Regulation and Impact of Hepatitis B x protein in persistence and oncogenesis of Hepatitis B Virus

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Running title- "HBx in Chronic Hepatitis B Infection"

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Abstract

Background: Chronic Hepatitis B (CHB) Virus infection is major etiological factor for liver cirrhosis and/ or liver cancer. The viral protein, major contributor in predisposition of chronicity and Hepatocellular Carcinoma (HCC) is Hepatitis B x (HBx) protein. Its dynamic subcellular distribution to an extent determines its multifactorial role. It is a regulatory protein which modulates viral as well as host machinery in favours to HBV persistence. An insight on HBx stabilising factors is critical for therapeutic purpose. The precise role of HBx in the pathogenesis of Chronicity of HBV is not known.

Summary: This review comprehensively summarizing different mechanisms and their regulation by HBx protein with respect to chronicity and HCC emphasising viral persistence.

Key Messages

1. **HBx is a key protein for viral persistence.**
2. **Dynamic subcellular distribution of HBx determines its function.**
3. **HBx modulates cellular machinery to favours HBV survival.**
4. **HBx affects various intermediary mechanisms contributing to disease progression.**
5. **HBx may be a potent target to prevent the disease progression towards HCC.**

Abbreviations. Hepatitis B Virus (HBV), Chronic Hepatitis B (CHBV), Hepatitis B surface antigen (HBsAg), Hepatitis B e antigen (HBeAg), Alanine Transaminases (ALT), covalently closed circular DNA-(cccDNA).

Introduction

Hepatitis B is a global health problem with 257 million carriers of chronic Hepatitis B Virus (HBV) infection worldwide[1]. In chronic HBV infections, persistent low grade liver inflammations may sometimes occur due to the host immune response[2–4], followed by fibrogenesis. This may subsequently lead to fibrosis and cirrhosis and further progress to decompensated liver disease and/or Hepatocellular carcinoma (HCC). Chronic HBV carriers

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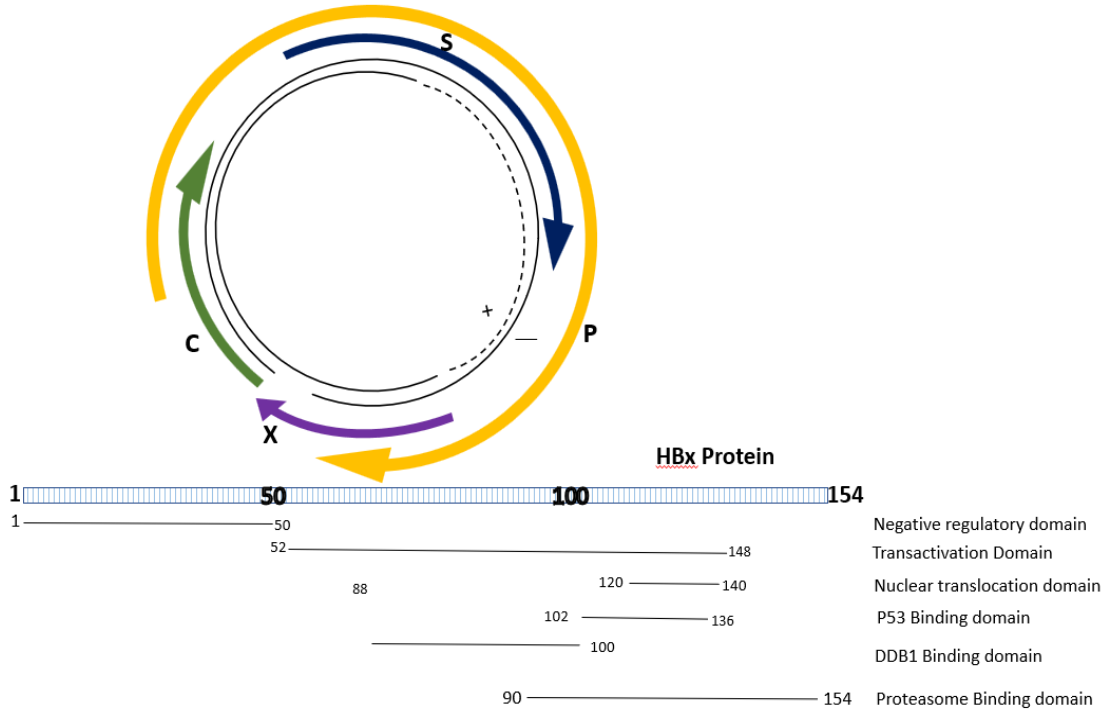
have a 20-60 fold higher chance of developing HCC with 50% cases of HCC worldwide being associated with chronic HBV infection [5,6]. The chronicity of HBV infection is a prolonged infection characterized by presence of HBsAg and/or HBV DNA for more than six months. During the natural course of chronic infection, the disease pass through four phases on the basis HBeAg positivity, Alanine Aminotransferase (ALT) levels and viral load[7].

