COOH-Terminal Truncated HBV X Protein Plays Key Role in Hepatocarcinogenesis

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Abstract  Purpose: X protein (HBx), a product of hepatitis B virus, has been closely associated with the development of hepatocellular carcinoma (HCC). Based on observations that the COOH-terminal truncated HBx was frequently detected in HCC, the aim of this study is to evaluate the function of COOH-terminal truncated HBx in hepatocarcinogenesis.

Experimental Design: Expression pattern of HBx was analyzed by immunohistochemistry on tissue microarray containing 194 pairs of HCCs and their matched nontumor liver tissues. MIHA and HepG2 cells transfected with full-length (X2) and COOH-terminal truncated HBx (X1) were tested for their ability to grow in soft agar and form tumors in vivo. Proliferation and apoptosis were assessed using 2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assays, respectively. To gain additional insight, the expression profile of HepG2-X2 and HepG2-X1 were compared using cDNA microarray.

Results: COOH-terminal truncated HBx was frequently detected in HCCs (79.3%, n = 111), and our in vitro and in vivo studies showed that the truncated rather than the full-length HBx could effectively transform immortalized liver cell line MIHA. Interestingly, expression profiling revealed differential expression of key genes implicated in the control of cell cycle and apoptosis.

Conclusions: These findings strongly suggest that the COOH-terminal truncated HBx plays a critical role in the HCC carcinogenesis via the activation of cell proliferation.

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third cause of cancer death in the world (1). The prognosis of HCC is very poor and the overall 5-year survival rate worldwide is estimated only ~ 3%, mainly because of late diagnosis (2). Although the molecular pathogenesis of HCC remains elusive, the etiologic association between hepatitis B virus (HBV) infection and hepatocarcinogenesis has been established (3). Epidemiologic studies have shown that the relative risk of HCC among HBV carrier is 10-fold higher compared to noncarrier (4). Among the four proteins translated with HBV, the X-gene product (HBx) has been closely associated with the HCC carcinogenesis (5).

HBx is a small protein with 154 amino acids, which is required for the establishment of viral infection. The correlation between HBx and HCC development has been extensively studied and oncogenic roles of HBx include the following: (a) the activation of a variety of transcription factors such as nuclear factor-kB (6), activator protein (7), and cAMP-responsive element binding protein/activating transcription factor 2 (8); (b) the interaction with cellular oncogenes such as Ras (9), Src (10), c-jun (11); and (c) the stimulation of cytoplasmic signal transduction pathways such as Ras-Raf-mitogen-activated protein kinase pathway (7, 9), and cell stress–induced MEKK1-p38-cJNK pathway (12). One important question that needs to be answered is why HCC occurred only in a small percentage of the HBV-infected patients although HBx expression was frequently observed in most of HBV-infected hepatocytes.

We and others reported observations that integration of HBV was detected in 80% to 90% of host genome of HBV-infected HCC cases, suggesting that viral DNA integration plays a critical role in the HCC carcinogenesis (13, 14). One important clue derived from studies of HBV integration was that 3’-end X gene was frequently deleted in HCC cells, leading to the...
COOH-terminal truncated HBx protein (15, 16). Accordingly, we proposed here that the truncated HBx protein plays a key role in the HCC carcinogenesis. To test this hypothesis, we investigated the HBx expression in 140 cases of HBV-related HCCs paired with the adjacent nontumor tissues, and compared an oncogenic role of the COOH-terminal truncated HBx with the full-length HBx protein. Our results reported here showed that the COOH-terminal truncated HBx, rather than the full-length HBx, is required and sufficient to cause HCC.

Materials and Methods

HCC samples and HCC cell lines. The specimens for tissue microarray (TMA) were recruited from archive paraffin blocks of 194 primary HCCs with positive serum HBsAg in the First Affiliated Hospital and Cancer Center of Sun Yat-Sen University between 1998 and 2002. Among these HCCs, frozen tissues (both tumor and matched nontumor liver tissues) were available for 20 cases that were collected at the time of surgical resections.

