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Hepatitis B virus-induced hepatocellular carcinoma: The role of the virus x protein

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Summary. – Hepatocellular carcinoma (HCC) is one of the most common malignant diseases and has the fourth highest mortality rate worldwide. Chronic hepatitis B virus (HBV) infection has been identified as a major risk factor in HCC. Currently available evidence support a critical role of hepatitis B virus x (HBx) gene and protein in the pathogenesis of HBV-induced HCC. HBx protein is a multifunctional regulator that modulates cellular signal transduction pathways, transcriptional regulations, cell cycle progress, DNA repair, apoptosis, and genetic stability by interacting with different host factors. This review describes the current state of knowledge about the biological roles of this protein in the development of HCC.

Keywords: hepatitis B x protein; hepatitis B virus; hepatocellular carcinoma; apoptosis; nuclear excision repair; epigenetic modifications

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Abbreviations: DNMTs = DNA methyltransferases; HBV = hepatitis B virus; HBx = hepatitis B x protein; HCC = hepatocellular carcinoma; MMP = matrix metalloproteinase; MTA = metastasis-associated protein; RAR-β2 = retinoic acid receptor β2; TSGs = tumor suppressor genes; XPB = xeroderma pigmentosum B

1. Introduction

With an annual incidence of more than 500,000 in the year 2000, HCC is considered to be one of the major malignant diseases in the world today (Motavaf and Alavian, 2012). Overall, 75–80% of global HCC cases are attributable to persistent viral infections with either HBV (50–55%) or hepatitis C virus (HCV) (25–30%) (Lu *et al*., 2006; Motavaf *et al*., 2012). Although this evidence indicates that HBV is a major etiologic factor in HCC (Alavian, 2011), the association of chronic HBV infection with HCC remains obscure. The hepatocarcinogenesis of HBV infection has been extensively analyzed, and multiple factors appear to play a role. Much of the evidence available supports the role of HBx gene and its expression product in the pathogenesis of HBV-related HCC. It is demonstrated that HBx gene is often included, and remains functionally active, in the HBV-DNA that is frequently integrated into cellular DNA during hepatocellular carcinogenesis. Several putative mechanisms by which HBx protein may contribute to the development of HCC have been investigated. While the specific mechanisms are still unknown, its criti-

cal role in hepatocarcinogenesis has been demonstrated by different *in vivo* and *in vitro* studies. Here, we attempt to summarize the current knowledge about carcinogenic pathways in HBV-induced HCC, with a focus on the role of HBx protein.

2. HBV-specific mechanisms involved in HCC development

Chronic HBV infection is the primary risk factor for the development of HCC (Mahboobi *et al.*, 2010). After decades of chronic hepatitis, about 30–40% of patients progress into liver cirrhosis, and of them, around 1–5% subsequently develop HCC (Liu and Kao, 2007).

Intensive research has focused on the role of HBV in hepatocarcinogenesis for the past decades. Current evidence points to two major HBV-specific mechanisms that contribute to the development of HCC. The first mechanism is the integration of HBV-DNA into host cellular DNA, which is observed in approximately 85% of HBV-associated tumors and is crucial during HBV chronic infection. Integration of HBV-DNA causes cis-effects, which by direct act in the genome disrupt or promote expression of cellular genes that are important in cell growth and differentiation. The second mechanism involves the expression of trans-activating factors encoded by HBV genome, which have the potential to influence intracellular signal transduction pathways and alter host gene expression indirectly. Several HBV trans-activating factors such as the HBx protein, the PreS2 activators, and a novel spliced transcript of HBV, referred to as the hepatitis B spliced protein (HBSP) have been found to be implicated in hepatocarcinogenesis (Su *et al.*, 2008). An increasing number of studies suggest that HBx protein may contribute to the development of HCC by interfering with different cellular activities.

