Digestive and Liver Disease 45 (2013) 1041–1048

Contents lists available at ScienceDirect

Digestive and Liver Disease

journal homepage: www.elsevier.com/locate/dld

Liver, Pancreas and Biliary Tract

Hepatitis B virus X protein co-activates pregnane X receptor to induce the cytochrome P450 3A4 enzyme, a potential implication in hepatocarcinogenesis

Yongdong Niu[a,b], Zheng Wu[a], Qiujin Shen[a], Jin Song[a], Qin Luo[a], Haiyan You[a], Ganggang Shi[b], Wenxin Qin[a,*]

[a] State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China
[b] Department of Pharmacology, Shantou University Medical College, Guangdong, China

ARTICLE INFO

Article history:
Received 18 April 2013
Accepted 10 June 2013
Available online 26 July 2013

Keywords:
Cytochrome P450 3A4
Hepatitis B virus X protein
Liver cancer
Pregnane X receptor

ABSTRACT

Background: Hepatitis B virus X protein is a key regulator of hepatocarcinogenesis. The pregnane X receptor is a xenobiotic nuclear receptor that plays a role in the regulation of drug-metabolizing enzymes including the cytochrome P450 3A4, an enzyme important for the bioactivation of the liver carcinogen aflatoxin B1.
Aims: To identify novel host factor that interacts with hepatitis B virus X protein and the functional interaction between hepatitis B virus X protein and pregnane X receptor in hepatocarcinogenesis.
Methods: Co-immunoprecipitation, glutathione *S*-transferase pull-down, and chromatin immunoprecipitation were utilized to assess the interaction between hepatitis B virus X protein and pregnane X receptor. The functional relevance of hepatitis B virus X protein–pregnane X receptor interaction was investigated in cell cultures and hepatocellular carcinoma samples.
Results: We observed that hepatitis B virus X protein and pregnane X receptor co-localize in hepatic cells. Pregnane X receptor interacted with hepatitis B virus X protein via the ligand-binding domain of pregnane X receptor. Functionally, hepatitis B virus X protein increased the transcriptional activity of pregnane X receptor. Pregnane X receptor was able to recruit hepatitis B virus X protein to the CYP3A4 gene promoter. In clinic samples, the expression of pregnane X receptor was high in hepatitis B virus-associated liver cirrhosis and stage I hepatocellular carcinoma, but low in state II and stage III hepatocellular carcinoma.
Conclusion: We revealed a novel function of hepatitis B virus X protein in co-activating pregnane X receptor. The increased expression of pregnane X receptor and its target gene CYP3A4 are potential biomarkers for the early stage of hepatitis B virus-associated hepatocarcinogenesis.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the leading malignancies with high rate of deaths worldwide [1]. Approximately 80% of HCC are attributable to chronic hepatitis virus infection [2,3]. Hepatitis B virus X protein (HBx) is considered to be a key regulator [4] in the development of hepatitis B virus (HBV)-associated HCC by virtue of its capacity to function as a deregulated trans-activator [5,6].

To investigate the molecular mechanism for the association between HBx and HCC, we tried to identify novel host factors that interact with HBx. There is increasing evidence that HBx may functionally interact with nuclear receptors, a family of ligand-dependent transcriptional factors. Moreover, the functional interaction between HBx and nuclear receptors may be involved in the pathogenesis of HBV-associated hepatocarcinogenesis [7–12]. The current study is aimed to further examine the role of HBx-nuclear receptor interaction in hepatocarcinogenesis.

Pregnane X receptor (PXR) is known as a xenobiotic nuclear receptor that is responsible for the metabolic activation or detoxification of many carcinogens. Recent evidence suggested that PXR may also play a role in carcinogenesis and tumour progression [13,14]. For example, PXR can affect the bioactivation of the liver

* Corresponding author at: State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiao Tong University School of Medicine, No. 25, Lane 2200, Xie-Tu Road, Shanghai 200032, China.
Tel.: +86 21 64436581; fax: +86 21 64432142.
E-mail addresses: ydniu@126.com (Y. Niu), tiger1403@163.com (Z. Wu), shenqiujinnsd@163.com (Q. Shen), songjin7191@gmail.com (J. Song), luoqin1989@163.com (Q. Luo), bettyyouyou@yahoo.com.cn (H. You), ggshi@stu.edu.cn (G. Shi), wxqin@sjtu.edu.cn (W. Qin).

1590-8658/$36.00
http://dx.doi.org/10.1016/j.dld.2013.06.004

carcinogen aflatoxin B1 (AFB1) [15], because cytochrome P450 3A4 (CYP3A4), a primary PXR target gene, is responsible for the bioactivation of AFB1 [16].

In this study, we demonstrated that HBx has a novel function in co-activating PXR and inducing the expression of CYP3A4. The increased expression of PXR and CYP3A4 are potential biomarkers for the early stage of HBV-associated hepatocarcinogenesis.

2. Materials and methods

2.1. Cells and cell culture

HepG2, Hep3B and HEK293T cells were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum at 37 °C in a 5% CO_2 incubator.

