HBP1-mediated transcriptional repression of AFP inhibits hepatoma progression
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Abstract
Background: Hepatoma is a common malignancy of the liver. The abnormal high expression of alpha-fetoprotein (AFP) is intimately associated with hepatoma progress, but the mechanism of transcriptional regulation and singularly activation of AFP gene in hepatoma is not clear.

Methods: The expression of transcription factor HBP1 and AFP and clinical significance were further analyzed in hepatoma tissues from the patients who received surgery or TACE and then monitored for relapse for up 10 years. HBP1-mediated transcriptional regulation of AFP was analyzed by Western blotting, Luciferase assay, Realtime-PCR, ChIP and EMSA. After verified the axis of HBP-AFP, its impact on hepatoma was measured by MTT, Transwell and FACS in hepatoma cells and by tumorregenesis in HBP1−/− mice.

Results: The relative expressions of HBP1 and AFP correlated with survival and prognosis in hepatoma patients. HBP1 repressed the expression of AFP gene by directly binding to the AFP gene promoter. Hepatitis B Virus (HBV)-encoded protein HBx promoted malignancy in hepatoma cells through binding to HBP1 directly. Icaritin, an active ingredient of Chinese herb epimedium, inhibited malignancy in hepatoma cells through enhancing HBP1 transrepression of AFP. The repression of AFP by HBP1 attenuated AFP effect on PTEN, MMP9 and caspase-3, thus inhibited proliferation and migration, and induced apoptosis in hepatoma cells. The deregulation of AFP by HBP1 contributed to hepatoma progression in mice.

Conclusions: Our data clarify the mechanism of HBP1 in inhibiting the expression of AFP and its suppression in malignancy of hepatoma cells, providing a more comprehensive theoretical basis and potential solutions for the diagnosis and treatment of hepatoma.

Keywords: HBP1, AFP, Transrepression, HBV, Hepatoma

Background
Hepatoma is the fifth largest cancer species in the world, over half a million new cases are diagnosed each year. Due to the poor prognosis, hepatoma is an important cause of cancer-related deaths, with an estimated 695,900 deaths per year [1, 2]. More than 90% of hepatoma is developed on the basis of chronic hepatitis, and the main cause is Hepatitis B Virus (HBV) or Hepatitis C Virus (HCV) infection [3, 4]. Hepatoma usually has no obvious symptoms at an early stage, so nearly 85% of patients are already in intermediate or advanced stage when they are diagnosed [5, 6].

Alpha-fetoprotein (AFP), a glycoprotein, is the most commonly used serum marker for the diagnosis of hepatoma in clinical practice and is widely used in screening, diagnosis, efficacy evaluation and recurrence assessment...
of hepatoma [7]. Many studies have demonstrated that AFP is an important intracellular signaling molecule involved in a variety of cellular processes, serving as a pro-cancer factor. Li et al. reported that the interaction of AFP and PTEN prohibits the inhibition function of PTEN on the PI3K/AKT pathway, leading to abnormal proliferation of hepatoma cells [8]. It is also reported that the interaction of AFP and caspase-3 blocks the caspase-3 signaling, thereby inhibiting the apoptotic signaling pathway [9]. Furthermore, AFP competitively binds to retinoic acid receptor (RAR), inhibiting the formation of ATRA-RAR complex and nuclear translocation of RAR, up-regulating the expression of survivin, down-regulating the expression of GADD153, Fn14 and GADD45a, resulting in the decrease of cell apoptosis [10–12]. AFP can also promote hepatoma cell migration and invasion by increasing the expression of cell migration-related genes such as K19, EpcAM, MMP2/9 and CXCR4 [13, 14]. These data indicate that AFP interferes with normal signaling networks and leads to tumorigenesis by binding to different signaling proteins. The abnormal high expression of AFP is intimately associated with hepatoma progress, but the mechanism of transcriptional regulation and singularly activation of AFP gene in hepatoma cells is not clear.

The transcription factor HBP1 belongs to the sequence-specific high mobility group (HMG) family and functions as a tumor suppressor. As a transcription factor, HBP1 inhibits its target genes by directly binding to specific affinity elements, such as N-MYC, C-MYC, p47phox, DNMT1 and EZH2 [15–19], all of which are oncogenes or genes that promote tumor development. In addition, HBP1 also transcriptionally activates several downstream genes, including genes encoding p16, p21, myeloperoxidase (MPO) and histone H1 [20–23]. In addition, multiple amino acid residues of HBP1 protein have different chemical modifications. HBP1 can be phosphorylated by p38 and Pim-1 [24, 25], and acetylated by p300/CPB [26], while GID/CTLIH and MDM2 can ubiquitinate HBP1 [27, 28]. The type and position of modifications affect the protein stability and transcriptional activity of HBP1. Since HBP1 is involved in the regulation of many important cell cycle regulators, the expression level of HBP1 in cells or tissues affects cell growth or tumorigenesis.

In this study, we found a transcriptional regulatory mechanism to regulate the progression of hepatoma between HBP1 and AFP. We noticed that HBP1 repressed the AFP promoter in a sequence-specific manner through a high affinity site. The repression of AFP by HBP1 inhibited the malignancy of hepatoma cells. In addition, we constructed HBP1-deficient mice and induced hepatoma model by DEN/CCl4. Compared to wild-type mice, HBP1-deficient mice developed more severe liver damage, liver fibrosis, and ultimately induced hepatoma with higher malignancy. Since HBPI and AFP play a key role in the development of hepatoma, studies on the regulation of HBPI and AFP are crucial in maintaining the normal physiological function of the liver and the diagnosis and treatment of liver cirrhosis and hepatoma.

