REVIEW

An insight into interaction of cell cycle regulating miRNAs and hepatitis B virus X protein

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The perspective of disease development can often be attributed for deregulation of gene expression. Hepatocellular carcinoma (HCC) has a high frequency of occurrence amongst all malignancies worldwide. Chronic Hepatitis B Virus (HBV) infection is one of the root causes of inception of this disease in humans. Information about microRNA mediated regulation of viral infections is just emerging. MicroRNAs (miRNAs) are about 19- 23 base pair long functional transcripts that govern gene expression by cleavage or translational repression of target mRNA. Oncogenic (or tumor suppressive) roles of miRNA in many aspects of cancer biology and wide spread differential expression of miRNAs in different stages of HBV associated Hepatocellular Carcinoma (HCC) compared with normal tissues are well documented. During HBV infection, perturbations of miRNA expression particularly cell cycle regulating miRNAs might have significant correlation with HCC development. Hepatitis B virus X protein (HBx) acts as a multifunctional protein that balances cell proliferation and programmed cell death by its anti-apoptotic and pro-apoptotic function. HBx interacts with various nuclear transcription factors and brings about transcriptional activation. It also modulates several cytoplasmic signaling pathways contributing to cell proliferation and survival. HBx is often referred as oncoprotein for its significant role during development of HCC. HBx-miRNA interaction in HBV related HCC has been at the core of much scientific interest over last few years. HBx is found to modulate several miRNAs that are associated with HBV related HCC. This review concentrates on the interaction of HBx protein with some of the miRNAs that are essentially associated with cell proliferation and found modulated in HCC. HBx-miRNA interactions provide a fresh understanding of the potential modus of viral protein action utilizing miRNA to control host functioning. Finally, the HBx-miRNA interaction could be utilized as a curative approach for management of HBV associated HCC.

Keywords: Hepatocellular carcinoma; HBV; HBx; miRNA

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Hepatocellular carcinoma (HCC) is a widely prevalent and potentially fatal malignancy. HCC accounts for 5.6% of all cancers [1]. The occurrence of HCC is the fifth highest amongst all cancers and is the third highest cause of deaths from cancer [2]. Death due to HCC is quite common in Asia and current estimate is that there are 450,000 people dying from this disease every year. The following conditions increase the likelihood of developing this disease: hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, intake of food adulterated with aflatoxin B1, excessive consumption of alcohol and nonalcoholic fatty liver disease [3]. Chronic Hepatitis B Virus (HBV) infection can lead to severe pathological conditions, the most severe ones being liver cirrhosis (LC) and HCC. Hepatitis B virus X protein (HBx) which is denoted by the X open reading frame (ORF) of the HBV genome is known to be involved in the viral