The first phase is HBeAg positive viral infection or immunotolerant phase, characterized by high levels of Hepatitis B surface Antigen (HBsAg) and HBV DNA, but low levels of Alanine Aminotransferase (ALT). In this phase, HBeAg is a key protein which generates tolerance towards adaptive immunity and suppresses it. The second phase is HBeAg positive viral hepatitis or immune active phase, characterized by low levels of HBsAg, fluctuating levels of HBV DNA, and high levels of ALT, released by cytolysis caused by activation of intrahepatic and peripheral HBV specific T cell response. The third phase is HBeAg negative viral infection or inactive carrier phase, characterized by low levels of HBV DNA and ALT, accompanied by disappearance of HBeAg and appearance of HBe Antibody (seroconversion). HBV specific immune response gets downregulated with low grade inflammation mainly due to Th1 cells. The fourth phase is HBeAg negative viral hepatitis or reactivation phase, characterized by re elevation of HBV DNA with increase in ALT and HBsAg positivity and T cell anergy[3,4,7,8].

The predisposition to HCC and HBV persistence is conciliated by a multifunctional, single regulatory protein of HBV called Hepatitis B virus X (HBx) protein. However, it is not possible to draw a line between mechanisms involved in viral persistence (chronicity) and oncogenesis. This review, comprehensively updates the major mechanisms involved in viral persistence and oncogenesis.

Life cycle

Fig 1. Genomic organization of Hepatitis B virus (HBV)



HBV is a prototype virus of the Hepadnaviridae family with a small genome of 3.2 Kb containing four Open Reading Frames (ORF), namely, S, P, C and X, which encode surface, polymerase, core/envelope and x proteins respectively (Fig.1) and exhibits retroviral mode of

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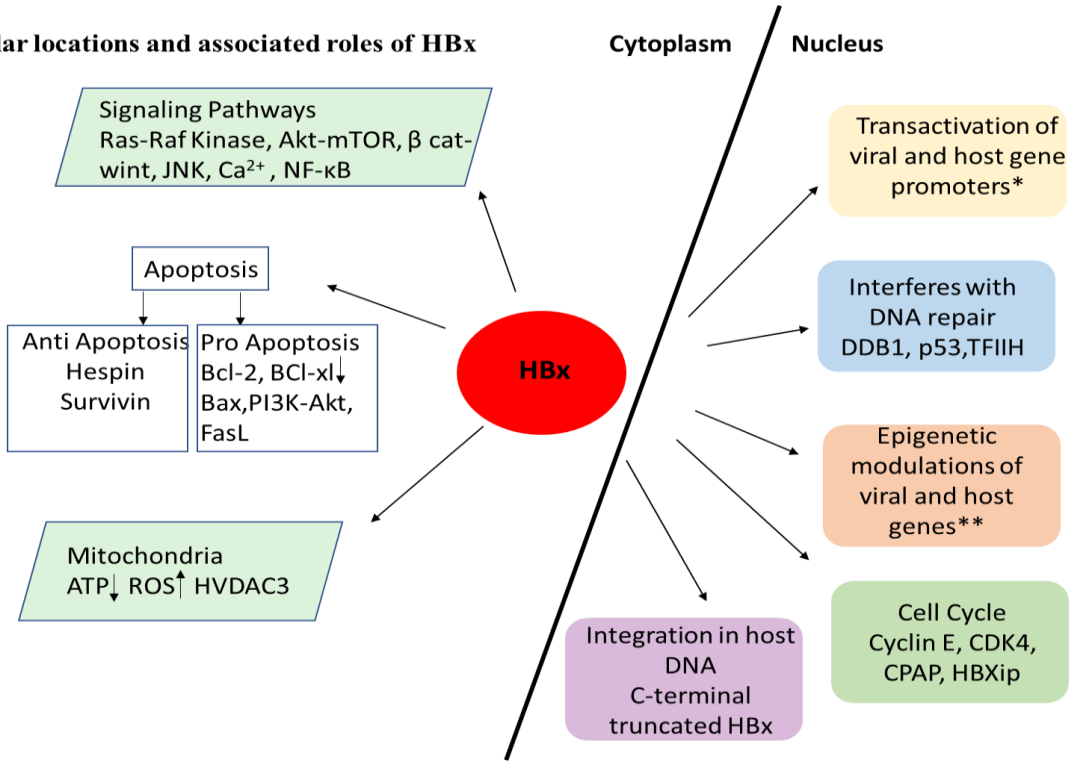
replication. The S ORF has three regions- Pre S1, Pre S2 and S which encode three proteins, the large (Pre S1+PreS2+S), medium (Pre S2+S) and small (S) proteins. The P ORF encodes a polymerase enzyme with RNA dependent and DNA dependent polymerase activity as well as RNase H activity. The C ORF encodes HBcAg and HBeAg when transcription starts from core region and precore region, respectively. The HBx protein is coded by X ORF[9,10].

