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MINI REVIEW

Interplay of Wnt β-catenin pathway and miRNAs in HBV pathogenesis leading to HCC

Muhammad Adeel Rana[a,b], **Bushra Ijaz**[b,*], **Muhammad Daud**[b], **Sommyya Tariq**[b], **Tariq Nadeem**[b], **Tayyab Husnain**[b]

[a] *Department of microbiology, Quaid-i-Azam University, Islamabad, Pakistan*
[b] *Centre of Excellence in Molecular Biology, University of the Punjab, Lahore Pakistan*

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Summary The prevalence of Hepatocellular carcinoma (HCC) has been identified world-wide. Plethora of factors including chronic infection of HBV/HCV has been characterized for the development of HCC. Although the onset and progression of HCC has been linked with awry of various signaling pathways but precise mechanism, still lies under the multitude layers of curiosity. HBV is spreading with insane speed throughout the world and has been found a main culprit in HCC development after regulating the several cellular pathways including Wnt/β-catenin, Raf/MAPK, Akt and affecting cell multiplication to genomic instability. The role of Wnt/FZD/β-catenin signaling pathway is centralized in liver functions and its anomalous activation leads to HCC development. β-catenin mainly plays a pivotal role in canonical pathway of the system. Altered mainly overexpression of β-catenin along its nuclear localization tunes the aberrations in liver functions and set disease progression. In the development of HCC, modulation of Wnt/FZD/β-catenin signaling pathway by HBV has been established. As HBV infects the cell it affects the miRNAs, the master regulators of cell. Previous studies showed the connection between HBV and cellular miRNAs. In the present review, we unveiled how HBV is deciphering the cellular miRNAs like miR-26a, miR-15a, miR-16-1, miR-148a, miR-132, miR-122, miR-34a, miR-21, miR-29a, miR-222 and miR-199a/b-3p to modulate the Wnt/FZD/β-catenin signaling pathway and develop HCC. These HBV mediated miRNAs may prove future therapeutic options to treat HBV-Wnt/FZD/β-catenin associated HCC.

Introduction

Hepatocellular carcinoma (HCC) is the 6th most common malignancy around the globe [1]. It lacks proper prognosis and irrevocable therapeutic options. In HCC development, the role of viral chronic infection such as HBV/HCV has been

* Corresponding author.
E-mail address: bijaz_009@yahoo.com (B. Ijaz).

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established [2]. A number of researchers have described the HCC progression under the umbrella of different dysregulated signaling pathways, which are important players in cellular proliferation to metastasis. But, the exact molecular pathogenesis of virus to induce HCC remains inscrutable. A setting of miRNAs in cell physiology as RNA-induced silencing has galvanized the new era of research [3]. miRNAs, due to their role as major regulators of cell physiology ranging from cell proliferation to apoptosis [4,5], hold centralized importance in the development of various diseases [6] like kidney, neurodegenerative diseases and in numerous cancers [7—10]. Many findings buttress a critical role of miRNAs in the modulation of the viral infection and viral related diseases. Thus, suggesting the deployment of miRNAs and their machinery by the viruses to make auspicious cellular environment for their survival and replication [11—14]. Moreover, HBx has been found to modulate signaling pathways by regulating their components including Akt, Wnt/β-catenin and p53, involved in HCC development [15—21]. Now researchers are in a race to explore the connection between miRNAs and HBV to induce HCC. Recently, a cryptic function of HBx as mediator of miRNAs expression has been unveiled. So, HBV modulates the expression of miRNAs that affect cellular pathways leading to HCC [22]. In our previous review paper, we explored the direct modulation of Wnt/β-catenin components by HBV to develop HCC [23]. In this review, we will uncover the possible cross-talk between HBV-induced miRNAs expression and Wnt/β-catenin pathway to cause HCC.

miRNAs at a glance

Mechanism of action

Micro RNAs consist of non-expressible class of RNAs with 20—22 nucleotides in length. The initiation of microRNAs biogenesis starts with the transcription of primary miRNA by RNA polymerase-II. Pri-miRNA transcribed by RNA polymerase, is the largest part and can hold massive nucleotides with stem loop structures [24]. After the synthesis of pri-miRNA, its cleavage is done to release pre-miRNA, a small hairpin structure [25]. The processing from pri-miRNA to pre-miRNA occurs within the nucleus by Drosha, an RNAse-III type protein [26]. To carry out the process of maturity, a cofactor named as the DiGeorge syndrome critical region gene 8 (DGCR8) is also required along with Drosha. In fact, a large complex known as microprocessor comes in existence by the combination of Drosha and DGCR8. Microprocessor creates a ∼70 nucleotide imperfect stem—loop structure of pre-miRNA by the cleavage of pri-miRNAs. This cleavage by microprocessor takes place without regarding the sequence of pri-miRNAs [26,27]. It is noteworthy that microprocessor is unable to process mirtron (intronic pri-miRNAs). The classic splicing machinery requires processing these precursors instead of Drosha cleavage [28]. After completion of pri-miRNAs processing into pre-miRNAs in the nucleus, these fledgling- miRNAs are transported to the cytoplasm by exportin-5, a RAN-GTP dependent nucleo-cytoplasmic cargo transporter [29,30]. In the cytoplasm, further processing takes place by RNA-III polymerase type Dicer protein. Dicer cleaves them into 21—23 nucleotides of RNA duplex comprising of passenger and mature miRNA strands along with 2 nucleotides overhang at 3' site [31,32]. A complementary interaction occurs between seed region of mature miRNA and 3'UTR of the target mRNA. Consequently, an RNA-induced silencing complex (RISCs) is formed, which results in cleavage of target mRNA or its translational inhibition [31—35]. Current studies have explored the dual nature of miRNAs including post-transcriptional regulation of gene expression as well as regulation of epigenetic machinery [36—38].

