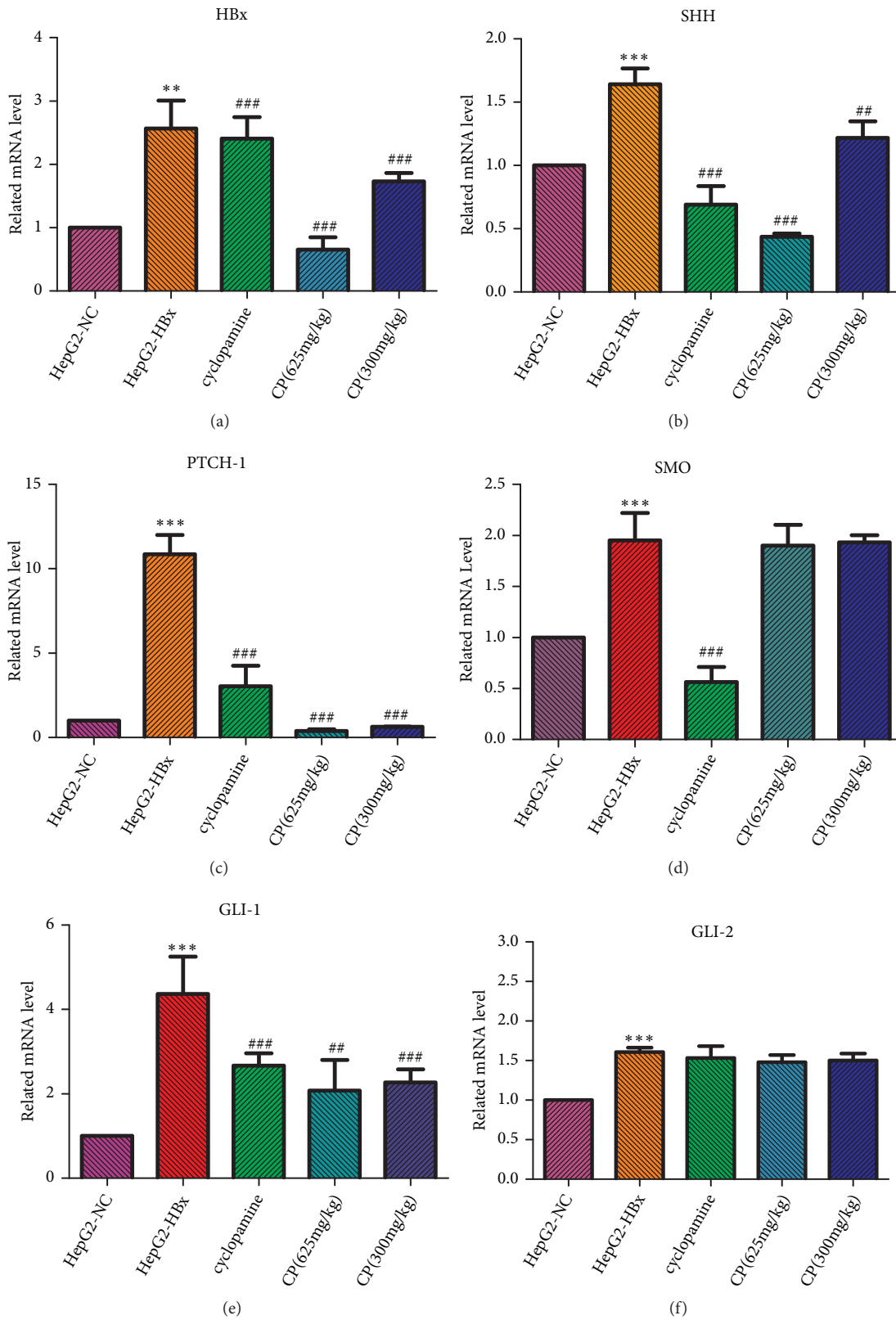


FIGURE 8: CP could inhibit the mRNA expression of SHH, PTCH-1, GLI-1, and HBx in vivo. The mRNA levels of HBx (a), SHH (b), PTCH-1 (c), SMO (d), GLI-1 (e), and GLI-2 (f) in tumors were measured by RT-PCR. \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$ , compared with the group of HepG2-NC. ##  $P < 0.01$  and ###  $P < 0.001$ , compared with the group of HepG2-HBx.