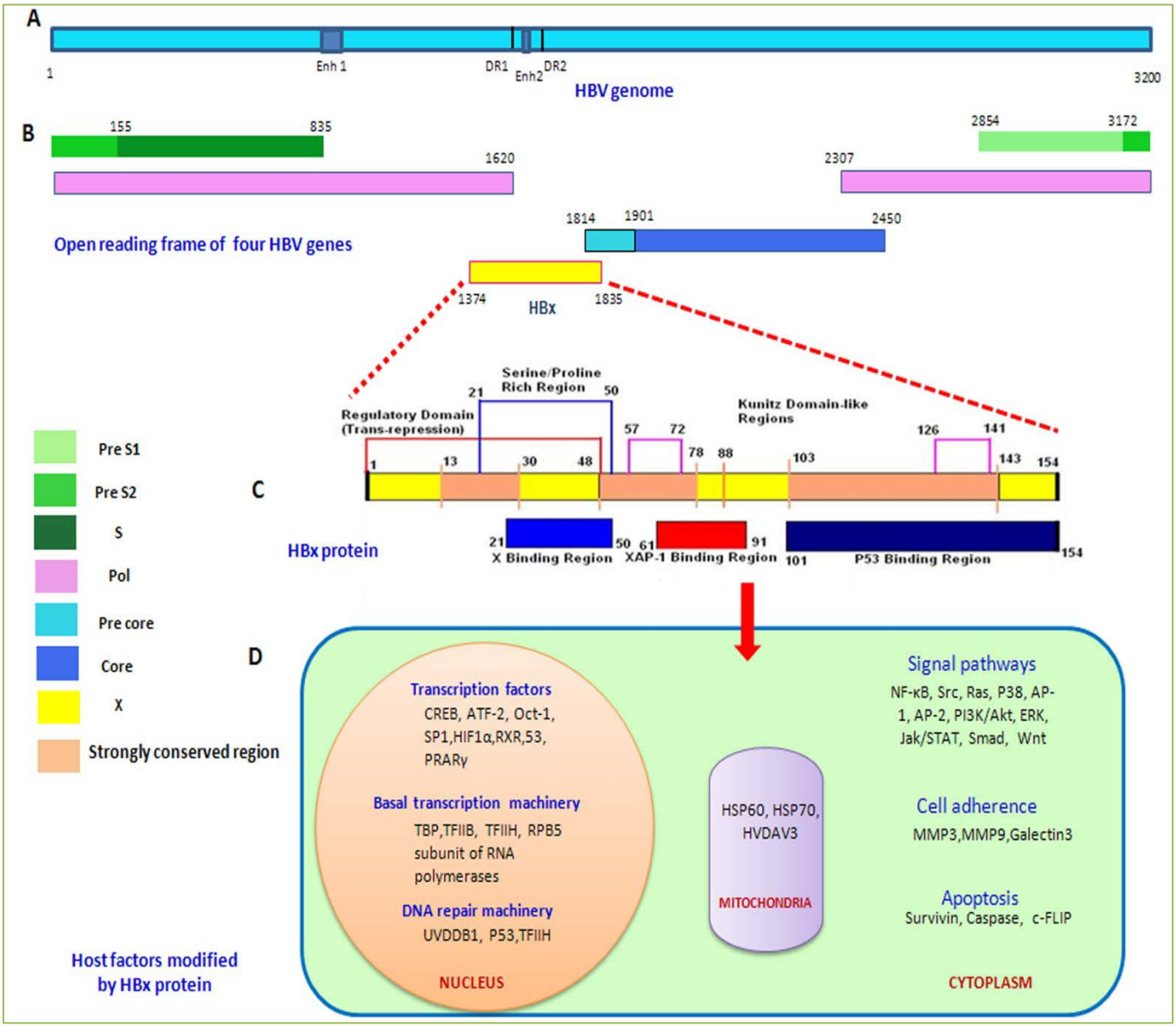

Figure 1. An overview of host factors modulated by HBx. **A.** Linear depiction of the HBV genome with regulatory elements such as DR1, DR2, Enh1, Enh2. Numbers indicate nucleotide position, using EcoRI-based numbering system. **B.** The overlapping open reading frame of Four HBV genes (C, S, Pol, and X) showed as coloured solid boxes. **C.** HBx protein. Numbers indicate amino acid position. **D.** Host factors modified by HBx protein.

infection, pathogenesis and replication though its precise function in viral replication is not yet established. HBV X protein has been in the spotlight in recent years because several reports documented its crucial role in the inception of HCC.

MicroRNAs (miRNAs) have been identified relatively recently and are a group of functional transcripts in eukaryotic cells [4]. miRNAs are 21 to 23-nucleotide long highly conserved RNA molecules that govern the stability or translational efficiency of target mRNAs [5]. Role of miRNA is being revealed in almost every field of research on malignancy, such as proliferation, tumorigenesis, invasion/metastasis, angiogenesis and apoptosis [6, 7]. Interaction between multiple intricate signal transduction pathways are regulated by these diminutive RNAs. Various studies have revealed significant differentiation in the expression of miRNA genes in malignant tissues in comparison to non-malignant tissues [8].

Fresh evidence is coming to light regarding the interrelationship between miRNAs and HBV X protein. Involvement of HBx in the modulation of several miRNAs that are found to associated with various types diseases especially cancer is well recognized. Several studies reported that miRNA-21, miRNA-222 and miRNA-145 are modulated

in hepatocellular carcinoma. HBx and miRNAs both are known to be critically involved in cell proliferation and neoplastic transformation. Though quite a few reports have revealed many aspects of HBx and miRNA interaction, present review focusses on involvement of HBx protein of HBV modulating the expression of miRNA-21, miRNA-222 and miRNA-145 in hepatic cancer cells thereby revealing miRNA mediated complex host viral interaction leading to cell proliferation that eventually culminate towards hepatic cancer.