miRNAs in action

These non-coding RNAs are highly conserved from evolutionary point of view. miRNA mediated regulation of different genes involves complementary interaction with their mRNAs. They play crucial role in basic cellular processes from cell multiplication to cell apoptosis [39]. The role of miRNAs was unveiled with the discovery of lin-4 gene encoded miRNA in nematode *Caenorhabditis elegans* in 1993. Lin-4 miRNA has been engaged in down regulating the expression of lin-14 mRNA during developmental stage of *C. elegans* [40]. A twist came in the story, when the 2nd miRNA Let7 with 21 nucleotides was discovered in 2000 within the same organism. The researchers reported the role of let-7 miRNA in the transition of L4 to adult stage of larval development [41]. The fact of the conserved sequence of let-7 among the different animals from flies to human [42] has drastically empowered the study of miRNAs in further livings [43]. Moreover, 12 human miRNAs joined the let-7 family on the basis of similarity with let-7 in 5' ends (seed regions) [44]. Conserved character of these small RNAs elucidated their roles in the regulation of developmental processes than previously alleged. Bioinformatics analysis of target sites revealed that miRNAs might be involved in the regulation of 1—3 of the human transcriptomes [45]. On miR-Data Base (mirbase.org), over 1920 and 1100 mature miRNAs have been reported in human and mice respectively [46]. The list is burgeoning in a quick response of time. As miRNAs are the classical regulators of signaling pathways, they can be easily tractable by HBV, and taming the cellular pathways to cause HCC.

miRNAs as Mediator of HBV Replication

HBV is a member of family *Hepadnaviridae*, comprises of enveloped circular DNA about 3.2 kb in size [47]. HBV loves the hepatocytes and gain entry into cells via the cellular receptor known as Sodium Taurocholate Co-transporting Polypeptide (NTCP) [48]. The four over lapping open (ORF) reading frames Tetra (S, C, P and X) structure the HBV genome [49,50]. The ORF-S translates into HBsAg, the viral envelop surface proteins. Based on structure and function, the ORF-S is segmented into three regions pre S1-S2 and S. Similarly, the ORF-C can be divided into two regions known as pre-core and core. Depending on the initiation of translation site either from pre-core or core regions, the ORF-C encodes HBeAg (Hepatitis-B e Antigen) or HBcAg (viral nucleo-capsid) proteins respectively. The C-terminus of core protein with the activity of RNA binding holds a cluster of highly basic amino acids. Moreover, core protein

has a self-assembling intrinsic property to organize a capsid like structure [51]. Endoplasmic reticulum mediates the formation of the secreted HBeAg by processing the translational product, directed by pre-core encoded signal peptide. Although the immune tolerance property of HBeAg has been established to augment persistent infection, but most of its functions remain to be shrouded [52]. The ORF-P encodes polymerase, a protein of 800 amino acids. Based on its function it is divided into 3 domains known as ribonuclease H, reverse transcriptase (RT) and terminal protein domain. The former domain (ribonuclease H) facilitates viral replication by degrading the pre-genomic RNA. The second domain (RT) functions as catalyzing the synthesis of genome. And the last domain (terminal protein domain) operates the initiation as well as encapsidation of (-) strand synthesis. The ORF-X translates into HBxAg protein of 17 kDa in size with peculiar sequence of amino acids. Viral subtypes are found to be conserved in HBxAg sequence. Additionally, in hepatocytes it is mainly localized in cytoplasm instead of nucleus. Although there is a lack of erudite knowledge about the role of HBxAg in the life cycle of virus but its biological role has been described in promoting the viral infection as well as replication. Furthermore, in nucleus the role of HBx has been observed in modulating a number of signaling pathways, after interfering with transcription factors to activate them. HCC development may trigger by HBx mediated modulation of Jak-Stat, Ras, protein Kinase C, NFkB, Src, phosphatiylinositol-3 kinase FAK, RAF as well as Wnt pathways [53—60]. HBV not only modulate the host signaling pathways but it also alters the miRNAs signature. A group of researchers applied the loss-of function methodology to screen out the effect of HBV on host miRNAs during its replication. They found the increased expression of HBsAg and HBV replication as a consequence of miR-199a-3p and miR-210 inhibition. Further, they validated their results by using GFP reporter assay and computational analysis, and revealed the HBV-S and pre-S1 regions as binding sites for miR-199a-3p and miR-210 respectively [61]. Another group found the translational interference of miR-125a-5p with HBV-S gene [62]. Wu et al [63], used more sophisticated target prediction softwares, and predicted the S or polymerase gene as target site for miR-511, miR-433, miR-196b and let-7, while X gene for miR-205 and preC gene for miR-345. In vitro replication of HBV was found to be suppressed by miR-224, miR-92a-1 (2 members of miRNAs, miR-20a and miR15a/miR-16-1), possibly they can directly target the HBV genes [64—66]. Additionally, some miRNAs modulate the host proteins to suppress as well as to support HBV replication. For-instance, miR-141 negatively regulates the HBV positive transcription factor, peroxisome proliferator activated receptor-α, and suppressed HBV replication [67]. Similarly, miR-122 has been involved in inhibition of HBV replication via two mechanisms. One study reported in vitro binding of miR-122 in regions of core (mRNA 3' UTR) and polymerase gene. The other study explored the role of miRNA-122 in down-regulation of Cyclin G1, and augmented the p53-mediated suppression of HBV [68,69]. Moreover, in human hepatic cells, miR-155 suppressed the cytokine signaling-1(SOCS1) suppressors, and consequently promoted the JAK/STAT pathway, resulted in increased antiviral immunity and mild inhibition of HBV infection [70]. Furthermore, miRNAs can also modulate the host proteins to support HBV infection. Like, miR-1 augmented the expression of farnesoid X receptor α, and increased HBV-core promoter activity, resulted in enhanced HBV replication [71]. Similarly, miRs-372/373 targeted the nuclear factor I/B, thus the production of HBV core related DNA and HBV proteins was stimulated in HepG2 cells [72]. In same series of cells, the role of miR-501 was observed in targeting the inhibitor of HBV replication namely HBXIP, and promoted HBV replication [73].

