

Role of hepatitis B virus X protein in liver cancer

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KEY MESSAGES

1. Full-length HBx and two natural C-terminal truncated HBx mutants (1-130 and 1-119 amino acid residues) do not alter the mitotic checkpoint control in hepatocytes or deregulate the expression of mitotic checkpoint proteins in hepatocellular carcinoma (HCC) cells.
2. The inducible gene expression systems are useful tools to characterise the role of HBx protein in

human HCC.

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Introduction

An estimated 350 million people worldwide are chronically infected with hepatitis B virus (HBV).¹ In Southeast Asia and Mainland China, including Hong Kong, the prevalence of HBV infection is high (10% in Hong Kong). Hepatocellular carcinoma (HCC) is a dreaded complication of chronic HBV infection; 55% of liver cancers worldwide occur in China including Hong Kong. HBV is a partial double-stranded DNA virus that comprises four known open reading frames: the viral DNA polymerase (P), viral envelope (surface antigens) proteins (PreS1, PreS2 or S), core protein (PreC or C), and HBV X protein (HBx). Among these four genes, HBx is the most frequently integrated and often considered an important factor in HBV-related hepatocarcinogenesis.² Integration of HBV DNA into the host genome is common in HCC and this may lead to alteration of the host cells by disrupting expression of cellular genes that are important for cell growth, survival, and cellular differentiation.^{3,4} Random HBV genome integration can also lead to truncation of the HBV genome, especially the HBx gene at the C-terminus.⁵⁻⁷ The function of HBx in HBV-associated HCC has been characterised, but its functional role in hepatocarcinogenesis remains unclear. This study aimed to (1) investigate the alteration by HBx of cell targets in HCC cells, (2) delineate the role of HBx in deregulation of mitotic checkpoint control, and (3) characterise the cellular effects of natural HBx mutants in HCC.

Methods

This study was conducted from September 2006 to August 2009.

In vitro functional characterisation of HBx in a HCC cell line and an immortalised normal liver cell line

Full-length HBx DNA (ayw subtype, GenBank No.:

U95551) was amplified from the HBx/pcDNA3.1+ plasmid⁸ and subcloned into Myc/pcDNA3.1+ and Myc/pLVX Tight Puro vectors. Two natural C-terminal truncation HBx mutants, HBxΔC1 (1 - 130 amino acid [aa]) and HBxΔC2 (1 - 119 aa) were generated and subcloned into Myc/pcDNA3.1+, Myc/pLVX Tight Puro, and Myc/pIND-Hygro vectors. Transient doxycycline- and ecdysone-inducible expression systems of full-length HBx, HBxΔC1 and HBxΔC2 were established for in vitro functional characterisation in the non-HBV infected immortalised normal liver cell line LO2 and HepG2 hepatoma cells.

RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from HCC cell lines using TriZOL (Invitrogen, Grand Island, NY, USA), according to the manufacturer's protocol. Primers for MAD1 (5'-CACCATGGTTTT ATCCACCC-3' and 5'-GCATCCAAGTTCTGCTGACA-3'), MAD2 (5'-CTTTTGTGTTTGTC TCCCTGGC-3' and 5'-GTCATTGACAGGAATTTGTAGG-3'), BUBR1 (5'- TGTAGCTCCGAGGGCAGG-3' and 5'-TTGAGAGCACCTCCTACACG-3'), BUB1 (5'-GTGGAGACATCCCATGAGGATC-3' and 5'-GGATCTTCTGCAATGGCAGCG-3') and BUB3 (5'-CGGGAAACGTTGCTTCTG-3' and 5'-TCTAGTCCCTCCACTCCAG-3') were used. β-Actin (primer set of 5'-GTGGGGCGCCCCAGGACACA-3' and 5'-CTCCTTAATGTCACGCACGATTC-3') was used as a reference for the amount of cDNA added in the PCR reactions.