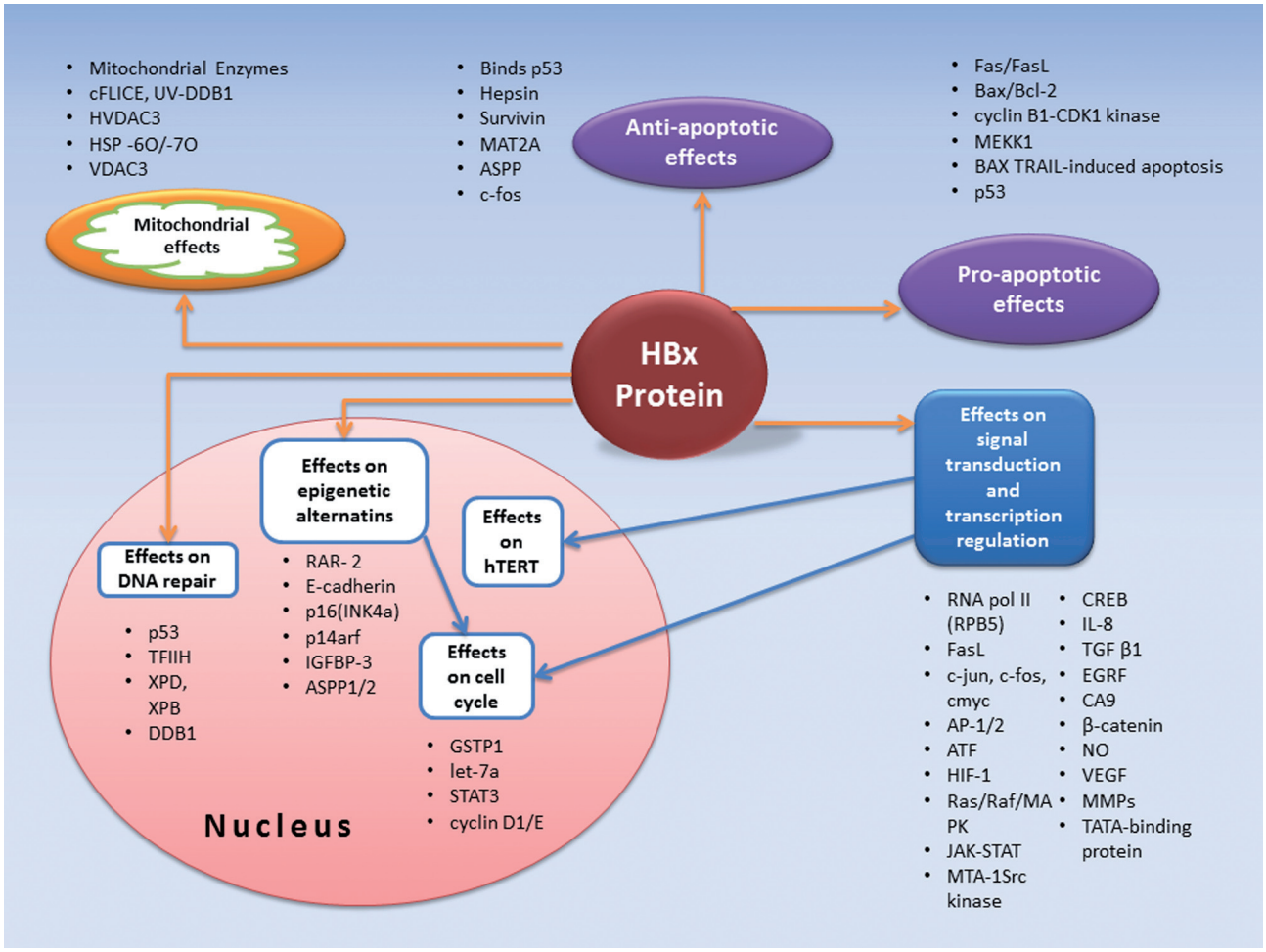

Fig. 1

Scheme of various targets of HBx protein in the cell

3. HBx gene and HBx protein

The HBV genome is a 3.2 kb circular, partially double-stranded DNA molecule with four overlapping open reading frames (ORFs), which code for the viral envelope (pre-S1/pre-S2/S), core proteins (pre-C/C), viral polymerase, and HBx protein. Among the proteins originating from the HBV genome, the HBx protein has drawn much attention for its pleiotropic functions as a viral trans-activator playing different roles in the development of HCC. The HBx gene that codes the HBx protein is phylogenetically highly conserved among all mammalian hepadnaviruses, strongly suggesting that it plays a critical role in viral life cycle. This gene comprises 452 nucleotides that encode HBx protein. The protein is 154 aa long with a molecular mass of 17 kDa. HBx protein operates as a multifunctional regulator that can modulate various cellular processes, including signal transduction cascades, transcriptional pathways, cell cycle progress, DNA repair, apoptosis, cell proliferation and genetic stability by interacting with different host factors. Currently available evidence supports multiple roles for the HBx protein in the pathogenesis of HBV-induced HCC (Fig. 1).

4. Different roles of HBx protein in hepatocarcinogenesis

4.1 Effects of HBx protein on epigenetic modifications

Epigenetic modifications refer to alterations in gene expression with no underlying changes in the genetic sequence itself. These alterations can switch genes on or off and determine which proteins are transcribed. Within cells, there are three systems that epigenetically silence the genes: DNA methylation, histone modifications, and RNA-associated silencing. Disrupting any of these three systems can cause abnormal activation or silencing of genes. Such disruptions have been associated with different disorders including cancer, syndromes involving chromosomal instabilities, and mental retardation. DNA methylation plays an important role for epigenetic gene regulation in development and disease. It is catalyzed by a family of DNA methyltransferases (DNMTs), including DNMT1, DNMT3a, and DNMT3b (Bestor *et al.