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Original contribution

Ground-glass hepatocytes co-expressing hepatitis B virus X protein and surface antigens exhibit enhanced oncogenic effects and tumorigenesis ☆

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Summary Hepatitis B virus (HBV) X protein (HBx) and pre-S2 deletion mutant large surface antigens are oncoproteins that induce hepatocellular carcinoma (HCC). The interaction of these two oncoproteins in hepatocytes and its significance in tumorigenesis remain to be elucidated. In this study, we observed the co-expression of HBx with surface antigens in ground-glass hepatocytes in 5 of 20 hepatitis B surface antigen-positive livers. In vitro, hepatocytes co-expressing HBx and a pre-S2 mutant showed enhanced expression of vascular endothelial growth factor–A, phosphorylated Akt 1/2/3, phosphorylated extracellular signal-regulated kinase 1/2, and phosphorylated mammalian target of rapamycin signals. Transgenic mice harboring both HBx and pre-S2 mutant construct plasmids developed HCCs at an average of 15.1 months, earlier than animals carrying either HBx (16.9 months) or pre-S2 mutant (24.5 months) alone. The oncogenic signals of vascular endothelial growth factor–A, phosphorylated Akt 1/2/3, phosphorylated extracellular signal-regulated kinase 1/2, and phosphorylated mammalian target of rapamycin were sequentially and differentially activated at different stages in tumorigenesis. Phosphorylated mTOR was consistently activated in transgenic and human HCCs. We conclude that ground-glass hepatocytes co-expressing HBx and surface antigens exhibit enhanced oncogenic effects and tumorigenesis in chronic HBV infections. The mTOR signal cascade may be the key regulator in HBV tumorigenesis and may be useful targets in the design of HCC therapy.

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1. Introduction

The association of hepatocellular carcinoma (HCC) with chronic hepatitis B virus (HBV) infection is well established [1]. However, the contribution of HBV to the development of HCC remains to be elucidated. Several theories have been proposed to explain the mechanism of HBV hepatocarcinogenesis, including CD8 immune-mediated and viral protein-driven tumorigenesis [2,3]. Two viral proteins, the hepatitis B X protein (HBx) and pre-S deletion mutants, have been considered to have either direct or indirect oncogenic effects in the liver [4]. The HBx protein mediates the activation of multiple signaling pathways, including Ras/Raf, nuclear factor-κB, phosphatidylinositol 3-kinase/protein kinase B (Akt), and recently, the mammalian target of rapamycin (mTOR) signal cascade [5–10]. Besides HBx, we previously identified the hepatitis B surface antigen (HBsAg) with deletions in the pre-S2 region in type II ground-glass hepatocytes from patients with chronic HBV infection [11].

The pre-S2 deletion mutant proteins are retained in the endoplasmic reticulum and induce oxidative DNA damage, activate cyclooxygenase-2 through the nuclear factor-κB and p-p38 mitogen-activated protein kinase (MAPK) pathways, and enhance an endoplasmic reticulum stress-dependent vascular endothelial growth factor–A (VEGF-A)/Akt/mTOR pathway [12–14]. The presence of pre-S mutants in serum carries a high risk of HCC development in patients with chronic HBV infection [15]. Furthermore, in type II ground-glass hepatocytes, pre-S2 mutants have been recognized as precursors of HBV-related HCC [4,16,17].

Although both HBx and pre-S2 deletion mutants exhibit oncogenic effects, the exact expression patterns in hepatocytes and the interaction and significance of these two oncoproteins in HBV tumorigenesis are unexplored. In this study, a newly developed monoclonal antibody designed specifically against HBx was used for immunohistochemical and double-labeled immunofluorescence studies, and the expressions of HBx and surface antigens were evaluated. In vitro studies and transgenic mouse models were used to examine the interaction and oncogenic effects of these two viral oncoproteins.

2. Materials and methods

2.1. Human HCC tissues

This study was carried out with the approval of the institutional research committee. The human liver specimens were collected retrospectively from our files and included 58 paired tumorous and nontumorous nonoptimal cutting temperature–embedded freshly frozen liver tissues and 20 formalin-fixed, paraffin-embedded tissues from HBV-related HCC in patients who underwent surgical resection at the National Cheng Kung University Hospital, Taiwan, from 1995 to 2007. The frozen tissues were used in Western blot analysis to explore signal activation profiles, whereas the 20 formalin-fixed, paraffin-embedded tissues were used for HBV viral protein expression studies.

2.2. Immunohistochemistry and double-labeled immunofluorescence studies

The staining protocol was described in a previous report [18]. The HBx mouse monoclonal antibody (clone 20F3) was kindly provided by Dr Quan Yua (Xiamen University, Xiamen, China). Polyclonal rabbit antibodies against HBV surface antigens were purchased from Abcam (Cambridge, UK). For HBx, strong (clearly visible at low power) cytoplasmic staining was considered a positive reaction.

2.3. Plasmid, cell line, and transient transfection

The plasmids of pIRES-Δ2, pIRES-X, pIRES-X fusion HA tag, and pIRES-Δ2+X express pre-S2 mutant (nt 4–57 deletion) large surface antigen, HBx, HBx fusion HA tag, and 2 viral proteins, respectively. Plasmid construction was described in a previous report [14]. The Huh-7 cell line was used. All transfections were performed with the Micro-Porator (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions.

2.4. Transgenic mice

Transgenic mice overexpressing HBx or pre-S2 mutant (nt 4–57 deletion) large surface antigen (Δ2) in their livers were established by Wu et al [19]. The double-construct (HBx and Δ2) transgenic mice were generated by mating HBx and Δ2 transgenic mice. The genotype of the transgenic mice was confirmed by polymerase chain reaction analysis. Six mice of all 3 types were sacrificed at each time point at ages 1, 3, 6, and 12 months. Twenty-five transgenic mice were followed up for as long as 30 months to evaluate tumor development.

During the follow-up period, the serum concentration of alanine transaminase was monitored monthly, and mice showing an alanine transaminase concentration >100 U/L were sacrificed regardless of age. Six paired tumorous and nontumorous freshly frozen liver tissues from HBx and double-transgenic mice were randomly selected for Western blot analysis. All the mice used in this study were male and were housed in a specific pathogen-free facility.

2.5. Western blot analysis

Our Western blot analysis technique was described in a previous report [20]. The primary antibodies used were as follows: anti-VEGF and anti–phospho-Akt (Ser473) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–phospho-Raf-1 (Ser338), anti–phospho-extracellular signal-regulated kinase (ERK) 1/2 (Thr212/Tyr214), and