



Title	Hepatitis B virus X gene in the development of hepatocellular carcinoma
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Hepatitis B virus X gene in the development of hepatocellular carcinoma

Key Messages

1. Deletion of the 3' end of the hepatitis B virus X gene (HBx) was frequently detected in clinical hepatocellular carcinoma (HCC) samples.
2. In vivo animal tumour xenograft experiments demonstrated the tumourigenic ability of the C-terminal truncated HBx.
3. cDNA microarray study suggested that the C-terminal truncated HBx played a critical role in the HCC development via activation of cell proliferation and inhibition of apoptosis.

Introduction

Hepatocellular carcinoma (HCC) has been the second commonest cancer in both Hong Kong and China since 1990s. Its overall 5-year survival rate worldwide is about 3%, mainly because of late diagnosis. Although the precise molecular switch that triggers HCC development remains elusive, the aetiological association between hepatitis B virus (HBV) infection and hepatocarcinogenesis has been established.¹ The relative risk of HCC in HBV carriers is 10-fold higher than in non-carriers, but only a small percentage of HBV carriers develop HCC.

One important clue has been derived from the Hepatitis B virus X protein (HBx), which is one of the four proteins encoded by HBV. Previous studies from our group and others have shown that integration of HBV is detected in 80 to 90% of the host genome of HBV-infected HCC cases, and often results in the C-terminal deletion of the protein HBx.² Thus, we speculated that truncated HBx may play a role in HCC development.

Frequent C-terminal truncation of hepatitis B virus X protein in hepatitis B virus-related hepatocellular carcinoma

By screening HCC tissue using a microarray containing 194 pairs of HCCs and their matched non-tumour liver tissues, C-terminal truncated HBx was frequently observed in HBV-related HCC tissues. In order to investigate the expression pattern of HBx in HCCs, two antibodies (Ab1 and Ab2) were generated. Their antigen position is shown in Fig. 1a. The antibodies Ab1 and Ab2 can recognise the full-length HBx, whereas the Ab2 cannot bind to the C-terminal truncated HBx. Immunohistochemistry results showed that the C-terminal truncated HBx was detected in 88/111 (79.3%) HCC tissues, whereas full-length HBx was observed in all 111 non-tumour liver tissues, but only in 23/111 (20.7%) of HCC tissues. The corresponding deletion of 3' within the X gene was further confirmed by polymerase chain reaction (PCR) analysis on 20 HCC cases using five pairs of primers encompassing the X gene (Figs. 1b and 1c).

C-terminal truncated HBx enhanced tumour development

We cloned and constructed mammalian expression vectors for ectopic expression of full-length HBx (X2) and C-terminal truncated HBx (X1) in human liver cell lines HepG2 and MIHA. Stable X1-expressing (HepG2-X1 and MIHA-X1) and X2-expressing (HepG2-X2 and MIHA-X2) cell lines were established and tested for their oncogenic ability. MIHA-X1 grew much faster than MIHA-X2 and MIHA-P. The colony formation in soft agar was 10-fold higher in MIHA-X1 than in MIHA-X2 and MIHA-P ($P < 0.001$, Student's *t* test). Tumour xenograft experiment was performed in nude mice to study the tumourigenicity of X1- and X2-expressing cells. Tumour formation in nude mice was observed in 7/10 and 1/10 of nude mice injected with MIHA-X1 and MIHA-X2 cells, respectively. To study the role of HBx in cell apoptosis, a TUNEL assay was employed to compare apoptotic frequencies among X1- and X2-expression cells, as well as vector-transfected mock control cells. Apoptotic frequencies were similar between X1-expression and vector-transfected cells. However, the apoptotic frequency in X2-expression cells was significantly higher than in X1-expression

