



Original Article

Interaction of Hepatitis B Virus X Protein with the Pregnane X Receptor Enhances the Synergistic Effects of Aflatoxin B1 and Hepatitis B Virus on Promoting Hepatocarcinogenesis

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Abstract

Background and Aims: Hepatitis B virus (HBV) infection has been found to increase hepatocellular sensitivity to carcinogenic xenobiotics, by unknown mechanisms, in the generation of hepatocellular carcinoma. The pregnane X receptor (PXR) is a key regulator of the body's defense against xenobiotics, including xenobiotic carcinogens and clinical drugs. In this study, we aimed to investigate the molecular mechanisms of HBV X protein (HBx)-PXR signaling in the synergistic effects of chemical carcinogens in HBV-associated hepatocarcinogenesis. **Methods:** The expression profile of PXR-cytochrome p450 3A4 (CYP3A4) signaling was determined by PCR, western blotting, and tissue microarray. Cell viability and aflatoxin B1 (AFB1) cytotoxicity were measured using the cell counting kit-8 assay. Target gene expression was evaluated using transient transfection and real time-PCR. The genotoxicity of AFB1 was assessed in newborn mice with a single dose of AFB1. **Results:** HBx enhanced the hepatotoxicity of AFB1 by activating CYP3A4 and reducing glutathione S-transferase Mu 1 (GSTM1) in cell lines. Activation of PXR

by pregnenolone 16 α -carbonitrile increased AFB1-induced liver tumor incidence by up-regulating oncogenic KRAS to enhance interleukin (IL)-11:IL-11 receptor subunit alpha-1 (IL11RA-1)-mediated inflammation in an HBx transgenic model. **Conclusions:** Our finding regarding AFB1 toxicity enhancement by an HBx-PXR-CYP3A4/ GSTM1-KRAS-IL11:IL11RA signaling axis provides a rational explanation for the synergistic effects of chemical carcinogens in HBV infection-associated hepatocarcinogenesis.

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Introduction

Hepatocellular carcinoma (HCC) is a primary malignancy with a high incidence of mortality rate worldwide.^{1,2} A specific association between hepatitis B virus (HBV) infection and aflatoxin B1 (AFB1) exposure in hepatocarcinogenesis has been suggested.^{3,4} AFB1, a potent hepatotoxicant and carcinogen, requires the bioactivation of AFB1 to the 8,9-epoxide form of AFBO, which is the most important risk factor for HCC due to its irreversible genotoxic effects.^{5,6} Epidemiological studies and a previous prospective case-control study⁷ have suggested a strong interaction between HBV and AFB1 exposure in the etiology of HCC.⁸ However, the mechanism underlying this synergistic interaction remains unclear.

The pregnane X receptor (PXR) is a ligand-dependent steroid and xenobiotic receptor that is responsible for the metabolic activation or detoxification of several carcinogens, and may play various roles in hepatocellular carcinogenesis.^{9,10} The metabolism of AFB1 *in vivo* is closely related to PXR, cytochrome P450 3A4 (CYP3A4), and glutathione S-transferase

Keywords: Liver cancer; Hepatitis B virus X protein; Pregnane X receptor; Aflatoxin B1; Hepatotoxicity.

Abbreviations: AFB1, aflatoxin B1; BW, body weight; CCK-8, cell counting kit-8; CYP1A2, cytochrome p450 1A2; CYP3A4, cytochrome p450 3A4; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; GSTM1, glutathione S transferase Mu 1; h, human; HBV, hepatitis B virus; HBx, hepatitis B virus X protein; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; IL, interleukin; IL11RA-1, IL-11 receptor subunit alpha-1; PCN, pregnenolone 16 α carbonitrile; PXR, pregnane X receptor; qRT-PCR, quantitative real-time polymerase chain reaction; RIF, rifampicin; Tg, transgenic.

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Mu 1 (GSTM1).¹¹ HBV X protein (HBx) is considered a key regulator in HCC, due to its capacity to function as a deregulated transcriptional activator. Although HBx alone rarely causes spontaneous liver cancer in our ATX-HBx/FXR^{-/-} mouse model,¹² it increases the incidence of G/C-to-T/A transversion mutations by approximately 2-fold following AFB1 exposure in ATX-HBx transgenic mice.¹³ In addition, p21-HBx knock-in transgenic (Tg) mice, in which HBx was knocked into the p21 allele, show spontaneous liver tumors at the age of 18 months, whereas p21 knockout does not increase their susceptibility to HCC.¹⁴ Considering that the induction of CYP3A4 by HBx is PXR-dependent and HBx is a PXR cofactor,¹⁵ we propose that HBx-PXR signaling increases the genotoxicity of AFB1 by disrupting the metabolism of AFB1 *in vivo*.

In this study, we demonstrate homeostatic disturbance of AFB1 metabolizing enzymes by HBx contributes to enhancing AFB1 genotoxicity, resulting in oncogenic KRAS signaling to induce hepatocarcinogenesis via activating a PXR signaling axis in HBx Tg mice. These results provide a possible mechanism for the synergism of HBV and AFB1 co-exposure in hepatocarcinogenesis.

Methods

Cell culture

HepG2 human HCC cells, Hepa1-6 murine hepatoma cells, and AML12 normal murine hepatocytes were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (i.e. DMEM), RPMI-1640 medium, or DMEM:F12 medium containing 10% fetal bovine serum, at 37°C in a 5% CO₂ incubator.

Reagents and plasmids

Pregnenolone 16 α -carbonitrile (PCN, a specific mPXR agonist), rifampicin (RIF, a human (h)PXR agonist), and AFB1 were obtained from the Sigma-Aldrich Corporation (St. Louis, MO, USA). The expression plasmids and vectors for mPXR, hPXR, and Flag-tagged HBx have been described previously.^{15,16}

Transient transfection

HepG2 cells were grown to 70–80% confluence in 6-well plates. Cells were transiently transfected with HBx and/or PXR using Lipofectamine™ 2000, as previously described.¹² Transfected cells were treated with drugs for 24 h before being lysed and for RNA extraction as described. Transfection experiments were performed at least three times in triplicate. Data are presented as fold induction over empty vector alone.

Reverse-transcriptase polymerase chain reaction, quantitative real-time polymerase chain reaction (qRT-PCR), and western blotting

Total RNA was prepared using TRIzol reagent. SYBR Green-based qRT-PCR was performed with an ABI 7500 Real-Time PCR System (Applied Biosystems Inc., Hercules, CA, USA). Data were normalized against a cyclophilin control. The qRT-PCR primer sequences are presented in Supplemen-

tary Table 1.

Specific antibodies against HBx (MAB8419; Millipore, Danver, MA, USA), PXR (PP-H4417-00; R&D Systems, Minneapolis, MN, USA), MDR1 (ab3366; Abcam, Cambridge, UK), CYP3A4 (H00001576-B01P; Novus Biologicals, Littleton, CO, USA), SULT2A1 (ab38416; Abcam, Cambridge, UK), CYP3A (sc-30621; Santa Cruz Biotechnology, Dallas, TX, USA), cytochrome p450 1A2 (CYP1A2) (AP11325c; Abgent, Suzhou, China), and GSTM1 (AP6896b; Abgent, Suzhou, China) were used.