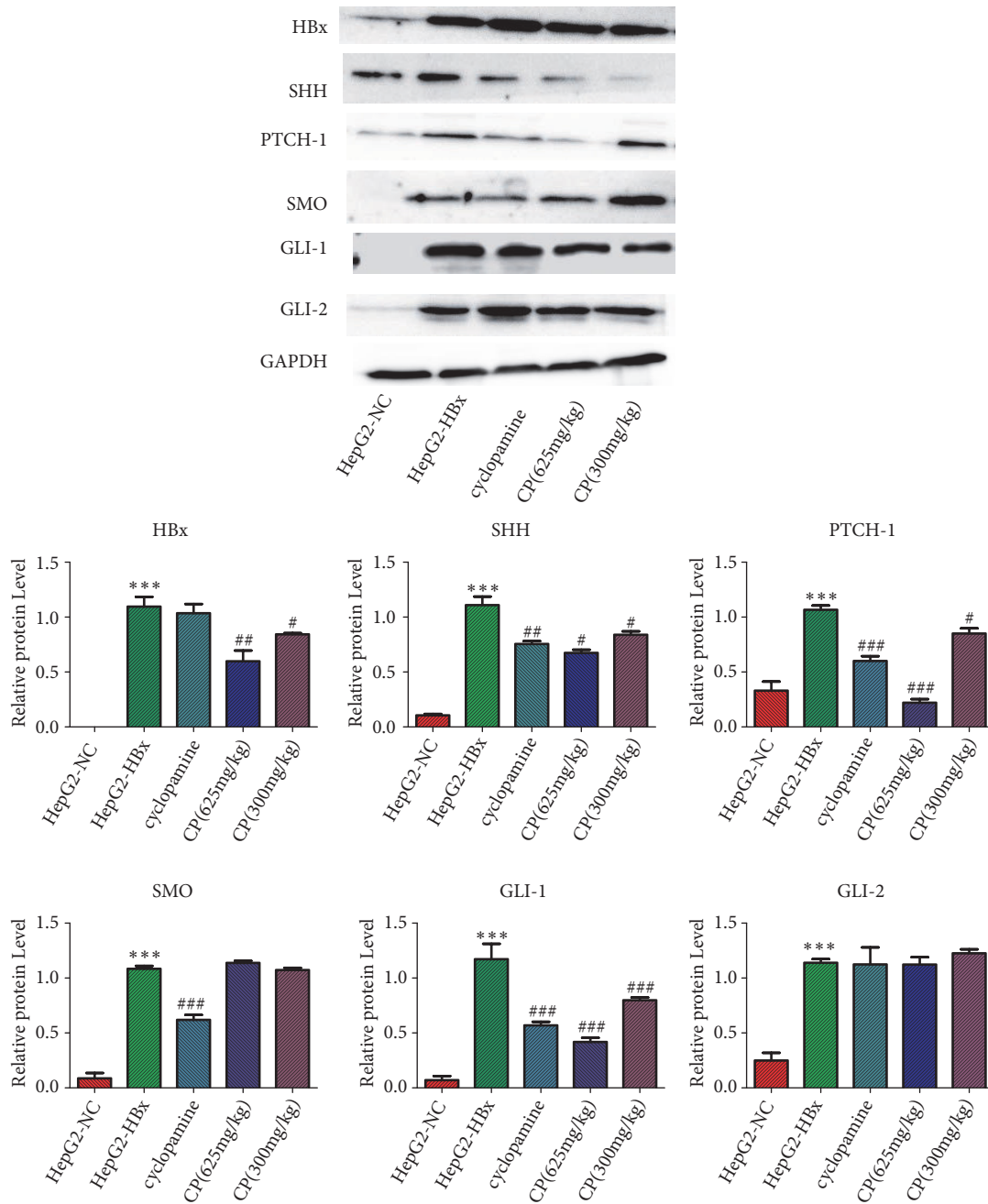


FIGURE 9: CP could inhibit the proteins expression of HBx, SHH, PTCH-1, and GLI-1 in tumors. We measured the proteins level of HBx, SHH, PTCH-1, SMO, GLI-1, and GLI-2 in vivo. \*\*\*  $P < 0.001$ , compared with the group of HepG2-NC; #  $P < 0.05$ , ##  $P < 0.01$ , and ###  $P < 0.001$ , compared with the group of HepG2-HBx.