Hepatitis B virus X protein and hepatocellular carcinoma

HBV genome contains four overlapping open reading frames among which the smallest X ORF denotes for hepatitis B virus X protein (HBx). HBx is 154 amino acid long 17 KDa protein that bears two functional domains with opposite functions - the N-terminal domain with negative regulatory / anti-apoptotic function and the C-terminal domain with pro-apoptotic / transactivation function (Figure 1). The HBx protein is found mainly in the cytoplasm and though in a small amount, the nucleus of hepatocytes. HBx is often referred as proto oncogenic factor as it modulates host gene expression by stimulating a number of cytoplasmic signaling pathways like NF-κB, Src, Ras, AP-1, AP-2, PI3K/Akt, Jak/STAT, Smad and Wnt [9, 10]. Multifunctional HBx protein acts as transcriptional transactivator by interacting with both nuclear transcription factors (e.g., CREB, ATF-2, Oct-1, TBP) and basal transcription factors [11] that causes increased cell proliferation and survival [12]. Other cellular processes that are modulated by HBx are reduction of DNA repair, activation of mitogen activated protein kinase (MAPK) pathways. HBx acts as pro apoptotic and anti apoptotic factor through induction of apoptosis by altering the TNFα and NF-κB signaling pathways [13, 14, 15] and inhibition of p53-mediated apoptosis by direct interface with p53 respectively [16]. Informations are present that HBx protein increases the expression of TERT and telomerase activity, thereby increasing the lifespan of hepatocytes thus converting to malignancies [17]. Several *in vivo* experiments were performed to examine the contribution of HBx in the development and progression of HCC. The first model of transgenic mouse contained HBx expression vector that included the HBV X coding sequence, the X gene promoter along with HBV enhancer I region and the polyadenylation signal. It was evident that tumor development in the liver tissue was directly associated with HBx expression [18]. Yu *et al* using an identical expression construct, later re-established this finding in another descent of transgenic mice [19]. A recent elaborate study by Ye H *et al* used hepatocyte-specific Kras (G12D) transgenic mouse model and found functional interaction between HBx and Kras (G12D) during the initiation and progression of HCC. They observed that the Akt, MAPK, p53 and TGF-β signaling pathways are more pronouncedly modulated in Kras (G12D) + HBx double transgenic mice than *in vitro* study of Kras (G12D)-driven HCCs [20]. Taken together, HBx may cause hepatocytes to become more susceptible to diverse carcinogenic signals. Such signals interact with the immune response of the host and along with the interplay between cellular proteins and HBx, may significantly increase the occurrence of hepatocyte transformation [21, 22, 23, 24] which advances towards HCC.

miRNA in hepatocellular carcinoma

The viewpoint of gene regulation has mainly emphasized on protein coding genes following the consecutive direction of DNA → RNA → protein. Recent advancement in technology, for example, parallel sequencing and high resolution microarray has enabled us to know that about 90% of the human genome transcribe into non-coding RNAs (ncRNAs) and less than 2% of the genome actually have the protein coding potential [25]. Depending upon size, ncRNAs are grouped into two categories- small ncRNAs (‹200bp) and long ncRNAs (lncRNAs). Among the small ncRNAs, miRNAs are the crucial regulator of gene expression and various cellular processes like proliferation, development, differentiation, apoptosis and tumorigenesis. Multiple studies indicate that miRNA expression is significantly affected in cancerous tissues as a result of numerous genomic and epigenetic modifications. The types of expression of miRNA are related to type of malignancy, its degree of advancement and other clinical factors. Analyses of miRNA expression have revealed that miRNAs can have both oncogenic and tumor-suppressive properties, depending upon target genes. Evidences have accumulated regarding modulated expression of miRNA in HCC. For example, miR-21[26, 27, 28, 29], let-7a [28] and miR-224 [29] are found elevated in HCC. Expression of miR-145 is found to be altered in HuH 7 cell line, human HCCs [30] and also in carcinomas from other tissues. Higher expression of miR-221 and miR-222 directly causes down regulation of the well-known tumor suppressor and cell cycle regulator gene p27 (Kip1) [31]. miR-1269 was found overexpressed in HCC cells and tissues. Ectopic expression of miR-1269 promoted proliferation, tumorigenicity and cell cycle progression of HCC cells, whereas inhibition of miR-1269 reduced the same in hepatoma cells [32]. miR-362-5p was found significantly elevated in HCCs and inhibition of miR-362-5p caused remarkable decrease in cell proliferation, migration, invasion and clonogenicity *in vitro* as also tumor growth and metastasis *in vivo*. This finding reveal oncogenic role of miR-362-5p that acts through CYLD (target mRNA) to activate the NF-κB signaling which contributes to progression of HCC [33]. miR-141 serves as a tumor suppressor in hepatocarcinoma cells through the impediment