Cell proliferation assay

Cell viability and AFB1 cytotoxicity were measured using the cell counting kit-8 (CCK-8) assay (Dojindo Laboratories, Rockville, MD, USA), as described in the following methods. HepG2 or PXR knockdown (HepG2) cells¹⁵ were trypsinized and seeded at 5×10³ cells/well in 96-well plates. After 24 h, AFB1 was added and incubated for another 24–96 h at 37°C. The effects of different AFB1 concentrations were evaluated in HepG2 cells after various exposure times. For longer treatment with AFB1, mycotoxin was added every 48 h, with each medium renewal. Then, 10 μ L of CCK-8 solution was added and plates were incubated for an additional 1–2 h. The optical density for each well was measured at a wavelength of 450 nm.

HBx Tg mice

Six- to eight-week-old HBx Tg mice were maintained on a 12 h light/12 h dark cycle. Animals were allowed food (standard chow) and water *ad libitum*. Mice were euthanized by CO₂ asphyxiation 24 h after treatment with 40 mg/kg of the mPXR agonist PCN by intraperitoneal injection. Livers were excised, snap-frozen on dry ice, and stored at -80°C until further analysis. All protocols and procedures were approved by the Institutional Animal Care and Research Advisory Committee of the Shanghai Cancer Institute. Expression analysis for PXR, MDR1, CYP3A11, SULT2A1, CYP3A, and GSTM1 was performed by qRT-PCR and immunohistochemistry (IHC).

Administration of toxins

Six pregnant HBx Tg female mice were used in the study. Pups (3–7 days of age) were inoculated intraperitoneally with a single injection of AFB1 at 7 mg/kg body weight (BW)¹⁷ with or without 40 mg/kg PCN per week. All dosing was administered by intraperitoneal injection based on BW at the time of treatment. Animals treated with solvent vehicle (dimethyl sulfoxide, commonly known as DMSO) were used as the controls. In the transient genotoxicity study with AFB1, 3- to 7-day-old animals ($n=20$, 5 in each group) were treated with a single dose of AFB1, then euthanized either at 24 h or 7 days to check for AFB1:DNA adducts. Six- to eight-week-old animals (16 HBx Tg mice and 6 wild-type mice) were treated with a single dose of AFB1 at 7 days posttreatment with PCN to assess the activity of PXR signaling. For the chronic AFB1-induced liver cancer HBx Tg mice model, HBx Tg offspring ($n=48$) were randomized into four groups: (1) vehicle ($n=7$); (2) AFB1 ($n=12$); (3) PCN ($n=15$); or (4) AFB1+PCN ($n=14$). Mice were euthanized at 14 months by CO₂ inhalation, and livers were collected for histopathology and qRT-PCR. All procedures were approved by the Ethics Committee of Shanghai Cancer Institute.

IHC and tissue microarray

Clinical samples were obtained from the First Affiliated Hospital and Second Affiliated Hospital of Shantou University Medical College after acquiring informed consent according to an established protocol approved by the Ethics Committee of Shantou University Medical College (SUMC-2015-07) and in conjunction with the ethical requirements of "The Operational Guidelines for Ethics Committees that Review Biomedical Research" and the Declaration of Helsinki.

Human HCC tissues and mouse liver tissues were analyzed using IHC, as previously described.¹² Tissues were first placed into paraffin blocks and dewaxed with xylene followed by rehydration, and antigen retrieval (1:25, DAKO) was performed according to standard procedures. The tissues were then sequentially blocked with 2% bovine serum albumin/0.1% Triton X-100 for 60 min at room temperature. Tissues were incubated with primary antibody against human PXR (1:40) (PP-H4417-00; R&D) or PXR (1:40) (H1-160, sc-25381; Santa Cruz Biotechnology, Dallas, TX, USA), HBx antibody (1:250) (ab39716; Abcam), CYP3A4 (1:500) (H00001576-B01P; Abnova), SULT2A1 (1:500) (sc-32941; Santa Cruz Biotechnology), CYP1A2 (1:50) (AP11325c; Abgent), GSTM1 (1:100) (AP6896b, Abgent), CYP3A (1:150) (L-14; Santa Cruz Biotechnology), or AFB1 (1:150) (6A10, Novus Biologicals LLC) at 4°C overnight, followed with biotinylated horseradish-peroxidase secondary antibody for 1 h. For controls, tissues were incubated without primary antibody.

Immunostaining with antibody against hPXR was performed on a tissue assay of HBV-associated diseases, representing 16 normal liver tissues, 24 hepatitis liver tissues, 32 hepatic cirrhosis liver tissues, and 32 HCC liver tissues (duplicate cores per case), according to the method described above. The results were observed and photographed with an Axioskop 2 microscope (Carl Zeiss, Oberkochen, Germany) and DP70 Imaging System (Olympus, Tokyo, Japan). Slides were graded as follows: -, 0–5% cells stained; +, 5–10% cells stained; ++, 10–50% cells stained; +++, >50% cells stained.

Data mining

Expression patterns of AFB1 metabolic and PXR signaling genes were analyzed in our previous data (GSE84402), which included 13 matched HCC samples and corresponding adjacent liver tissues with HBV infection. Similar information can also be found in the GSE14520 dataset, which includes 35 paired clinical early-stage HBV-associated HCC samples and corresponding adjacent liver tissue.² The relative genes were downloaded and parsed into Excel for analysis. To uncover the prognostic value of AFB1 metabolism relative to PXR signaling genes in the cohort, a Kaplan-Meier plotter was used (<http://kmplot.com/analysis/>). Additionally, the HCCDB3 (GSE25097) dataset (<http://lifeome.net/database/hccdb/home.html>), which includes 268 HCC tissues, 243 adjacent liver tissues, 40 cirrhotic liver tissues, and 6 healthy tissue samples, was used.¹⁸

Statistical analysis

Data were expressed as the mean±standard deviation of three independent experiments. Significance of the differences was determined by Student's *t*-test. Clinical data were analyzed using the Wilcoxon signed-rank and Mann-Whitney *U* tests. The Kaplan-Meier method with log-rank analysis was used to obtain estimates of overall survival. A

p-value <0.05 indicated significant mean differences.

Results

Deregulation of PXR-CYP3A4 signaling is involved in HBV-associated hepatocellular carcinogenesis

We have previously reported that the PXR mRNA level is substantially lower in intermediate and end-stage HBV-associated HCC samples compared to normal and adjacent noncancerous tissue samples.¹⁵ To determine whether PXR signaling is dysregulated in HCC, the dynamic expression of PXR was examined in 104 HBV-associated clinical samples by tissue array. Lower expression was observed in HCC samples than compared to normal controls, adjacent noncancerous tissues, and early small tumors. At the advanced or end stage, there was a general decrease or even absence of PXR in tumor tissues (Fig. 1A and Supplementary Table 2). These results suggested that PXR or PXR signaling is dysregulated in HCC.

We also evaluated the PXR mRNA levels in fresh samples from patients with HBV-associated liver disease (Fig. 1B). The association of PXR with pathological stage was analyzed in six pairs of HCC stage I and HCC stage II tissue samples, by western blotting (Supplementary Fig. 1A). HBx shared similar strong costaining in cirrhosis but showed low co-expression with PXR in HCC sections, as observed by IHC in serial sections (Fig. 1C). Down-regulation of both PXR and CYP3A4 was observed in 24 paired HBV-associated intermediate and advanced HCC tissues compared to corresponding adjacent noncancerous tissues (Fig. 1D and Supplementary Fig. 1B). Moreover, a clinical association between PXR and CYP3A4 with liver disease statuses was found in the HCCDB dataset HCCDB3 (Fig. 1E and Supplementary Fig. 1C; *p*<0.0001).