HBV enters into hepatocytes via a specific receptor sodium taurocholate co-transporting polypeptide (NTCP) which binds with the pre S1 region of large surface protein of HBV[11,12]. After attachment, HBV is endocytosed by the asialoglycoprotein receptor (ASGPR)[13,14]. As the relaxed circular DNA of HBV reaches the nucleus, the polymerase associated with the negative strand of partially double stranded genomic DNA converts it into a covalently closed circular DNA (cccDNA) minichromosome. cccDNA is transcribed using host transcription factors and forms RNA of different sizes i.e. 3.5 kb pgRNA, 3.5 Kb precore mRNA, 2.4 kb and 2.1Kb surface mRNA and 0.7kb X mRNA[15,16]. These poly adenylated RNAs translocate to the cytoplasm and are translated. In the cytoplasm, pgRNA is encapsidated with core protein. Inside the capsid, the polymerase associated with pgRNA reverse transcribes it to rcDNA and forms complete infectious virions (Dane particles) which egress through the multivascular secretory pathway[17]. Some of the rcDNA is trafficked back to the nucleus to maintain the cccDNA pool. The HBV DNA, mainly X ORF, also integrates into the host genome. The HBV related entities present in the blood stream are – complete virions (dane particle), capsid bounded pgRNA, capsid bounded HBsAg, empty nucleocapsids, HBeAg, and P22cr[8].

HBx: Intracellular localization and function

HBx is a multifunctional regulatory protein of 17 KDa size and 154 amino acid length, encoded by the X ORF of the HBV genome. HBx protein is conserved in mammalian hepadnavirus [18] and plays a major role in HBV replication, persistence and hepatocyte transformation to HCC.

Fig 2 Subcellular locations and associated roles of HBx



HBx has been reported to exhibit nuclear, cytoplasmic as well as mitochondrial localization which determines its function and effect on cells. (Fig 2) HBx localizes to nucleus at low levels

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and to cytoplasm when its level increases[18]. In the nucleus, it promotes transcription of cccDNA, stabilizes its minichromosomal form, supports replication by transactivation of enhancers/promoters of HBV DNA and mediates epigenetic modulations[19].In the cytoplasm, it facilitates the reverse transcription of pregenomicRNA (pgRNA), and hence, the replication and persistence of HBV[20]. Here, it can also induce transformation and apoptosis whereas. HBx has mitochondria targeting signals leads to its mitochondrial localization[21] where it interferes with ATP production and pushes the cell towards metabolic reprogramming during HCC progression[22,23].

Prieto et al., demonstrated through their study on human hepatocarcinoma cells, phosphorylation of the serine 31 and 41 of the conserved region of HBx regulates its cytoplasmic presence whereas point mutation at these sites localizes HBx to nucleus[24]. A recent study by Hernández et al., on hepatoma cells, demonstrated the presence of three isoforms of HBx: XF (full-lengthisoform), XM (medium-length isoform), and XS (short-length isoform) having different subcellular localization and effect on viral replication[25].

HBx regulated mechanisms of viral persistence

The primary contributing mechanisms for viral persistence are: replication of HBV and transcription of cccDNA in part regulated by HBx.

HBx is detected early infection and persists in all phases of chronic infection and known to be responsible for initiation and enhancement of viral replication[26,27]. HBx facilitates viral replication by various mechanisms such as transcriptional activation of cccDNA, triggering autophagy, various signaling pathways, and by regulating epigenetic machinery. HBx also prevents its own degradation and supress agents directly targeting pregenomic RNA (pgRNA).

Silencing of host restriction factors –

Keasler et al., has shown HBx is a short live protein primarily localized in nucleus[28]. HBx does not have nucleus localization signal, though it is primarily localized in nucleus[29], may be due to its binding to certain cellular proteins, stabilizes and localizes it to nucleus. It was known that HBx protein is expressed very early in infection. Niu et al., showed HBV RNA is delivered to HBV infected cells through extracelluar vesicles and leads to the expression of HBx. HBx promotes the transcription of cccDNA by degrading smc5/6[30]. HBx stabilises itself by binding with DDB1. HBx-DDB1 complex promotes the ubiquitin mediated proteolysis of smc5/6, and relieves transcriptional inhibition of cccDNA minichromosome promotes replication[31–33].(Fig.3) similarly, apolipoprotein B mRNA editing catalytic polypeptide-like (APOBEC) is also an intracellular restriction factor the degrades cccDNA. Chen et al., in his work on transfected Huh-7 cells demonstrated that HBx selectively decreases the expression of APOBEC3G and increases its externalization via exosomes thus decreasing its intracellular concentration[34]. In vitro silencing of some other host restriction factors like stromal interaction molecule 1 (STIM1), zinc finger E-box binding homeobox 2 (ZEB2), and proteasome activator subunit 4 (PSME4), also promotes HBV replication, and they are found to be down regulated by HBx[35].