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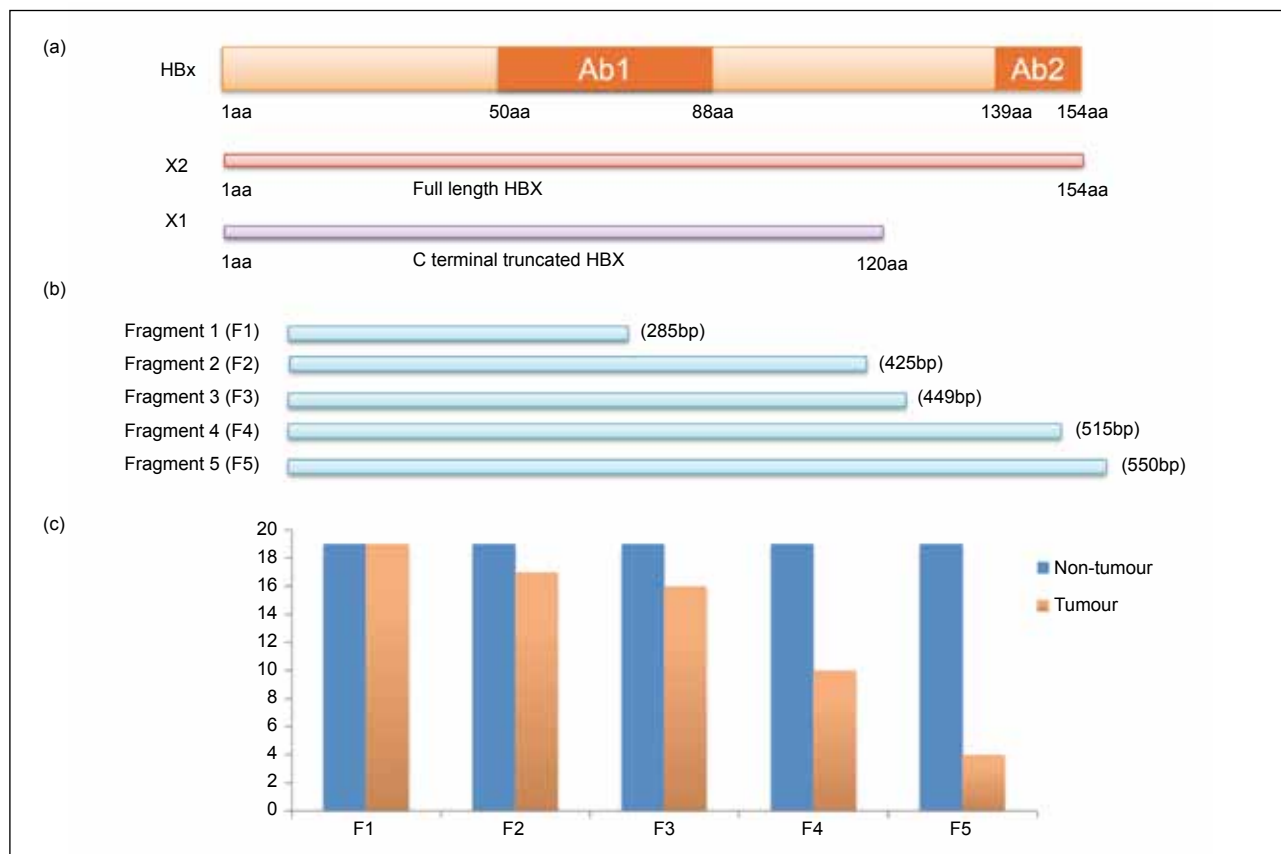


Fig 1. Detection of C-terminal truncation of hepatitis B X protein (HBx) in primary hepatocellular carcinoma (HCC)
 (a) Recognition sites of two anti-HBx antibodies (Ab1 and Ab2) on HBx. (b) Polymerase chain reaction (PCR) amplification fragments by five pairs of primers used for detecting 3'-end deletion of X gene. (c) Summary of truncation frequencies in HCC/non-tumour pairs in different regions of HBx