Methods

Cell culture, transfection, and lentivirus

HepG2, and PLC5 (PLC/PRF/5) cells were purchased from the National Institute of Biological Products, Beijing, China. All the cells were cultured in DMEM containing 10% fetal bovine serum. The plasmids were transiently transfected into cells utilizing turbofect transfection reagent (Thermo Scientific) according to the manufacturer’s instruction. The transfection efficiency was detected by western blotting after 48 h post-transfection. The lentivirus plasmid pLL3.7-shHBPI expresses shRNA that targets HBPI mRNA (5′-ACTG TGAGTGCCACTTCTC-3′), pLL3.7-shAFP could express shRNA that targets AFP mRNA (5′-GAACGTGG TCAATGTATAA-3′).

Western blotting and antibodies

Cells were lysed in RIPA buffer (Thermo Scientific) containing protease inhibitor cocktail (Sigma), and BCA protein analysis kit (Pierce) was used to measure the protein concentration. Twenty to Sixty μg of total protein was separated by 8–12% SDS-PAGE and transferred to nitrocellulose membranes (Pall). These primary antibodies we used as follow: anti-HBP1 (11746–1-AP, Proteintech), anti-AFP (14550–1-AP, Proteintech), anti-AKT (#4060, Cell Signaling Technology), anti-AKT (#9272, Cell Signaling Technology), anti-MMP9 (10375–2-AP, Proteintech), anti-Caspase-3 (#14220, Cell Signaling Technology), anti-Type III (17590–1-AP, Proteintech), anti-Flag (#1140, Sigma-Aldrich), anti-HA (MMS101P, Covance), and anti-GAPDH (KM9002, Sangene). And these secondary antibodies we used as follow: anti-mouse IgG antibody DyLight 800-conjugated (610–145-121, Rockland) and anti-rabbit IgG DyLight 800-conjugated (611–145-002, Rockland). Odyssey infrared imaging system (LI-COR Bioscience, Lincoln, NE) was used to acquire the infrared fluorescence image.

Realtime PCR

Total RNA was extracted utilizing the RNAsimple Total RNA kit (Tiangen). The ReverAid First Strand cDNA Synthesis kit (Thermo Scientific) was used to synthesize cDNA. The mRNA expression level was analyzed by real-time quantitative PCR instrument (Applied...
Biosystems) with Maxima SYBR Green qPCR Master Mix (Thermo Scientific). The primers’ sequences were shown as follows: human AFP, 5′-GCAGTGAAATC TACAGGGACGC-3′ and 5′-ATCCTGATCCAAACCA TCACC-3′; human HBP1, 5′-GGAAGCTTGGATGATA TGGAGGAAATG-3′ and 5′-CATAGAAGGGGTGGTC CAGCTTA-3′; and human GAPDH, 5′-CCATGG AGAAGGCTTGGG-3′ and 5′-GCAAATTTTCTCATG AGTACC-3′; mouse HBP1, 5′-GGGAAATGGAAGA ACAAT-3′ and 5′-TGACAGGGAGGACATACA-3′; mouse AFP, 5′-AACCTCCAGGCAACAAACA-3′ and 5′-ACTCCAGCGAGTTTCTCTTG-3′; mouse IL-1β, 5′-GAAATCCACCTTGTAGCAGT-3′ and 5′-TGGATGCTCTCATCAGGACAG-3′; mouse TNF-α, 5′-CAGGCGGTTGCTACTGTC-3′ and 5′-CGATCA CCCGAAGTTCATGAG-3′; mouse type I collagen, 5′-GGTCTCTCTATGGGGCCACT-3′ and 5′-CCACGT CTACCACTTGGG-3′; mouse type III collagen, 5′- AGGTAGTAATGGATGAGTTGCAG-3′ and 5′-GGGT TGGGGCAGTCTCAG-3′; mouse β-actin, 5′-AGCC ATGTAGTGACCATC-3′ and 5′-GCTGTGGGTTTG TGAAGCTGTGA-3′.

Chromatin immunoprecipitation (ChIP)
The ChIP assays were performed as described previously [19]. The primers’ sequences for AFP promoter were 5′-ACCTCTTTCTGTAGACGTACG-3′ and 5′-GCAGTC ATCGTGAAGGTCGT-3′.

Electrophoretic mobility shift assay (EMSA)
The EMSA assay was performed as described previously [19]. The probes’ sequences were as follow:

- AFP-WT: 5′-TGGAACAAAAATCAACAAAATGAA TGGGAATGTAGTTGCTAGCAT-3′ and 5′-ATGC TAATCAATGCAATCTCACCATTCTTGTAGTTT TTGTCCA-3′.
- AFP-MT: 5′-TGGAACAAAAATCAACAAAATGAA TGGGAATGTAGTTGCTAGCAT-3′ and 5′-ATGC TAATCAATGCAATCTCACCATTCTTGTAGTTT TTGTCCA-3′.

Reporter gene assay
The plasmids were transfected into 293 T cells for individual experiments. Cell lysates were prepared at 24 h after transfection utilizing the Dual Luciferase Reporter Assay Kit (Promega). The Firefly luciferase activity measurement was standardized to the same sample’s Renilla luciferase activity.

Immunoprecipitation
The plasmids were transfected into 293 T cells for individual experiments. Cells were harvested and lysed in IP lysis buffer (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, and 5% glycerol) containing protease inhibitor cocktail. The lysates were incubated with primary antibodies and protein A-Sepharose (GE Healthcare) with constant rotation overnight at 4°C. The samples were washed with IP lysis buffer three times and the relevant protein binding was detected by Western blotting analysis.