Wnt signaling

In a climax of science and research, researchers and scientists have put a lot of effort in order to explore the organism's development related basic mechanisms and pathways. Aberrations in these fundamental mechanisms may foment the different pathological conditions including cancer. Prevention and treatment of these pathological conditions is possible by understanding these mechanisms, how do they regulate, and what are the factors behind them. Much effort has been done on understanding a central pathway called ''Wnt Pathway'', it is the essence of organism's development. Wnt signaling pathway is evolutionary conserved and present at the nexus of others crucial pathways, which participate in embryonic development by regulating the organogenesis, cell migration, insulin sensitivity, cell polarity, neural patterning, determining cell fate, tooth morphogenesis and bone development [74—78]. Similarly, pathological conditions in a severe form including cancer in human can arise due to any deviation from normal cascade. Wnt signaling is regulated under the tight regulation of its components ranging from ligands/Wnt proteins to receptors and agonists to its antagonists [79,80]. In human 19 Wnt ligands and ten different FZD receptors have been identified. Wnt-FZD interaction triggers the Wnt pathway. A binding of a single Wnt protein with multiple Frizzled proteins can occur and vice versa so, this interaction appears promiscuous [81]. Wnt- FZD binding attracts another co-receptor of the LRP family, which is a single-pass transmembrane molecule. This molecule is known with different names, like in Drosophila it's tagged as Arrow [82], while LRP5/6 in vertebrates [83,84]. Wnt signaling pathway can be categorized into 3 sub types depending upon their regulatory players. Among these, the first one is Wnt-canonical pathway (Wnt/βcatenin), the 2nd Wnt-calcium pathway and the last is planner cell polarity stream. The former is β-catenin (CTNNB1 gene encoded protein) dependent for activation while the other two are non-canonical pathways i.e. β-catenin independent. β-catenin dependent and independent pathways differ in their receptors as well as in target genes. Ror2/Ryk as co-receptors place the Lrp5/6 in non-canonical signaling cascade. On the other hand, coupling of FZD-ligand in cell polarity pathway initiates the MAP kinase activation, which ends with the accomplishment of gene expression associated with AP1 [85,86]. Out of these pathways, Wnt/β-catenin pathway holds prime importance in regulating the number of processes. In canonical signaling (Wnt β-catenin) binding occurs among Wnt ligands, FZD receptors and their co-receptors Lrp5/6. As a result, a ternary complex (Wnt/FZD/Lrp) is formed. Dishevelled (Dvl/Dsh), a scaffolding plasma phosphoprotein is recruited at the plasma protein by this ternary

complex. Consequently, cytosolic β-catenin skip proteasomal degradation, since axin-bounded-Gsk3β is trapped by plasma membrane bounded Dvl/Dsh. Stabilized β-catenin is translocated into nucleus in order to bind with transcription factors such as TCF/LEF. After binding with transcription factors, a transcriptionally active complex is formed with BCL9, pygopus (Pygo) and CBP (CREB-Binding Protein) [87]. Moreover, description of four TCF genes in mammals makes the stream of Wnt canonical more complex [88].

Wnt regulation through miRNAs

E-cadherin/β-catenin complex is an important key player in maintaining the integrity of epithelial cell adhesion by tight regulation of Wnt signaling pathway. Dissociation of this complex leads to up-regulation of β-catenin, as it happens in case of epithelial to mesenchymal transition (EMT), fibrosis and tumorigenesis [89]. miR-200a is found to accelerate the E-cadherin availability for β-catenin binding, targeting ZEB1/ TCF8 and ZEB2/ SIP1 which are known as E-cadherin suppressors and embolden the formation of cell-cell adhesion complex [90]. Down-regulation of miR200a relates to up-regulated cytoplasmic and nuclear accumulation of β-catenin, and ultimately initiates the EMT induction [91,92]. Additionally, Sydam et al., have proposed critical role of miR-200a in meningioma disease. They demonstrated that miRNA-200a down-regulation and up-regulation of β-catenin has direct correlation in most of human meningioma samples. Down-regulation of miRNA-200a results in increased β-catenin dependent transcription, which consequently leads to cell proliferation. Moreover, miRNA-200a directly targets the mRNA of β-catenin and emerges as tumor repressor miRNA in meningioma via exerting effects on both Wnt and E-cadherin signaling pathways. On the other hand, β-catenin expression was not affected by miR-200b and c, members of miR-200 family [93]. miR-21 was found to repress the Wnt1 expression by targeting 3' UTR of Wnt1. miR-21 is important in human monocyte-derived dendritic cell differentiation (MDDC). MDDC differentiation was inhibited by applying miRNA-21 inhibitors or external addition of Wnt1, revealing the leading role of miR-21 [94]. Wnt transcription factor Lef1 is the target of miR-203 in zebrafish regeneration process. miR-203 represses Lef1 via recognizing its two 3'UTR miRNA recognition elements (MREs) and blocks regeneration. To confirm this interaction, Lef1 mRNAs lacking 3′ UTR recognition sites were introduced in the presence of miR-203, and observed no effects of miR-203. This suggests that regulation of Lef1 by miR-203 plays a vital role in the production of regeneration related effects. Moreover, miR-203 mediated suppression of Lef1 results in blockage of fin regeneration while inhibition of miR-203 promotes high expression of Lef1 and fin overgrowth [95]. miR-8 family members have evolutionary conserved role in wingless (Wg) signaling regulation and enhance adipogenesis by inhibiting this pathway. Possibly, miR-8 family members regulate Wnt pathway by three potential mechanisms. One of them is to direct target the wntless, a gene essential for Wg secretion, while other mechanism involves downstream repression of TCF protein levels by miR-8. Finally, miR-8 regulates Wnt pathway as positive regulator through the CG32767 [96]. miR-135a and b target 3' UTR of APC, and ensures the stabilization of β-catenin. Up-regulated miR-135a and miR-135b expression is correlated with low APC miRNA levels in the advancement of colorectal cancer. It is a conserved interaction and does not depend on APC mutational status in the cancers [97]. Additionally, miR-315 activates Wnt pathway by direct targeting the Axin and Notum which negatively regulates Wg signaling [98]. miR-199a is found to be down-regulated frequently in HCC cells and its down-regulation indicates poor prognosis and oncogenicity. However, upon restoration, miR-199a targets FZD7 along with its down-stream genes like c-Myc, β-catenin and Cyclin D1, and inhibits HCC cells proliferation [99]. HCC cell lines which were resistant to chemotherapy drugs showed down-regulation of miR-27a, and its restoration can make the cells sensitive to drugs via down-regulating/targeting the FZD7/β-catenin pathway [100]. While, miR-126-3p and miR-202 have shown the suppression in HCC cell proliferation and metastasis to angiogenesis through targeting the LRP6 [101,102]. Moreover, miR-432 and miR-610 inhibit canonical pathway by targeting the LRP6, and their down-regulation was associated with enhanced HCC cells proliferation [103,104]. Additionally, SNP of miR-1269a not only lose its anti-cancerous property but also failed to target LRP6, and promoted the setting and progression of HCC cells [105]. BMI1 is a poly-comb protein and has been found to repress the DKK family and activate the Wnt pathway. The over expression of this activator is reported in HCC. miR-200b was found to target BMI1, and dramatically repressed the characters of malignancy including cell proliferation and invasion in HCC cells [106]. Moreover, miR-218 played a role as a tumor suppressor in HCC pathology by inhibiting the expression of BMI1 [107,108]. On the other hand, miRNAs can act as oncogenic and activating the Wnt/β-catenin pathway through targeting pathway antagonists. For instance, miR-522 positively regulates the proliferation of HCC cells regulating the Wnt/β-catenin signaling antagonists DKK1 and SFRP2 [109]. Thus, miRNAs can activate or de-activate the Wnt signaling pathway, and determine the fate of the disease including HCC.