Activation of PXR by its agonist and/or HBx enhances the toxicity of AFB1

The contribution of HBV infection to hepatocarcinogenesis following AFB1 exposure is related to the sensitization of hepatocyte susceptibility to AFBO cytotoxicity.¹⁹ We have previously reported that CYP3A4 is up-regulated in the pre-HCC stage and adjacent nontumor tissues, and that induction of CYP3A4 by HBx is PXR-dependent.¹⁵ This suggests that the activation of PXR by HBx will affect the metabolism of AFB1 in the liver. As shown in Figure 2A, prolonged exposure to AFB1 led to a significant increase in the sensitivity toward of AFB1 toxicity. Treatment of HepG2 cells with RIF, to activate the PXR, increases AFB1 metabolism by inducing CYP3A4.²⁰ Activation of PXR by HBx produced a similar effect, i.e. the toxicity of AFB1 was enhanced by RIF (Fig. 2B). PXR and HBx co-overexpression also increased AFB1 hepatotoxicity, especially in the presence of RIF (Fig. 2C). Moreover, this effect was abolished by PXR knockdown (Fig. 2D). These results suggest that atypical activation of PXR by HBx is a potent promoting factor that affects AFB1 hepatotoxicity.

Molecular mechanism of AFB1 toxicity enhancement by HBx-PXR interaction

To understand the induction of AFB1 hepatotoxicity by HBx-PXR interaction, we measured the expression of AFB1 metabolizing enzymes in HepG2 cells transfected with PXR and/or HBx. Figure 3A shows the key AFB1-metabolizing

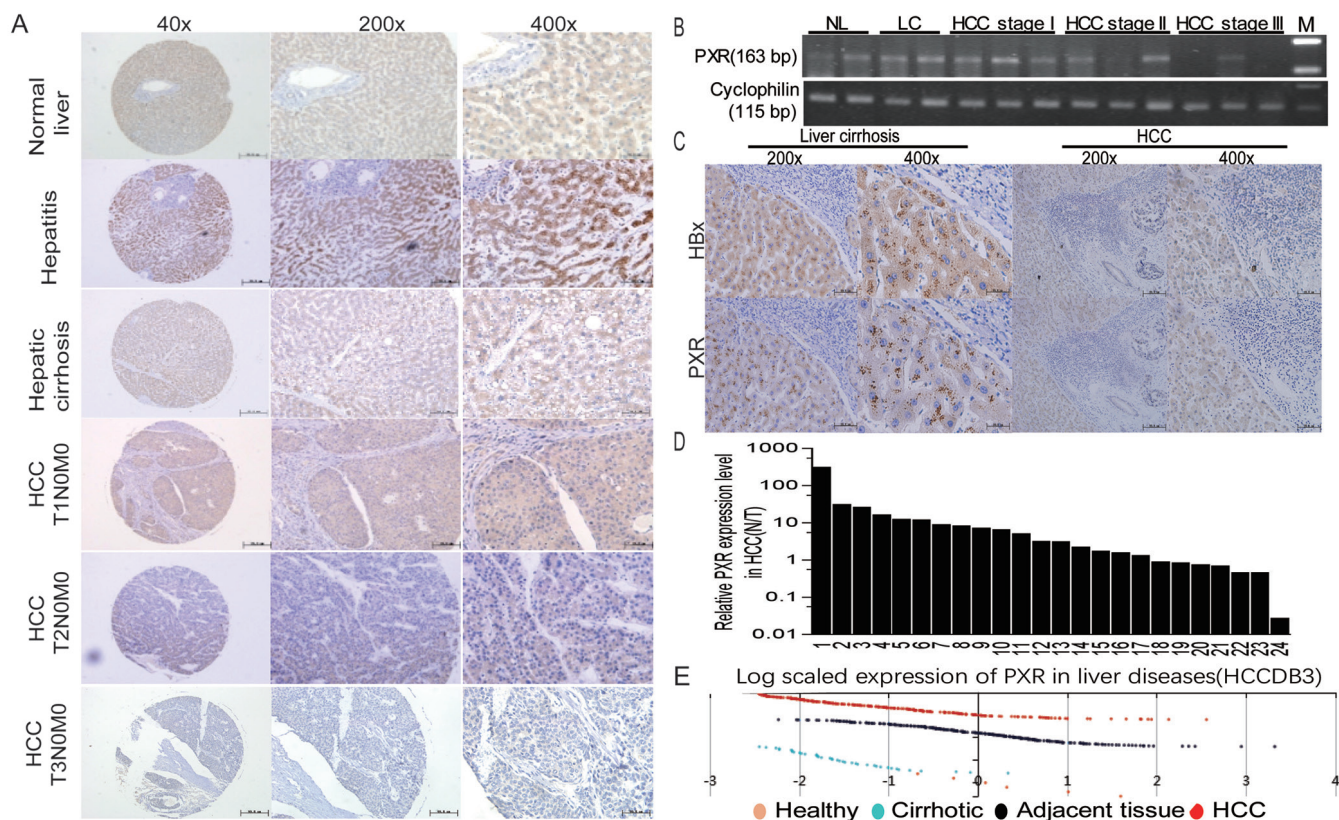


Fig. 1. Ectopic expression model of PXR in liver specimens of HBV-associated liver diseases. (A) IHC of PXR in HBV-associated liver diseases ($n=104$). Representative samples for each stage are shown, and include normal liver tissues, hepatic cirrhosis liver tissues, and HCC clinical pathology stage I-III liver tissues. Scale bar, 100 μm . Original magnification, $\times 40$, 200 and 400. (B) Relative PXR mRNA level in HBV-associated liver diseases. RT-PCR was performed from two normal livers (NL), two cirrhotic livers (LC), three HCC stage I, three HCC stage II, and three HCC stage III cases. (C) IHC showing the same expression pattern of HBx and PXR in hepatic cirrhosis and HCC tissues. Scale bar, 100 μm . (D) PXR expression in 24 paired HCC and non-tumor tissues by qRT-PCR. (E) PXR mRNA levels in a cohort from the HCCDB database (Accession No. HCCDB3).

enzymes involved in hepatocarcinogenesis. CYP3A4, the most important enzyme involved in converting AFB1 to AFBO, was significantly up-regulated in HepG2 cells in the presence of RIF/PXR and AFB1 (Fig. 3B-D). High expression of CYP3A4 and cytoplasmic immunoreactivity was also shown in our previous studies on HBV¹⁵ and HCV cirrhosis, respectively. CYP1A2, which is also responsible for the formation of AFBO, remained unchanged, in urine of patients with HCC and chronic HBV, based on comparisons of the amounts of the AFB1 metabolic product AFM1 and AFB1-N7 guanine adducts. CYP1A2 remained unchanged or slightly decreased in HepG2 cells overexpressing PXR and/or HBx after AFB1 exposure (Supplementary Fig. 2A), and GSTM1, a major factor responsible for the enzymatic detoxification of AFBO,²¹ dramatically decreased (Fig. 3C). EPHX1, which is responsible for AFB1 8,9-epoxide hydrolysis, has been proposed to be important. However, the rapid nonenzymatic hydrolysis is difficult to perform *in vitro*.²²

Additionally, the key enzymes metabolizing AFB1 in the presence of RIF were analyzed in HepG2 cells. Dramatically increased CYP3A4 was found in both the RIF and HBx treatment groups, whereas CYP1A2 was induced by HBx but reduced by HBx and RIF. The change in GSTM1 was not significant (Fig. 3D).