CP is made up of *Phyllanthus urinaria L*, *Astragalus membranaceus (Fisch.) Bunge*, *Scutellaria barbata D. Don*, *Curcuma zedoaria (Christm.) Rosc*, and *Crematista appendiculata (D. Don) Makino*. It is a formula used for HCC, especially HBV-related HCC in China. In our previous studies, we have elucidated that CP could reduce the expression of HBxAg in transplant tumors, and we also verified that CP could inhibit HBV-DNA replication and reduce the expression of URG4, URG7, URG11, and URG9, preventing and delaying the development of HBV-related HCC in patients treated

with CP for two years [16–18]. Other scholars proved that CP could inhibit expressions of HBsAg and HBeAg, and it also could enhance the immune response in vivo and in vitro [19]. *Phyllanthus urinaria L* extract could reduce the expression of HBx and also could deregulate miR-21 and upregulate miR-145 [14, 20]. As the constituents of CP, gallic acid, quercetin, and luteolin have anticancer activities. Quercetin can suppress miR-21 to alleviate TGF- $\beta$ -induced fibrosis and activate the JNK pathway to induce apoptosis in KRAS-mutant colorectal cancer cells and also could inhibit

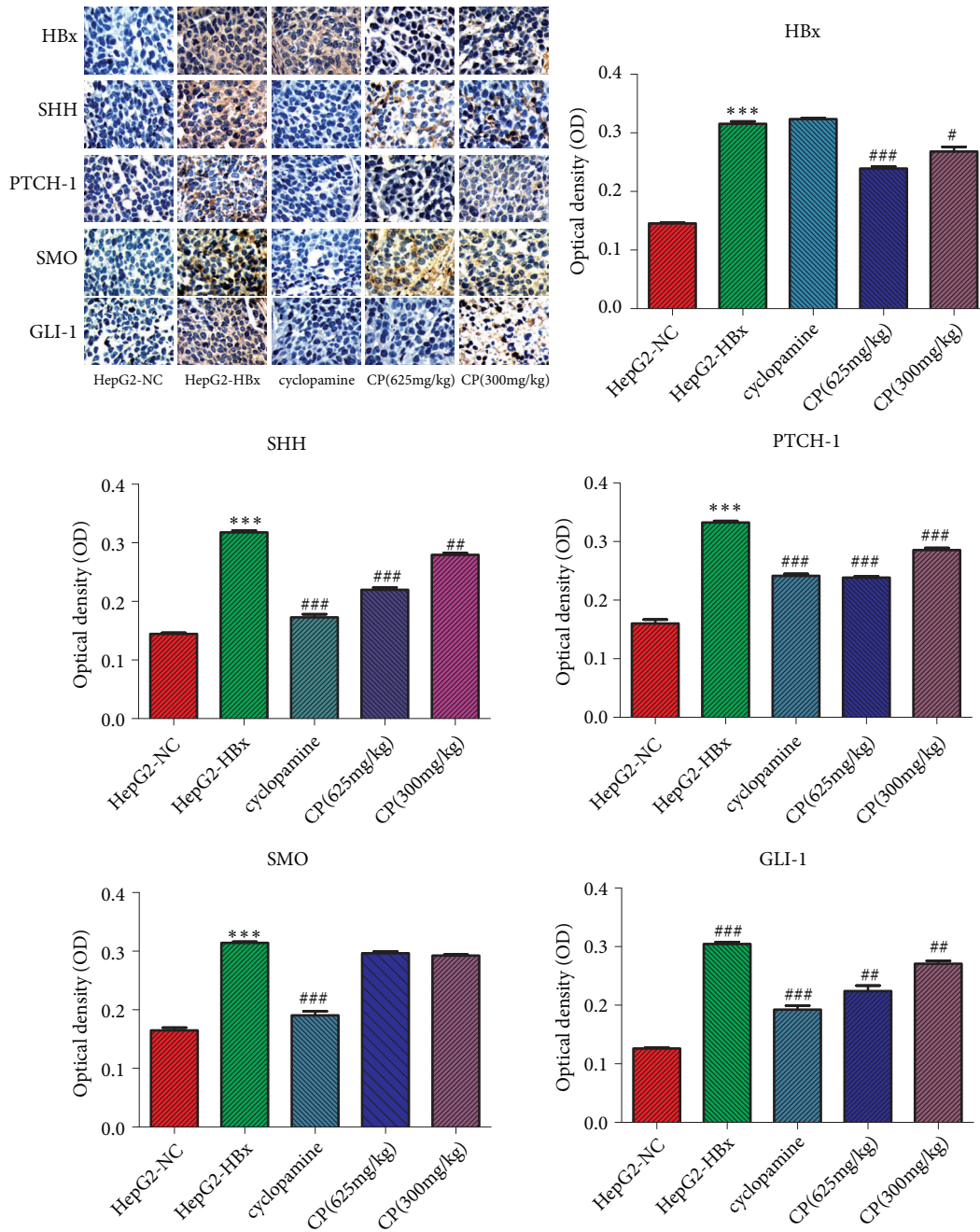


FIGURE 10: CP could inactivate the SHH pathway in tumors. We measured the expression levels of HBx, SHH, PTCH-1, SMO, and GLI-1 in tumors with immunohistochemistry. \*\*\*  $P < 0.001$ , compared with the group of HepG2-NC; #  $P < 0.01$  and ###  $P < 0.001$ , compared with the group of HepG2-HBx.

the SHH pathway to regulate pancreatic cancer stem cell characteristics [21]. Gallic acid could inhibit the proliferation and induce apoptosis in HepG2 and SMMC-7721 cells via mitochondrial-mediated pathway, and it also can reduce the replication of HCV [22]. Luteolin could promote cells apoptosis by inducing autophagy in SMMC-7721 cells [23].

Although CP and its constituents present the capability of anticancer, the molecular mechanism of CP on HBV-related HCC remains not fully uncovered. HBx, as an oncogene,