HBV, Wnt/β-catenin pathway and miRNAs

As, miRNAs are important in numerous biological processes/mechanisms, it is not a matter of surprise that their dys-regulation is associated with multiple human disorders including chronic HBV (CHBV) infection and HCC [110]. Cellular miRNAs regulate the expression of HBV genes either positively or in a negative way. Some cellular miRNAs regulate HBV gene expression and replication via targeting the HBV required important transcription factors or directly target the HBV [110,111]. Researchers have described the ability of HBV to alter the expression patterns of cellular miRNAs [112,113]. According to immune response characteristics, acquired CHBV infection holds four stages known as immune tolerance, immune control, activation and immune reaction [114]. Xing et al., [115] documented the role of miR-548 in various pathways including Wnt, MAPK and TGF-β signaling pathway. They demonstrated that up-regulation of miR-548ah-p can play a leading role in altering the immune status from tolerance to activation in chronic-HBV infec-

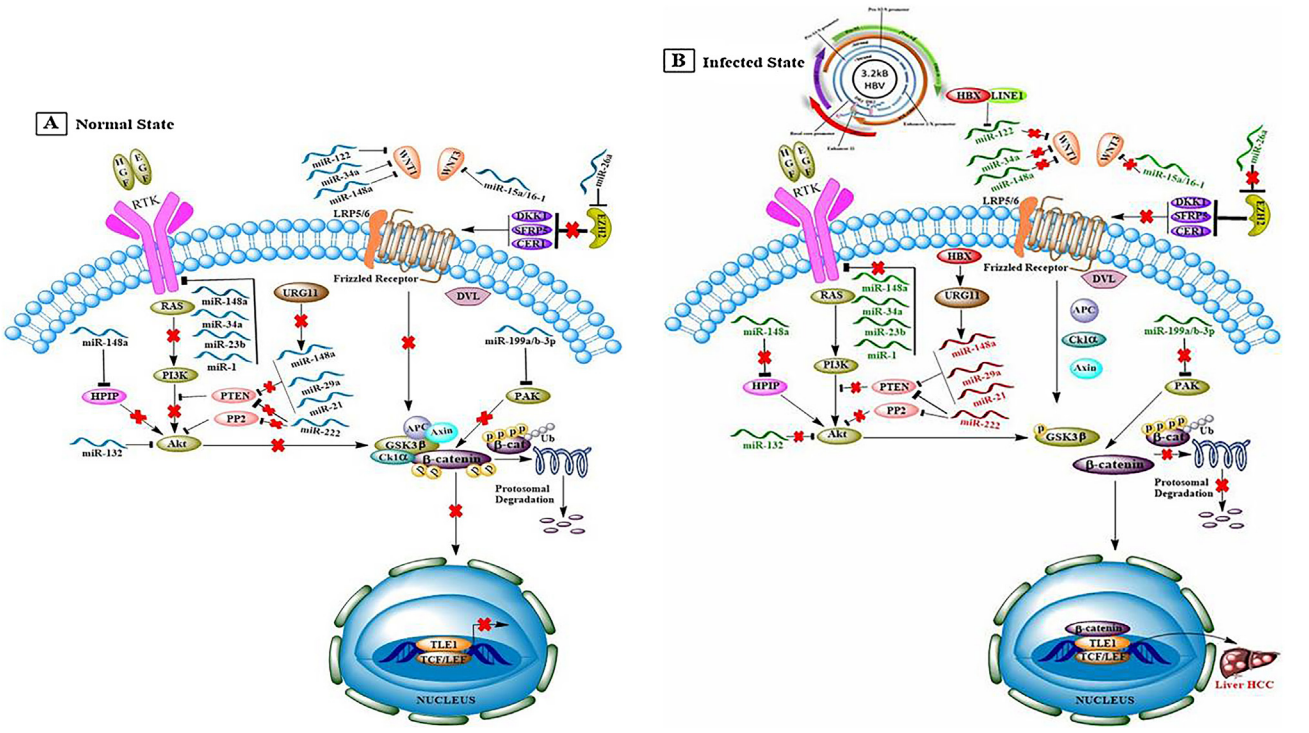

Figure 1 A. (Normal State): when Wnt/β-catenin pathway is not activated, a destruction complex is formed by GSK3β after the recruitment of APC, Axin and Ck1α. Then, destruction complex subsequently phosphorylates the β-catenin, and tag it for ubiquitinated mediated proteosomal degradation. Thus, β-catenin is not trans-located into nucleus to activate TLE1/TCF/LEF complex for cell proliferation. miR-148a, miR-34a, miR-23b and miR-1 can negatively regulate the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, an RTK-related pathway, by targeting the c-Met, which is a RTK (Receptor Tyrosine Kinase) for hepatocyte growth factor (HGF) and epidermal growth factor (EGF), as a result they may prevent the Wnt/β-catenin pathway activation through Ras-PI3K-Akt and GSK3β route. Moreover miR-148a can act as negative regulator of pre-B cell leukemia transcription factor-interacting protein (HPIP), an activator of Akt, thus it may inhibit the activation of Wnt/β-catenin cascade via HPIP-Akt and GSK3β path. Another, miRNA-132 prevents the activation of pathway by inhibiting the Akt. Moreover, miR-148a, miR-122 and miR-34a may in-activate the Wnt/β-catenin pathway by targeting the Wnt1, an activator of Wnt/β-catenin cascade. Similarly, miR-15a/16-1 can target Wnt3, another activator of Wnt/β-catenin cascade, and may negatively regulate the pathway. Further, miR-26a can target EZH2, a negative regulator of DKK1, SFRP5 and CER1 (Wnt antagonists), and may inhibit the activation of Wnt/β-catenin stream. miR-199a/b-3P can target PAK (p21 protein (Cdc42 or RAC)-activated kinase), and may obstruct the Wnt/β-catenin pathway activation by suppressing the PAK- β-catenin interactions. In normal cell (non-infected), phosphatase and tensin homolog (PTEN) may inhibit Wnt/β-catenin pathway by preventing the PI3K mediated activation of Akt-GSk3β. Similarly, PP2 inhibits Akt mediated activation of GSk3β, and may modulate the β-catenin degradation. In a normal state, miR-148a, miR-29a, miR-21 and miR-122 are unable to target PTEN and PP2. Similarly miR-122 is unable to target PP2 simultaneously. So, in homeostatic cell (non-infected) miRNAs can avert the activation of Wnt/β-catenin pathway. Bars show inhibition, whereas the arrows indicate the activation effects. While, the Cross mark (X) on the Bars and Arrows indicate the in-activation of the phenomena. B. (Infected State): In activated Wnt/β-catenin pathway, destruction complex is not formed and β-catenin skips for degradation, which