To determine whether PXR signaling indeed regulates key AFB1-metabolizing enzymes, we used short hairpin RNA targeting human PXR. We failed to observe a significant induction of CYP3A4 by HBx in PXR knockdown cells, even

upon RIF treatment. In contrast, we observed the induction of CYP3A4 but not GSTM1 in rescue experiments in PXR knockdown cells by HBx-PXR interaction (Fig. 3E).

Activated HBx-PXR signaling promotes AFB1: DNA adducts by disrupting key AFB1-metabolizing enzymes

To explore whether the activation of PXR by HBx can induce AFB1 genotoxicity *in vivo*, we analyzed the expression of AFB1-metabolizing genes. CYP3A11, GSTM1,2 AND CYP2E1 levels were dramatically increased, while those of Gsta2, which is the murine glutathione S-transferase isozymes alpha class 2, and Gsta3 decreased in mouse hepatoma Hepa1-6 cells transfected with hPXR and HBx, and treated with RIF (Fig. 4A, B). Additionally, the expression of CYP3A11 and GSTM1 increased, but that of GSTA3 decreased in AML12 cells transfected with HBx and treated with AFB1 compared to cells transfected with the empty vector (Supplementary Fig. 2B). To demonstrate the activation of PXR by HBx *in vivo*, we showed that there is no difference in mPXR and mRXR between wild-type and HBx Tg mice (Fig. 4C), and the expression of CYP3A11 was increased in both HBx Tg and wild-type mice (Fig. 4D, E). To further confirm the activation by HBx on mPXR, the expression of Mdr1 and Sult2a1 was observed in both HBx Tg and control mice (Fig. 4D, E).

To determine the genotoxicity of AFB1-exposed mice, we

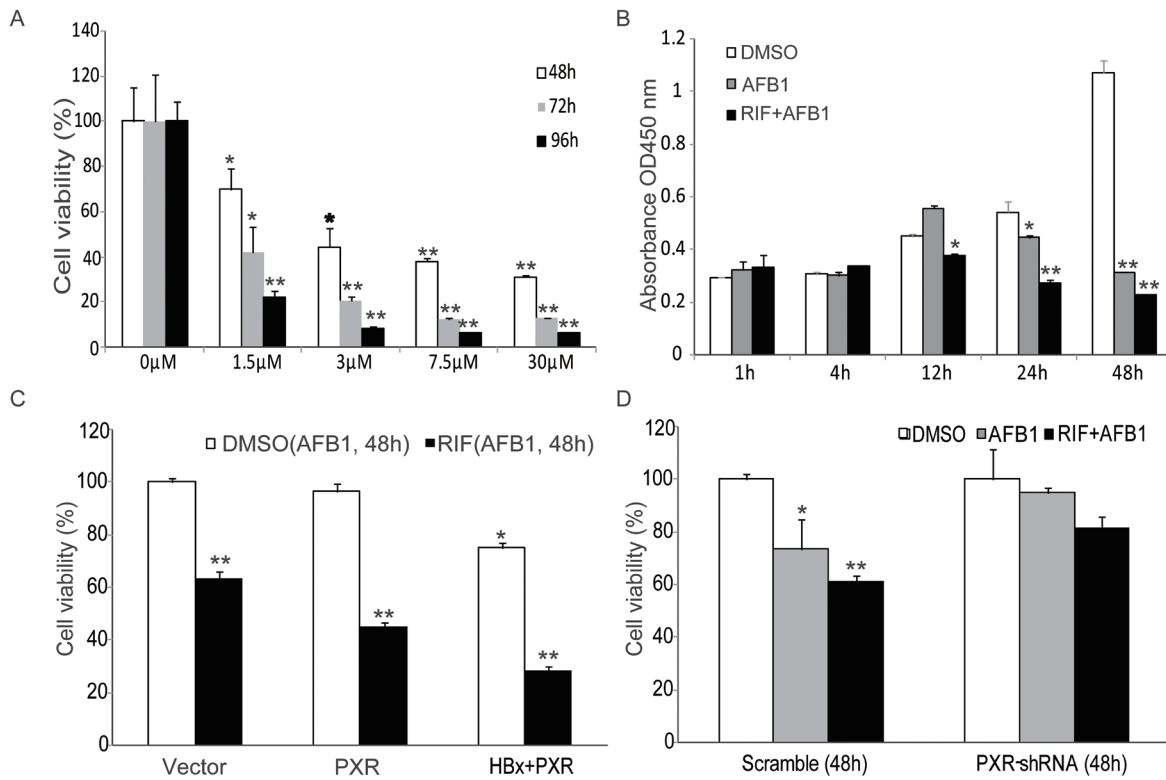


Fig. 2. Activated PXR signaling enhances the liver toxicity of AFB1. (A) Dose-dependent and time-dependent response curves of AFB1-induced toxicity in HepG2 cells. (B) Activated endogenous PXR signaling by RIF enhanced liver cell toxicity of AFB1. (C) Co-overexpressed hPXR and HBx enhanced the liver toxicity of AFB1. HepG2 cells were treated with RIF (10 μM) and AFB1 (3 μM) after transient transfection of PXR and/or HBx. (D) Toxicity of AFB1 can be blocked by knockdown of PXR. Comparative toxicity of AFB1 in PXR knockdown cells. AFB1 cytotoxicity was assayed using a CCK-8 assay. Three independent experiments were performed. * $p < 0.05$, ** $p < 0.01$, treated vs. control.

measured AFB1:DNA adducts by histological evaluation of newborn mice with and without PCN treatment (Fig. 4F). We failed to find significant differences between the AFB1 and AFB1+PCN groups in terms of forming AFB1:DNA adducts in the liver biopsies of HBx Tg mice; although, there could have been a marginal increase in AFB1:DNA adducts in the HBx Tg (AFB1+PCN) group compared to that in the wild-type (AFB1+PCN) group (Fig. 4F).

CYP1A2 and CYP3A11 increased, while GSTM1 decreased in HBx Tg mice compared to wild-type mice after treatment with a single dose of AFB1 (Fig. 4G). Notably, adult mice could better tolerate aflatoxin-induced genotoxicity, possibly due to the increase in the number of GST genes. Here, *Gsta1*, *Gsta2* and *Gsta3*, which are responsible for AFB1 detoxification in mice,²³ dramatically increased in HBx Tg mice (Fig. 4G). Also, a significant change occurred in AFB1-metabolizing enzymes in both AFB1-exposed HBx Tg mice and control mice, especially after PCN treatment (Fig. 4H, J), confirming that PXR signaling is involved in the potentiation of AFB1 genotoxicity *in vivo*, and suggesting that HBx-PXR-AFB1:DNA adduct signaling might serve as a driver in hepatocarcinogenesis.