*, 1988; Okano *et al.*, 1998). DNMT1 is responsible for the maintenance of DNA methylation after each round of replication. DNMT3a and DNMT3b are the main players involved in *de novo* methylation during early development. This family catalyzes the transfer of a methyl group to the C-5 position of cytosine in CpG dinucleotide, using S-adenosyl methionine as the methyl donor. The increased expression of DNMT1 and DNMT3a has been shown to be associated with increased cell proliferation, tumorigenesis, and tumor progression. Thus, elucidation of the mechanism for DNMT1 and DNMT3a up-regulation may provide an important clue for the understanding of epigenetic alterations in tumors.

Studies indicated that DNMT1 and DNMT3a expression is significantly higher in HCC when compared with non-neoplastic liver tissues (Saito *et al.*, 2001). HBx protein has been contributed to the up-regulation of DNMT1 and DNMT3a expression at both the mRNA and protein levels (Jung *et al.*, 2007). By virtue of this ability, HBx protein is suggested to be involved in epigenetic modifications during hepatocarcinogenesis.

DNA in CpG rich islands (genomic regions that contain a high frequency of GC dinucleotide) in the promoter regions of the TSGs is a common target for methylation by DNMT1. One of the important results of this methylation is inactivation of TSGs, leading to tumor development by eliminating negative regulatory proteins. The observation of methylation in 82% of at least one TSG promoter during hepatocarcinogenesis demonstrates the importance of this mechanism in HCC development (Yang *et al.*, 2003). Methylation of the p16INK4A promoter, a potent TSG is an important early event in carcinogenesis. It has been demonstrated that up-regulation of DNMT1 and DNMT3a by HBx protein is one of the leading causes of p16INK4A promoter methylation resulting in its inactivation (Zhu *et al.*, 2010). P16INK4A is one of the cyclin-dependent kinase inhibitors (CDKIs) and acts as a negative cell cycle regulator. Its functional inactivation is shown to be one of the most frequent alterations in HCC.