plays a vital role in pathogenesis and tumorigenesis in HBV-related HCC. Cross-talking with hypoxia-inducible factor-1 alpha (HIF-1alpha) via MAPK pathway, HBx leads to activate of Hif-1 alpha target genes, which contribute to HBV-related HCC development [24]. And HBx also can interact with absent in melanoma 2 (AIM2) to lead to HCC metastasis [25]. And *Kim et al.* confirmed that HBx could stimulate Hedgehog-Gli pathway activation to promote the tumorigenesis [26]. At the same time, we found that

HBx has a positive correlation with the SHH pathway in liver tissues of HBV-related HCC patients. Several lines of evidence confirm that abnormality of the SHH pathway plays an important role in the liver diseases. *Chen et al.* validated that deregulating the GLI-1 could inhibit the migration and invasion in hepatocellular carcinoma. *Kim et al.* have found that hepatic stellate cells could stimulate the proliferation and migration of cholangiocarcinoma cells through HH signaling activation [27]. As the SHH pathway inhibitor, cyclopamine could induce cell apoptosis by downregulating BCL-2 in HCC [28].

Based on our previous studies, we predicted that CP has an anti-HBV-related HCC capability, which might be regulated by inactivation of HBx-SHH pathway axis. As expected, in our studies, all of data in vivo and in vitro further confirmed that HBx could increase the expression of the SHH pathway compositions: SHH, PTCH-1, SMO, GLI-1, and GLI-2, contributing to HCC development. More importantly, CP not only reduced the expression of HBx, but also could inactivate the SHH, PTCH-1, and GLI-1 to inhibit the tumors growth. However, CP could not decrease the expression of SMO and GLI-2.

Notably, from proteins analysis (Figures 5 and 9) and immunofluorescence staining (Figure 6), we found that the SHH pathway components were also detected in HepG2-NC cells, which implied that maybe the SHH pathway also was activated in naïve HepG2 cells. So, it is worthwhile to explore the effect of CP on naïve HepG2 cells, which would confirm whether there is effect of CP on SHH pathway just through the inhibition of HBx or not.