2.2. Reagents and plasmids

Pregnenolone 16α carbonitrile (PCN) and rifampicin (RIF) were purchased from Sigma. The reporters tk-3A23-Luc, MH100(UAS)x4-tk-Luc and the expression vectors for mouse PXR (mPXR), mPXR ligand-binding domain (LBD), hPXR, pCMX-β-gal, Gal-mPXR, and HA-tagged mPXR (HA-mPXR) have been previously described and they are generous gifts from Dr. Yonggong Zhai from the Beijing Normal University [14,17,18]. Flag-tagged HBx (Flag-HBx) was constructed by the insertion of HBx cDNA into the pcDNA3.0-Flag vector. pCMX-GAL4-HBx was constructed by inserting HBx cDNA into pCMX-GAL4 vector. Glutathione *S*-transferase (GST)-mPXR LBD was constructed by inserting mPXR LBD cDNA into pET-mPXR vector. GST-HBx was constructed by inserting HBx cDNA into pGEX-2 T vector. All new constructs were verified by DNA sequencing.

2.3. Reverse-transcriptase polymerase chain reaction (RT-PCR), quantitative real-time polymerase chain reaction (qRT-PCR), and Western blotting

Total RNA was extracted using TRIZOL reagent. SYBR Green based real-time PCR was performed with the ABI 7300 Real-Time PCR System. Data were normalized against the control cyclophilin. The PCR primer sequences are listed in Table 1. HBx, hPXR, and CYP3A4 monoclonal antibodies were purchased from Millipore, R&D, and Abnova, respectively.

2.4. Transient transfection, luciferase reporter gene, and β-galactosidase analysis

HepG2 cells were grown to 70–80% confluence in 48-well plates. Cells were transiently transfected, using Lipofectamine™ 2000, with pcDNA-HBx in the presence or absence of pCDG-mPXR. Transfected cells were then treated with vehicle or ligands for 24 h before harvesting for luciferase assay. The luciferase activities were normalized against the β-galactosidase activities from the co-transfected pCMX-β-gal plasmid. Transfection experiments were performed at least three times in triplicate.

2.5. Mammalian two-hybrid assay

HepG2 or HEK293 cells were seeded in 48-well plates, and cells were transiently transfected with Gal4-mPXR and pcDNA-HBx, or Gal-HBx and VP-mPXR/VP-hPXR, as well as the MH100(UAS)x4-tk-Luc reporter plasmid. After 48 h, cells were harvested and the luciferase activities were measured.

2.6. Immunofluorescence staining

Indirect immunofluorescence for HBx and PXR was carried out in Hep3B or HepG2 cells. Cells were fixed with 3.7% paraformaldehyde on the coverslips for 30 min at room temperature, permeabilized with 0.2% Triton X-100 at room temperature for 20 min, and blocked with 1% donkey serum for 1 h at 37 °C. HBx was detected with anti-Flag (M2) (Sigma) and visualized with donkey anti-mouse IgG: FITC (LifeSpan BioSciences). HA-mPXR was detected with anti-HA (Cell Signaling) and visualized with donkey anti-rabbit IgG: CY3 (Santa Cruz, CA). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Photographs were taken with the Nikon ECLIPSE TE300 microscope.

2.7. Confocal laser scanning microscopy

Five thousand HepG2 cells were seeded per 3.5-cm dish, transfected and immunofluorescence staining as described above. HBx and PXR were detected by anti-Flag, anti-HA, anti-human PXR/NR1I2 (R&D), and anti-HBx (Millipore), visualized with goat anti-mouse IgG (H + L): Alexa flour 594 (Invitrogen) and goat anti-rabbit IgG (H + L): Alexa Fluor® 488 (Invitrogen). Nuclei were stained with Hoechst (Invitrogen). The cells were imaged by confocal laser scanning.

2.8. Co-immunoprecipitation assay

The co-immunoprecipitation assays were performed as described [19]. 2.5 μg plasmid DNAs of HA-PXR or/and Flag-HBx were transfected into HEK293T cells. 48 h after transfection, cells were collected by scraping and incubated in 500 μl modified RIPA buffer containing protease inhibitors. Clear lysates were pre-cleared by adding 25 μl protein G beads slurry. Supernatants were incubated with 1 μg of rabbit anti-HA antibody with rotation overnight in cold room, followed by an additional incubation for 1–2 h with protein G beads. The beads were washed 3 times with RIPA buffer before loading SDS–PAGE gels for Western blot analysis.

2.9. GST pull-down assay

The GST pull-down assays were performed as described [20]. The recombinant fusion protein was purified by glutathione-sepharose-4B beads. Recombinant ^{35}S-methionine-labelled mPXR/PXR LBD was produced by *in vitro* translation using the TNT-coupled transcriptional translation system from Promega. GST beads bound proteins were eluted in sample buffer and analysed by SDS–PAGE and visualized by autoradiography.

2.10. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed according to the published methods [21–23]. 6 μg of Flag-HBx expression plasmid was transfected into HepG2 cells. 24 h after transfection, cells were treated with vehicle or RIF. ChIP assays were performed using EZ ChIP™ Chromatin Immunoprecipitation Kit (Millipore). Normal mouse IgG or anti-Flag was used for immunoprecipitation. DNA flanking the region of the ER6/DR3/dXREM/ER6 binding sites on the human CYP3A4 gene promoter was amplified by PCR for 30 cycles. PCR primers sequences are listed in Table 1. Real-time PCR was performed using 1 μl of DNA sample. 1% of the total cell lysate was used as the "input" control.