By inducing DNMT1 transcription, HBx protein also represses the expression of the E-cadherin gene (Lee *et al.*, 2005). E-cadherin plays a well-established role in cell-cell adhesion, ensuring that cells within tissues are bound together. The adhesive function of E-cadherin is dependent on its binding to the cytoplasmic α- and β-catenin proteins, two components of adherence junctions, which serve as a link between E-cadherin and the cytoskeleton. In a variety of cancers, including HCC, reduced expression of E-cadherin has been correlated with disruption of cell-cell contacts and enhanced cancer cell invasion. Binding of E-cadherin to β-catenin also suppresses cell growth and transformation. In addition, β-catenin plays an essential role in the Wnt signaling pathway. Activation of the Wnt signaling pathway leads to nuclear translocation of β-catenin where it functions as transcriptional regulator. Suppression of E-cadherin has important ramifications upon β-catenin function and consequently Wnt signaling pathway. Normally by binding to β-catenin, E-cadherin sequesters it at the membrane and keeps it away from the nucleus resulting in inhibition of β-catenin/Wnt signaling pathway. When E-cadherin expression is suppressed, β-catenin is released and translocates to the nucleus. Excessive accumulation of β-catenin in nucleus induces overactivation of β-catenin/Wnt signaling pathway, which results in enhanced cell growth and malignant cel-

lular transformation. This suggests that HBx protein may promote elevated β-catenin/Wnt signaling, in part, by down-regulation of E-cadherin.

By up-regulating DNMTs expression, HBx protein also induces methylation of the insulin-like growth factor binding protein 3 (IGFBP-3) promoter, which suppresses IGFBP-3 expression (Park *et al*., 2007). IGFBP-3 is an anti-proliferative, pro-apoptotic, and anti-tumor protein. Thus, its suppression can be associated with cancer progression.

The ankyrin-repeat-containing, SH3-domain-containing, and proline-rich-region-containing protein1 (ASPP1) and ASPP2 family of proteins regulate apoptosis through interaction with p53 and enhancing its binding to promoters of pro-apoptotic genes. The expression of ASPP2 and ASSP1 genes is frequently down-regulated by DNA methylation via increased DNMT expression in HBV-positive HCC, which may play important role in the development of HCC. (Samuels-Lev *et al*., 2001; Zhao *et al*., 2010).

HBx modulates the function of retinoic acid (RA) with up-regulation of retinoic acid receptor (RAR) β. RA regulates important biological processes such as cell proliferation, cell differentiation and cell death. It has been reported that RA acts as inhibitor of carcinogenesis by blocking the promotion of cell-transformation via induction of apoptosis, inhibition of further growth of abnormal cells and induction of abnormal cells to re-differentiate back to normal. Their signal can be transduced through two families of receptors including RAR (alpha, beta and gamma) and retinoid X receptor (RXR). HBx protein has been shown to induce promoter methylation of RAR-β2 via up-regulation of DNMT1 and DNMT3a resulting in down-regulation of its expression in human HCC cells. Because RAR-β2 is a major executor of the anti-tumor activity of retinoic acid, its down-regulation is likely to be an important event in HCC (Jung *et al*., 2010). Methylation also abolishes the potential of retinoic acid to down-regulate levels of G1-checkpoint regulators including p16, p21, and p27. This leads to activation of transcription factor E2F1, which plays a crucial role in the control of cell. As a result, in the presence of HBx protein, cells are less susceptible to RA-induced growth inhibition.

It has recently been demonstrated that HBx protein is able to deregulate cellular micro-RNA expression. miRNAs are small noncoding RNA molecules that inhibit gene expression by interacting with target mRNAs. miRNAs have been shown to exhibit regulatory functions in numerous cellular processes, including proliferation, differentiation, and apoptosis. They can function as either oncogenes or tumor suppressors through the suppression of protein-coding genes involved in cancer development and progression. Different studies provide evidence for the ability of HBx protein to deregulate cellular miRNAs expression (Wang *et al*., 2010; Xu *et al*., 2013). Although the function of many of these miRNAs remains largely unknown, but functional studies illustrated the role of a number of them in hepatocarcinogenesis. For instance miRNA let-7, a negative regulator of cellular proliferation is one of these miRNAs, which expression was shown to be down-regulated by HBx protein (Wang *et al*., 2010).