In conclusion, our data presented here indicated that CP could repress expression of HBx and inactivate the SHH pathway in a dose-dependent manner, which might be one of underlying mechanisms of CP anti-HBV-related HCC.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Conflicts of Interest

None of the authors have any financial interests or conflicts of interest with this article.

## Acknowledgments

This work was supported by Shenzhen Science and Technology Innovation Committee (JCYJ20160428175717223; JCYJ20150401163247220).

## References

- [1] M. C. Kew, "Hepatitis B virus x protein in the pathogenesis of hepatitis B virus-induced hepatocellular carcinoma," *J. Gastroenterol. Hepatol.*, vol. 26, 1, pp. 144–152, 2011.
- [2] S. Nayagam, M. Thursz, E. Sicuri et al., "Requirements for global elimination of hepatitis B: a modelling study," *The Lancet Infectious Diseases*, vol. 16, no. 12, pp. 1399–1408, 2016.
- [3] A. Arzumanyan, H. M. G. P. V. Reis, and M. A. Feitelson, "Pathogenic mechanisms in HBV- and HCV-associated hepatocellular carcinoma," *Nature Reviews Cancer*, vol. 13, no. 2, pp. 123–135, 2013.
- [4] J.-M. Su, X.-M. Lai, K.-H. Lan et al., "X protein of hepatitis B virus functions as a transcriptional corepressor on the human telomerase promoter," *Hepatology*, vol. 46, no. 2, pp. 402–413, 2007.
- [5] W. Li, S. Miao, M. Miao et al., "Hedgehog signaling activation in hepatic stellate cells promotes angiogenesis and vascular mimicry in hepatocellular carcinoma," *Cancer Investigation*, vol. 34, no. 9, pp. 424–430, 2016.
- [6] L. G. Harris, R. S. Samant, and L. A. Shevde, "Hedgehog signaling: Networking to nurture a promalignant tumor microenvironment," *Molecular Cancer Research*, vol. 9, no. 9, pp. 1165–1174, 2011.
- [7] D. M. Fink, M. R. Sun, G. W. Heyne et al., "Coordinated D-cyclin/Foxd1 activation drives mitogenic activity of the Sonic Hedgehog signaling pathway," *Cellular Signalling*, vol. 44, pp. 1–9, 2018.
- [8] S.-N. Tang, J. Fu, D. Nall, M. Rodova, S. Shankar, and R. K. Srivastava, "Inhibition of sonic hedgehog pathway and pluripotency maintaining factors regulate human pancreatic cancer stem cell characteristics," *International Journal of Cancer*, vol. 131, no. 1, pp. 30–40, 2012.
- [9] M. Hui, A. Cazet, R. Nair, D. N. Watkins, S. A. O'Toole, and A. Swarbrick, "The Hedgehog signalling pathway in breast development, carcinogenesis and cancer therapy," *Breast Cancer Research*, vol. 15, no. 2, 2013.
- [10] M. Kebenko, A. Drenckhan, S. J. Gros et al., "ErbB2 signaling activates the Hedgehog pathway via PI3K-Akt in human esophageal adenocarcinoma: Identification of novel targets for concerted therapy concepts," *Cellular Signalling*, vol. 27, no. 2, pp. 373–381, 2015.
- [11] J. Jung, N. K. Kim, S. Park, H.-J. Shin, S. G. Hwang, and K. Kim, "Inhibitory effect of Phyllanthus urinaria L. extract on the replication of lamivudine-resistant hepatitis B virus in vitro," *BMC Complementary and Alternative Medicine*, vol. 15, no. 1, 2015.
- [12] G. D. Tong, X. Zhang, D. Q. Zhou et al., "A clinical study of treatments on 52 patients with hepatitis B virus-associated precancerous stage hepatocellular carcinoma," *Chinese Journal of Hepatology*, vol. 10, pp. 781–782, 2008.
- [13] L. Yuhui and F. Linchun, "Experimental study on the Anti-HBV activity of Compound Phyllanthus urinaria L in vitro," *China Tropical Medicine*, vol. 7, pp. 196–203, 2007.
- [14] P. Lisheng, H. Jinsong, T. Guangdong et al., "Inhibition of extract of phyllanthus urinaria L on hepatitis B virus and hepatitis B X gene in vitro," *Chinese Journal of Integrated Traditional and Western Medicine on Liver Diseases*, vol. 6, pp. 340–343, 2006.
- [15] L. Zhou, H. Chen, Q. He, C. Li, L. Wei, and J. Shang, "Evaluation of hepatotoxicity potential of a potent traditional Tibetan medicine Zuotai," *Journal of Ethnopharmacology*, vol. 234, pp. 112–118, 2019.
- [16] G.-D. Tong, X. Zhang, D.-Q. Zhou et al., "Efficacy of early treatment on 52 patients with preneoplastic hepatitis B virus-associated hepatocellular carcinoma by compound Phyllanthus Urinaria L.," *Chinese Journal of Integrative Medicine*, vol. 20, no. 4, pp. 263–271, 2014.

