

# C-terminal truncated HBx reduces doxorubicin cytotoxicity via *ABCB1* upregulation in Huh-7 hepatocellular carcinoma cells

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Hepatitis B virus (HBV) encoding the HBV x protein (HBx) is a known causative agent of hepatocellular carcinoma (HCC). Its pathogenic activities in HCC include interference with several signaling pathways associated with cell proliferation and apoptosis. Mutant C-terminal-truncated HBx isoforms are frequently found in human HCC and have been shown to enhance proliferation and invasiveness leading to HCC malignancy. We investigated the molecular mechanism of the reduced doxorubicin cytotoxicity by C-terminal truncated HBx. Cells transfected with C-terminal truncated HBx exhibited reduced cytotoxicity to doxorubicin compared to those transfected with full-length HBx. The doxorubicin resistance of cells expressing C-terminal truncated HBx correlated with upregulation of the ATP binding cassette subfamily B member 1 (ABCB1) transporter, resulting in the enhanced efflux of doxorubicin. Inhibiting the activity of ABCB1 and silencing *ABCB1* expression by small interfering ribonucleic acid (siRNA) increased the cytotoxicity of doxorubicin. These results indicate that elevated ABCB1 expression induced by C-terminal truncation of HBx was responsible for doxorubicin resistance in HCC. Hence, co-treatment with an ABCB1 inhibitor and an anticancer agent may be effective for the treatment of patients with liver cancer containing the C-terminal truncated HBx. [BMB Reports 2019; 52(5): 330-335]

## INTRODUCTION

Hepatocellular carcinoma (HCC), one of the leading causes of cancer-related deaths worldwide, is mainly associated with

chronic hepatitis B virus (HBV) infection (1, 2). The HBV genome encodes the HBV core surface, DNA polymerase, and X (HBx) proteins (3). The HBx protein activates numerous signal transduction proteins which are involved in cell proliferation and survival (4). In addition, the HBx protein has been associated with HCC development (4, 5). A transgenic study showed that an HBx-expressing transgenic mouse developed HCC (5). These observations indicate that HBx plays an important role in HCC development.

According to a recent report, mutant forms of HBx were found in HBV-infected patients with HCC (6). Specifically, C-terminal truncated HBx mutants were frequently observed in tissues from patients with HCC (7, 8). C-terminal truncated HBx was detected only in tumorous liver tissues, whereas full-length HBx was found in both non-tumorous and tumorous liver tissues (6). The abnormal expression of truncated HBx protein has been implicated in HCC (6). Furthermore, C-terminal truncated HBx activated cell division and transformation by increasing the expression of ras and myc oncogenes (9) and was also associated with tumor metastasis and promotion of the invasive ability of cells (9). C-terminal deletion of HBx accelerated cell proliferation, migration, and hepatocellular carcinogenesis (10-13), deregulated the FXR/RXR pathway, and has been associated with drug resistance (14).

The molecular mechanisms of drug resistance, in general, include ATP-dependent drug efflux, increases in drug detoxification, alteration of drug targets, changes in DNA damage repair, failure of cell cycle arrest regulation, and modification of Bcl-2 expression. Among these, an increase in drug efflux is a very common drug resistance mechanism in cancer cells (15).

Increases in drug efflux can be mediated by ATP-binding cassette (ABC) transporters. The most important and effective transporters in multidrug resistance include ABCB1, ABCC1, and ABCG2. The ABC transporter ABCB1, also known as p-glycoprotein (p-gp) or multidrug resistance 1 (MDR1), is encoded by the *ABCB1* gene (16). ABCB1, ABCC1, and ABCG2 are crucial for and more effective in multidrug resistance compared to other transporters (15). Overexpression of *ABCB1* confers significant resistance to various neutral and cationic hydrophobic chemotherapeutic agents, including anthracyclines (e.g., doxorubicin and daunorubicin) (17).