In vivo ubiquitination assay
Two hundred ninety-three T cells transfected with HA-HBP1 were treated with or without Icaritin for 6 h. We harvested and lysed cells by IP lysis buffer. The lysates were incubated with anti-HA antibody and protein A-Sepharose (GE Healthcare). Western blotting was performed with the indicated antibodies.

MTT assay
HepG2 cell sublines were constructed by lentivirus infection. Cells were seeded into 96-well plates 1000 cells per well. Fifteen μl of MTT solution (5 mg/ml) was added to each well after culturing for 1–7 days separately. Four h later we removed the medium and added 200 μl DMSO to each well to dissolve the formazan crystals. We measured the absorbance each sample at 490 nm utilizing the microplate reader.

Transwell assay
HepG2 cell sublines were constructed by lentivirus infection. 5 × 10^4 cells were added to each chamber with 100 μl serum-free medium, and 1 ml of serum-containing medium was added to the lower layer. After continuing to culture for 12 h, the cells in the chamber were wiped with a cotton swab and fixed with 4% paraformaldehyde for 30 min. Stain with 0.5% crystal violet stain for 20 min, then rinse with water to remove excess dye solution. The number of cells passing through the membrane was observed under a microscope, and the number of cells in at least 5 fields of view was counted.

Apoptosis assay
HepG2 cell sublines were constructed by lentivirus infection. For induction of apoptosis, cells were seeded into 6-well-plate and treated with 150 μM H_2O_2 for 24 h. The cells were trypsinized, washed, and then stained with FITC-Annexin V (640,906, Biolegend) and propidium iodide (PI) for 10 min in the dark at room temperature. 1 × 10^5 cells per sample were acquired and analyzed using the FACS Canto II flow cytometer (BD).

Animals
The experimental animal facility has been accredited by the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International). All mouse experiments conformed to the Guide for the Care and Use of Laboratory Animals of the Health Science
Fig. 1 (See legend on next page.)
Center of Peking University. Mice were housed in groups with 12-h dark-light cycles and had free access to food and water. Mice were sacrificed under the isoflurane inhalation and followed by cervical dislocation. All mouse lines were maintained in a C57BL/6J genetic background. Primer 5′-CAGCCTCTGACCTCTTCTAGT-3′ and 5′-TATGAAGTGAGGACAAACGT-3′ were used for PCR analysis to confirm the success of HBP1 knockout.

To induce HCC, 8 weeks old HBP1-knockout male mice and their wild-type (WT) littermates were intraperitoneally injected with DEN at a dose of 100 mg/kg per mouse, and 0.9% saline was injected into the control group. Two weeks later, DEN was intraperitoneally injected at a dose of 50 mg/kg per mouse, and the control group was injected with 0.9% saline. Thereafter, the CCl4 and the olive oil were mixed at a ratio of 1:4, and the mice were intragastrically administered twice a week, 100 μl each time, and the control group was intragastrically administered with 100 μl of olive oil for 16 weeks. At the same time, the drinking water of the experimental group was changed to 8% ethanol, and the control group was given normal drinking water.

Tumor tissues and immunohistochemistry staining

Human tissue microarrays of hepatoma patients (Cat No. LVC-1608) were purchased from Shanghai Superbiootek Pharmaceutical Technology Co. Ltd. (Shanghai, China). The information and samples of these patients were collected from 2008 to 2017. The tissue microarray slides were dewaxed, antigen retrieved and incubated with anti-HBP1 or anti-AFP specific antibodies, followed by biotin labeled secondary antibodies and colored by streptavidin-ALP system.

Statistical analysis

SPSS 17.0 software was used for statistical analysis. The data are expressed as the mean ± SD (standard mean error) of at least three independent experiments. The data in statistical tests were in accordance with normal distribution and the variance was similar. The statistical analysis was conducted by Student's t test. *, p < 0.05, **, p < 0.01.

Results

HBP1 regulates AFP expression

We applied survival analysis to the data on 71 hepatoma patients who received conservative surgery and then were monitored for up to 10 years. We observed a significant inverse correlation between HBP1 and AFP protein levels in hepatoma tissues by immunohistochemical staining (Fig. 1a). As the degree of malignancy increased, the expression of HBP1 gradually decreased from phase I to phase IV under the TNM staging system. In contrast, AFP gradually increased from phase I to phase IV. We defined HBP1 or AFP expression greater than mean as HBP1 or AFP high expression group, and vice versa low expression group. Survival analysis demonstrated that HBP1 high expression, or AFP low expression was associated with higher survival rate (Fig. 1b, top panel). The expression level of HBP1 decreased with the increase of tumor TNM stage, and the survival rate after surgery also decreased (Fig. 1b, middle and bottom panels). In addition, HBP1 protein level in HBV positive (HBs Ag+) hepatoma patients was lower than that in HBV negative (HBs Ag-) hepatoma patients (Fig. 1c). The data suggest that the inverse expressions of HBP1 and AFP correlate with relapse and survival in hepatoma patients. HBP1 may be an inhibitor in the development of HBV-associated hepatoma.

To determine whether HBP1 represses AFP expression, we overexpressed HBP1 in HepG2 and PLC5 cells (Fig. 1d). AFP protein (in cytoplasm or supernatant) (left panel) and mRNA (right panel) levels were reduced by HBP1 overexpression in both HepG2 and PLC5 cells. To confirm the endogenous regulation of HBP1 on AFP expression, we knocked down the expression of HBP1 using short hairpin RNAs (shRNAs). As shown in Fig. 1e, HBP1 knockdown resulted in increased levels of AFP protein (in cytoplasm or supernatant) (left panel) and...
Fig. 2 (See legend on next page.)
mRNA (right panel) in the two cell lines. These results suggest that HBP1 inhibits AFP expression at the transcriptional level.