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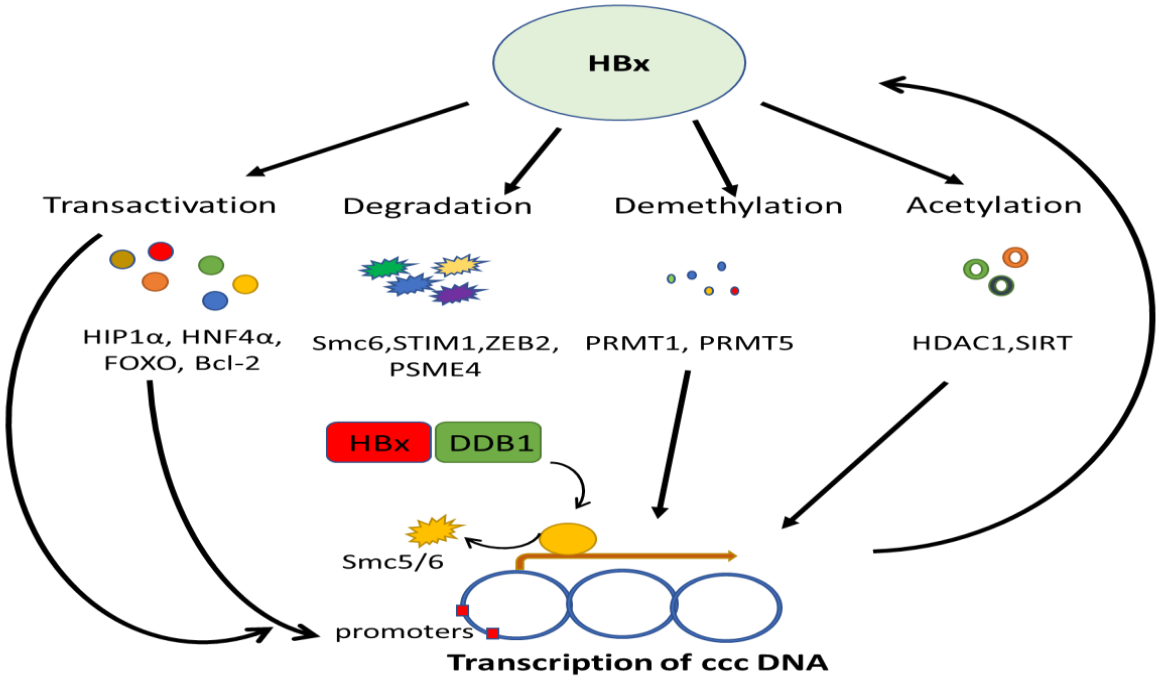
Fig 3 HBx mediated regulation of viral transcription



Use of Host transcription factors-

HBV genome has four promoters (PreS1, Pre S2, Core and X) and two enhancers (ENHI and ENHII). Enhancers have synergistic effect, ENHI has 3' end overlapping with X promoter and mainly responsible for the enhanced expression of HBx. It combines various liver enriched transcription factors to increase the expression of HBx[36]. HBx also transactivates these transcription factors, for instance transcriptional activation of Hypoxia induced factor 1a (HIF1α) and NFκB by HBx to promote viral transcription, which often leads to HBV persistence responses[37–39]. Cyclic adenosine monophosphate (cAMP) response element–binding protein (CREB), a transcription factor responsible of regulating expression of several genes in liver has also been shown to mediate transcription of HBV cccDNA in hepatoblastoma cells[11,40]. HBx favours CREB mediated transcription by recruiting CREB-binding protein (CBP)/P300 acetyltransferase to phosphorylated CREB and facilitates its binding to cccDNA. HBx also inhibits dephosphorylation of CREB by Protein phosphatase 1 (PP1) sustaining its activation for transcription of target genes[41] (Fig 3)

Combating proteasomal machinery to stabilises itself-

HBx modulates cellular machinery not only to support its expression but also to prevents its degradation. Certain protein-protein interactions stabilize HBx and promotes replication. Saeed and colleagues, showed that HBx interacts with multifunctional protein of gene PIN 4; parvulin 14 (Par14) and parvulin 17 (Par17). These proteins are involved in protein folding, chromatin modelling, ribosomal biogenesis and cell cycle progression. Their interaction stabilizes HBx, promotes its traslocation to nucleus and stimulates HBV replication, ccc DNA formation and virion secretion[42]. Proteins like Proteasomal activator 28 gamma (PA28γ), USP15, CUL4B stabilizes HBx by preventing its proteasomal degradation, thus limits the inhibition of HBV replication[43–45].Liu et al., reported in Huh-7 cells, E3 ligase HDM2 promotes binding of HBx to Ubiquitin Like Proteins (ULPs) i.e. SUMO and NEDD8 (SUMOylation and NEDDylation of HBx) which prevents its proteasomal degradation, and foster its chromatin localization[46].

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Modulating noncoding RNAs

HBx also manipulates long noncoding RNAs in favours to replication. An *in vitro* study by Salerno et al., showed that HBx enhances expression and accumulation of DLEU2, long noncoding RNA, and HBx-DLEU2 complex relieves the suppression of cccDNA transcription from PRC2. DLEU2 is also found to be upregulated in HCC[47]. Different studies also reported other lncRNAs as lncIHS[48], UCA1[49] regulated by HBx and accumulate in HCC. In addition, in vitro and in vivo study found, HBx also downregulates the suppressor of tumor lncRNA Dreh[50]

Preventing pgRNA

Recent studies showed m6A methylation of viral transcripts have dual regulatory function, lack of m6A methylation of viral transcripts stabilises and activates protein expression while HBx recruits m6A methylatransferase complex (METTL3/14) and promotes reverse transcription of pgRNA[51]. HBx represses transcription of microRNAs like miR-138, miR-224, miR-576, miR-596 that directly target the HBV pgRNA and would inhibit HBV replication[37,52].