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<https://doi.org/10.5483/BMBRep.2019.52.5.312>

Received 20 December 2018, Revised 13 February 2019,  
Accepted 12 March 2019

**Keywords:** Apoptosis, C-terminal truncated HBx expressing cells, Cytotoxicity, Drug efflux, Hepatocellular carcinoma

In this study, we explored the role of C-terminal truncated HBx in HCC malignancy and found that C-terminal-truncated HBx-expressing cells were more resistant to doxorubicin than cells expressing full-length HBx. Doxorubicin resistance was due to increased *ABCB1* expression, which could play an important role in the malignant phenotype of cells expressing the C-terminal-truncated HBx. Thus, our findings provide new insight into the role of C-terminal-truncated HBx on HCC malignancy and suggest novel treatment methods for liver cancer containing the C-terminal-truncated HBx.

## RESULTS

### C-terminal-truncated HBx reduces cytotoxicity of doxorubicin in Huh-7 HCC cells

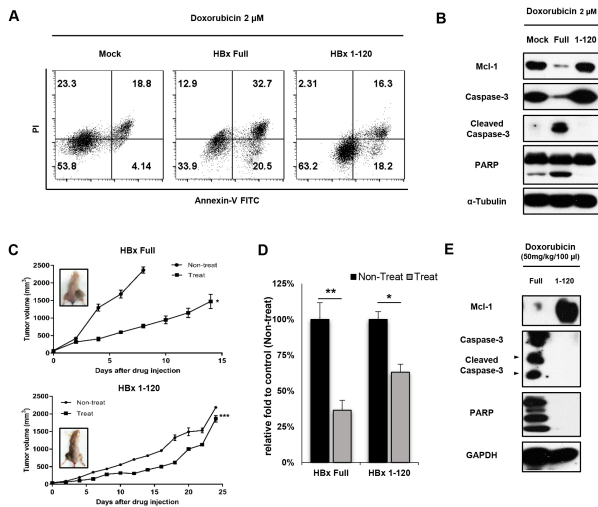
C-terminal-truncated HBx proteins are frequently found in tissues from patients with HCC (7, 8) and have been associated with the malignancy of HCC (6, 9). To investigate the role of mutant HBx protein lacking 34 amino acids on the C-terminal (HBx 1-120) in HCC malignancy, we first tested the cytotoxic effect of the anti-cancer drug doxorubicin by MTT assay using cells stably transfected with either full-length HBx (HBx Full) or HBx 1-120. The viabilities of the mock and HBx

Full-expressing cells decreased to 65% and 69%, respectively, following doxorubicin treatment, while cells transfected with HBx 1-120 exhibited 81% survival (Supplementary Fig. 1). In addition, annexin-V/PI staining was assessed using flow cytometry to confirm the induction of apoptosis by doxorubicin in the Huh-7 stable cell lines. As expected, an elevated survival rate was observed in cells expressing HBx 1-120. These cells showed a survival rate of 63.2%, in contrast to 33.9%, for HBx Full-expressing cells (Fig. 1A).

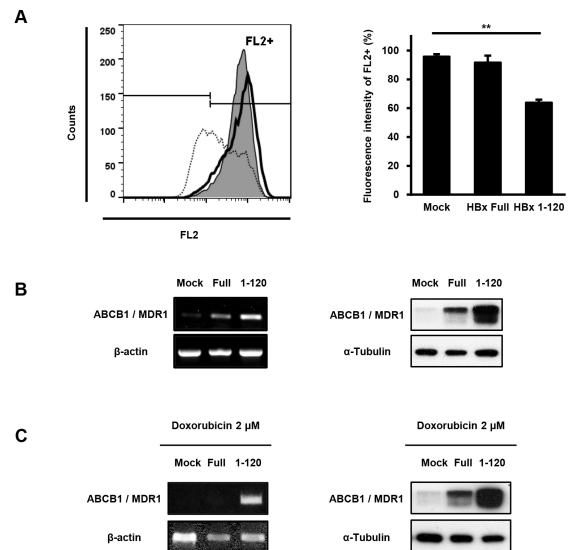
We also examined changes in apoptosis-associated molecules using Western blot analysis (Fig. 1B). When the cells expressing HBx-Full were treated with doxorubicin, the level of the anti-apoptotic Bcl-2 family protein Mcl-1 was conspicuously decreased, and cleaved isoforms of both caspase-3 and PARP levels were increased, whereas no changes in apoptosis markers were observed in cells expressing HBx 1-120 (Fig. 1B). Thus, these results indicate that the C-terminal region of HBx played a role in apoptosis and that HBx 1-120 reduced doxorubicin cytotoxicity in HCC cells.