HBP1 represses the AFP gene through binding an affinity site in the AFP promoter

We next investigated whether HBP1 regulates the promoter activity of AFP in a DNA-binding-dependent manner. We found that the AFP promoter contains an affinity site (TGAATGGG) for HBP1 at position -1512 to -1505 bp from the transcriptional start site. We coinfected 293T cells with plasmids expressing HBP1 and different deletion mutants of AFP promoter reporter genes. HBP1 inhibited the reporter genes containing the HBP1 affinity site (-1871 to +134 and -1545 to +134), whereas HBP1 had no effect on the reporter genes lacking the HBP1 affinity site (-1500 to +134, -1004 to +134, -448 to +134 and -215 to +134). HBP1 also inhibited AFP promoter activity in a dose-dependent manner (Fig. 2 b).

To further verify the DNA binding requirement for the effects of HBP1, we constructed a mutant reporter for the AFP promoter with point mutations in the binding element at -1510 (changing AA to CC). HBP1 inhibited the activity of the wild-type AFP promoter, but had no effect on the mutant (Fig. 2 c). To further investigate whether the transcriptional repression of HBP1 depends on DNA binding, we used two mutants of HBP1: pmHMG, which has three amino acid mutations in the HMG domain and lacks DNA binding ability, and DelEx7, which was isolated from breast cancer tissue and lacks the DNA binding domain and part of the repression domain [17, 20]. As shown in Fig. 2 d and e, wild-type HBP1 decreased the activity of AFP promoter and protein level, but overexpression of pmHMG and DelEx7 had no effect. Since HBP1 inhibited the activity of the AFP promoter, we tested whether HBP1 directly binds to the AFP promoter. Electrophoretic mobility shift assay (EMSA) (Fig. 2 f) and chromatin immunoprecipitation (ChIP) assay (Fig. 2 g) demonstrated that HBP1 bound directly to the specific affinity site in the AFP promoter in vivo and in vitro. In contrast, pmHMG and DelEx7 did not bind the AFP promoter.

HBP1 acetylation at K419 is required for HBP1 suppression of AFP transcription

Our previous studies showed that HBP1 is acetylated by p300/ CBP at five lysine sites (K292, K305, and K307 in the Repression domain, and K171 and K419 in the P domain). Acetylation of HBP1 is required for its transcriptional activity [26]. To investigate whether acetylation of HBP1 affects its transcriptional repression of AFP, we used various HBP1 mutants: K171R, K419R, 2KR (K171/419R), 3KR (K297/305/307R), and 5KR (K171/419/297/305/307R) (Fig. 3 a). We co-infected the AFP luciferase reporter with either wild-type HBP1 or the HBP1 acetylation mutants into 293T cells. Wild-type HBP1 repressed AFP promoter activity. The 3KR acetylation-deficient mutant in the Repression domain showed similar results as wild-type HBP1, whereas mutant 2KR and 5KR showed no repression of the AFP promoter-luciferase construct (Fig. 3 b, left panel). Consistent with these results, wild-type HBP1 and mutant 3KR decreased AFP mRNA and protein levels, however, overexpressing mutant 2KR or 5KR had no effect on AFP expression compared with control vector (Fig. 3 c and d, left panel). We further found that HBP1 acetylation at K419 was required for its suppression of AFP transcription (Fig. 3 b, right panel). Likewise, the regulation of AFP mRNA and protein levels (Fig. 3 c and d, right panel) depended on acetylation of HBP1 at K419. These data indicate that HBP1 acetylation at K419 is crucial for its repression of AFP transcription.
HBx attenuates the suppression of HBP1 on AFP through inhibiting HBP1 binding to AFP promoter

The development of hepatoma is closely related to hepatitis B virus (HBV) infection [29]. HBx is a HBV regulatory protein that up-regulates AFP expression and promotes the development of HBV-associated hepatoma [30, 31]. To determine whether HBx promotes the development of hepatoma through inhibiting the down-regulation of AFP by HBP1, we overexpressed HBP1 with or without HBx in HepG2 cells. As shown in Fig. 4 a–4c, HBx rescued the HBP1-mediated decrease of AFP protein level, mRNA level and promoter activity. We next examined whether HBx affects the binding of HBP1 to the AFP promoter. ChIP results confirmed that HBx

![Diagram of HBP1 acetylation at K419](image)

**Fig. 3** HBP1 acetylation at K419 is required for HBP1 suppression of AFP transcription. a Schematic diagram of wild-type HBP1 and associated point mutants. b The acetylation of HBP1 at K419 is indispensable for its suppression of AFP promoter. Two hundred-ninety three T cells were co-transfected with AFP promoter and HBP1 or mutant expression plasmids. Luciferase activity was determined after transfection. c The acetylation of HBP1 at K419 is indispensable for its suppression of AFP mRNA. The mRNA level of AFP was measured by Realtime-PCR in HepG2 cells transfected with HBP1 or associated mutant vectors individually. d The acetylation of HBP1 at K419 is indispensable for its suppression of AFP protein. HepG2 cells were transfected with HBP1 or associated mutant vectors. The protein levels of HBP1 and AFP were measured by Western blotting. Error bars represent S.D. **, p < 0.01
reduced the binding of HBP1 to the AFP promoter (Fig. 4 d). We also performed co-IP assays and found that exogenous HBx could interact with HBP1 (Fig. 4 e). Therefore, we speculated that HBx may inhibit the binding of HBP1 to the AFP promoter by interacting with HBP1. Together with Fig. 1 c, HBP1 protein level in HBs Ag+ hepatoma patients was lower than that in HBs Ag- hepatoma patients, indicating that the inhibition of AFP
Fig. 5 (See legend on next page.)
Icaritin promotes the suppression of HBP1 on AFP through enhancing HBP1 binding to AFP promoter