consequently trans-located into nucleus and increase cell proliferation by interacting with TLE1/TCF/LEF complex. HBV after infection modulate the Wnt/β-catenin signaling by deciphering the expression of the miRNAs, which may involve in tight regulation of this pathway. In figure green color miRNAs are down regulated while red miRNAs are up-regulated in response of HBV infection to cause HCC andEMT. In HBV infection, miRNAs 148a, 34a, 23b and miR-1 are down regulated, consequently RAS-PI3K-Akt-GSK3β path is activated and destruction complex is not formed to grasp β-catenin. Moreover, miR-148 unable to inhibit HPIP, which activates Akt that subsequently phosphorylates GSK3β and activate the pathway. Similarly, HBV mediated down regulation of miR-132 fail to inhibit Akt, which may result in activation of pathway by the track of Akt-GSK3β. Additionally, miRNA-148a as well as miRNA-34a are incapable of targeting Wnt1 which may bind to Frizzled and LRP55/6 to activate the cascade. While, HBx and LINE1 (long non-coding RNA) may activate the pathway by sequestering the miR-122 and hijack its ability to target Wnt1. Similarly, down-regulation of miR-15a/16-1 leads the activation of Wnt3, which may bind to receptor and may activate the signaling pathway. HBV mediated down regulation of miR-126a results in activation of EZH2, which target Wnt antagonists (DKK1, SFRP5 and CEK1) and may activate the Wnt/β-catenin pathway. Similarly, miRNA-199a/3b-p fails to inhibit PAK-β-catenin interactions, which results to skip β-catenin degradation and its translocation into nucleus to activate the complex (TLE1/TCF/LEF). On the other hand, HBx augments URG11 gene which enhances the expression of miR-148a, and the up-regulated miRNA148a targets PTEN (inhibitor of PI3K-Akt activation) and may activate the Wnt/β-catenin pathway via the path of PI3K-Akt-GSK3β. Similarly, up-regulated miRNAs, miR-29a,-21 and miR-222 target PTEN and activate the pathway by using the same path as used by miRNA-148a. Further, miR-222 also targets PP2, an inhibitor of Akt activation, and may activate the pathway by Akt-GSK3β route.

Table 1 List of Hepatitis B virus mediated miRNAs that may have role in direct or indirect modulation of Wnt β-catenin pathway to cause HCC.

miRNA	Model of study	Expression	Confirmed targets	Method of detection	Ref
miR-26a	HCC-LM3 or MHCC97-H cells, HEK293 T cells	Down	ERα, IL-6, Cyclin D2 and Cyclin E2	qRT-PCR, Luciferase Reporter Assay	[201]
miR-15a/16-1	HBx-expressing HepG2/Huh7/SKHEP-1 cells	Down	CCND1	Micro- array, qRT-PCR	[145]
miR-34a	HCC tissues, HepG2 cells	Down	CCL22, MET	qRT-PCR	[202]
miR-23b	Human tumor, HCC cells (SKHep1C3)	Down	uPA, MET	qRT-PCR	[203]
miR-1	HEK293 T cell- line	Down	HDAC4,MET,E-DN1/PI3K-Akt	qRT-PCR, Western-blot	[71,204]
miR-199a/b-3p	HCC liver samples (HBV and non-HBV	Down	PAK4	NGS, qRT-PCR	[197]
miR-132	HCC cells, HBV-related HCC tissues	Down	Akt	methylation-specific PCR (MSP), real-time PCR	[38]
miR-122	HBV-transfected HepG2/Huh7/ SK-Hep-01, HBV-transgenic mice, HBV-infected liver tissue	Down	PBF, ADAM10 Cyclin G1, Igf1R, ADAM17, NDGR3, β-catenin/E-cadherin gene,	qRT-PCR	[68,171]
miR-148a	LO2 cells transfected with HBx or empty vector, HepG2 cell, nude mice	Down	HPIP/AKT/ER-K/FOXO4/ATF-5/mTOR	QRT-PCR, Luciferase reporter assay.	[149]
miR-148a	HepG2X, HepG2URG11 and HepG2CAT	Up	PTEN/PI3K/Akt/β-catenin	SYBR green qRT-PCR	[146]
miR-29a	HepG2.2.15, HBx-expressing HepG2, HBx-transgenic mice	Up	PTEN	qRT-PCR	[190]
miR-21	HBx-expressing HepG2/Huh7	Up	PDCD4, PTEN	qRT-PCR	[205]
miR-222	(Hep3B, HKCI-4, and HKCI-9) cell lines, HCC tissue biopsy	Up	PPP2R2 A	qPCR	[191]