Western blot analysis

Cells were lysed in sodium dodecyl sulphate-containing buffer and equal amounts of protein were separated on SDS-PAGE gel for western blot analysis. Immunodetection was performed using anti-c-Myc (Santa Cruz, CA, USA), anti-Flag (Sigma-

Aldrich, St Louis, MO, USA), anti-p53 (Santa Cruz), anti-p21 (Santa Cruz), anti-NF- κ B p50 (Santa Cruz), anti-NF- κ B p65 (Santa Cruz), anti-MAD1 (Bethyl Laboratories, Montgomery, TX, USA), anti-MAD2 (BD Transduction, San Jose, CA, USA), anti-BUBR1 (BD Transduction), and anti- α -tubulin (Sigma-Aldrich) antibodies.

Mitotic index measurement for analysis of mitotic checkpoint competence

Two $\times 10^5$ cells were seeded in six-well plates overnight and medium containing nocodazole at 0.2 μ g/mL or colcemid at 0.1 μ g/mL was applied to the cultures. Cells were collected at 24-hours, fixed in 3.7% of formaldehyde, stained with 4',6-diamidino-2-phenylindole (DAPI), and examined under an inverted fluorescence microscope. Cells with condensed nuclear DNA were considered as undergoing mitosis. To measure the mitotic index (percentage of viable cells arrested in mitosis), at least 300 cells were counted for each experiment using fluorescence microscopy. Data points represent the average results from two independent experiments. A cell line with mitotic index $<50\%$ following nocodazole or colcemid treatment was regarded as having mitotic checkpoint incompetence.⁹

Results

Inducible HBx-expression systems in HCC and immortalised normal liver cell lines

Transient overexpression of the full-length form of HBx (ayw subtype) caused a marked induction of cell death in the colony formation assay. Therefore, to stably express HBx for further functional studies, an inducible system—the doxycycline-inducible mammalian expression system—was used to study the effects of HBx. Adding doxycycline to the culture medium induced expression of the HBx protein in HepG2 and LO2 cells. The level of HBx expression closely correlated with withdrawal of doxycycline (data not shown). In addition, an ecdysone-inducible expression system was established in LO2 cells. Adding ponasterone A to the culture medium induced expression of HBx in LO2 cells. These inducible mammalian expression systems were useful to study the cellular effects of HBx.

Effect of full-length HBx on cellular targets, p53, p21 and NF- κ B, in HepG2 and LO2 cells

No significant change was noted in the protein expression of cell cycle regulators, p53 and p21/waf1/cip1, in HepG2 or LO2 cells with induced expression of full-length HBx. Similarly, there was no significant change in the protein expression levels of NF- κ B p65 or p50.

Effect of full-length HBx, HBx Δ C1 and HBx Δ C2 on mitotic checkpoint control

To determine whether HBx impaired mitotic checkpoint control, the mitotic checkpoint competence was examined by treating cells with either nocodazole or colcemid, both being microtubule-disturbing agents. A cell line with mitotic index $<50\%$ after nocodazole or colcemid treatment was considered to have mitotic checkpoint incompetence.⁹ There was no significant difference in the mitotic index between the LO2 cells stably expressing Myc-tagged full-length HBx and myc-tagged vector control (cell lines were treated with oxycycline for at least 7 days), indicating that full-length HBx had no significant effect on the mitotic checkpoint competence of LO2. There was also no significant difference in the mitotic checkpoint control in the presence of nocodazole or colcemid in LO2 cells expressing HBx Δ C1 or HBx Δ C2, compared with those expressing full-length HBx or vector control. These results suggest that the inducible expression of both full-length and C-terminal truncated HBx does not alter the mitotic checkpoint control of LO2 cells. As HepG2 cells were mitotic checkpoint incompetent,⁹ the mitotic checkpoint competence assay was not performed on HepG2 cells expressing the various forms of HBx.

Effect of full-length HBx, HBx Δ C1 and HBx Δ C2 on expression of mitotic checkpoint genes

To further investigate whether full-length HBx, HBx Δ C1, and HBx Δ C2 altered the expression of important mitotic checkpoint gene regulators (such as MAD1-3, BUB1, and BUB3), semi-quantitative RT-PCR and western blot analysis were performed. There was no significant change in expression at both mRNA and protein levels of MAD1, MAD2, and MAD3/BUBR1 in LO2 cells expressing full-length HBx, HBx Δ C1, or HBx Δ C2, compared with the vector control. There was also no significant difference in mRNA expression of other mitotic checkpoint genes, BUB1 and BUB3, LO2 cells expressing the various forms of HBx, compared with the vector control.