Icaritin is an active ingredient of the Chinese herb epimedium that has been proven to have a wide range of biological and pharmacological functions, such as enhancing immunity and antioxidative and anticancer functions [32]. Previous studies reported that Icaritin inhibits the growth of liver cancer cells through promoting the apoptosis of hepatoma cells by activating the caspase pathway and inhibiting the IL-6/Jak2/Stat3 signaling pathway [33, 34]. Zhang et al. demonstrated that Icaritin reduces the expression of AFP by promoting miRNA-mediated degradation of AFP mRNA [35]. We thus next examined whether Icaritin interferes with the suppression of HBP1 on AFP. We overexpressed HBP1 with or without Icaritin treatment in HepG2 cells and examined the protein level, mRNA level and promoter activity of AFP. As shown in Fig. 5a–5c, HBP1 more significantly inhibited AFP expression in the presence of Icaritin compared with HBP1 alone. ChIP assay showed that Icaritin enhanced the binding of HBP1 to the AFP promoter (Fig. 5d). To further investigate how Icaritin enhances the transcriptional repression of HBP1, we evaluated the protein and mRNA levels of HBP1 in HepG2 cells after Icaritin treatment. As shown in Fig. 5e, Icaritin increased HBP1 protein expression but had no effect on mRNA level. Furthermore, Icaritin inhibited HBP1 ubiquitination-mediated proteasome degradation (Fig. 5f). Thus, we concluded that Icaritin increases HBP1 expression by inhibiting the ubiquitination of HBP1, thereby enhancing HBP1 binding to AFP promoter.

The repression of AFP by HBP1 attenuates AFP effect on PTEN, MMP9 and caspase-3 protein levels in hepatoma cells

Previous studies reported that AFP exerts its tumor promotion through interacting with PTEN, MMP9, and caspase-3 and blocking their functions on the PI3K/AKT, metastasis and caspase signaling [8, 9]. We then tested whether the repression of AFP by HBP1 attenuates AFP effect on PTEN, MMP9 and caspase-3 in HepG2 cells. As shown in Fig. 6a, HBP1 overexpression increased PTEN protein expression and decreased phosphorylated AKT level. Meanwhile, HBP1 increased pro-caspase-3 processing to cleaved-caspase-3 and decreased the protein level of MMP9, whereas co-expressing AFP rescued the HBP1-mediated changes in PTEN, caspase-3, and MMP9. Furthermore, knockdown of HBP1 increased AFP expression, thereby decreased PTEN and cleaved-caspase-3 levels, and increased phosphorylated AKT and MMP9 levels, but there was no effect when AFP was also knocked down (Fig. 6b). We also examined the K419R mutant and found that it had no effect on expression of the proteins (Fig. 6e). These results indicated that HBP1 increases protein levels of PTEN and cleaved-caspase-3 and decreases protein level of MMP9 through suppressing AFP, and HBP1 exerts the role depending on its acetylation at K419.

We also tested the role of HBx or Icaritin in regulating HBP1-mediated expression of these proteins. As shown in Fig. 6c and d, HBx rescued HBP1-mediated expression of these proteins, while Icaritin enhanced HBP1-mediated expression of these proteins, suggesting that HBx or Icaritin influences PTEN/AKT, caspase-3, and MMP9 signals through regulating HBP1-AFP axis.

HBP1 inhibits malignancy through suppressing AFP in hepatoma cells

Since HBP1 upregulates AKT and caspase-3 signals and decreases metastasis-related protein of MMP9 through suppressing AFP, we next investigated the role of HBP1 suppression on AFP expression in cell viability, migration and apoptosis in HepG2 cells. Consistent with previous studies, HBP1 decreased cell viability and cell migration as shown by growth curve (Fig. 7a, top panel) and Transwell assay (Fig. 7a, middle panel), also increased cell apoptosis response to H2O2 induction, as

by HBP1 might reduce in HBs Ag+ hepatoma patients because of low HBP1, thereby promoting hepatoma progression.
Fig. 6 (See legend on next page.)
demonstrated by FACS (Fig. 7 a, bottom panel). AFP rescued the HBPI-induced decrease of cell proliferation and migration, and also rescued the increase of cell apoptosis (Fig. 7 a). HBPI-knockdown cells showed higher growth rate and cell migration ability, but lower apoptosis rate response to H2O2 induction, while AFP knockdown by shRNA in HBPI-knockdown cells rescued the elevated proliferation and migration, and the decreased apoptosis induced by knockdown of HBPI (Fig. 7 b).

To further determine whether HBx or Icaritin treatment alters the degree of malignancy in hepatoma cells, HepG2 cells were stably transfected with HBPI with or without lentivirus expressing HBx or Icaritin treatment. As shown in Fig. 7 c and d, HBx rescued HBPI-mediated decrease of cell proliferation and migration, and the increase of cell apoptosis, while Icaritin enhanced HBPI-mediated decrease of cell proliferation and migration, and the increase of cell apoptosis. In addition, Icaritin inhibited tumorigenesis of HepG2 cells in the nude mice (Fig. 7 f). These results indicated that HBPI inhibits malignancy in hepatoma cells through suppressing AFP, and HBx or Icaritin influences hepatoma cell malignancy through regulating HBPI-AFP axis. We also examined the K419R mutant and confirmed that the effect of HBPI on cell proliferation, migration and apoptosis is dependent on its acetylation of K419 (Fig. 7 e).