Epigenetic regulation of cccDNA transcription

HBx also epigenetically modulates the expression of cccDNA through broadly two mechanisms- Firstly by DNA methylation, at CG islands of viral genome as well as at other sites but HBx ORF remains unmethylated even if other loci are methylated[53].Secondly by, Modifications of the proteins associated with viral DNA (i.e., Histone and HBc Viral protein). Histone acetylation and methylation of the lysine 4 of the histone H3 (H3K4me) are markers of active viral transcription, whereas histone hypoacetylation, H3K9me and H4R3me are transcription repression markers. Recruitment of acetyltransferases (HATs) like CREB-binding protein, p300 and p300/CBP-associated factor (PCAF) promotes transcription[54]. Whereas Histone deacetylases (HDAC1 and hSirt1) are associated with hypoacetylation and low viremia in chronic hepatitis B (CHB) patients[55,56]. Zhang and colleagues found positive correlation between HDAC, acetylated H3 activity and HBV DNA replication in CHB patients[57]. Histones H3 and H4 along with non-canonical variant H3.3 were found to be related to the regulation of transcription[58]. A study on hepatoblastoma cell line showed Sirtuin 2 (SIRT2), a class III histone deacetylase promotes hepatocarcinogenesis and reported to be up regulated by HBx[59].(Fig.3)

Acetylases and deacetylases also cooperate for other post translation modifications of histones like methylation[60]. Arginine methyltransferase (PRMT1, 5) introduces inhibitory marker H4R3me on cccDNA and inhibits transcription. HBx binds to PRMT1 and relieves inhibition of replication[61]. DNA methyltransferases (DNMTs) generally suppress transcription. Intriguingly, elevated DNMTs has been reported not only in cancerous liver tissue but also in cirrhotic and normal HBV infected liver tissue[62]. A study by Oropeza et al., on HBV transgenic mice showed that postnatal increased viral biosynthesis with progressive loss of DNA methylation was associated with decline of DNMT and Forkhead box protein A (FoxA) mediated recruitment of ten-eleven translocation (Tet) and methylcytosine dioxygenase[63]. HBx can modify transcription of DNMT1 and DNMT3 to epigenetically silence critical genes of adherence like E-cadherin, thus promoting invasiveness and metastasis[64,65].

Metabolic Reprogramming and cell survival

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Altered metabolome during the natural course of chronicity was previously reported which leads to reprogramming with progression towards HCC[66] Recently Lan et al., in mice model found HBV induced metabolic reprogramming at extrahepatic sites like heart, kidney and spleen[67]. Major pathways affected by HBx includes gluconeogenesis, ATP production, lactate production, fatty acid oxidation and AMPK/mTOR pathway[68].

HBX upregulated glucose-6- phosphate dehydrogenase (G6PD) and other enzymes to regulate gluconeogenesis[69]. HBx translocate to the mitochondria where it interferes with proton gradient formation, ATP generation and induces oxidative stress through mechanisms like altering the permeability of mitochondrial membranes, binding to voltage gated channel 3 and changes membrane potential, blocking of proton transfer across the membrane, binding to Cytochrome C oxidase III (COXIII), hinders electron transfer chain leads to excess Reactive Oxygen Species (ROS) generation and reduced ATP production[21]. Along with COX HBx also reduces the expression of Superoxide dismutase, a scavanger of ROS. The reduced ATP production leads to increased ROS generation and switching of cell towards glycolysis for ATP generation[70].

Moreover, HBx sustains cell survival under glucose deprived conditions through activating AMP-activated protein kinase (AMPK) pathway. Activation of AMPK, supports cell survival through maintaining intracellular ATP and NADPH pool. AMPK switches off anabolic processes (uses ATP) and switches on catabolic processes (generates ATP). HBx directly and indirectly activates AMPK[26]. HBx uses upstream kinases i.e., liver specific Liver Kinase B1 (LKB1) senses ATP imbalance, calcium/calmodulin-dependent protein kinase kinases (CaMKKs) senses cytoplasmic calcium level, Transforming growth factor-β-activated kinase 1 (TAK1) responses towards inflammatory cytokines[71,72]. The HBx modulated activation of AMPK affects downstream signallings to promote viral replication. mTOR and mTORC are two downstream targets of AMPK, both accelerates protein synthesis. Hepatoma cell linebased studies showed depleted glucose level activates AMPK/mTOR/ULK1/autophagy axis and promotes viral replication in contrast, higher glucose level activates Akt/mTOR/autophagy axis which supresses viral replication[68,73,74].

Bagga et al., demonstrated in rat hepatocytes that HBx activates mTORC1 which inhibits viral replication in contrast, it also activates AMPK which inhibits activation of mTORC1 thus promotes viral replication[68]. Like other vires, a possibility of simultaneous activation of AMPK and mTORC1 by HBx to balance viral replication and cell survival results in viral persistence. Wang et al., in his work on HBx expressing hepatoma cells and HCC liver specimens showed HBx utilises AMPK mediated activation of fatty acid oxidation to curb energy demand and cell survival under glucose depleted condition[75].