Activated PXR signaling promotes AFB1-induced liver tumorigenesis in HBx transgenic mice

To determine the possible effect of HBx in potentiating the ability of AFB1 to induce tumorigenesis via PXR signaling, we established a chronic AFB1-induced liver tumor HBx Tg mouse model (Fig. 5A). We treated newborn mice with a

single dose of AFB1, PCN, or vehicle neonatally. A total of 48 HBx Tg mice were euthanized and examined for liver tumors at 14 months of age. As shown in Figure 5B, approximately 42.9% (6/14) of mice treated with AFB1+PCN and 20.0% (3/15) of those treated with only AFB1 developed identifiable liver tumors, whereas no visible neoplasms or preneoplastic lesions were observed in the vehicle or PCN group for up to 14 months. Tumorigenesis in both the AFB1 and AFB1+PCN groups were confirmed by hematoxylin and eosin staining (Fig. 5C). Tumor numbers (6/6) and liver index (ratio of liver weight to BW) in male mice in the AFB1+PCN group were higher than the tumor numbers (3/8) of their male counterparts in the AFB1 group (Fig. 5D, E). Similarly, tumor incidence in the female AFB1+PCN group was higher than that in their female counterparts in the AFB1 group (Fig. 5F).

To gain insight into the heightened carcinogenesis in the AFB1+PCN group, we observed the up-regulation of the oncogene *KRAS*, which is consistent with tumor incidence. Oncogenic Ras can induce interleukin (IL)11 production in both mouse NIH3T3 and human pancreatic carcinoma cells.²⁴ However, we failed to show a significant change in IL11 (Fig. 5G). Considering the function of IL11 is dependent on the binding of IL-11 receptor subunit alpha-1 (*IL11RA-1*), which is reportedly involved in liver fibrosis, hepatocyte death, and inflammatory response,²⁵ we assessed the expression of *IL11RA-1* and showed a similar pattern between *KRAS* and *IL11RA-1* in AFB1-induced liver tumorigenesis animal models (Fig. 5G). Since *IL11* is a canonical member of the *IL6* family, which has been identified as a key driver of hepatocarcinogenesis,^{26,27} we also examined the expression of *IL6*, which is substantially increased in mouse-liver tumori-

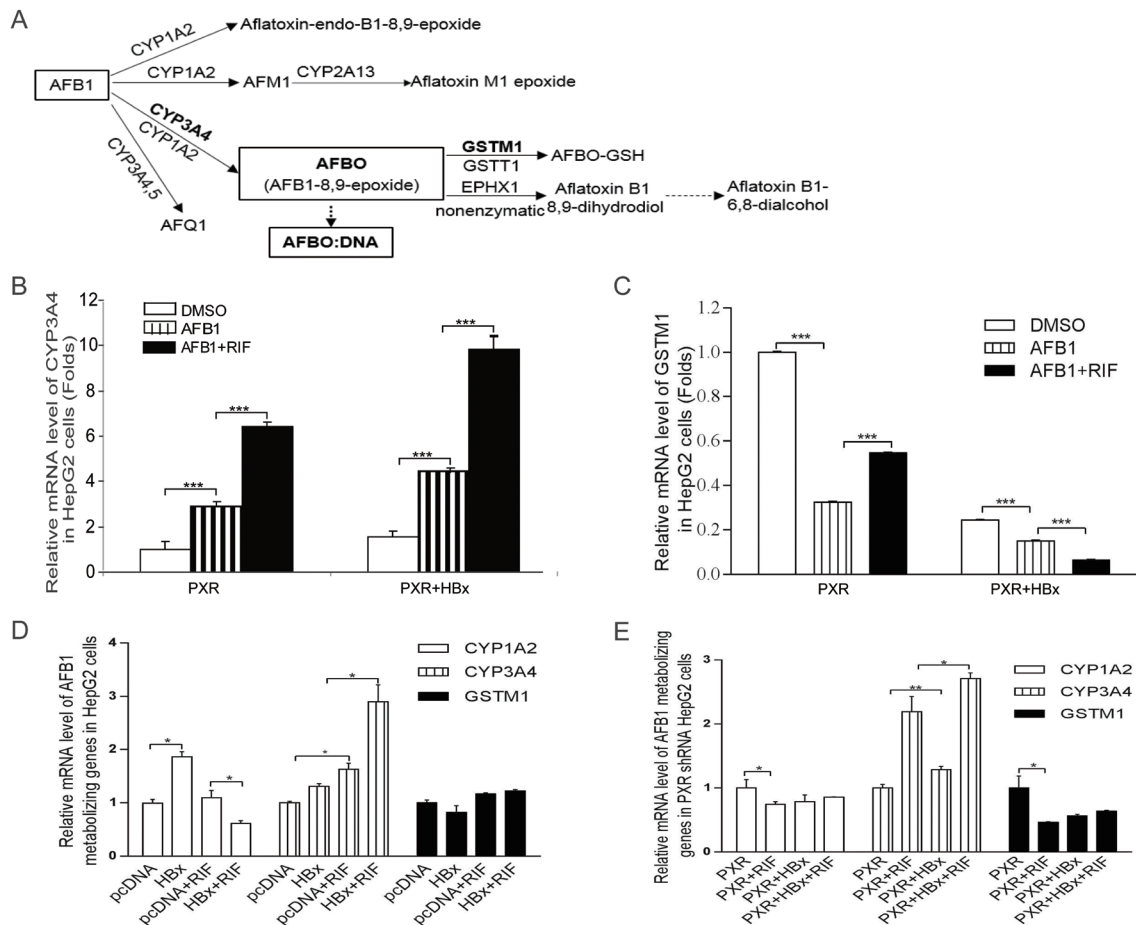


Fig. 3. Relationship of AFB1-metabolizing genes and the activated hPXR signaling pathway in HCC. (A) Schematic showing the key enzymes or steps in the metabolism of AFB1 in liver. (B,C) AFB1 and/or RIF enhanced CYP3A4 and GSTM1 expression. HepG2 cells were transiently transfected by PXR alone or PXR and HBx, and treated with RIF and/or AFB1 (3 μM), and then mRNA was isolated for qRT-PCR analysis of CYP3A4 and GSTM1. (D) Relationship of AFB1-metabolizing genes and the activated endogenous PXR signaling pathway in HepG2 cells. (E) Expression of genes related to AFB1 metabolism in cells overexpressing PXR with or without HBx.

genesis. The expression of *IL6* receptors showed either an opposite or no change (Fig. 5H).

Clinical implications of an HBx-PXR-AFB1-metabolizing signaling axis in patients with virus-associated HCC

To assess the clinical significance of AFB1-metabolizing genes in HBV-associated HCC, we compared the ratio of AFB1-metabolizing genes in 24 paired HBV-associated intermediate and advanced HCC clinical samples (Fig. 6A). The expression patterns of *PXR*, *CYP3A4*, *GSTM1*, *KRAS*, and *IL11RA* in HBV-associated HCC were further confirmed by data mining in the GSE84402 dataset (Fig. 6B). Consistently, we observed possible clinically associated expression patterns of *PXR*, *CYP3A4*, *GSTM1*, *KRAS*, and *IL11RA* in GSE14520, which included 35 paired clinical early-stage HBV-associated HCC samples and corresponding adjacent liver tissue samples (Fig. 6C and Supplementary Fig. 3A–J). Additionally, to further uncover the possible prognostic value of *PXR*, *CYP3A4*, *GSTM1*, *KRAS*, *IL11*, and *IL11RA* in the cohort, RNA-seq data from 93 Asian patients with HCC and hepatitis virus infection were analyzed by Kaplan-Meier plots with a log-rank test. We found that poor overall survival of HCC patients was associated with high *CYP3A4* and

GSTM1 expression levels (Fig. 6D, E; log-rank $p < 0.05$) but not with *PXR*, *KRAS*, *IL11*, and *IL11RA* (Supplementary Fig. 4A–D).