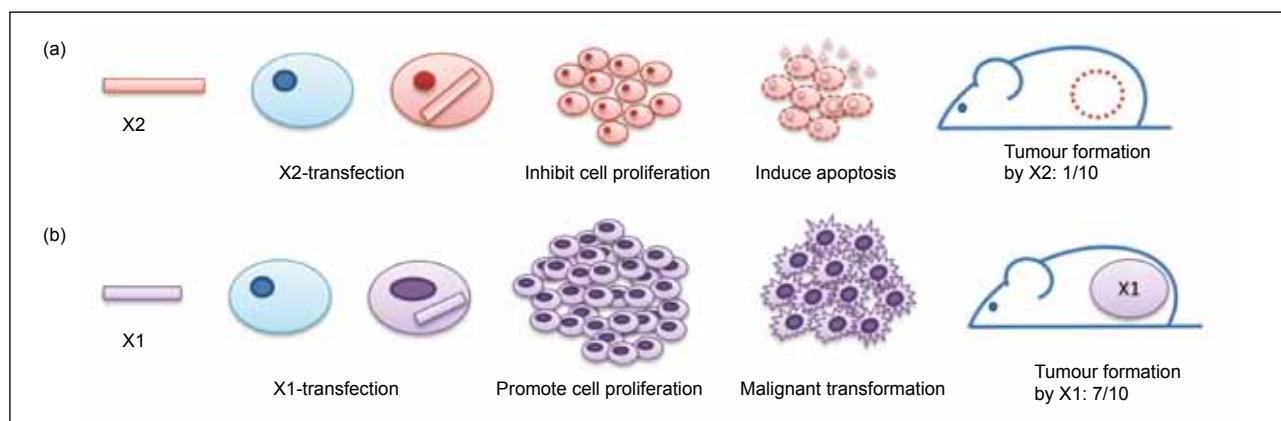


Fig 2. Truncated-X enhanced tumorigenicity
 Ectopic expression of X1 and X2 were established in HepG2 and MIHA cells by stable transfections. Ectopic expression of X1 dramatically increased cell growth and colony formation rates, whereas overexpressing X2 inhibited cell growth and induced apoptosis. MIHA-X1 cells were able to promote tumourigenesis in xenograft model

or vector-transfected cells ($P < 0.001$, Student's *t* test). The results are summarised in Fig 2.

cDNA microarray analysis pinpointed key molecules involved in the process

Using a cDNA microarray analysis, we identified the gene signatures contributing to either full-length

HBx inducing apoptosis or C-terminal truncated HBx promoting tumourigenicity. Of 12 000 human genes used to compare gene expression profiles between HepG2-X1 and HepG2-X2, 59 genes with significant changes were identified, including 38 genes up-regulated by X1 and 21 genes up-regulated by X2. The most relevant and interesting finding was that genes up-regulated by X1 gene have known functions either promoting proliferation (TFDP1,