HBx favours cell survival also by inhibiting apoptosis. Li et al., in hepatoma cell line showed HBx induces activation of AMPK/manganese superoxide dismutase (MnSOD) axis to prevent apoptosis[76]. Lee and colleagues found an antiapoptotic mechanism of HBx by inhibition of Caspase 3 (CPP32) in chang liver cells (CHL) through activation of PI3K/Akt pathway which was also observed by Gottlob in rat fibroblasts and hepatoblastoma cells[77,78]. Moreover, increase in the concentration of anti-apoptotic protein SURVIVIN along with HBXIP is probably another contributor to cell survival[79].

Calcium Signalling

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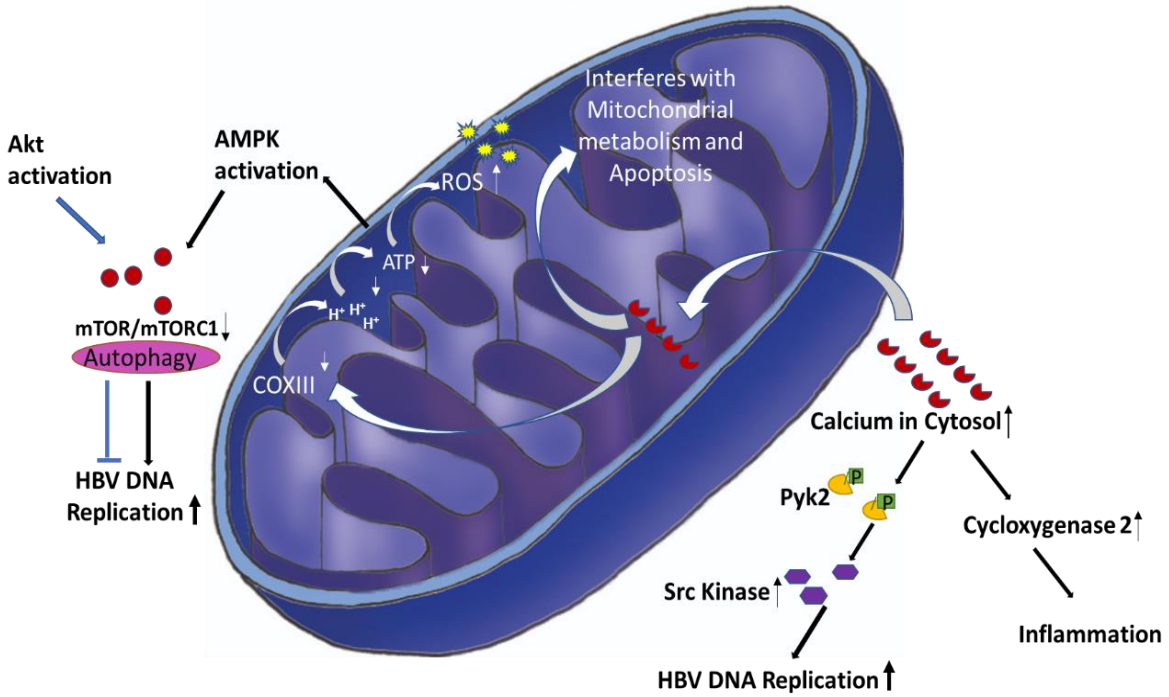
in vitro studies showed that both mitochondrial ROS and cytoplasmic calcium signaling is required for cyclooxygenase 2 expression (COX2) mediated inflammation[80]. HBx expressing hepatocytes exhibited higher cytosolic calcium levels. HBx modulates cytosolic calcium through store operated calcium channels (SOC) present on the plasma membrane. As the cytosolic calcium increases, mitochondria absorbs excess cytoplasmic calcium which interferes with mitochondrial metabolism and sensitizes cells towards apoptosis[81]. HBx also interferes with feedback mechanism of calcium uptake in Endoplasmic reticulum (ER)[82,83] HBx mediated increase in cytosolic calcium auto phosphorylates the PyK2, a cytoplasmic calcium activated kinase, which then stimulates the Src kinase and its downstream effectors like JNKs. Activation of Src promotes reverse transcription and replication of HBV. (Fig. 4) HBx protein also induces the calcium-stimulated transcription factor NFAT[22,84]. HBx uses calcium signalling to enhance its replication, it also alters cellular proliferation and apoptosis[85]. (Fig.4)

Fig 4 HBx mediated mechanisms of oxidative stress, mitochondrial dysfunction and autophagy



Autophagy

Autophagy is a process through which cells degrade aged proteins or organelles which can be used as nutrients by starving cells. According to one view, HBV triggers autophagy resulting in release of amino acids and nucleotide required for viral replication which is a precursor for to chronic liver disease and progression to HCC. HBX induces autophagy to support its replication via the PI3K/AKT/mTOR pathway, mitogen-activated protein kinase pathway (MAPK) and AMPK/mTOR pathway and ROS/JNK pathway[75,86,87]. Beclin1 was the first to identified autophagy stimulating gene (ASG) overexpressed in HBV induced HCC. Zhong and colleagues in their study on hepatoblastoma cell lines showed that the class III PI3K(VPS34)/beclin-1 (ROS/JNK) pathway is crucial for HBx mediated autophagy[87]. Nutritional starvation also induces autophagy through upregulating beclin1[88]. Deregulating autophagy in HCC may also be due to Nrf2/p62 pathway activated in response to oxidative stress in hepatocarcinoma cells[69,89]. According to other view, HBx induced autophagy restricts viral replication. Xie et al., demonstrated ROS/PRKAA/AMPK induced autophagy restricts