Human hepatoblastoma cell line HepG2 was obtained from the American Type Culture Collection. An immortalized human liver cell line MIHA was kindly provided by Dr Chowdhury (Albert Einstein College of Medicine, New York; ref. 17).

Construction of TMA and immunohistochemistry. A TMA block containing 194 pairs of HCCs and their matched nontumor surrounding liver tissues was constructed as described previously (13). Among the 194 HCCs, serum HBsAg was detected in 146 cases. Multiple sections (5 μm) were cut from the TMA block and mounted on microscope slides.

Immunohistochemistry (IHC) studies were done using standard streptavidin–biotin–peroxidase complex method (13). In brief, TMA sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 15 min. For antigen retrieval, TMA slides were microwave treated in 10 mmol/L citrate buffer (pH 6.0) for 10 min. Nonspecific bindings were blocked with 10% normal rabbit serum for 10 min. Two anti-HBx antibodies were used in this study. HBx-Ab1 is a monoclonal anti-HBx antibody (Chemicon International, Inc.), which recognizes aa50-88 of HBx, whereas HBx-Ab2 (Boster Biotechnology Co. Ltd.) is a polyclonal anti-HBx antibody that recognizes aa139-154. HBx-Ab2 was obtained by immunizing rabbit with the peptide of aa139-154 of HBx. Two adjacent TMA slides were incubated with HBx-Ab1 (1:100 dilution) and HBx-Ab2 (1:100 dilution) at 4°C overnight in a moist chamber, respectively. The slides were sequentially incubated with goat anti-mouse antibody and streptavidin–biotin–peroxidase complex method (13). In brief, TMA slides were incubated with HBx-Ab1 (1:100 dilution) and HBx-Ab2 (1:100 dilution) at 4°C overnight in a moist chamber, respectively. The slides were sequentially incubated with goat anti-mouse antibody and streptavidin–biotin–peroxidase complex method (13).

Detection of X gene deletion by PCR. To validate the COOH-terminal truncation of HBx detected by IHC, deletion of X gene in 20 pairs HBV-related HCCs and matched nontumor tissues were investigated by PCR using 5 pairs of primers encompassing the entire and different lengths of X gene. Primer sequences used in this study were listed in Supplementary Table S1. Twenty-eight cycles were done for all PCR reactions (denaturing at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min). PCR products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide.

Establishment of full-length and 3-deleted X gene–expressing cell lines. X2 (nt1082-1973) containing the full-length X gene (nt1374-1838, 155aa) and X1 (nt1082-1734) containing a 3-deleted X gene (nt1374-1734, 120aa) were amplified by PCR using HBV subtype adr (HBV-ASA-EX3) as a template. PCR products were cloned into expression vector pcDNA3.1(+)(Invitrogen) and then transfected into HepG2 and MIHA cells, respectively, using lipofectamine (Life Technologies Bethesda Research Laboratories) according to the manufacturer’s instructions. Sequences of PCR primers for X1 and X2 were also listed in Supplementary Table S1. Stable expressing cell lines HepG2-X1, HepG2-X2, MIHA-X1, and MIHA-X2 were selected using Geneticin (Life Technologies Bethesda Research Laboratories) at a concentration of 800 μg/mL, and the expression level of X gene in each clone was determined by Northern blot analysis and IHC. For IHC detection, X1- and X2-expressing cell lines were plated and grown on 22 × 22 mm coverslips. Cells were fixed with 4% paraformaldehyde in PBS for 15 min and subsequently treated with HBx-Ab1 and HBx-Ab2 (1:100 dilution), respectively, overnight at 4°C. Blank vector pcDNA3.1(+)(Invitrogen) was also transfected into HepG2 and MIHA cells (HepG2-P and MIHA-P) as controls.

Cell growth assay. Cell growth rates of X1- and X-transfected cells were detected by XTT assay. Briefly, cells were seeded onto 96-well plate at a density of 1 × 104 cells per well and incubated in 100 μL DMEM with or without serum for 1 to 5 d. The cell growth rate was detected using cell proliferation XTT kit (Roche) according to manufacturer’s instructions. Triplicate independent experiments were done.