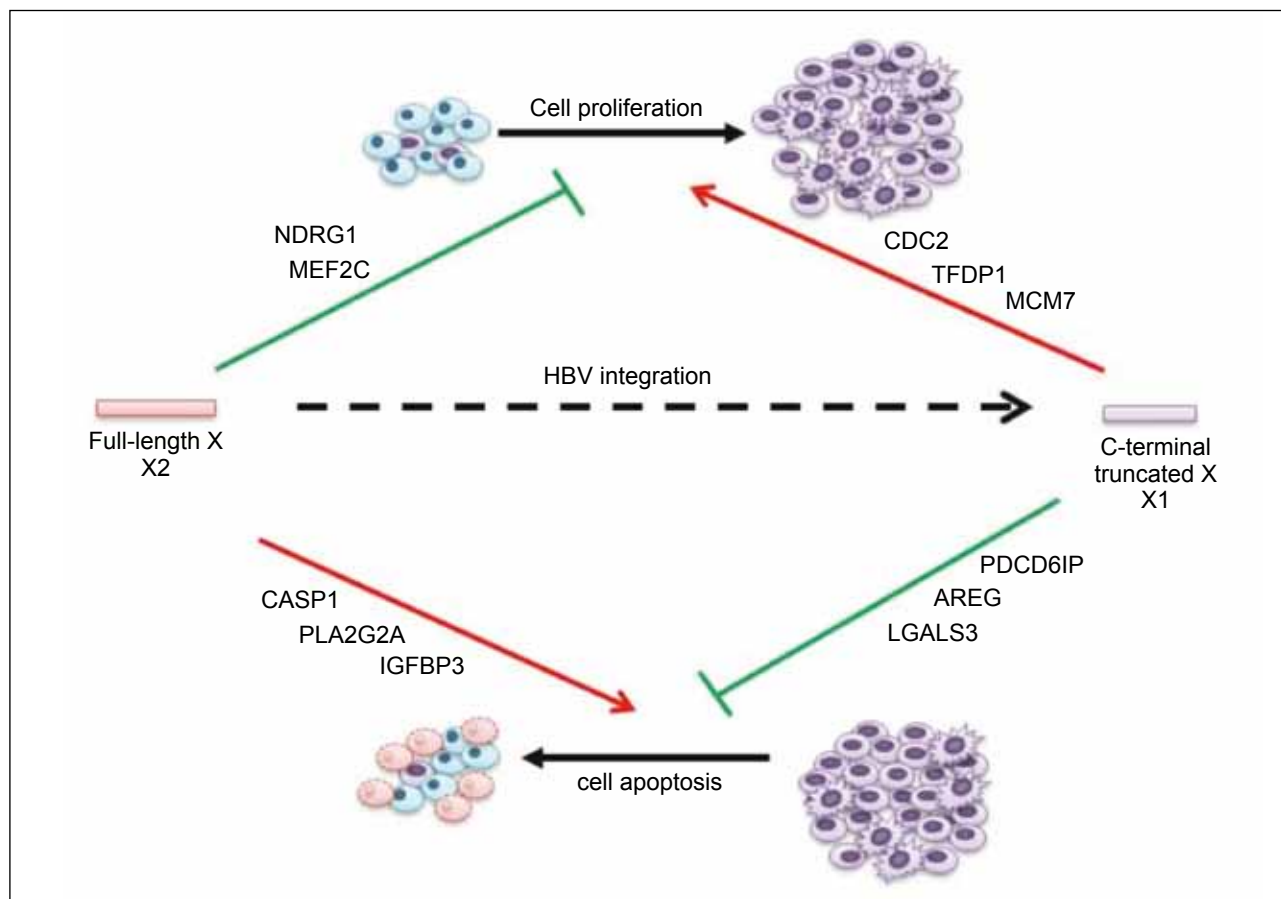


Fig 3. A proposed model for the role of C-terminal truncated hepatitis B X protein (HBx) in hepatocellular carcinogenesis
 C-terminal truncated HBx caused by hepatitis B virus (HBV) integration can promote cell proliferation and inhibit cell apoptosis

CDC2, CDC20, CDCA7, and MCM7) or anti-apoptosis (AREG, PDCD6IP, IER3, and LGALS3). In contrast, genes up-regulated by X2 gene have the opposite function like anti-proliferation (MEF2C, NDRG1, and IGFBP3) or pro-apoptosis (CASP1, PLA2G2A, and PLA2G6) [Fig 3].

Discussion

Alteration of HBV X gene has been closely associated with HCC pathogenesis. The mechanism of HBx in HCC development remains unclear. In this study, the C-terminal truncated HBx was frequently detected in HCC tissues (79.3%, n=111), which could also be confirmed by PCR with five pairs of primers encompassing the entire and different lengths of X gene. C-terminal truncated HBx, rather than the full-length HBx, could effectively transform normal liver cell lines and increase the tumourigenicity in cell models by a series of in vivo and in vitro experiments.

HBx plays an important role in HCC pathogenesis by interacting with cellular oncogenes and its functional domain involved in oncogenesis is at the middle of HBx protein. HBx can also induce apoptosis.³ In the present study, the apoptotic frequency was significantly higher in X2-expression cells than in X1-expression and mock

control cells (P<0.001). The induction of apoptosis by the full-length HBx, but not by the C-terminal truncated HBx, strongly suggests the C-terminal peptide is required for HBx pro-apoptotic function.

Based on our observations, there are two important functional domains located within the full-length HBx. One is an oncogenic domain (the N-terminal through middle peptide) and the other is a pro-apoptotic domain (the C-terminal peptide). During infection, HBV survival is probably related to a balance between these two functions. When the pro-apoptotic domain is lost, which is probably through viral integration, the balance is broken. Subsequently, the dominant oncogenic domain accelerates the development of HCC.

The hypothesis of two functional domains of HBx protein was supported by our cDNA microarray results, although the molecular mechanism underlining the truncation of C-terminal HBx has yet to be determined. Consistent with the two-domain hypothesis, cDNA array revealed five genes with known functions of promoting cell proliferation were up-regulated in X1-expression cells, and three genes with known negative functions on cell proliferation were down-regulated at least two fold. These results suggest that

the tumourigenic role of the C-terminal truncated HBx is via the activation of cell proliferation and inhibition of cell apoptosis (Fig 3). Our molecular dissections of the two-domain peptides and functions are in progress.

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