### C-terminal-truncated HBx reduced doxorubicin cytotoxicity in HCC xenograft model

To evaluate the contribution of C-terminal-truncated HBx to



**Fig. 1.** Doxorubicin cytotoxicity was reduced in C-terminal-truncated HBx-expressing cells. (A) The effect of C-terminal-truncated HBx 1-120 on doxorubicin cytotoxicity was determined by flow cytometric analysis. (B) Western blot analysis of apoptosis markers (Mcl-1, caspase-3, cleaved caspase-3, and PARP) in Huh-7 HBx cells cultured with doxorubicin. (C) Changes in tumor xenograft volumes after initiating the doxorubicin treatment. Circle, non-treatment; square, doxorubicin treatment. (\* $P < 0.05$ , non-treatment vs doxorubicin in HBx Full; \*\*\* $P < 0.005$ , non-treatment vs doxorubicin in HBx 1-120) (D) Relative tumor volume at the endpoint of the non-treatment group. Relative tumor volumes are presented as mean  $\pm$  SD ( $n = 10$ ). \*\* $P < 0.005$  (HBx Full), \* $P < 0.05$  (HBx 1-120) (E) Western blot analysis of apoptosis markers in xenograft tumor tissues.



**Fig. 2.** C-terminal-truncated HBx upregulated ABCB1 expression. (A) Flow cytometry analysis of Huh-7 mock (grey-filled solid line) or those stably expressing HBx-Full (bold, solid line), or HBx 1-120 (dashed line) treated with rhodamine 123. Rhodamine 123 fluorescence was measured by fluorescence-activated cell sorting (FL2 = 575 nm). (B) RT-PCR and Western blot analysis were used to measure the mRNA and protein levels of ABCB1 in stable Huh-7 cells. (C) RT-PCR and Western blot analysis were used to measure the mRNA and protein levels of ABCB1 in stable Huh-7 cells after doxorubicin treatment.

the reduction of doxorubicin cytotoxicity *in vivo*, we developed a human HCC xenograft model. Tumor formation in mice injected with HBx 1-120 was significantly delayed compared to those injected with HBx-Full (Fig. 1C). Consistent with this result, a published report showed that tumor sizes were smaller in patients expressing C-terminal-truncated HBx, compared to those in patients expressing full-length HBx (9). When injected with doxorubicin, the growth of tumors expressing HBx-Full was dramatically decreased. However, the growth of tumors expressing HBx 1-120 was only slightly reduced by doxorubicin injection (Fig. 1C and 1D).

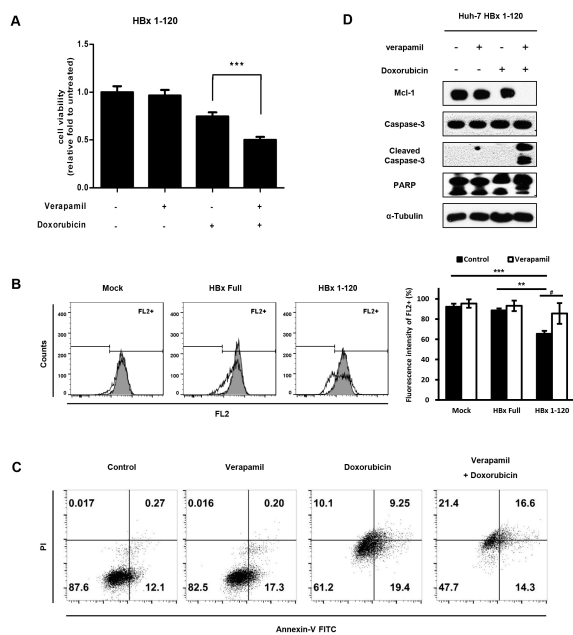
The expression pattern of apoptosis markers in the xenograft tumor tissues was consistent with the results of our *in vitro* study. Caspase-3 and PARP were not cleaved in tumor tissues from mice injected with HBx 1-120 (Fig. 1E). Our results, therefore, indicate that C-terminal-truncated HBx reduced doxorubicin cytotoxicity *in vivo*, as well as *in vitro*.