of Hepatocyte Nuclear Factor-3β (HNF-3β) translation. HNF-3β is crucially involved during hepatocyte differentiation and controls expressions of liver-specific genes during HCC development [34]. All of these evidences reveal that multiple genomic and epigenetic modifications are responsible for the differential expression of microRNAs between malignant versus normal tissues.

HBx and miRNA

HBx is found to be associated with the modulation of several miRNAs that are found to be connected with molecular pathogenesis of various types diseases particularly cancer. There are a number of recent publications reporting on the interplay between miRNA and HBx protein. HBx is reported to have produced extensive modification of miRNAs in HepG2 cells. The first report of HBx induced modulation of miRNAs in HepG2 cells was revealed by Wang *et al* [35]. A number of studies emphasized about interaction between HBx protein and miR-15a/16. Along with a number of miRNAs, reduced expression of the miR-16 family was noted in HBx expressing hepatoma cells (HepG2, Huh7 and SK-HEP-1) by microarray technique and quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Moreover, down regulation of miR-15a/16 in HBx expressing HepG2 cells was found host c-Myc gene mediated [36]. An Interesting study by Wang *et al* [37] revealed that HBx transcript mediate the repression of miR-15a/miR-16-1 through the microRNA targeting sequence in the HBV RNA. Recently, Li *et al* [38] demonstrated that HBx causes up regulation of miR-146a expression through NF-κB mediated activation of miR-146a promoter which in turn reduced the expression of its direct target, complement factor H (CFH). The reduced CFH expression is found to be associated with liver inflammation. HBx induced down regulation of miR-101 elevated expression of DNA methyl transferase 3A which is responsible for aberrant DNA methylation of many tumor suppressor genes [39]. HBx, either viral transcript or protein is able to control the expression of specific microRNAs, thus expanding the biological and pathological properties of the oncogenic HBX gene in the HBV biology.

Hepatocellular tumor development is a multistep process and involves several structural, genetic and epigenetic alterations. Three main basic aspect of neoplastic growth are increased cell proliferation, decreased apoptosis and increased angiogenesis. We emphasized our discussion on HBx mediated modulation of few miRNAs that are involved in cell proliferation process through regulation of their target genes.

HBX down regulated miRNA -21 in malignant hepatocytes

miR-21 is often referred as oncomiRNA as its expression is frequently found increased in various types of cancers like hepatocellular carcinoma, cholangiocarcinoma [40], glioblastoma [41], pancreatic cancer [42], breast cancer [43], stomach cancer [42], thyroid cancer [44], colon cancer [45] and prostate cancer [42]. miR-21 stimulated cell proliferation, migration and invasion In HCC tissues and multiple HCC cell lines by suppressing the expression of widely acknowledged tumor-suppressor genes such as Phosphatase and Tensin homologue (PTEN) and Programmed Cell Death Protein-4 (PDCD4) [46], [47]. New reports are emerging about HBx miR-21 interaction and involvement of HBx protein in the regulation of hepatoma cell proliferation. A recent study by Damania *et al* [48] showed that HBx can induce cell proliferation through modulation of miR-21 expression, which consecutively prevents PDCD4 and PTEN expression and stimulates Akt. Li *et al* demonstrated that miR-21 expression is dependent upon HBx protein of HBV and over expression promotes development of HCC in hepatocytes through HBx-induced IL-6 pathway following STAT3 activation [49].