Soft agar assay and tumorigenicity in nude mice. Anchorage-independent growth assay in X1- and X2-transfected cells was carried out by growing 1 × 105 cells in 0.4% bactoagar on a bottom layer of solidified 0.6% bactoagar in 6-well plates. After 2 wk, colonies formed in the top layer were counted and colony formation rate was calculated as percentage of total seeded cells. Triplicate independent experiments were done.

Tumorigenicity of X1- and X2-transfected cells were investigated by tumor xenograft experiments. About 1 × 106 X1-expressing cells and the same amount of X2-expressing cells were injected s.c. into the left and right hind legs of 4 to 6-wk-old nude mice (20 mice for HepG2 cells and 10 for MIHA cells), respectively. HepG2-P (5 mice), HepG2 cells (3 mice), and MIHA cells (5 mice) were used as negative controls. Tumor formation in nude mice was monitored over a 6-wk period.

Detection of apoptosis by terminal deoxynucleobrontide transferase-mediated dUTP nick-end labeling assay. Morphologic changes in the nuclear chromatin undergoing apoptosis were detected by terminal deoxynucleobrontide transferase-mediated dUTP nick-end labeling assay according to the manufacturer’s protocol (Roche). Apoptotic nuclei after staining by dianinobenzidine (0.025%) were examined by light microscopy. Apoptotic bodies were counted among at least 1,000 cells and expressed as a percentage value. Triplicate independent experiments were done.

cDNA microarray and gene expression data analysis. In-house made cDNA microarrays on glass slides were designed and fabricated by the previously published methods (18). cDNA clones of human genes (12,000) were purchased from Invitrogen and were sequence-verified by the Company. Gene symbols, names, and functional information were routinely updated based on Human UniGene database. Total RNA for microarray hybridization was extracted from HepG2-X1, HepG2-X2, and parental HepG2 cells using Trizol reagent (Life Technologies Bethesda Research Laboratories) and purified using RNeasy kit (Qiagen), according to the manufacturer’s instructions. For Reverse transcription labeling, 50 μg of total RNA from test or control RNA samples were labeled with Cy3-dUTP or Cy5-dUTP using Micromax kit (Perkin-Elmer Life and Analytical Sciences). Fluorescently labeled targets were combined and followed by overnight hybridization at 65°C. After hybridization, the slide was washed and then scanned using the ScanArray Express Microarray Scanner (Perkin-Elmer Life and Analytical Sciences). To ensure the reproducibility of the microarray results, we repeated each experiment duplicate (HepG2-X1 versus HepG2 and HepG2-X2 versus HepG2) or triplicate (HepG2-X1 versus HepG2-X2).

To analyze the gene expression data, a database was compiled in FileMaker Pro (FileMaker, Inc.) and included individual expression files

and a master relational expression file. The average intensities and sample/control ratios of genes were calculated for differentially expressed genes. The spots with signal intensities that were at least 2-fold different from control levels in all repeat experiments were designated as genes that are differentially expressed between the two cell lines.

**Real-time quantitative reverse transcription-PCR.** Real-time reverse transcription-PCR was used to verify two differentially expressed genes (CDC2 and AREG) between HepG2-X1 and HepG2-X2 cells. Commercially available TaqMan gene expression assay (CDC2: Hs00938777_m1 and AREG: Hs00950669_m1) was used to determine the expression-level of the target genes. Gene expression values were represented by the threshold cycle of amplification (Ct), which is inversely correlated to the initial amount of target cDNA being amplified. The expression levels of CDC2 and AREG were then normalized using the housekeeping gene (GAPDH: Hs99999905_m1) and calculated using the comparative ΔCt method (ΔCt = Ct\_target gene - Ct\_GAPDH). Real-time reverse transcriptipn PCR was done on a Chromo 4 Thermal Cycler (MJ research) for 40 cycles (95°C for 15 s and 60°C for 60 s).

**Statistical analysis.** Statistical analysis was done with the SPSS software (SPSS Standard version 8.0). The difference of the frequency of truncated HBx between HCCs and nontumor liver tissues was evaluated by Fisher’s exact test, and significant difference was considered when P value was <0.05. Two-tailed unpaired Student’s t test was used to assess the difference of cell growth rate and apoptotic frequency between HepG2-X1 and HepG2-X2 cells. P values of <0.05 designated as statistically significant.