### C-terminal truncated HBx enhanced the expression of the ABCB1 transporter

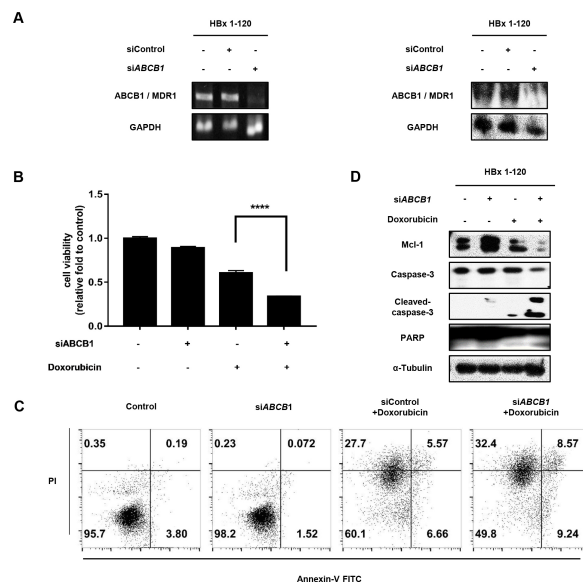
Several studies have reported that doxorubicin resistance was

predominantly associated with the upregulation of ABCB1/MDR1 (18). This transporter reduces intracellular drug accumulation in various human cancers by increasing drug efflux. Many types of multidrug resistance proteins, such as ABCB1, MRP1, and BCRP, can transport a wide range of substances, including doxorubicin and rhodamine 123. A typical method used to examine multidrug resistance protein activity is the rhodamine 123 assay (16, 19). To determine whether HBx 1-120 promoted doxorubicin efflux, we measured the retention of rhodamine 123. There was no significant difference in fluorescence between Huh-7 mock cells and those expressing HBx-Full but fluorescence was clearly decreased in cells expressing the C-terminal-truncated HBx compared to mock cells or those expressing HBx-Full (Fig. 2A). These results suggest that C-terminal truncated HBx promoted the activity or expression of multidrug resistance proteins.

We then examined the *ABCB1* mRNA and protein levels. Compared to mock cells and cells expressing HBx-Full, the *ABCB1* mRNA and protein levels were markedly increased in cells expressing C-terminal-truncated HBx (Fig. 2B). We also observed a similar increase after doxorubicin treatment (Fig. 2C). We did not observe any changes in other well-known transporter proteins, including MRP1 and BCRP (Supplementary Fig. 2). These data indicate that HBx 1-120



**Fig. 3.** Inhibition of ABCB1 activity increases the cytotoxicity effect of doxorubicin. (A) The effect of the ABCB1-specific inhibitor verapamil on doxorubicin-induced cytotoxicity was examined by MTT assay (\*\**P* < 0.005, doxorubicin vs verapamil and doxorubicin). (B) The effect of verapamil on the efflux of rhodamine 123 was determined by flow cytometric analysis. Solid line, control; gray-filled solid line, verapamil treatment. Rhodamine 123 fluorescence was measured by fluorescence-activated cell sorting (FL2 = 575 nm). (C) The effect of verapamil on doxorubicin-induced cytotoxicity was examined by annexin-V/PI staining. (D) Western blot analysis of apoptosis markers after treatment with doxorubicin and verapamil.



**Fig. 4.** Silencing of the ABCB1 expression by siRNA increases the cytotoxicity of doxorubicin. (A) RT-PCR and Western blot analysis to confirm the siRNA-mediated depletion of *ABCB1*. (B) The effect of *ABCB1* siRNA on doxorubicin-induced cytotoxicity was examined by the MTT assay (\*\*\*\**P* < 0.005, doxorubicin vs siABC B1 and doxorubicin). (C) The effect of *ABCB1* siRNA on doxorubicin-induced cytotoxicity was examined by annexin-V/PI staining. (D) Western blot analysis of apoptosis markers after treatment with doxorubicin and siRNA.

may have reduced doxorubicin cytotoxicity via efflux of the drug through upregulation of *ABCB1*.