A study from our lab revealed that expression of miR-21 was found reduced in HBx transfected HepG2 cells [50]. HepG2 cells when transfected with full length HBV genome, exhibited the similar result.. HepG2.2.15 cell line is a modified HepG2 cell that contains a plasmid carrying four $5^{/}$-$3^{/}$ tandem copies of full length HBV genome. In HepG2.2.15 cells we observed a down regulation of miR-21 expression too. PTEN is one of the reported, valid targets of miR-21. PTEN is a phosphatase that dephosphorylate PIP_3 producing the bisphosphate product PIP_2. This dephosphorylation causes inhibition of the AKT signaling. Accordingly PTEN expression was found elevated in HBx transfected HepG2 cells and HBV replicating HepG2.2.15 cells. When 3 cellular system i.e. HBx transfected HepG2, HBV genome transfected HepG2 and HepG2.2.15 cells were treated with HBx-mRNA specific siRNA, we could found that miR-21 expression was restored at different time points compared to the control cell line. In addition, significant down regulation of miR-21 was observed in advanced liver disease patients or its subsets LC and HCC groups. We would like to mention that our study was performed using HBx of HBV genotype D. The results of our study are, therefore, reflective of the responses characteristic of the HBV genotype D.

miRNA-222 was down regulated by HBx protein in transiently transected HepG2 cells and in constitutively HBV producing HepG2.2.15 cells

miR-221 and miR-222 are found on the X-chromosome and positioned in tandem. Various studies have demonstrated

that miR-222 expressions are often modulated in HCC. Guanine nucleotide binding protein, alpha inhibiting activity polypeptide 3 (GNAI3) prevents migration and invasion of HCC cell. By software predictions and experimental screening, Zhang *et al* [51] very recently found that miR-222 could directly bind to GNAI3 mRNA and decrease GNAI3 protein expression in HCC cells. Their study collectively demonstrated that GNAI3 deters cell migration and invasion in HCC and is post-transcriptionally controlled by miR-222. The p27 (Kip1) gene is one of the targets of miR-222. p27 belongs to the Cip/Kip family of cyclin dependent kinase (CDK) inhibitors. It is a cell cycle regulatory protein that binds to CDK2 and cyclin E complexes and inhibit cell cycle progression at G1 [52]. Sage *et al* [53] have proved that miR - 221& miR - 222 can suppress p27 (Kip1) expression and in that manner are responsible for cancer development. miR-221, miR-222 can be attributed as a novel member of oncogene family as overexpression of these miRNAs can cause down regulation of the p27(Kip1) mRNA which is critically involved in cell cycle regulation and tumor suppression. Our study with miR-222 revealed that miR-222 is found down regulated in HBx and HBV transfected HepG2 cells and also in HepG2.2.15 cell. Consequently, increased p27 mRNA and protein expression was observed in these aforementioned cell lines. Interestingly, when HBx transfected HepG2 cells and HBV transfected HepG2 cells were treated with HBx specific siRNA, miR-222 expression was reestablished in those transfected HepG2 cells than their control cells. Likewise, miR-222 expression was restored in HBx silenced HepG2.2.15 cells. Our results demonstrated that HBx is responsible for the suppression of miR-222 expression, in that way increasing the expression of p27. Other group of researchers with their *in vitro* study and experiments on mouse primary hepatocytes previously proved that HBx can increase the expression of p27 which in turn can cause cell cycle arrest in G1-S phase [54, 55]. Moreover, our study on HBV infected advanced liver disease patients with different clinical outcomes such as LC and HCC subsets revealed that miR-222 expression was reduced compared to healthy individuals.