4.2 Effects of HBx protein on apoptosis

Maintenance of normal tissue homeostasis mainly depends on the balance between cell proliferation and programmed cell death (apoptosis). Apoptosis is responsible for the removal of infected, damaged, cancerous cells and cells that are simply in the wrong place during development. Apoptosis can be mediated through various extrinsic or intrinsic signal pathways, with activation of caspases and the possible involvement of mitochondria. The effect of HBx protein on apoptosis is one of the most fully documented mechanisms by which it contributes to the development of HCC. These effects are complex, because HBx protein has both anti-apoptotic and pro-apoptotic effects.

4.2.1 Anti-apoptotic effects of HBx protein

A number of ways in which HBx protein may induce anti-apoptotic effects have been described in different studies. HBx protein has been demonstrated to function as inhibitor of p53-mediated apoptosis. The main role of p53 is maintaining the integrity of the genome in response to stress signals including oxidative stress, metabolic stress, and DNA damage by inducing growth arrest, or apoptosis. In response to these stress signals, p53 is imported into the nucleus, where it induces cell cycle arrest, allowing for repair of damage or p53-dependent apoptosis (Ellis *et al*., 1991). It is suggested that HBx protein reduces the concentration of nuclear p53 by cytoplasmic sequestration of this apoptosis inducer (Wang *et al*., 1995). One consequence of this effect is the failure of p53 to up-regulate genes involved in apoptosis (Feitelson, 2006). p53 also plays a role in induction of apoptosis via binding to the xeroderma pigmentosum B (XPB) and XPD components of the transcriptional factor II H (TFIIH), which are involved in induction of apoptosis. Binding of HBx protein to the C-terminal of p53 and inhibiting its binding to XPB and XPD, may disrupt p53 induced apoptosis.

HBx protein can also exert anti-apoptotic functions independently of p53 via modulating activities of the serine protease hepsin and up-regulation of survivin (Zhang *et al*., 2005b). Survivin is a member of the apoptosis-inhibitor protein family, which is implicated in both the control of cell division and the inhibition of apoptosis. By inhibiting apoptosis and stimulating mitosis, survivin simplifies cancer cell survival and growth. Furthermore, survivin can form complexes with HBx-interacting protein (HBxIP), a cellular protein which was originally recognized for its association with HBx protein. Survivin-HBxIP complexes bind to procaspase 9, preventing its recruitment to apoptotic protease

activating factor 1 (Apaf1), and thereby suppressing initiated mitochondrial apoptosis pathway. HBx protein is able to interact with such complexes and suppress caspase activation in a surviving-dependent way. Findings show that HBx protein is a potent caspases-3 inhibitor as well (Gottlob *et al.*, 1998). HBx protein may inhibit apoptosis by activating methionine-adenosyltransferase II alpha (MAT2A) expression through NF-κB and cAMP-response-element-binding protein (CREB) signaling pathways, resulting in the decrease of S-adenosyl-methionine production (Liu *et al.*, 2011).