- [17] D. Q. Zhou, G. D. Tong, and J. S. He, "The effect of Compound *Phyllanthus urinaria* L on the expression of HBxAg in transplant tumors," *China Journal of Chinese Materia Medica*, vol. 131, pp. 1649–1651, 2006.
- [18] D. Q. Zhou, G. D. Tong, X. Zhang et al., "The clinic study about the intervene of precancerosis in HBV-related HCC patients," in *The 17th National Academic Conference of Integrated Chinese and Western Medicine in Liver Diseases*, pp. 50–55, 2008.
- [19] Y. Zhong, C. Zuo, F. Li et al., "Chemical constituents of *Phyllanthus urinaria* L. and its antiviral activity against hepatitis B virus," *China Journal of Chinese Materia Medica*, vol. 23, no. 6, pp. 363–364, 1998.
- [20] H. Jinsong, W. Chunshan, T. Guangdong et al., "Effect of Aqueous extract of *phyllanthus urinaria* L on aberrant miR-21 and miR-145 expression induced by HBx gene in hepatoma cells," *Chinese Journal of Integrated Traditional and Western Medicine on Liver Diseases*, vol. 6, pp. 352–355, 2015.
- [21] Y. Yang, T. Wang, D. Chen et al., "Quercetin preferentially induces apoptosis in KRAS-mutant colorectal cancer cells via JNK signaling pathways," *Cell Biology International*, vol. 43, no. 2, pp. 117–124, 2019.
- [22] G. Sun, S. Zhang, Y. Xie, Z. Zhang, and W. Zhao, "Gallic acid as a selective anticancer agent that induces apoptosis in SMMC-7721 human hepatocellular carcinoma cells," *Oncology Letters*, vol. 11, no. 1, pp. 150–158, 2016.
- [23] Z. Cao, H. Zhang, X. Cai et al., "Luteolin promotes cell apoptosis by inducing autophagy in hepatocellular carcinoma," *Cellular Physiology and Biochemistry*, vol. 43, no. 5, pp. 1803–1812, 2017.
- [24] S.-L. Chen, L.-L. Liu, S.-X. Lu et al., "HBx-mediated decrease of AIM2 contributes to hepatocellular carcinoma metastasis," *Molecular Oncology*, vol. 11, no. 9, pp. 1225–1240, 2017.
- [25] J.-S. Chen, H.-S. Li, J.-Q. Huang et al., "Down-regulation of Gli-1 inhibits hepatocellular carcinoma cell migration and invasion," *Molecular and Cellular Biochemistry*, vol. 393, no. 1–2, pp. 283–291, 2014.
- [26] H. Y. Kim, H. K. Cho, S. P. Hong, and J. Cheong, "Hepatitis B virus X protein stimulates the Hedgehog-Gli activation through protein stabilization and nuclear localization of Gli1 in liver cancer cells," *Cancer Letters*, vol. 309, no. 2, pp. 176–184, 2011.
- [27] Y. Kim, M.-O. Kim, J. S. Shin et al., "Hedgehog signaling between cancer cells and hepatic stellate cells in promoting cholangiocarcinoma," *Annals of Surgical Oncology*, vol. 21, no. 8, pp. 2684–2698, 2014.
- [28] X.-L. Chen, Q.-Y. Cheng, M.-R. She et al., "Expression of sonic hedgehog signaling components in hepatocellular carcinoma and cyclopamine-induced apoptosis through Bcl-2 downregulation in vitro," *Archives of Medical Research*, vol. 41, no. 5, pp. 315–323, 2010.