miRNA-145 is differentially modulated by HBx protein in malignant hepatocytes

miR-145 is widely acknowledged as a tumor suppressing miRNA, as there are ample evidence of under expression in many types of tumors [56]. Sachdeva *et al* performed elaborate work on miR-145. They have shown that miR-145 is a direct target of p53. p53 binds to a p53 response element (p53RE) present in the miR-145 promoter region and induces the transcription of miR-145. Notably, miR-145 has a negative relation with oncogene c-Myc as elevated level of miR-145 reduces c-Myc expression, while inactivation of endogenous miR-145 increases c-Myc expression. The inhibition of tumor cell growth, both *in vitro* and *in vivo* can be explained, at least partly, by miR-145 mediated post transcriptional regulation of c-Myc gene by p53. [57]. However, limited information is available regarding down regulation of miR-145 in tumors. Sachdeva *et al* have identified the putative CCAAT/enhancer binding protein beta (C/EBP-β) binding site in the miR-145 promoter region and C/EBP-β acts as a negative regulator for miR-145 expression by direct interaction with it. In the presence of wild-type p53 allele, C/EBP-β thwarts the ability of p53 to induce miR-145. In addition, C/EBP-β can suppress miR-145 expression in the mutant p53 background, signifying the p53 independent regulation of miR-145. They have further showed that, the antioxidant resveratrol possess the ability to suppress pAkt and phosphorylation of C/EBP-β and simultaneously, it can induce miR-145. Taken together, their experimental findings demonstrate a miR-145 regulatory system involving the Akt and C/EBP-β, which may explain the reduced miR-145 expression in cancer cells [58].

We found reduced expression of miR-145 when HepG2 cells were transfected with HBx plasmid and full length HBV genome. HBx protein is known to activate Ras-GTP complex and can establish Ras Raf MAP kinase signal cascade [59]. Previous study reported HBx can stimulate Ras-activating proteins of the Src family of tyrosine kinases, which passes signal to Ras [60]. Our result is in synchronization with aforesaid study as reduced expression of miR-145 by HBx caused increased expression of MAP3K (Raf 1) which is known to be effectively involved in cellular growth and proliferation by modulating downstream signaling molecules. Therefore, it appears from our study that HBx induces cell growth and proliferation by suppressing the expression of miR-145.

However, miR-145 was found over expressed in HepG2.2.15 cell line than control HepG2 cells. Its target MAP3K (Raf 1) protein expression was found decreased in HepG2.2.15 cells when compared with control HepG2 cell line. An early study by Jiang *et al* [61] showed that the expression of miR-145 is increased by more than 5 fold in HepG2.2.15 than in HepG2 cells. HepG2.2.15 cells carry HBV DNA as chromosomally integrated sequences and episomally as relaxed circular DNA as well as covalently closed form of HBV genome (CCC) [62]. Evidences confirm that chronic HBV induced HCC involves both integrated HBV DNA as well as CCC episomal HBV genome. Integration into the host chromosome exerts *cis* effects resulting in interruption of cellular genes that are crucial for cell growth and proliferation. Alternatively, *trans*-activating factors like HBx from episomal HBV DNA are responsible for modulation of various cell signaling pathways in the

cytoplasm leading to hepatocarcinogenesis [63]. It can be presumed that the HBV uses both the mechanisms in HBV related HCC leading to a difference in expression of miR-145 between transiently transfected HepG2 cells and HBV genome integrated HepG2.2.15 cell line. Still, future mechanistic study is required to explain the exact reason behind the modulation of miR-145 expression.

Conclusion and future direction

Overall, we summarized the involvement and mechanism of HBx mediated regulation of miRNAs that are associated with HBV related hepatocarcinogenesis. The process of cancer development is dependent on a number of factors, which include direct and indirect mechanisms which sometimes operate in a coordinated manner. Available experimental evidences indicate that there a number of molecules and pathways that influence the inception and advancement of HCC, however, the specific modus through which HBx causes carcinogenesis continues to be inconclusive. Along with cell-transforming potential, interaction with numerous transcription factors and diverse cell signalling pathways, modulating apoptosis and the immune system, interaction of HBx protein with regulatory RNAs like miRNAs is also a crucial event during development of hepatic cancer.

HBx-miRNA interactions provide new understanding of the likely action of viral protein on microRNA and the resultant impact on functioning of the host cell. Disease severity and development of hepatocarcinogenesis is often associated with viral genotype of HBV. The interdependence between cellular miRNA and HBx protein having its genesis in remaining genotypes of HBV remains a subject of further investigation. Furthermore, future studies need to be conducted to understand how the HBx-miRNA interactions can be used as a curative strategy to restrict the inception and growth of HBV related HCC and investigation of these interactions *in vivo* should be the focus of such studies.

Competing interests

The authors declare that they have no competing interests.

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