5-fluorouracil (5-FU) is an anti-liver cancer drugs widely used in clinic. In order to test if 5-FU displays similar effects of Icaritin on HBPI or AFP, we performed Western blotting, Realtime PCR, ChIP, and MTT assays with HBPI-overexpressing HepG2 cells treated with or without 5-FU. As shown in Fig.S1A-1F, 5-FU also increased HBPI protein expression by inhibiting HBPI ubiquitination-mediated proteasome degradation, thereby enhances HBPI repression on AFP promoter. Furthermore, 5-FU enhanced HBPI-mediated expression of PTEN/AKT, caspase-3, and MMP9 proteins (Fig.S1G), and enhanced HBPI-mediated decrease of cell proliferation (Fig.S1H). These results indicated that 5-FU, which displays similar effects of Icaritin on HBPI or AFP, may inhibit hepatoma progression though regulating HBPI-AFP axis.

HBPI deletion aggravates DEN/CCl4-induced hepatoma
To further investigate the function of HBPI in the development of hepatoma, we constructed HBPI knockout mice using the CRISPR/Cas9 system. The removal of 194 base pairs in the third exon of the HBPI gene prevented the HBPI gene transcription, resulting in the deletion of HBPI protein. After genetic identification of newborn mice, we selected two groups of wild-type and HBPI-deficient mice and randomly assigned six wild-type and six HBPI-deficient mice per group. One group was treated with DEN/CCl4 to construct a mouse hepatic fibrosis and hepatoma models, and the other group was treated with saline/olive oil as a negative control. About 12 weeks later, the mouse hepatic fibrosis model was firstly established. We sacrificed the mice and excised the livers for analyses. As shown in Fig. S2A, more inflammatory cell infiltrations and necrotic foci were formed in the liver of HBPI-deficient mice treated with DEN/CCl4 compared with that of HBPI wild-type mice. Masson staining and Sirius Red staining showed that hepatic fibrosis only occurred in the mouse group treated with DEN/CCl4, and more severe hepatic fibrosis occurred in HBPI-deficient mice (Fig. S2B). There was no liver damage and fibrosis in wild-type or HBPI-deficient mice in the groups not treated with DEN/CCl4 (Fig. S3A, S3B). We then detected the protein and mRNA levels of several inflammatory factors associated with hepatic fibrosis in the liver of mice. As shown in Fig. S2C and S2D, the protein and mRNA levels of TypeIcollagen, Type III collagen and TNF-α were increased in the liver of HBPI-deficient mice, while IL-1β expression was unchanged compared with levels in wild-type HBPI mice. We also tested ALT and AST levels in mice serum. ALT and AST levels increased after DEN/CCl4 treatment and both levels increased more significantly in HBPI-deficient mice compared with wild-type mice (Fig. S2E), indicating that HBPI-deficient mice had
Fig. 7 (See Fig. 7 continuation on next page.)
Fig. 7 HBP1 inhibits malignancy through suppressing AFP in hepatoma cells. a AFP rescues HBP1-inducing decrease in cell growth and migration, and increase in cell apoptosis. MTT, Transwell and FACS assays were conducted with HepG2 cells stably transfected with control vector, HBP1, or HBP1 + AFP. b AFPshRNA rescues HBP1shRNA-inducing increase in cell growth and migration, and decrease in cell apoptosis. MTT, Transwell and FACS assays were conducted with HepG2 cells stably transfected with control vector, HBP1shRNA, or HBP1shRNA + AFPshRNA. c HBx rescues HBP1-mediated decrease of cell proliferation and migration, and the increase in cell apoptosis. MTT, Transwell and FACS assays were conducted with HepG2 cells stably transfected with control vector, HBP1, or HBP1 + HBx. d Icaritin enhances HBP1-mediated decrease of cell proliferation and migration, and the increase in cell apoptosis. MTT, Transwell and FACS assays were conducted with HepG2 cells stably transfected with control vector, HBP1 with or without Icaritin treatment. e HBP1 acetylation at K419 is indispensable for its inhibition of cell growth and migration, and for its promotion of apoptosis. MTT, Transwell and FACS assays were conducted with HepG2 cells stably transfected with control vector, HBP1, K419R. f Icaritin inhibits tumorigenesis of HepG2 cells in nude mice. HepG2 cells were subcutaneously injected into nude mice. Icaritin was given at a dose of 100 mg/kg via intraperitoneal injection three times a week. The control group was given an equivalent amount of vehicle solvent. Four weeks after injection, the tumors were weighed, and size was measured. Data are shown as mean ± S.D. (n = 5). * p < 0.05, **, p < 0.01. Scale bar, 200 μm.
more severe liver function damage after DEN/CCl₄ treatment, whereas ALT and AST levels did not increase in the group without DEN/CCl₄ treatment (Fig. S3C).

In separate experiments, mice were treated with DEN/CCl₄ or saline/olive oil (as a negative control) for longer (about 20 weeks) to induce hepatoma. HBP1-deficient mice had more liver tumor tissue blocks, and the tumor tissue volume was larger compared with wild-type mice (Fig. 8 a). In addition, multiple nodules were clearly visible on the HBP1-deficient mouse liver surface, while the wild-type mouse liver was relatively smooth. As shown in Fig. 8 b and c, the protein level and mRNA level of AFP were increased in the liver of HBP1-deficient mice. Since AFP is a serum marker of primary liver cancer, we evaluated the AFP content in mouse serum using ELISA and found that serum AFP level of HBP1-deficient mice was higher than that of wild-type mice (Fig. 8 d). IHC staining of paraffin sections showed that Ki67 expression was higher in HBP1-deficient mice compared with wild-type mice (Fig. 8 e), indicating that HBP1-deficient mouse hepatoma was more malignant in the DEN/CCl₄-induced hepatoma model. Together, the data suggest that HBP1 deletion aggravates DEN/CCl₄-induced liver damage, hepatic fibrosis and hepatoma in mice. Our results are thus consistent with a model in which HBP1 inhibits hepatoma by repressing the expression of AFP (see model in Fig. 8 f).