### ***ABCB1* is responsible for the reduced doxorubicin cytotoxicity**

Based on the above results, *ABCB1* may be responsible for reducing the doxorubicin cytotoxicity of cells expressing HBx 1-120. To test this hypothesis, we used the specific *ABCB1* inhibitor verapamil to repress *ABCB1* activity. Verapamil treatment alone did not affect cell viability (Fig. 3A). When the cells expressing C-terminal-truncated HBx were treated with both doxorubicin and verapamil, doxorubicin-induced cytotoxicity was remarkably enhanced. In addition, the efflux of rhodamine 123 in cells expressing the C-terminal-truncated HBx was reduced significantly by verapamil (Fig. 3A). When cells expressing the C-terminal-truncated HBx were treated with both verapamil and doxorubicin, fluorescence intensity was 1.3-fold higher than that of the control cells (Fig. 3B). We observed similar results with C-4, another *ABCB1* inhibitor (Supplementary Fig. 3). We also measured doxorubicin-induced cell death by annexin-V/PI staining, with and without the *ABCB1* inhibitor verapamil, to verify that *ABCB1* was responsible for the doxorubicin resistance in the cells expressing HBx 1-120. The results showed 61.2% survival, which was significantly decreased to 47.7% by the addition of verapamil (Fig. 3C). When the cells were treated with either doxorubicin or verapamil, there was no change in the anti-apoptotic protein Mcl-1 but a combination of doxorubicin and verapamil abolished Mcl-1 expression in cells expressing HBx 1-120. In addition, the expression levels of the apoptosis markers cleaved caspase-3 and cleaved PARP were restored by the combined treatment (Fig. 3D).

Since the combined treatment of *ABCB1* siRNA and anticancer drugs has been shown to decrease multidrug resistance in cancer (20, 21), we used an siRNA against *ABCB1* to confirm that HBx 1-120 induced doxorubicin resistance through the upregulation of *ABCB1* expression. As shown in Fig. 4A, both the mRNA and protein levels of *ABCB1* were markedly reduced by siRNA transfection. The viability of doxorubicin-treated HBx 1-120-expressing cells was significantly decreased by si*ABCB1* transfection compared to scrambled siRNA-transfected cells (Fig. 4B). Annexin-V/PI staining showed a 60.1% survival rate, which was decreased to 49.8% in cells treated with both doxorubicin and si*ABCB1* (Fig. 4C). The anti-apoptotic protein Mcl-1 was reduced by the combined treatment with doxorubicin and si*ABCB1* in cells expressing HBx 1-120 (Fig. 4D). These data, therefore, confirm that HBx 1-120 induced doxorubicin resistance by upregulating *ABCB1* expression. Taken together, our results indicate that C-terminal-truncated HBx played an important role in the doxorubicin-resistant phenotype via upregulation of *ABCB1* expression.

## **DISCUSSION**

Chronic HBV infection has been associated with the development of HCC. During HBV infection, the HBV genome is frequently integrated into the host genome and HBx mutations, including C-terminal deletion in the integrated DNA, have been reported (2). Such C-terminal-deleted HBx was detected only in tumorous liver tissues, whereas HBx Full was found in both non-tumorous and tumorous liver tissues (6). Several reports have indicated that the C-terminal-truncated HBx protein was responsible for the malignant phenotype, including the enhanced invasiveness of HCC (9, 12, 14).

Our previous report demonstrated that cells transfected with mutant HBx protein lacking 34 amino acids on the C-terminal (termed HBx 1-120) did not show a significant increase in reactive oxygen species (ROS), while cells transfected with full-length HBx showed elevation of intracellular ROS, which may induce mitochondrial DNA damage (22). Thus, ROS production may not be a crucial factor in HBx 1-120-induced HCC malignancy.

The aim of the present study was to further explore the role of C-terminal-truncated HBx in HCC malignancy. We investigated doxorubicin resistance with respect to drug resistance in general, as well as the malignant phenotype and its treatment. Doxorubicin promoted nuclear DNA damage through DNA intercalation and topoisomerase II inhibition and produced a variety of cellular effects, including ROS generation. It is commonly used to treat many cancer types, including hepatocellular carcinoma (23-27).