Discussion

Viral infection alone is rarely oncogenic in hepatocarcinogenesis,²⁸ although integration sites in the human genome of HBV have been observed more frequently in tumors.²⁹ The TERT promoter, Wnt, TP53, and MLL4 confer risk for HBV-related HCC, as determined through a genome-wide association study³⁰ and exome sequencing.³¹ However, HBV and its DNA replication fail to increase DNA damage and TP53 mutation by AFB1 in HepaRG cells, which have hepatocyte-like morphology, effectively metabolize AFB1 and support HBV infection.³² Aflatoxin exposure to individuals with HBV infection increases the risk of HCC by at least 3-fold.³³ Hepatic oval cells, considered to be liver stem cells, cause liver tumors in an overexpressing HBx xenograft tumor model combined with AFB1 exposure.³⁴ The collaborative effect of HBx and AFB1 in causing hepatic steatosis in zebrafish and HBV transgenic mice has been reported.³⁵ Our results suggest an HBx-PXR-CYP3A4/GSTM1-AFB1 genotoxicity-KRAS-IL11:IL11RA signaling axis to explain the synergistic effect of chemical and infectious liver carcinogens

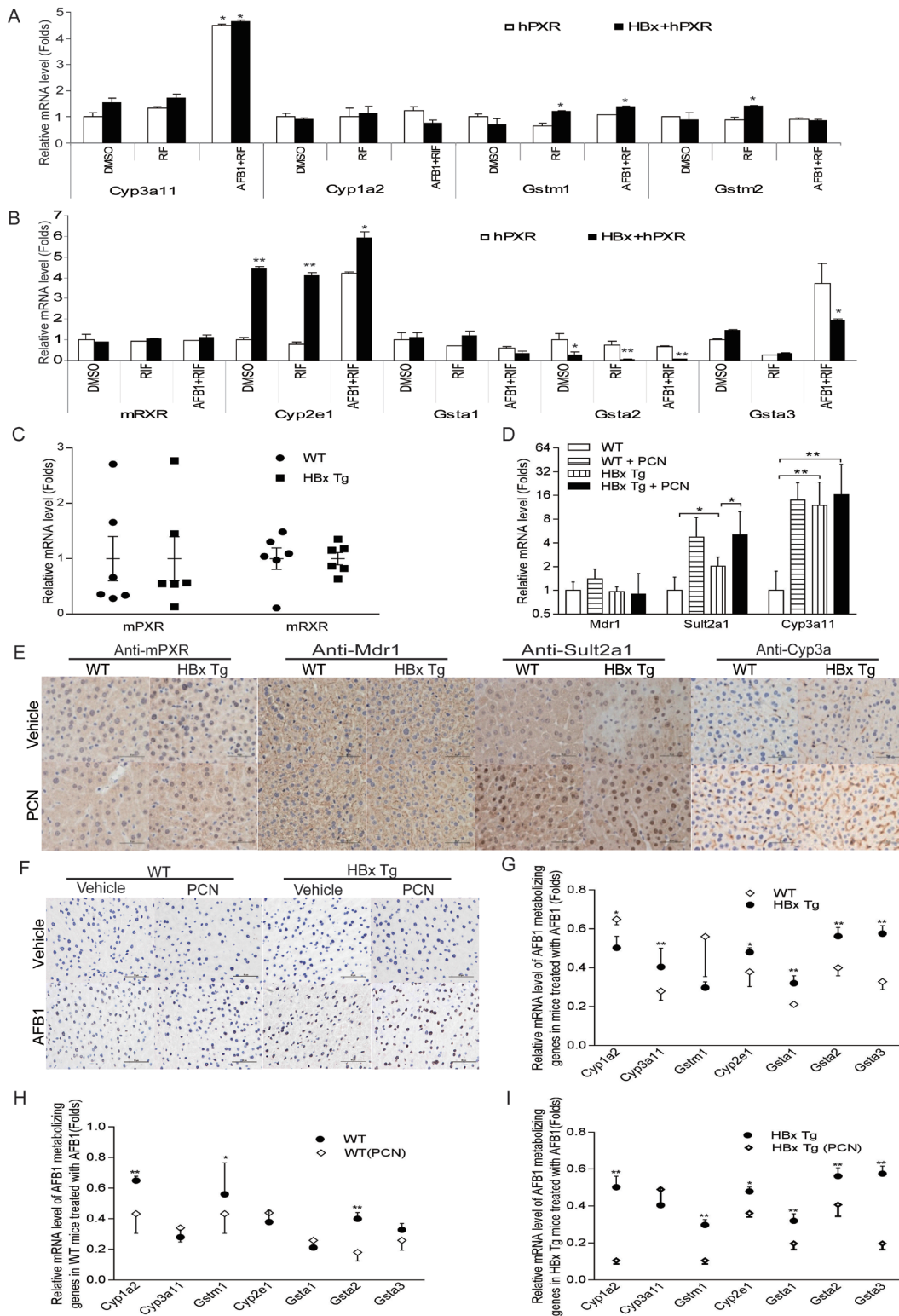


Fig. 4. PXR-AFB1-metabolizing genes (Cyp3a11/Gstm1)-AFB1:DNA adduct signaling in HBx Tg mice. (A, B) Relationship of AFB1 metabolizing genes and HBx. Mice Hepa1-6 cells were treated with RIF and AFB1 after transient transfection of HBx and hPXR. Expression of CYP3A11, CYP1A2, GSTM1, mRxx, CYP2E1, Gsta1, Gsta2, and Gsta3 was analyzed by qRT-PCR. (C) Expression of mPXR and mRxx in HBx Tg mice treated with PCN. (D, E) Expression of PXR signaling genes in HBx Tg mice after PCN treatment, determined by qRT-PCR and IHC. Representative immunostaining of mPXR, Mdr1, Sult2a1 and CYP3A (instead of CYP3A11) in control mice and HBx Tg mice. (F) IHC showing induction of AFB1:DNA adducts by activated PXR in newborn HBx Tg mice. (G-I) Representative expression of genes responsible for AFB1 metabolism in both control mice and HBx Tg mice with or without PCN treatment. * $p < 0.05$, ** $p < 0.01$, treated vs. control.

