C-terminal truncated hepatitis B virus X protein regulates tumourigenicity, self-renewal, and chemoresistance via STAT3/Nanog signalling pathway: abridged secondary publication

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KEY MESSAGES

1. HBx-ΔC1 (a major C-terminal truncated form reported with a breakpoint at 130aa) regulates cancer stem cell properties including tumour initiation, self-renewal, drug resistance, and invasiveness.
2. HBx-ΔC1 regulates liver cancer stem cells through Stat3/Nanog cascade, which provides a new insight for the therapeutic intervention for hepatitis B virus–related hepatocellular carcinoma.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide. Hepatitis B virus (HBV) DNA is often integrated and highly rearranged within the host DNA in HCC. These templates frequently produce the HBV viral oncoprotein (HBx), which is active in transactivation assays. The sustained production of HBx is associated with hepatocellular transformation and represents a major contribution of HBV to HCC.[1] HBV integration is detected in 80% to 90% of host genomes from HBV-infected HCC cases, and the HBx gene is partially deleted frequently during the integration process, causing the C-terminal truncation of HBx.[2,3] In addition, C-terminally truncated HBx (HBx-ΔC) plays a critical pro-oncogenic role in hepatocarcinogenesis and metastasis.[2,3] These results demonstrate that, in addition to the full-length HBx, the HBx-ΔC also plays an important role in HCC development. Among various truncated mutants of HBx, c-terminal truncation with a breakpoint of 130aa (HBx-ΔC1) is the major truncated form of HBx existing in our HCC samples.[3] However, the molecular mechanism whereby HBx-ΔC1 contributes to HCC remains largely unknown. Recent evidence supports the existence of cancer stem cell (CSC) / tumour-initiating cell (T-IC) model in leukaemia and a wide range of solid tumours, including HCC.[4] CSCs are believed to possess both cancer cell– and stem cell–like characteristics, including tumour initiation, self-renewal, and differentiation. HBV can induce stem cell-like and CSC-like signatures in HCC. Despite these findings, direct evidence demonstrating a functional role for HBx-ΔC in promoting liver carcinogenesis and through regulation of stemness factors in HCC is lacking, and the molecular mechanisms are unknown. In this study, we aim to evaluate the clinical relevance and prognostic significance of stemness factors and HBx-ΔC1 expression at different HCC development stages in the presence or absence of HBV infection and delineate the role of HBx-ΔC1 in regulating Nanog and its tumour-initiating properties.

Methods

Total RNA of human HCC liver tissues was extracted using TRIzol (Invitrogen, Carlsbad, CA), according to manufacturer's protocol. For polymerase chain reaction (PCR) amplification of HBx, sets of PCR primers (1425F: 5'-TCCTTTGTTTACGTCCCGTC-3', 1840R:-5'-TTAGGCAGAGGTGAAAAAGTTG-3' and 1661R: 5'-GAATTCCTTATGTAAGACCTTGGG CAACAT-3') were used for full-length and COOH-truncated HBx, respectively.

Full-length HBx DNA (GenBank no.: U95551) was amplified from the HBx/pcDNA3.1+ plasmid[3] and sub-cloned into Myc/pLVX-Tight Puro and Myc/pcDNA3.1+ vectors. HBx truncation mutant (named HBx-ΔC1) with 24 C-terminal amino acid of HBx deleted was made and sub-cloned into Myc/pLVX-Tight Puro and Myc/pcDNA3.1+ vectors. Bel-7402 and SMMC-7721 cell line were first transfected with pLVX Tet-On Advanced vector (Clontech Laboratories, Mountain View [CA], USA) using Lipofectamine 2000 (Invitrogen), according to manufacturer's protocol. tTA(Tet-On)-expressing Bel-7402 and SMMC-7721 cells were selected

with G418 at 700 μg/mL and 400 μg/mL for 14 days, respectively. To obtain stable inducible HBx-expressing cells, lentivirus containing full-length and C-terminal truncated HBx in Myc/pLVX-Tight Puro vector was infected into tTA-expressing Bel-7402 and SMMC-7721 cells and selected with puromycin at 1 μg/mL for 7 days. In vitro and in vivo functional assays were used to evaluate the effect of transgene on self-renewal, tumourigenicity, drug resistance, and cell migration.