**Results**

**COOH-terminal truncation of HBx in HBV-related HCC.** To evaluate the expressing pattern of HBx in HBV-related HCC, an HCC TMA containing 194 pairs of HCCs and their matched nontumor liver tissues including 146 HCC tissues from patients
with the HBsAg-positive serum was studied by IHC staining with HBx-Ab1 and HBx-Ab2 (Fig. 1A). The full-length HBx can be recognized by both HBx-Ab1 and HBx-Ab2, whereas the COOH-terminal truncated HBx can be bound only by HBx-Ab1. Our IHC staining successfully detected HBx expression in 111 of 140 (79.3%) of informative nontumorous tissues. The result showed that the full-length HBx was observed in all 111 nontumorous liver tissues but only in 23 of 111 (20.7%) of HCC tissues. Eighty-eight of 111 (79.3%) HCC tissues were considered positive for truncated HBx, including 68 cases with HBx-Ab1(+)/HBx-Ab2(-) and 20 cases with HBx-Ab1(-)/HBx-Ab2(-). For these 20 cases with double negative result, both antibody recognizing sites might be deleted or rearranged during the HBV integration. No positive staining was observed in serum HBsAg-negative HCCs. Figure 1B shows a representative example of HBx expression in a pair of HCC and its matched nontumor liver tissue detected by HBx-Ab1 and HBx-Ab2.

To confirm the HBx-Ab1–positive and HBx-Ab2–negative cases were due to a deletion of the 3’-end of X gene, we conducted PCR analysis on the X gene of 20 HCC cases using 5 pairs of primers encompassing the X gene (Fig. 1C). The PCR results found that the X gene deletion was observed in 15 of 19 (78.9%) cases, which is very similar to those observed by IHC (79.3%; Table 1). In the case number 17, the HBx expression was detected by IHC, whereas the X gene DNA was not amplified by PCR. In the case number 20, the X gene was detected neither by IHC nor by PCR. Figure 1D showed examples of 3’-end deletion of X gene detected by PCR.

**Generation of X1- and X2-expressing cell lines.** To investigate biological function of the full-length and truncated HBx, mammalian expression vectors containing either X1 (3’-end–deleted X) or X2 (full-length X) gene were transfected into HepG2 and MIHA cells. Stable X1-expressing (HepG2-X1 and MIHA-X1) and X2-expressing (HepG2-X2 and MIHA-X2) cell lines were established. Expression of X1 and X2 proteins were studied by Northern blot (Fig. 2A) and IHC (Fig. 2B). As expected, HBx-Ab2 did not detect X1 transfected protein.

One interesting finding is that deleted X gene–transfected HepG2 and MIHA cells grow obviously faster than full-length X gene–transfected cells during the first six to eight cell passages, and this difference became inconspicuous after eight passages (data not shown). This phenomenon was observed in several independent experiments. Because full-length X gene–transfected cells grew too slow during the first four passages, cells from passages five to six were used for all functional studies.

**Oncogenic ability of 3’-deleted X gene.** The oncogenic role of X1 and X2 was compared between X1 and X2 transfectants. Cell growth assay showed that the cell growth rate in MIHA-X1 was significantly higher than those in MIHA-X2 and MIHA-P \((P < 0.05; \text{Fig. 2C})\). Soft agar assay showed that the colony formation in soft agar was >10-fold higher in MIHA-X1 than that in MIHA-X2 and MIHA-P \((P < 0.001; \text{Fig. 2D})\). Tumor xenograft experiment showed that tumor formation in nude mice was detected in 7 of 10 and 1 of 10 of nude mice injected with MIHA-X1 and MIHA-X2 cells, respectively (Fig. 2E). No tumor was formed in four nude mice injected with parental MIHA cells.