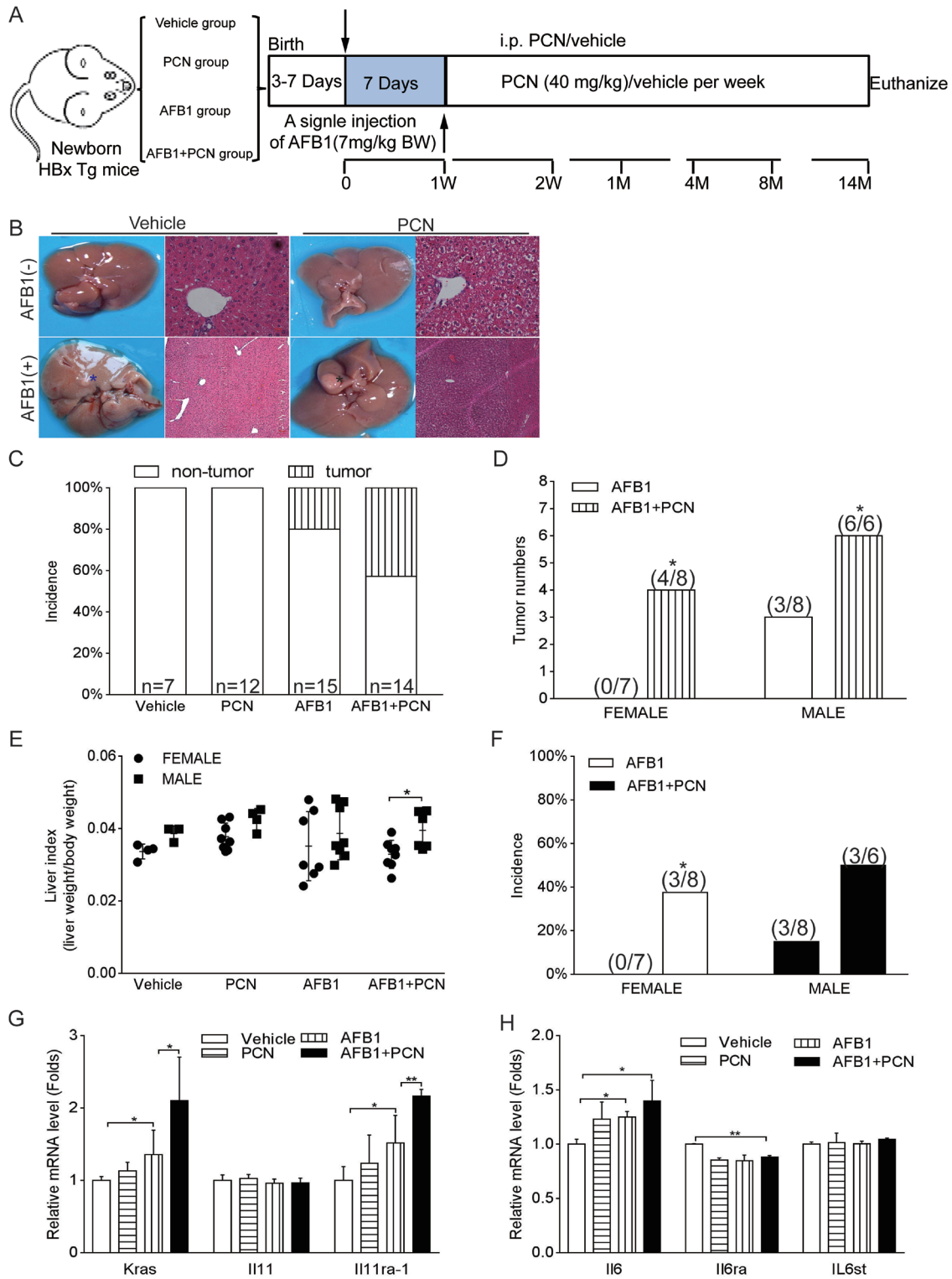


Fig. 5. Molecular mechanism of tumorigenesis due to HBx and AFB1 co-exposure in mice. (A) Scheme of the experimental protocol for a single injection of AFB1 (7 mg/g) in newborn HBx Tg mice. All mice were first treated with PCN (40 mg/kg of BW) or vehicle on day 7 post-injection of AFB1, and then were administered intraperitoneal injections every 7 days. Mice were euthanized at 14 months. (B) Representative gross appearance and hematoxylin and eosin-stained sections (×100 magnification) of livers from 14-month-old HBx Tg mice. Asterisks denote visible tumors between the groups as indicated. Representative hematoxylin and eosin staining from HBx Tg mice/(AFB1, AFB1+PCN) groups showing liver tumorigenesis *in situ*. (C) Prevalence of tumorigenesis in vehicle (n=7), PCN (n=12), AFB1 (n=15), and AFB1+PCN (n=14) HBx Tg mice. (D-G) Tumor number, liver index and prevalence of tumorigenesis in male and female HBx Tg mice. (H) Expression of inflammatory genes was measured by qPCR. *p<0.05, **p<0.01.