The effect of HBx and HBx-ΔC1 on Stat3/Nanog signalling pathway in both Bel-7402 and SMMC-7721 was evaluated by western blot analysis using specific antibodies against Stat3, p-Stat3 (Y705), and Nanog. The specific effect on Stat3 activation was further evaluated by immunofluorescence staining. The involvement of Stat3 in HBx-ΔC1-mediated self-renewal was investigated by a rescue experiment using Stat3 inhibitor (S3I-201). The clinical correlation between HBx-ΔC1 and Nanog was investigated in a cohort of 107 HCC patient samples by semi-quantitative RT-PCR analysis of HBx and HBx-ΔC1 as described above and qPCR analysis using specific primer of Nanog (F: 5'- CCTGTG ATTTGTGGGCCTG-3', R: 5'- GACAGTCTC CGTGTGAGGCAT-3'). Student's t was used for continuous data wherever appropriate. A P value of <0.05 was considered statistically significant.

Results

HBx- ΔC1 is correlated with stemness factors including Sox2/Nanog

Using lentiviral based Tet-On overexpression approach, we established inducible expression of HBx-FL and HBx-ΔC1 in Bel-7402 and SMMC-7721 cells upon addition of doxycycline at 1 μg/mL. By qPCR analysis, we found preferential induction of certain stemness related genes, including Nanog, Sox2, SMO, and ABCB5, in HBx-ΔC1 expressing HCC cells, when compared with HBx-FL and empty vector control. Interestingly, we found that overexpression of HBx-ΔC1 increased the expression of liver CSC markers including CD133 and CD47 when compared with control and HBx-FL expression in Bel-7402 and SMMC-7721 by qPCR and flow cytometry analyses. In a cohort of 107 HCC patient samples, we examined the expression of Klf4, c-myc, Oct4, β-catenin, Nanog, and Sox2 in HCC clinical samples by qPCR. Consistently, Nanog and Sox2 expression was up-regulated in tumour samples detected with HBx-ΔC1 when compared to those with full length of HBx and without HBV infection.

HBx- ΔC1 overexpression induced CSC properties in HCC

Upon transfection of HBx and HBx-ΔC1 into Bel7402 and SMMC-7721, HBx-ΔC1 transfectants exhibited increased self-renewal ability, as evidenced by the increase in the number and size of the spheres formed in sphere formation assay. We then examined whether C-terminal truncated form of HBx was more tumourigenic than full length of HBx in vivo by tumour forming assay with HBx-FL expressing and HBx-ΔC1 expressing Bel-7402 and SMMC-7221 cells. Cells at density of 5000, 10 000 and 50 000 were inoculated subcutaneously into NOD/SCID mice. A significant increase in tumour incidence and size was observed in HBx-ΔC1 expressing cells when compared with control and HBx-FL expressing cells. Our previous studies demonstrated that liver CSCs were more chemoresistant to chemotherapeutic drugs.[4] Thus, we assessed whether HBx-ΔC1 conferred chemoresistance to HCC cells. We treated transfectants of control, HBx-FL, and HBx-ΔC1 derived from Bel-7402 and SMMC-7721 with cisplatin and doxorubicin and subjected them to Annexin V staining assay. HBx-ΔC1 transfectants were more chemoresistant to cisplatin and doxorubicin when compared with EV and HBx-FL transfectants in both Bel-7402 and SMMC-7721 cells. In addition, overexpression of HBx-ΔC1 conferred greater migratory ability when compared with control and HBx-FL overexpression. Previously, we found that liver CSCs were enriched upon sorafenib treatment, as evidenced by increase in abilities in self-renewal and tumourigenicity in sorafenib-resistant cells.[5] Based on this finding, we hypothesise that HBx-ΔC1 overexpressing cells are more resistant to sorafenib treatment. We compared the sensitivity of sorafenib among transfectants of control, HBx-FL, and HBx-ΔC1 derived from Bel-7402 and SMMC-7721. By Annexin V staining, HBx-ΔC1 transfectants were more resistant to sorafenib when compared with EV and HBx-FL transfectants in both Bel-7402 and SMMC-7721 cells

HBx-ΔC1-driven tumour initiation and self-renewal through Stat3-Nanog signalling.