The oncogenic role of 3’-deleted X1 gene was also studied using a hepatoma cell line HepG2. Cell growth assays showed that HepG2-X1 cells grew much faster than HepG2-X2 cells and the vector-transfected mock control cells in serum-free culture medium (Fig. 2F). Tumor formation in nude mice was detected in all 20 mice injected with HepG2-X1 cells, whereas only in 3 of 20 mice injected with HepG2-X2 cells after inoculating the cells for 4 to 6 weeks. No tumor formation was observed in

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Abbreviations: N, nontumor liver tissue; T, tumor tissue.

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**Table 1. Detection of HBV X gene in 18 HCC cases by IHC and PCR**

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nude mice injected with HepG2-P (five mice) and parental HepG2 cells (three mice).

**Cell apoptosis induced by full-length HBx.** To study the role of HBx played in cell apoptosis, terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling assay was used to compare apoptotic frequencies among X1- and X2-expressing cells, as well as the vector-transfected mock control cells. The results showed that the apoptotic frequencies were similar between X1 expression and the vector-transfected cells (Fig. 3A). However, the apoptotic frequency in X2-expressing cells was significantly higher than that in X1- and vector-transfected mock control cells. Two X1- and two X2-expressing clones were tested. E, representative examples of tumor formation in nude mice induced by MIHA-X1 (left dorsal flank) and MIHA-X2 (right dorsal flank). F, X1-transfected HepG2 cells grow much faster than that in X2- and vector-transfected mock control HepG2 cells in culture medium without serum.

**Tumor xenograft of full-length X gene.** X gene status in one tumor caused by MIHA-X2 and three tumors caused by HepG2-X2 cells was further characterized by Northern blot analysis, PCR with five pairs of primers encompassing the different length of X gene, and IHC with HBx-Ab1 and HBx-Ab2. Interestingly, a smaller size RNA was detected in 2 of 3 tumors grown from HepG2-X2 by Northern blot analysis (Fig. 3C), suggesting that a deletion had happened in X gene caused by unknown reason. 3'-deleted X gene was detected in these two tumors by PCR (Fig. 3D). The COOH-terminal truncation of HBx (HBx-Ab1 positive and HBx-Ab2 negative) was also observed in these two tumors (Fig. 3E).

In another tumor caused by HepG2-X2 and the tumor caused by MIHA-X2, the full-length X gene was detected by Northern blot analysis and PCR. IHC study showed that positive staining was observed by both HBx-Ab1 and HBx-Ab2. No mutation was found in these two tumors by DNA sequence analysis.

**cDNA microarray.** To identify genes regulated by the full-length or COOH-terminal truncated HBx, cDNA microarrays containing 12,000 human genes were used to compare gene expression profiles between HepG2-X1 and HepG2-X2. After
filtering and normalizing data, differentially expressed genes between HepG2-X1 and HepG2-X2, including 38 genes up-regulated by X1 and 21 genes up-regulated by X2, were identified (Supplementary Table S2). The most relevant and interesting finding was that genes up-regulated by X1 gene have known functions either pro-proliferation (TFDP1, CDC2, CDC20, CDC7, and MCM7) or anti-apoptosis (AREG, PDCD6IP, IER3, and LGALS3). In contrast, Genes up-regulated by X2 gene have the opposite function, that is, antiproliferation (MEF2C, NDRG1, and IGFBP3) or proapoptosis (CASP1, PLA2G2A, and PLA2G6). Northern blot analysis was used to validate the cDNA microarray results, and the results agreed with that of microarray (Fig. 4A). Two differentially expressed genes (CDC2 and AREG) detected by microarray were also validated by real-time reverse transcriptipn PCR. The results showed that expression levels of CDC2 and AREG mRNA in HepG2-X1 were much higher than those in HepG2-X2 (Fig. 4B).

Discussion

Alteration of HBV X gene has been detected more frequently in tissue samples of cirrhosis and/or HCC than in those of mild liver disease (19, 20). However, the mechanism of HBx in HCC carcinogenesis is still unclear, although many studies have associated it to ability of HBx to activate cellular onco-genes and signaling cascades that stimulate cell proliferation and lead to HCC carcinogenesis (6–11). One critical question is why HCC occurs in only a small percentage of HBV-infected patients, although the expression of HBx was detected in almost all HBV-infected hepatocytes. Therefore, there must be an unknown event that triggers the carcinogenic cascades. Recently, we and others found that X gene is often deleted during the HBV integration into host genome and causes the COOH-terminal truncation of HBx (14–16). Consequently, we proposed that the COOH-terminal truncated HBx plays a critical role in HCC carcinogenesis.