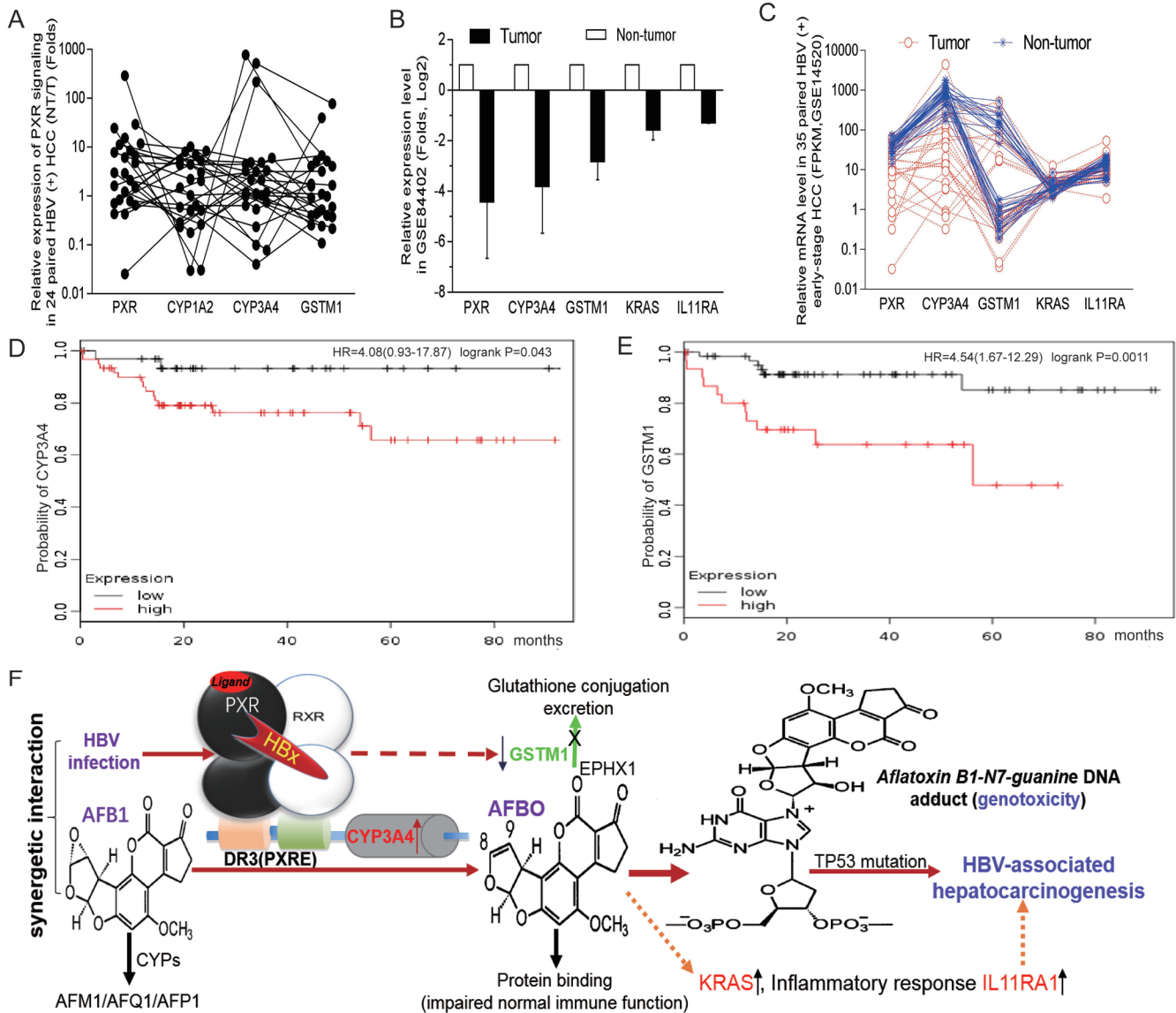


Fig. 6. Clinical correlation of the HBx-PXR-AFB1-metabolizing signaling axis in HBV-associated HCC. (A) Expression of PXR, CYP1A2, CYP3A4 and GSTM1 in 24 paired HBV-associated HCC samples. The value represents the ratio of gene expression in adjacent nontumor tissues to that in the corresponding HCC. (B) Expression of PXR, CYP3A4, GSTM1, KRAS and IL11RA in HBV-associated HCC from the GSE84402 dataset. (C) Expression of PXR, CYP3A4, GSTM1, KRAS and IL11RA in the early-stage HBV-associated HCC dataset (GSE14520). (D, E) Prognostic value for survival time of CYP3A4 and GSTM1 in the Asian HCC cohort with hepatitis. (F) Schematic diagram illustrating AFB1 exposure combined with ectopic activation of PXR signaling by HBx in hepatocarcinogenesis. Under the conditions of AFB1 and HBV co-exposure, the binding of agonist-activated PXR with RXR to the DR3(PXRE) region of the CYP3A4 promoter is enhanced by HBx coactivation. Bioactivation of AFB1 to AFBO, which is catalyzed by CYP3A4, is augmented as a result of enhanced transactivation of the CYP3A4 gene. Alternatively, the detoxification pathway through EPHX and GSTM1 is compromised by the molecular interaction of PXR and HBx due to transcriptional suppression of GSTM1. As a result, genotoxic AFBO accumulates to form DNA adducts or binds to proteins and causes genomic instability, which may lead to mutations in p53 and/or an increased activity of the KRAS-IL11:IL11RA-1 axis.

in hepatocarcinogenesis (Fig. 6G), although the findings are not likely the only reasonable explanation for hepatitis virus-associated HCC (Fig. 6F).

PXR can increase toxic xenobiotic-induced hepatotoxicity in the liver.^{10,36} CYP3A4 increases liver toxicity by metabolizing xenobiotics to carcinogens, which has been shown to have clinical significance.³⁷ PXR-CYP3A11 might be involved in the inflammatory response to tumorigenesis in AFB1-induced liver tumor models.³⁸ HepG2 cells with activities of various phase I, II, and III enzymes represent a good cellular model to investigate the activation and detoxification of genotoxic procarcinogens.³⁹ Our findings

that RIF increases susceptibility to AFB1 in HepG2 cells but not PXR knockdown cells also support our hypothesis that PXR is involved in the activation and detoxification of AFB1. Iterative HBV infection leads to interrelated changes in CYP3A4 involved in the carcinogenic activation of AFB1 in patients with hepatitis or cirrhosis.⁴⁰ The induction of P450s by HBx has been associated with the bioactivation of AFB1 to AFBO.⁴¹ Subsequently, we showed that HBx increases AFB1 cytotoxicity via PXR-CYP3A4 signaling. Moreover, chromatin immunoprecipitation-sequencing data show GSTM1, a phase II metabolism gene closely related to HCC,⁴² is regulated by PXR.⁴³ Of note, in China

in early 1995, individuals from a high AFB1-exposed population with mutant genotypes at EPHX1 and GSTM1 were reported to be at greater risk for HCC due to p53 mutations at codon 249. EPHX1 is speculated to be a novel PXR target gene.⁴⁴ However, the EPHX1 locus did not appear to be related to HCC in a human study including 231 HCC cases and 256 controls.⁴⁵ Despite the fact that HBx might be involved in the induction of phase I and II metabolic enzymes responsible for the bioactivation and elimination of AFB1,⁴⁶ HBx alone is insufficient to induce most CYPs and GSTs to alter the antioxidant system in most cell studies and animal models.⁴⁷ Mariana *et al.*⁴⁸ reported that, via a meta-analysis, the evidence for HBV-aflatoxin interaction with TP53 mutation in HCC is weak. Sulforaphane inhibits the formation of AFB1:DNA adducts, which is dependent on transcriptional repression of AFB1 metabolic enzymes rather than direct inhibition of catalytic activity, and GSTM1 has strong protective effects against DNA damage by AFB1 in the human liver.⁴⁹ Similarly, the synergistic interaction between virus and AFB1 exposure does not provide direct evidence to elucidate the synergistic effect of co-exposure of HBx and chemical carcinogens.

Based on our observations, HBx-PXR signaling can increase AFB1 hepatotoxicity in HepG2 cells, and activated PXR can promote the incidence and number of liver malignancies resulting from AFB1-induced carcinogenesis in HBx Tg mice by the age of 14 months, as observed in comparison to our cohort of paired only AFB1 exposure, vehicle, and PCN groups. Therefore, we propose that abnormal transactivation of PXR by HBx and/or AFB1 may promote subsequent carcinogenesis, despite the fact that we did not show whether there is a difference in AFB1:DNA adducts between the AFB1+PCN group and AFB1 treatment only group in the initiation stage of carcinogenesis. Moreover, sex disparity is a remarkable feature of inflammation-driven HCC.¹ IL-6 has been identified as a key regulator of male-predominant liver carcinogenesis.²⁶ We also showed that males develop liver neoplasms with much higher frequency than females, and KRAS-mediated canonical IL-6 family member IL11:IL11RA-1 signaling may be a major driver of hepatocellular carcinogenesis in our animal models; although, we still cannot discount the interference from IL6:IL6RA signaling.

In conclusion, by exploring the relationship between HBx-PXR interaction and AFB1 metabolism, we propose that sustained activation of PXR, especially by HBx, might aggravate the hepatotoxicity or genotoxicity of AFB1 by inducing CYP3A4 and reducing GSTM1. Furthermore, increased tumor development may be linked to an oncogene KRAS-mediated IL11:IL11RA inflammatory response to induce HCC. Overall, we uncovered an HBx-PXR-CYP3A4/GSTM1-AFB1 genotoxicity-KRAS-IL11:IL11RA signaling axis to explain the synergistic effect of chemical and infectious liver carcinogens in hepatocarcinogenesis, though the exact relationship of IL11RA signaling, synergistic effects of aflatoxin B1 and HBV in promoting hepatocarcinogenesis remains complicated.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceived the project (YN, WX, GS), designed the experiments (YN, SF), performed the experiments (QL, LC, DH, SF, WC), performed analyses (YN, SF, WC), oversaw the writing of the manuscript (YN, WX, GS). All authors read and approved the final manuscript.

Data sharing statement

All data are available upon request.

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