Previously, we found that Stat3-Nanog pathway was activated in liver CSCs contributing to tumourigenicity and stemness.[4] In addition, Stat3 signalling activity was enhanced in HBx expressing cells and HBx transgenic mice leading to carcinogenesis, and therefore, we explored whether Stat3-mediated Nanog regulation was involved in truncated HBx-induced stemness. By western blotting, HBx-ΔC1 preferentially induced expression of Stat3 (Y705) and stem cell transcriptional factor, Nanog, in Bel-7402 and SMMC-7721. We determined Stat3 activity by qualifying p-Stat3 (Y705) level using immunofluorescence staining. Consistently, HBx-ΔC1 stimulated stat-3 activity indicated by higher fluorescent intensity for p-Stat3

(Y705) staining when compared with control and HBx-FL. To further examine whether the HBx-induced response is stat3 dependent, we examined p-Stat3 (Y705) and Nanog expression in response to a STAT3 inhibitor (S3I-201) in Bel-7402 and SMMC-7721 cells. By XTT assay, the cytotoxic effect of Stat3 inhibitor on Bel-7402 and SMMC-7721 cells was examined and IC50 was around 575 μM and 700 μM, respectively. By western blot analysis, Nanog expression was downregulated upon S3I-201 treatment. Addition of S3I-201 led to abolishment of HBx-induced self-renewal indicated by the sphere-forming assay. Taken together, these findings suggest that HBx-ΔC1 regulates liver CSCs through Stat3-mediated Nanog regulation.

Discussion

Recent evidence supports the presence of CSCs contributing to resistance to conventional therapies and tumour relapse. HBx has been implicated as playing an oncogenic role in the development of HBV-associated HCC. Recent studies have reported that HBx-enhanced expression of stemness and CSC markers including Oct4, Nanog, Klf-4, and EpCAM in vitro and in vivo contributes to HCC. Consistent to previous findings, we found that HBx enhanced expression of stemness genes including Nanog, Sox2, SMO, and ABCB5. COOH-truncated form of HBx has been shown to have more aggressive behaviour in the development of HCC. In this study, in addition to full length of HBx, we also study the role of COOH-truncated HBx, with a breakpoint at 130aa (HBx-ΔC1) in regulation of cancer stemness. Interestingly, we demonstrated that HBx-ΔC1 regulated the liver CSC properties including enhanced expression of stemness genes, self-renewal capacity, and resistance to chemotherapeutic drugs, and driving tumourigenesis; the effect is more prominent than HCC cells transfected with full length of HBx. These results have demonstrated the distinct role of HBx-ΔC1 in regulation of liver CSCs. In addition, we showed that X gene of HBV integrated into the host liver DNA prior to the appearance of tumour, perhaps up-regulation of stemness factors such as Sox2 and Nanog would be more important to tumour pathogenesis at an early, preneoplastic stage. However, the use of the stemness factors for diagnostic and prognostic purpose is limited by low abundance in HCC samples. Cross-talks of stemness factors have been reported to regulate the stemness of HCC. For instance, Oct4 was found to regulate β-catenin activation in non-canonical manner.[6] In addition, Oct4 was found to regulate chemoresistance through regulation of Akt pathway.[7]

By western blot analysis and immuno-fluorescence staining, we found upregulation of nuclear stat3, p-Stat3 and Nanog expression in HBx-expression cells. We found that Stat3-Nanog signalling pathway was preferentially activated in HBx-ΔC1 transfectants, which was indicated by the enhanced activity of Stat3 and expression of Nanog observed in HBx-ΔC1-expressing Bel-7402 and SMMC-7221 cells, when compared with HBX-FL and control cells. Furthermore, the role of HBx-ΔC1-induced self-renew was further confirmed by treatment of Stat3 inhibitor. We found that the inhibition of Stat3 activation using a Stat3 specific inhibitor abrogated the effect of the HBx-ΔC1-induced self-renewal capacity. These findings suggest that Stat3-Nanog signalling plays a crucial role in regulating the stemness properties mediated by HBx-ΔC1.

Conclusion

HBx-ΔC1 plays critical role in HCC development and progression via the regulation of cancer stemness, which involves preferential activation of the Stat3-Nanog pathway. A better understanding of the molecular mechanism of HBx-ΔC1 will improve our knowledge of HCC pathogenesis, with the goal of developing more-effective management. Our data give a new insight on developing targeted therapies against HBx-ΔC1–induced Nanog and identifying novel markers to predict disease outcome and tumour recurrence.

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Disclosure

The results of this research have been previously published in:
(1) Ching RHH, Sze KMF, Lau EYT, et al. C-terminal truncated hepatitis B virus X protein regulates tumorigenicity, self-renewal and drug resistance via STAT3/Nanog signaling pathway. Oncotarget 2017;8:23507-16.

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