We treated HBx Full- or HBx 1-120-expressing cells with doxorubicin and measured cell viability. Viability in the presence of doxorubicin was higher in cells expressing HBx 1-120 than in mock or HBx Full-expressing cells (Fig. 1A and 1B). Furthermore, HBx 1-120-expressing cells showed doxorubicin resistance *in vivo* (Fig. 2). Interestingly, HBx 1-120 expression resulted in smaller tumors compared to HBx Full expression in mouse xenograft model experiments. Consistent with this result, another report associated C-terminal-truncated HBx with smaller tumor sizes than full-length HBx in patient tissues (9). After doxorubicin injection, the growth rate of HBx Full-expressing tumors was dramatically decreased, whereas the growth rate of HBx 1-120-expressing tumors was only slightly reduced (Fig. 2B). When we examined apoptosis and anti-apoptosis molecules after doxorubicin treatment, we observed the anti-apoptotic Mcl-1 protein, but not the cleaved forms of either apoptotic markers Caspase-3 or PARP, in HBx 1-120-expressing cells. In HBx Full-expressing cells, the Mcl-1 protein was hardly detected and the cleaved forms of both Caspase-3 and PARP were increased (Fig. 2C). These results clearly indicate that C-terminal-truncated HBx 1-120 reduced doxorubicin cytotoxicity.

HBx 1-120 could have several roles in the reduction of

cytotoxicity by doxorubicin. According to some reports, HBx suppressed apoptosis induced by anticancer drugs and activated *ABCB1* expression at both the transcriptional and translational levels. Therefore, we tested the activity and expression of *ABCB1* and examined the effect of *ABCB1* on doxorubicin-induced apoptosis in Huh-7 cells expressing HBx Full or HBx 1-120. When we measured the retention of rhodamine 123, there was no significant difference in fluorescence between HBx Full-expressing and mock cells. However, the fluorescence was clearly decreased in HBx 1-120-expressing cells (Fig. 2A), indicating rhodamine 123 efflux. We, therefore, examined the intracellular levels of *ABCB1*. Compared to Mock and HBx Full-expressing cells, the mRNA and protein levels of *ABCB1* were markedly increased in HBx 1-120-expressing cells (Fig. 2B and 2C). When *ABCB1* activity was inhibited by verapamil treatment or expression of the *ABCB1* gene was inhibited by siRNA, the cytotoxicity induced by doxorubicin was remarkably enhanced in HBx 1-120-expressing cells (Fig. 3 and 4). In addition, co-treatment with doxorubicin and verapamil or siRNA resulted in an increase in the anti-apoptotic Mcl-1 protein and a decrease in the apoptotic forms of cleaved caspase-3 in HBx 1-120-expressing cells (Fig. 3D and 4D). These results indicate that the role of C-terminal truncated HBx 1-120 in the doxorubicin-resistant phenotype was mediated by the upregulation of *ABCB1* expression. However, we do not have any evidence for a causal relationship between C-terminal deletion and increased *ABCB1* expression. Therefore, the exact mechanism for the upregulation of *ABCB1* expression by C-terminal-truncated HBx remains unknown.

In a previous study, we showed that full-length HBx protein resulted in the production of mitochondrial ROS and induced mitochondrial DNA damage (22). In these processes, which may constitute a potential route to the development of HCC, the C-terminal region of HBx was thought to play an important role. Interestingly, the C-terminal truncated forms of HBx were more frequently observed in cancerous regions than in non-cancerous regions of patients with HCC (6). It seems likely that DNA damage accumulation mediated by full-length HBx and other micro-environmental conditions may lead to truncation of the HBx gene and the subsequent development of cancer.

In this study, we showed that C-terminal-truncated HBx, but not full-length HBx, resulted in a reduction of doxorubicin cytotoxicity through drug efflux associated with increased expression of *ABCB1*. Taken together, our results suggest the hypothesis that while full-length HBx is associated with the development of HCC, C-terminal-truncated-HBx promoted HCC malignancy.

## MATERIALS AND METHODS

A Materials and Methods section is available in the Supplementary Material.

## ACKNOWLEDGEMENTS

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2013R1A1A2012964). All animal experimental procedures complied with the Pusan National University IACUC in accordance with institutional guidelines (PNU-2015-0948).

## CONFLICTS OF INTEREST

The authors have no conflicting interests.

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