In this report, we presented the evidence of frequent detection of the COOH-terminal truncated HBx (79.3%; n = 111) in HCC tissues. These results were also confirmed by PCR with five pairs of primers encompassing the entire and different lengths of X gene. We also uncovered molecular functions of the HBx and its truncated proteins in tumorigenesis by in vitro and in vivo studies, including cell proliferation, colony formation in soft agar, tumor formation in nude mice, and cell apoptosis assays. The results showed that the COOH-terminal truncated HBx, rather than the full-length HBx, could effectively transform an immortalized human liver cell line MIHA cells and increase the tumorigenicity of HepG2 and MIHA cells. The fact that 2 of 3 tumors formed in nude mice inoculated with the full-length X gene–transfected cells (HepG2-X2) had the 3’-end deleted of the X gene and the COOH-terminal truncation of HBx indicated that this truncation is required and sufficient to cause tumor formation in MIHA and HepG2 genetic backgrounds.
Fig. 4. A, Northern blotting was used to validate cDNA microarray results in G2-X1 and G2-X2 cells. Example of three up-regulated genes (CDC2, CDC20, and AREG) and one down-regulated gene (MEF2C) in HepG2-X1 cells were shown. Gel image of 18S and 28S rRNA was included as loading reference. B, real-time reverse transcription PCR results showed that the CDC2 and AREG expression levels in G2-X1 cells were ~1 Ct and 1.5 Ct lower than those in G2-X2 cells, indicating a 2-fold higher expression level of CDC2 and 3-fold that of AREG in G2-X1 cells compared with G2-X2 cells, respectively.

Many studies showed that HBx plays an important role in HCC pathogenesis by interacting with cellular oncogenes (21–23) and that its functional domain involved in oncogenesis is at the middle of HBx protein (24, 25). Several studies have also shown that HBx can induce apoptosis (26–29). In the present study, we found that the apoptotic frequency was significantly higher in X2-expressing cells than those in X1-expressing and mock control cells (P < 0.001). In addition, it was very difficult to establish stable X2-expressing cell line, and the cell growth rate of X2-expressing cell lines was much slower than those of X1-expressing cell lines during the first six to eight passages. Moreover, rapid growing X2-expressing cells (passage 9 HepG2-X2) were associated with the decrease in X2 expression. The induction of apoptosis by the full-length HBx, but not by the COOH-terminal truncated HBxs, strongly suggests the COOH-terminal peptide is required for the HBx proapoptotic function.

Therefore, we propose that the full-length HBx contains two function domains: oncogenic domain (the NH2 terminal through middle peptide) and proapoptotic domain (the COOH-terminal peptide). There is a balance between these two functions in HBV-infected hepatocytes. When the proapoptotic domain is deleted by an unknown mechanism during the viral integration, the balance is broken and the oncogenic function becomes dominant, leading to the subsequent development of HCC.

The hypothesis of two functional domains of HBx protein was supported by our cDNA microarray results. Comparing expression profiles between X1- and X2-expressing cells, seven differentially expressed genes have known functions involved in apoptosis. Interestingly, all four up-regulated genes in X1-expressing cells have antiapoptotic function, and all three down-regulated genes have proapoptotic function. In consistency with the two-domain hypothesis, five genes with known functions of promoting cell proliferation were up-regulated in X1-expressing cells, and three genes with known negative functions on cell proliferation were down-regulated.

In summary, our study showed that COOH-terminal truncated HBxs plays critical role in the HCC development carcinogenesis via the activation of cell proliferation and loss of proapoptotic ability. Better understanding of the mechanisms of the COOH-terminal truncated HBx may lead to better management of patient with HBV infection, via early detection of the oncogenic alterations in HBx and molecularly targeted treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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