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viral replication in hepatoma cell lines[86]. However, the pretumor or antitumor role of autophagy is yet to be explored. Several studies suggest that autophagy promotes HBV persistence, however, autophagy was found to be downregulated in the liver of HCC patients warranty further investigation of relationship of HBx with autophagy and chronicity[90]. HBx induced ROS generation causes dysfunctionality of mitochondria which may lead to cell death. Hence, for sustained survival of infected cells, HBx induces mitochondrial fission and mitophagy[91]. (Fig. 4)

HBx in oncogenesis-

HBx has pleiotropic roles in the pathogenesis of HCC. HBx is a potent oncoprotein and can promote the transformation of hepatocytes and cancer progression. Mechanisms involved in viral persistence along with others also contributes in oncogenesis. Some in vivo studies found that HBx promotes "stemness" in liver by elevating cancer stem cells properties in hepatocytes[92,93]. In addition, higher HBx ORF integration in host genome is found in HCC patients. This integration leads to deletion in 3' end, which results in C-terminal truncated protein that promotes transformation[94]. HBx promotes transcription and replication of HBV leading to chronicity, which may then progress to HCC by several HBx mediated intermediary mechanisms like metabolic reprogramming and repression of apoptosis etc. contribute to the malignancy of hepatocytes.

HBx regulates Tumour Suppressor Genes

Although HBx is not a mutagen, it promotes mutations in the cell by compromising DNA damage pathways. It supports cell survival even after DNA damage by inhibiting P53-mediated apoptosis and tumour suppressor genes like phosphatase and Tensin homolog (PTEN), which gets down regulated in HCC[95]. PTEN inhibits Akt/mTOR pathways which is associated with a variety of cancers[96]. Tian et al., showed PTEN is epigenetically downregulated by miR-181a[52]. Knoll et al., proposed a model in which HBx inhibits apoptosis by interfering with nuclear localization and functioning of p53 and p73 in non-tumour cells and promotes transformation while showing opposite effect in tumour cells. They found p53 was elevated in HCC and induces degradation of HBx[97].

HBx induces genetic instability

Hepatoblastoma cells and biopsy specimens of HCC based study demonstrated that HBx increases expression of human Telomere reverse transcriptase (hTERT) leading cells towards immortalization and predisposition of HCC[98]. HBX causes genetic instability by promoting aneuploidy with aberrant chromosomal segregation, abnormal centrosome numbers, multipolar spindle through various mechanisms like activation of the Ras-MEK-mitogen-activated protein kinase signalling pathway[99], by sequestering nuclear transport receptor Crm1 in the cytoplasm,[100] by deregulating hepatitis B XIP (HBXIP) which regulates centrosome duplication and by interfering with S phase progression by deregulating DDB1, a cell cycle regulator[79]. PAX8 is a transcription factor found to be elevated in many cancers but its role is not clear. HBx also stabilizes PAX8 by inhibiting its ubiquitin mediated degradation and promotes hepatoblastoma[101]. HBx can also transcriptionally up-regulate the centrosomal P4.1-associated protein (CPAP) which has roles in cell division and apoptosis. Overexpressed CPAP directly interacted with HBx to promote HBx-mediated cell proliferation and migration[102].

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Antiresection activity of HBx has been reported by ren et al., in primary human hepatocytes during the natural course of chronicity with progression to HCC[103].

Emergence of C-Terminal Truncated HBx

The HBx protein in HCC is predominantly found in the C-terminal truncated HBx (trHBX) form, but is not present in non-tumour cells. Studies on tumour hepatoblastoma cells showed that trHBx has antiapoptotic roles and promotes transformation whereas wild type HBx (wtHBx) has role in tumour progression and metastasis. wtHBx was found to prevent degradation and transcriptional activation of HIFa, which is a transcription factor for angiogenesis and vascular endothelial growth factor (VEGF) expression. In addition, it disrupts adherence junction of hepatocytes and facilitates their metastasis. trHBX exhibits nuclear localization which restricts its role in apoptosis and promotes its contribution towards transformation[93,104].

The 3' truncated HBx protein introduces metabolic reprogramming in hepatocytes by downregulating Thioredoxin interacting protein (TXNIP) mRNA which is a redox regulator and glucose sensor, which thus negatively affects aerobic glycolysis[105]. This reprogramming also increases HCC stem cells by BCL2 Interacting Protein 3 Like (BNIP3L) dependent mitophagy, which upregulates glycolytic metabolism[106]. Contributing pathways to metastasis like wnt/ β-catenin,caveolin-1/LRP6/β-catenin/FRMD5 and PDK1-WNK1are also get activated by trHBX[107–109].

Mutations in HBx-

Mutations reported in HBx found to play a significant role in oncogenesis, were summarized in Table 2.