Up: upregulated; Down: downregulated; Erα: estrogen receptor; IL-6: interleukin-6; Cyclin D2: Cyclin E2; CCND1: Cyclin D1; CCL22: chemokine (C-C motif) ligand 22; uPA: urokinase-type plasminogen activator; HDAC4: histone deacetylase 4; EDN1: Endothelin 1; PI3K: phosphatidylinositol-3-kinase; Akt: protein kinase B; PAK4: p21 protein (Cdc42/Rac)-activated kinase 4; PBF: pituitary tumor-transforming gene 1 binding factor; ADAM: disintegrin and metalloprotease; Cyclin G1; IGF1R: insulin-like growth factor 1 receptor; NDGR3: N-myc downstream-regulated gene; HPIP: hematopoietic pre-B cell leukemia transcription factor-interacting protein; mTOR: mechanistic target of rapamycin; PTEN: phosphatase and tensin homolog; PDCD4: programmed cell death 4; PPP2R2A: protein phosphatase 2A subunit B.

tion. Similarly, HBV and its proteins, specifically HBx can modulate Wnt/β-catenin pathway by altering the miRNAs signature as shown in Table 1. The aberrant activation of Wnt/β-catenin has been captured in numerous cancers including HCC. Here, we explained the possible direct or indirect dys-regulation of Wnt/β-catenin pathway by HBV induced miRNAs to cause HCC as shown in Fig. 1.

Role of miR-26a

Enhancer of zeste homolog 2 (EZH2) has been identified as a conserved gene with homology in structural domains and motifs in Drosophila to human [116]. The involvement of EZH2 in establishment and progression of cancer has been indicated due to its elevated expression and mutations in different tumors [117]. Different studies indicate an increased expression of the EZH2 in HCCs. The high expression level was helpful in differentiating the pre-neoplastic/dysplastic lesions, and also linked with poor prognosis and aggressiveness of HCCs. The experiments conducted in a nude mouse revealed that the reduced level of EZH2 can help in reversion of tumorgenesis. Hence, tagging the EZH2 as a crucial therapeutic candidate in HCC inhibition [118]. The researchers have shown the miRNAs mediated expression level of EZH2. Particular miRNAs have ability to interact with EZH2 RNA-transcript, and can affect its protein expression after modulating the EZH2 RNA translation, integrity as well as stability [119]. The role of miRA-26a has been described in the suppression and progression of cancer via targeting different cancer related

factors. The interaction of EZH2 and miRNA-26a has been reported by numerous researchers in different cancers. For instance, in vitro experiments showed that miRNA-26a post-transcriptionally repressed the EZH2 in human lung and hepatocellular carcinoma cells, which results in inhibition of epithelial-mesenchymal transition (EMT), and up-regulation of tumor suppressor RUNX3 and DAB2IP genes [120]. Another study demonstrated the ability of miRNA-26a in impeding the EMT process in HCC via down-regulating the expression of EZH2 [121]. A recent study disclosed that miRNA-26a in Wnt pathway can directly target CDK8 and EZH2, and mediate the suppression of both c-Myc and EZH2 in hepatocellular carcinoma. They also showed that AAVs based delivery of miRNA-26a can significantly reverse the primary and metastatic HCC. Thus, suggesting the role of miRNA-26a as a target of EZH2 particularly to cope up with Wnt associated HCC [122]. miRNA-26a as a metastamiR is under preclinical trials for the development of anti-HCC drug [123]. It is believed that suppression of specific miRNA is the preliminary requisite for Carboxyl-Terminal Truncated HBx associated HCC. It was observed that miR-26a induced tumor specific apoptosis, inhibited cancerous cells proliferation and decreased the disease progression in murine model of HCC [124]. The enhancer of zeste homolog 2 (EZH2) represses the Wnt/β-catenin antagonists (including DKK1, CER1, SFRP5, AXIN2, CK1α, PPP2CB, PPP2R2B, NKD1, PRICKLE1 and NLK), resulting in enhanced β-catenin dependent HCC [125]. The above discussion indicates that EZH2 oncogene is over expressed in various tumors. Yip and coworkers have shown the over expression of histone methyltransferase EZH2 in human hepato-carcinogenesis. They found that over expression partially activates the Wnt/β-catenin which augments HCC cell growth and carcinogenicity/tumorigenicity. Moreover, Ct-HBx can deregulate miR-26a and its control over the EZH2 epigenetic machinery for Wnt activation to develop HCC [126]. This idea was further strengthen by a study on patients suffered from HBV-related HCC. Patients with constantly low expression of miR-26a had shorter survival as compared to those with high expression [127]. HBV down regulates the expression of miR-26a [127,128]. Thus, HBV modulates the Wnt/β-catenin pathway to develop HCC, by suppressing miR-26a expression, which results in up-regulation of EZH2 that targets Wnt antagonists.

Role of miR-15a and miR-16-1 cluster, member of miR-16 family

Other miRNAs that play role in HBV infection include miR-15a-b, miR-16-1 and members of miR-16 family. Gene clusters of miR-15a/16-1 and miR-15b/16-2 are positioned at human chromosomes 13q and 3, and co-transcribed with DLEU2 and SMC4 respectively [129]. This family targets CCNE1, CDK6 and CCND1-3 and are critical modulators of the G1/S cell-cycle checkpoint [130,131]. The down-regulation of miR-15b has been associated with the up-regulation of Wnt7A in ovarian cancer [132]. As described previously that chromosome 13q is the location/position of miR-15a/16-1 gene cluster. Its deletion is associated with de-regulation of genes which play crucial role in cell cycle, proliferation in de-differentiated HCCs [133] and aggressive HCC behavior [134]. Researchers have shown that miR-15a and 16-1clsuter also targets Wnt3A and CCND1 (encoding Cyclin D1) mRNAs other than BCL2 [135—144]. Moreover, decreased expression level of miR-16 family has initiative and progressive role in HBV/HBx-induced HCC. HBV RNA is thought to be involved in the down-regulation of miR-15a/16-1 cluster in hepatocytes [64] Furthermore, in HepG2 cells, down-regulation of miR-15a/16 was induced by HBx through c-Myc axis [145]. So, in the course of HCC development, HBV /HBx can up-regulate the Wnt β-catenin pathway by down regulating the miR-15a and miR-16-1 gene cluster that targets Wnt3A.