4.2.2 Pro-apoptotic effects of HBx protein

In addition to its anti-apoptotic effects, HBx protein may also induce apoptosis by different mechanisms such as deregulating Fas/FasL death receptor pathway, Bax/Bcl-2 family-induced mitochondrial pathway, activity of cFADD-like interleukin-1 beta-converting enzyme (cFLICE), expression of heat shock protein 60 (HSP60) and HSP70, expression of DNA damage-binding protein 1 (DDB1) and activity of NF-κB (Kim *et al.*, 2008; Kim and Seong, 2003; Kim *et al.*, 2005). It is demonstrated that the HBx protein sensitizes cells to apoptotic killing when expressed during viral replication. Cells that were resistant to apoptotic killing by high doses of TNFα were made sensitive to very low doses of TNFα by HBx protein (Su and Schneider, 1997). Production of TNFα is often part of the immune responses associated with liver damage during HBV infection. HBx protein sensitizes cells to TNFα killing by prolonged stimulation of n-myc (a proto-oncogene) and the stress-mediated mitogen-activated-protein kinase kinase 1 (MEKK1) pathway, but it has no effect on regulation of TNFα receptors.

Interaction of HBx protein with mitochondria, which is associated with the abnormal aggregation of mitochondrial structures in the cell (Takada *et al.*, 1999) is another possible mechanism underlying HBx-related apoptotic cell death. It is also indicated that by causing the loss of mitochondrial membrane potential, HBx protein induces mitochondria-dependent apoptosis (Shirakata and Koike, 2003). Furthermore, HBx protein can form a complex with mitochondrial proteins, HSP60 and HSP70 that exerts effects on mitochondria-dependent apoptosis (Zhang *et al.*, 2005a). HBx protein also may alter mitochondrial function by association with a member of the family of human mitochondrial voltage-dependent anion channel (HVDAC3) (Rahmani *et al.*, 2000). Such functional roles of HBx protein, resulting in mitochondrial dysfunction and structural changes, have implications for HBV-induced liver injury and the development of HCC.

4.3 Effects of HBx protein on DNA repair

DNA repair systems are fundamental to the maintenance of genomic integrity through the recognition and repair of damaged or altered DNA. The nucleotide excision repair (NER) pathway is responsible for the repair of a number of DNA lesions. DNA repair is achieved by the collaboration of the products of as many as 30 genes. The transcriptional factor II H, which includes XPB and XPD subunits, is involved in NER. It is thought that by interacting with XPB/XPD and inhibiting their translocation from sites of damaged DNA, p53 stabilizes the formation of repair complexes. Both *in vitro* and *in vivo* studies have shown that HBx protein can bind and disrupt the p53 binding to XPB and XPD. The binding domain of p53 for interaction with HBx protein has been mapped between aa 293–393 (Abdel-Hafiz, 2011), which also binds to XPB and XPD. Thus HBx protein may interfere with the NER pathway by covering this domain and blocking p53 from binding to XPB and XPD.

HBx protein in liver cells is also able to down-regulate XPB and XPD expression in specificity protein 1 (Sp1)-dependent manner. Sp1 is one of the transcription factor responsive elements that are present in both XPB and XPD promoters. Sp1 has been shown to be a specific target for HBx protein resulting in impairment of its DNA binding properties and down-regulation of XPB and XPD.

4.4 Effects of HBx protein on signal transduction and transcription regulation

The HBx protein is known as a dual-specificity transactivator exerting both in the cytoplasm, via modulating intracellular signal transduction cascades, and in the nucleus, via interfering directly with transcription factors. Nuclearly localized HBx protein has been shown to up-regulate the expression of a number of cellular and viral genes including the HBV enhancers, RPB5 subunit of RNA polymerase II, TATA-binding protein, and proto-oncogenes such as c-jun, c-fos, and c-myc. Furthermore, HBx protein has been shown to activate transcriptional factors such as NF-κB, activator protein 1 (AP-1), AP-2, and activating transcription factor (ATF)/ CREB (Balsano *et al.*, 1991; Chirillo *et al.*, 1996; Kim *et al.*, 2010; Twu *et al.*, 1993). HBx protein also activates promoters of cellular genes associated with cell proliferation, such as IL-8, TNF, TGF-β1, and early growth response factor (EGRF) (Andrisani and Barnabas, 1999). TGF-β1 signaling involves phosphorylation of Smad3 at serine residues 208 and 213 in the linker region (Smad3L) and serine residues 423 and 425 in the C-terminal region (Smal3C). Smad3C pathway inhibits growth of cells *in vivo*, but Smad3L-mediated signaling promotes tumor cell proliferation by up-regulating c-myc oncoprotein. It has been observed that HBx protein shifts hepatocytic TGF-β1 signaling from the tumor-suppressive pSmad3C pathway to the oncogenic pSmad3L pathway in early carcinogenesis (Murata *et al.*, 2009). HBx protein localized in the cytoplasm, has been shown to activate signal transduction pathways such as