**Discussion**

It has been reported that HBP1 maintains a proliferation barrier through regulating G1 progression in differentiated liver tissue, but the mechanism is unclear [36]. Our current study expands the possibilities for the negative regulation of proliferation by HBP1 through its inhibition of AFP expression. Here, we show that HBP1 regulates AFP expression at the transcriptional level and inhibits the development of hepatoma. The inverse relationship of HBP1 and AFP in hepatoma patients correlates with hepatoma relapse. HBP1 low expression and/or AFP high expression in hepatoma can be correlated with low survival rate. Our experimental data demonstrate that AFP is a target gene of the transcription factor HBP1. HBP1 directly binds to the promoter of AFP to inhibit AFP gene expression. Using multiple point mutants of HBP1, we found that transcriptional inhibition of AFP by HBP1 is dependent on HBP1 acetylation at K419 (Figs. 1, 2, 3). In addition, HBx, a HBV regulatory protein that promotes the development of HBV-associated hepatoma, can interact with HBP1, inhibiting the binding of HBP1 to the AFP promoter region and leading to up-regulated AFP expression. Icaritin, an active ingredient of the Chinese herb epimedium, inhibits the ubiquitination of HBP1 and enhances HBP1 protein stability, resulting in downregulated protein level of AFP. Together, HBx or Icaritin mediates proliferation and migration of hepatoma cells through regulating the HBP1-AFP axis (Figs. 4-5). The repression of AFP by HBP1 attenuates AFP effect on PTEN, MMP9 and caspase-3, thus inhibits proliferation and migration, and induces apoptosis response to oxidative stress in hepatoma cells (Figs. 6-7). We constructed HBP1-deficient mice to investigate the role of HBP1 in the development of hepatoma. We found that AFP protein level increased in serum and liver in HBP1-deficient mice after DEN/CCl₄ induction. Furthermore, HBP1-deficient mice with DEN/CCl₄ induction developed more severe liver damage, fibrosis, and tumorigenesis (Fig. 8). Thus, the variation in the expression levels of HBP1 and AFP, which are both associated with cell proliferation, could have a lasting impact on tumorigenic progression.

In previous studies, HBP1 was described as a tumor suppressor that is activated by p38-MAPK and Pim-1, leading to premature cell decay and apoptosis [24, 25]. HBP1 expression is inhibited in a variety of human malignancies, and HBP1 gene mutation is found in breast cancer, suggesting that the mutation or loss of HBP1 may be one of the causes of cell transformation. In the present study, we found that HBP1 expression was also downregulated in hepatoma tissues, and overexpression of HBP1 in hepatoma cells transcriptionally inhibited AFP expression and decreased cell proliferation and migration. In the process of establishing a mouse hepatoma model, we found that the expression of HBP1 was gradually reduced, while AFP expression increased gradually (Fig. S4), suggesting that the function of HBP1 is gradually suppressed in the process of hepatitis-cirrhosis-hepatoma, and the deregulation of the HBP1-AFP axis contributes to hepatoma progression.

Chronic hepatitis caused by HBV infection is considered to be the main cause of hepatoma. HBV can activate the oncogenic pathway by integrating DNA into the genome of the host cell and causing gene mutations. Previous studies have reported that HBx can interact with the tumor suppressor p53, block p53 nuclear translocation, and abrogate the transcriptional repression of p53 on AFP [36–39]. In this study, we used HBx to mimic the effects of HBV infection. We demonstrated that HBx directly binds to HBP1, inhibits the binding of HBP1 to the AFP gene promoter and up-regulates AFP expression. Our study highlights a novel mechanism underlying HBx regulatory effects in hepatoma. In addition, previous studies showed HBx can inhibit apoptosis and promote tumorigenesis through other mechanisms [40, 41]. Therefore, we believe that due to the presence of HBx, treating HBV with drugs that only target HBV replication cannot completely eliminate the effects of HBV in promoting hepatoma. Alternate
Fig. 8 (See legend on next page.)
strategies should be pursued to inhibit the protein function of HBx, thereby reducing the possibility of hepatitis B patients developing hepatoma.

In this study, DEN/CCl₄ was used to induce a mouse hepatoma model. DEN is a strong carcinogen, and long-term stimulation with CCl₄ causes acute/chronic hepatitis in mice. Studies have shown that 70–90% of patients with hepatitis show symptoms of cirrhosis, and more than 10% of patients with cirrhosis eventually develop hepatoma [42–44]. Our study found that HBP1-deficient mice developed more severe liver fibrosis. This indicates that HBP1 has a certain protective effect on the normal function of the liver in the hepatitis caused by DEN/CCl₄ treatment. Deletion of HBP1 is more likely to cause collagen (Type I and Type III) secretion and deposition, leading to the occurrence of cirrhosis in mice. However, under normal growth conditions, HBP1-deficient mice did not show abnormalities compared with wild-type mice (Fig. S3). This suggests that HBP1 may not affect the growth and development of the liver, but instead HBP1 has a protective effect on the liver when liver cells are exposed to external stimuli such as inflammatory factors and carcinogens. Our results indicate that the presence of HBP1 protects the liver and delays the progression of hepatitis to cirrhosis, finally inhibiting tumorigenesis.