Role of miR-148a

HBV associated miR-148a regulation to modulate Wnt/β-catenin pathway, remains elusive. In a study, HBx and URG11 showed alteration in multiple miRNAs expression level. miRNAs array analysis revealed that both (HBx and URG11) enhanced the miR-148a expression. In fact, HBx stimulates URG11, which in turn up-regulates the miR-148a. Up-regulated miR-148a targets PTEN, a tumor suppressor gene. As a result, PTEN mediated PI3K blockage is suppressed. So, PI3K/Akt influences Gsk3β and results in increased β-catenin level. This shows an overall mechanism of HBV mediated miR-148a to modulate Wnt/β-catenin pathway in HCC development. Anti-miR148a was found to inhibit cell cycle progression, cell proliferation, in SCID mice model. Additionally, it also increased PTEN on both translational and transcriptional level. Enhanced PTEN was found to block PI3k-Akt mediated Gsk3β inhibition, in GSK3β mediated β-catenin degradation [146]. While in other study, HBV was found to down-regulate the expression of miR-148a, and tuned the up-regulation of Wnt/β-catenin pathway to cause HCC. Role of miR-148a in direct and indirect modulation of Wnt/β-catenin pathway can be described in three different ways. In direct regulation, miR-148a has been found in targeting the Wnt-1(Wnt signaling ligand), thus prevent the activation of Wnt/β-catenin pathway and EMT related HCC [147]. Not only miRNA-148a, but another member miRNA-148b has also been confirmed in regulating the Wnt-1/β-catenin pathway. Like miRNA-148a, miRNA-148b is found to be down-regulated in HCC tissues and its up-regulation is associated with better prognosis. Therefore, miRNA-148b holds onco suppressive character in case of HCC through the Wnt-1/β-catenin signaling. So, miRNA-148a and miRNA-148b can directly modulate the Wnt/β-catenin pathway by targeting Wnt-1 [148]. While, in indirect regulation of Wnt/β-catenin pathway, miR-148a inhibits RTK and Hematopoietic pre-B cell leukemia transcription factor-interacting protein (HPIP) mediated activation of PI3K-Akt, a GSK3β inhibitor. HPIP activates Akt [149], which subsequently inhibits GSK3β. miR-148a directly inhibits HPIP and suppresses the Akt. Thus, miR-148a inhibits the Wnt activation by inhibition of the HPIP mediated Akt-GSK3β activation. In HBV-HCC, miR-148a down regulation has been reported that enhance HPIP expression and ultimately PI3K-Akt mediated activation of Wnt/β-catenin pathway. So, HBV manipulates the expression of miR-148a, which directly or indirectly activates the Wnt/β-catenin pathway to cause HCC.

Role of miR-132

miR-132 acts as an onco-suppressor in versatile cancers including breast [150] and HCC [151] and, its down-regulation is associated with poor outcomes. In vitro study by Wei, X., et al., showed the involvement of miR-132 in cell proliferation and colony formation. They revealed that miR-132 inactivates the Akt pathway for tumor suppression. Further, they analyzed the down regulated expression of p-Akt, p-GSK3β, β-catenin and Cyclin D1, after the transfection of miR-132. However, the expression of miR-132 has to be down regulated by HBx via inducing the hyper-methylation of its promoter. This hyper-methylation favors the Akt pathway to induce the proliferation of hepatoma cells [38]. Thus, this study suggests the involvement of HBV associated HBx mediated epigenetic repression of miR-132, which results in activation of Akt pathway to cause HCC. Here, Akt activates (phosphorylates) the GSK3β which is a member of β-catenin degradation complex. So, HBx can activate the Wnt β-catenin pathway by snatching the control of miR-132 on Akt, which pulls the GSK3β from destruction complex and ultimately skips the β-catenin proteosomal degradation, and facilitates its translocation into nucleus to cause HCC.

Role of miR-34a

Viruses can also tame miRNAs for the establishment and progression of diseases including cancers. Multiple studies revealed the tumor suppressor activity of miRNA-34 family members including miR-34a/c and -449c. The down-regulation or deletion of miR-34a has been frequently observed in tumors like colon cancer [152], breast cancer [153] and esophageal squamous cancer [154]. Studies in different animal models demonstrated the anti-oncogenic potential of miRNA-34a against various types of cancers, including lung and prostate [155—157]. However, the modulation of miRNA-34a in HBV associated HCC remains enigmatic. A recent study describes the molecular mechanism of miRNA-34a in HBV associated HCC. They investigated the expression level of miRNA-34a in HBV related HCC tissues and in HepG2 cells, after transfection with HBx containing vector. In HCC cells, the down-regulation of miRNA-34a while up-regulation of MAP4K4 by HBx was observed [158]. The direct interaction of HBV with Wnt pathway to cause HCC, has been established. How does HBV decipher the miRNA-34a for the modulation of Wnt pathway, is a subject of extensive study, as the expression of miRNA-34a is crucial in different cancers including HCC. miRNA-34a mediates the various components of Wnt/β-catenin pathway. Smith et al, currently, demonstrated the antiviral activity of miRNA-34a especially in flaviviruses through repressing the Wnt components and promoting the interferon signaling [159]. They observed the significant inhibitory effects on the production of Wnt1, Wnt2, Wnt3, LEF1 and CTTNB1, after the transfection with miRNA-34a. Moreover, miR-34a targets Wnt1 and negatively regulates the Wnt/β-catenin pathway in dendritic cell differentiation [94]. In HCC, the repression of Wnt1 was restored after the down-regulation of miR-34a. Additionally, Wnt/β-catenin pathway has been reported to play a role in maintaining stemness other than invasion and metastasis [160—162]. In HBV infection, miR-34a is down-regulated. This provides a possible mechanism that HBV suppresses the expression of miR-34a, and can modulate the up-regulation of Wnt/β-catenin pathway by indirect up-regulation of Wnt1 to cause HCC. The overall discussion shows a possible link among miRNA-34a, HBV and Wnt components particularly Wnt1 in HBV derived HCC. The expression level of miRNA-34a holds great importance in case of HCC. Currently, a team of researchers has conducted phase I study of a liposomal miRNA-34a mimic (MRX34) in patients with advanced solid tumors including HCC, and showed promising results [163]. Alone or in combination, miRNA-34a based therapy might be effective in treating HBV-HCC after tight regulation of Wnt pathway components.