Discussion

HBV genomic DNA integration or mutation leads to COOH-truncation of the HBx protein in human HCC.^{5-7,10} COOH-truncated HBx DNA has been identified in 46% (23/50) of HBV-associated HCC tissue from HCC patients with chronic HBV infection.¹¹ About 79% of human HCCs from China have COOH-truncated HBx transcript in tumour tissue,⁵ suggesting that COOH-truncation of HBx is frequent in HCC in the Hong Kong population.

Cell invasiveness and up-regulation of uroplasinogen (uPA) is enhanced via NF-kappa B activation in HepG2 cells expressing full-length HBx.⁸ To determine whether HBx deregulated cellular targets in HCC, the protein expression of NF-κB and some cell cycle regulators (such as p53 and p21) were evaluated. There was no significant alteration in the expression of p53, p21, or NF-κB (p50/p65) when full-length HBx was expressed in HepG2 and LO2 cells with tetracycline inducible systems. Different subtypes or protein sequences of HBx can behave differently in repressing p21 protein expression in HCC cells¹²; the subtypes of HBx may be an influential factor.

DNA aneuploidy is frequent in human HCC.¹³ One of the leading causes of DNA aneuploidy is dysfunction of mitotic checkpoint regulation including alteration in abundance of mitotic checkpoint proteins or disruption of protein-protein interaction by viral proteins such as Tax protein in human T cell leukaemia virus type 1.^{9,14} In this study using Co-IP assays, full-length HBx interacted with the full-length form of MAD3/BUBR1 and p55cdc, but not with MAD1 or MAD2. As BUBR1 can interact with p55cdc when the mitotic checkpoint control is activated, we investigated whether the interaction between HBx and BUBR1 involved p55cdc, and a BUBR1 mutant, in which the putative p55cdc binding sites at BUBR1 protein were mutated, was constructed. The BUBR1 mutant could still interact with full-length HBx in the Co-IP assay, suggesting that HBx-BUBR1 interaction does not require the presence of p55cdc protein (data not shown). This is in line with a finding that HBx might alter the interaction between BUBR1 and p55cdc in mitotic checkpoint activation.¹⁵ Nonetheless, ectopic expression of HBx did not change the mitotic checkpoint status of LO2 cells, and the result differed from that observed in the HBx-expressing Chang liver cell line.¹⁵ Such discrepancy may be due to differences in the cell lines and the subtype of HBx (ayw subtype) used in the experiments.

To investigate the effect of C-terminal truncated HBx on mitotic checkpoint control, two natural C-terminal truncated forms of HBx found in human HCC were used: one with a breakpoint at 130 aa (HBxΔC1) and the other at 119 aa (HBxΔC2).^{5,7,16} Similar to full-length HBx, no significant difference was noted in the expression of mitotic checkpoint genes or alteration in mitotic checkpoint control in HBxΔC1- or HBxΔC2-expressing LO2 cells after treatment with nocodazole or colcemid. To determine whether HBx affects HCC tumour growth in nude mice, previous study has established full-length HBx stable knockdown cell in PLC/PRF/5 HCC cells and shown that knockdown of HBx inhibited HCC tumour growth.¹⁷ In our study, the HepG2 and LO2 cells with inducible expression of

full-length HBx and two truncated HBx forms were established to test the tumourigenicity in nude mice; neither HepG2 nor LO2 cells were tumourigenic. Therefore, the effect of C-terminal truncated HBx in tumour development requires further investigation using other cell line models. Our data suggested that neither full-length HBx (ayw subtype) nor natural C-terminal truncated HBx (1-130 and 1-119 amino acid) altered mitotic checkpoint control in hepatocytes.

Conclusions

Random HBV integration leads to C-terminus truncation of HBx in HCC. Our data suggest that neither full-length HBx (ayw subtype) nor natural C-terminal truncated forms of HBx (1-130 and 1-119 amino acid) alter the mitotic checkpoint control in hepatocytes or deregulate the expression of mitotic checkpoint proteins/genes in HCC cells.

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