We also found that high expression of HBP1 correlated with increased survival and prognosis in the hepatoma patient group examined in this study. Our results demonstrate that Icaritin enhances the stability of HBP1 and up-regulates the protein level of HBP1, thereby exerting anticancer activity.

Conclusions

The deregulation of AFP by HBP1 is associated with hepatoma cell metastasis and poor survival of hepatoma patients. Under normal conditions, HBP1 inhibits tumorigenesis by repressing AFP expression. During hepatoma progression, this biological process could be reversed by aberrantly low HBP1 expression, which promotes AFP effect on PTEN, MMP9 and caspase-3, thus induces proliferation and migration, and inhibits apoptosis in hepatoma cells. The identification of a mechanism by which high HBP1 expression inhibits hepatoma progression opens up new therapeutic avenues for hepatoma therapy. We suggest that HBP1 expression level may be a useful indicator in the treatment and prognosis monitoring of hepatoma to better improve the prognosis of patients with hepatoma.

Supplementary Information

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Additional file 1: Figure S1. 5-FU promotes the suppression of HBP1 on AFP through enhancing HBP1 binding to AFP promoter. A–C 5-FU promotes the suppression of HBP1 on AFP protein (A), mRNA (B) and promoter (C). HepG2 cells were transfected HBP1 with or without 5-FU treatment. The protein levels of HBP1 and AFP were measured by Western blotting. The mRNA level of AFP was measured by Realtime-PCR. The luciferase activities were detected in 293 T cells co-transfected with AFP promoter and HBP1 with or without 5-FU treatment and expressed as the means ± S.D. of the means from three experiments. (D) 5-FU promotes the interaction between HBP1 and AFP promoter. Two hundred ninety-three T cells were transfected HA-HBP1 with or without 5-FU treatment. The region from position –1600 to position –1448 contains the HBP1 affinity site and was analyzed by specific PCR. (E) HBP1 protein level is elevated in the presence of 5-FU. HepG2 cells were treated with different concentrations of 5-FU. The protein level was measured by Western blotting (top panel). The mRNA level was measured by Realtime-PCR (bottom panel). (F) 5-FU inhibits HBP1 ubiquitination-mediated proteasome degradation. HepG2 cells were treated with 5-FU with or without MG132. The protein level was measured by Western blotting (top panel). Two hundred ninety-three T cells were transfected HA-HBP1 with or without 5-FU treatment for 24 h and then exposed to MG132 for another 6 h prior to lysis. HBP1 protein was isolated by immunoprecipitation and analyzed by anti-IgB antibody (bottom panel). (G) 5-FU enhances the effect of HBP1 on PTEN, caspase-3, and MMP9 protein levels. HepG2 cells transfected with HBP1 were treated with or without 5-FU. The protein levels were measured by Western blotting. (H) 5-FU enhances HBP1-mediated decrease of cell proliferation. MTT assay was conducted with HepG2 cells stably transfected with control vector, HBP1 with or without 5-FU treatment. Error bars represent S.D. *, p < 0.05, **, p < 0.01.

Additional file 2: Figure S2. HBP1 deletion aggravates DEN/CCl₄-induced hepatic fibrosis. (A) Representative H.E. staining of liver sections from 12 weeks after DEN/CCl₄ treatment in wild type or HBP1−/− mice. Scale bar, 100 μm. (B) Representative images of Masson staining and Sirius red staining of liver sections from the mice described in Figure S2A. Scale bar, 200 μm. (C) The protein levels of HBP1, TypeⅢcollagen, TypeⅠcollagen, IL-1 and TNF-α were measured by Realtime-PCR in the liver described in Figure S2A. Serum ALT and AST levels of the mice described in Figure S2A were measured by Western blotting. β-actin was used as a loading control. (D) The mRNA levels of TypeⅢcollagen, TypeⅠcollagen, IL-1 and TNF-α were measured by Realtime-PCR in the mice described in Figure S2A. (E) Serum ALT and AST levels of the mice described in Figure S2A. The means ± S.D. are shown (n = 5). Error bars represent S.D. *, p < 0.05, **, p < 0.01.

Additional file 3: Figure S3. HBP1 deletion has no effect on liver morphology and function in mice without DEN/CCl₄ treatment. (A) Representative H.E. staining of liver sections from 12 weeks after saline treatment in wild type or HBP1−/− mice. Scale bar, 100 μm. (B) Representative images of Masson’s trichome staining and Sirius red staining of liver sections from the mice described in Figure S3A. Scale bar, 100 μm.
Additional file 4: Figure S4A. There is a negative correlation between the expression of HBp1 and AFP in DEN/CCl4-induced hepatoma in mice. The protein levels of HBp1 and AFP in the liver were measured by Western blotting during DEN/CCl4 induction. β-actin was used as a loading control.

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Authors’ contributions
Zhengyi Cao and Yuning Cheng performed most experiments and statistical analysis. Jinyi Wang generated lentivirus gene expression plasmids. Yujuan Liu and Ruixiang Yang performed ChIP. Wei Jiang contributed to the in vivo ubiquitination assay. Xiaowei Zhang and Hui Li conceived the study and wrote the paper. All authors read and approved the manuscript.

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Availability of data and materials
Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Ethics approval and consent to participate
All animal experiments were conducted in accordance with the principles and procedures approved by the Committee on the Ethics of Animal Experiments of Peking University.

Consent for publication
All authors reached an agreement to publish the study in this journal.

Competing interests
The authors declare that they have no competing interests.

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