Ras-Raf-mitogen-activated protein kinase (MAPK) pathway (Benn and Schneider, 1994), JAK/STAT pathway (Lee and Yun, 1998), the cell stress-induced MEKK1-p38-c-Jun N-terminal kinase (JNK) pathway (Benn *et al.*, 1996).

HBx protein may also contribute to tumorigenesis in HCC through modulation of the angiogenesis pathway. It is demonstrated that HBx protein up-regulates the transcription of vascular endothelial growth factor (VEGF), a potent angiogenic factor, by stabilizing or even up-regulating the hypoxia inducible factor 1 (HIF-1) (Lee *et al.*, 2000). HIF-1 is also the main transcriptional activator of carbonic anhydrase 9 (CA9). CA9 is involved in pH regulation, which helps tumor cells overcome intracellular acidosis and survive extended periods of time in hypoxic conditions. Thus, increased expression of CA9 results in the development of HCC by contributing to the survival of hepatocytes infected with HBV in the fibrotic liver parenchyma (Holotnakova *et al.*, 2010). Moreover, HBx protein plays an important role in facilitating invasion and metastasis by enhancing cellular migration through up-regulation of transcription of matrix metalloproteinase 2 (MMP-2), MMP-9, MMP-3, MMP14, MT1-MMP, and cyclooxygenase-2 (COX-2) (Lara-Pezzi *et al.*, 2002; Liu *et al.*, 2010b; Ou *et al.*, 2007). MMPs degrade extracellular matrix components, thereby contributing to physiological events (wound healing, and angiogenesis) and pathological conditions (cancer and arthritis).

HBx protein has been shown to induce the expression of metastasis-associated protein 1 (MTA1) co-regulator, via NF-κB signaling in hepatic cells. It is a component of the nuclear remodeling and histone deacetylase complex involved in carcinogenesis. MTA1 is a positive regulator of inducible nitric oxide synthase (iNOS) transcription as well (Bui-Nguyen *et al.*, 2010). The HBx/MTA1 complex stimulates the production of nitric oxide (NO°), by stimulation of iNOS expression in an NF-κB-dependent manner. NO° is a free radical, producing many reactive intermediates that account for NO°-mediated DNA damage or inhibition of DNA repair.

HBx protein has also been shown to up-regulate the expression and activity of human telomerase reverse transcriptase (hTERT) (Liu *et al.*, 2010a; Zou *et al.*, 2004), which is the one of the primary mechanisms underlying the proliferation, differentiation and tumorigenesis. hTERT is a catalytic subunit of the enzyme telomerase that maintains DNA telomere ends. Telomerase activity is associated with the number of times a cell can divide playing an important role in the immortality of cancerous cell lines.

5. Conclusion

HCC is one of the most prevalent and lethal cancers worldwide. Considerable efforts are currently aimed at unraveling the underlying molecular mechanisms of HCC in order to design better treatments, or even to prevent the disease. Chronic HBV infection is a major risk factor for HCC, but the pathogenesis of HBV-mediated HCC is not completely understood. Evidence suggests that the HBx protein plays a crucial role in hepatocarcinogenesis. Although further research is needed, these data give us insight into understanding the cell-transforming potential of HBx protein. Elucidating the role of HBx protein in hepatocarcinogenesis may ultimately lead to novel therapeutic strategies in the management of patients with HCC.

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