Table 2: Mutations of HBx and its significance in oncogenesis

Mutations	Significance	ref
C1485T	Activates Wnt and JNK signaling pathways, deactivates NF-κB signaling pathways, and contributes in ROS generation.	[110]
C1497T, A1630G, G1721A, A1762T/G1764A and A1774G	HBx integration in host genome	[111]
F30V	Decreases HBV replication efficiency and promotes cell survival thus contributes in viral persistence	[112]
K130M/V131I	activates AKT/FOXO1 pathway, alters arachidonic acid (AA) metabolism leads to inflammation	[113]
HBxΔ31	More prevalent in tumors tissue (70.6%) then nontumors (29.4%) and promotes metastasis	[104]
M130K and V131I (T-A) in genotype F	Associated with severe liver damage	[114]

HBx as Therapeutic Target Therapeutic targeting of transcripts or proteins of HBV may lead to reactivation. Therefore, therapeutics for silencing of cccDNA, entry inhibitors or

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immunomodulators are primarily investigated. Still there are continuing efforts of developing new drugs like nitazoxanide (NTZ), a thiazolide, which is an efficient inhibitor of HBx-DDB1 protein interaction and restores smc5 protein levels, which suppresses viral transcription in vitro[115]. Similarly, dicoumarol, an inhibitor of NAD(P)H quinone oxidoreductase 1 (NQO1), significantly reduces HBx expression in vitro and in mice by promoting its proteasomal degradation[116], and could be a therapeutic candidate. Various agents controlling intracellular calcium to prevent HBx dependent replication like BAPTA-MA, Rubiadin, Cyclosporine A and Cyclopiazonic Acid etc. has been proposed[117]. *in vitro* study showed Pevonedistat, an NEDD8 activating enzyme inhibitor found to restore smc5/6 function and inhibits transcription[118]. In addition, Immunotherapeutic cure for HBx yet to be uncovered[119–121].

Conclusion- HBx plays a multifactorial role in the natural course of HBV infection. Various *in vitro* studies show that HBx is critical for viral replication, transcription and persistence but its role may differ significantly *in vivo*. The current lack of an *in vivo* HBV model limits the uncovering of HBx mediated pathogenesis. At present, it remains difficult to target a specific function of HBx for therapeutics. Therefore, the pleiotropic role of HBx on different cellular pathways needs to be further investigated.

Figure legends

Fig 1. (A) Genomic organization of Hepatitis B virus (HBV), P- Polymerase ORF, C- Core ORF, S- Surface ORF and X- X ORF. (B) Different domains of HBx protein [11].

Fig 2 Subcellular locations and associated roles of HBx. Protein Kinas B (Akt), Mechanistic target of rapamycin (mTOR), Wingless and Int-1 (wnt), c-Jun N-terminal kinases (JNK), Nuclear Factor-Kappa B (NFκB), SURVIVIN or Baculoviral Inhibitor of apoptosis Repeat-Containing 5 (BIRC5), B cell lymphoma (Bcl-2), B cell lymphoma extra-large (Bcl-xl), Bcl-2 associated X protein (Bax), Phosphoinositide 3-kinase (PI3K), Adenosine Triphosphate (ATP) Reactive Oxygen Species (ROS), voltage-dependent anion channel (VDAC3), DNA Damage binding protein (DDB1), Transcription Factor IIIH (TFIIH), Cyclin Dependent Kinase 4 (CDK4), Centrosomal P4.1-associated protein (CPAP), Hepatitis B X-interacting protein (HBXIP).* viral promoters (preS2, preS1, Core, and X) and enhancer elements (ENHI and ENHII),** miR-138, miR-224, miR-576, miR-596

Fig 3 HBx mediated regulation of viral transcription, HBx activates transcription of cccDNA through transactivation of Hypoxia Induced Factor 1α (HIF1α), Hepatocyte Nuclear Factor 4α (HNF4α), Forkhead Box o (FOXO), B cell lymphoma-2 (Bcl-2) which induces different pathways. Degradation of restriction factors; structural maintenance of chromosomes protein 6 (SMC6) and stromal interaction molecule 1 (STIM1), zinc finger E-box binding homeobox 2 (ZEB2), and proteasome activator subunit 4 (PSME4). Histone modification by Histone Acetyltransferase (HATs), Histone Deacetylase (HDAC1), Inhibitory histone and DNA methyltransferase (DNMTs), protein arginine methyltransferase 1 (PRMT1), protein arginine methyltransferase 5 (PRMT5).

Fig 4 HBx mediated mechanisms of oxidative stress, mitochondrial dysfunction and autophagy. Cytochrome Oxidase III (COXIII), Reactive Oxygen Species (ROS), Protein Tyrosine Kinase (PyK2), AMP-activated protein kinase (AMPKF, Protein Kinas B (Akt), Mechanistic target of rapamycin (mTOR)

Acknowledgments. NA

Conflict of Interest statement: The authors have no conflicts of interest to declare.

Funding Sources: Department of Science and Technology, Govt. of India. Grant No. [DST/INSPIRE/04/2018/000019] and DST Core Grant [CRG/2020/003856].

Author Contributions: Conceptualization, Manuscript writing, graphical and tabular presentation: Garima Garg. Reviewing and editing: Dr. Shashank Purwar, Dr. Anirudh K. Singh, Dr. Ashish Kumar Vyas

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 doi:10.20944/preprints202207.0341.v1