Role of miR-122

miR-122 possesses dual behavior in hepatitis associated viral pathogenesis [164]. It supports HCV replication [165—167]. On the other side, it can suppress HBV replication via p53 mediated inhibition of HBV transcription. P53 inhibits HBV replication by binding with its enhancer elements. This binding is blocked by Cyclin G1. But, miR-122 targets Cyclin G1 and prevents its interaction with p53, result in the suppression of HBV replication [68]. A study documented that miR-122 can directly modulate the Wnt/β-catenin pathway by targeting the Wnt1, and subsequently induce HCC cell apoptosis, and suppression of cell proliferation [168]. Another study also confirms the Wnt-1 as a direct target of miRNA-122 in HCC [169]. miRNA-122 also inhibits the Wnt pathway in glioma cells. Hence, a strong interaction exists between miRNA-122 and Wnt pathway [170]. The miR-122 complementary sites in all HBV mRNAs, make the endogenous sequestration of miR-122 favorable [111,171]. Moreover, an interaction between HBx and non-coding RNAs has been delineated by the number of authors [22,172,173]. Recently, Liang, Wang et al reported that lnRNA HBx-LINE1 sequesters the cellular miR-122, and activated the Wnt/β-catenin pathway by targeting Wnt1 RNA [174]. Thus, HBx interacts with one ncRNA to sequester the other ncRNA, and promotes the tumor-genesis by activating the Wnt/β-catenin pathway. This provides another insight of miR-122 down regulation in HBV associated HCC-EMT. Over all, HBV down regulates the expression of miR-122 either directly or indirectly for the sake of Wnt/β-catenin pathway activation, which results in HCC-EMT development.

Role of miR-21,-29a and miR-222

Commonly, cancer is characterized with the down-regulation of miRNAs [175]. However, the up-regulation of certain miRNAs have been documented consistently by the researchers in case of HCC [176]. Different miRNAs including miR-21, miR-29a and miR-222 play pivotal role in the progression of tumors including HCC [177]. Either miRNAs directly activate the oncogenic pathway (Wnt pathway) by targeting its antagonists or indirectly by targeting other pathways or factors, which are linked to oncogenic pathway. miR-21 directly targets the TGFβ-R2, an inhibitor of Wnt/β-catenin pathway, and augments the colon cancer [178]. In hFObI cells, miR-29a promotes osteoblast differentiation by

targeting the Wnt inhibitors such as SFRP2, Kremen2 and DKK1 [179]. A current study suggested the miR-29 family as a target of GSK3β for the activation of Wnt/β-catenin pathway in MEF-cells [180]. While, miR-222 targeted DKK2 in glioma cells and enhanced the β-catenin expression constitutively [181]. Normally, protein phosphatase 2A (PP2A) and phosphatase and tensin homolog (PTEN) negatively regulate the PI3K/AKT signaling. Silencing of these two regulators can activate the PI3K/AKT pathway, and can promote HCC-metastasis. A number of researchers have shown the interaction among these miRNAs and PTEN, a tumor suppressor gene. In HCC, miR-21 inhibits PTEN gene activity and promotes metastasis [182]. miR-21 can also enhance proliferation of cancer cells and metastasis by targeting the PTEN and activating the Akt pathway. Moreover, a positive relationship was found among miRNA-21 and Wnt/β-catenin pathway components (β-catenin and Cyclin D1) in mice and human lung cancer cells [183]. Similarly, miR-222 also targets the PTEN and makes the tumor cells more resistant to apoptotic factors [184]. Investigators have reported the cross-talk among PTEN, PI3K and Wnt pathway. Persad and his colleagues reported the interaction between Wnt pathway and PTEN/PI3K for the regulation of activated β-catenin (ABC) at nuclear level [185]. Another latest report disclosed the cross-talk among Wnt/β-catenin, PI3K/Akt pathways and PTEN in tumor cells for the induction of their reprogramming. They demonstrated that melanoma cells alter their invasive potential under the influence of Wnt/β-catenin signaling after the deletion of PTEN. On the other hand a significant reduction in metastasis was observed in PTEN wild type cells [186]. Moreover, the nuclear accumulation of β-catenin is antagonized by PTEN [187,188]. In HBV associated HCC, PTEN down-regulation can result in up-regulation of miR-222, miR-29a and miR21 expression [184,189,190] and PP2A can be a target of miR-222 simultaneously [191] which consequently can lead to increased metastasis. Hence, the above discussion clears the cross-talk/connection among miRNAs (−21,29a and −222), PTEN, PI3K/Akt and Wnt pathways. So, HBV modulates the up-regulation of these miRNAs, target the Wnt antagonists and activate Wnt pathway or target the PTEN and PP2A, and use the PI3K/Akt axis to inactivate the GSK3β, and can promote the Wnt-β-catenin to develop HCC.

Role of miR-199a/b-3p (A member of miR-199 family)

For oncogenesis, signaling pathways require intracellular protein kinases such as p21-activated kinases (Paks), which are serine/threonine-specific and are placed at the nexus of these pathways. On the basis of structural and sequential characters, 6-mammalian Paks are classified into 2 subgroups: Pak1-3 as group I and Pak 4-6 as group II. Paks have been engaged in activation of components of cell progression pathways such as Wnt, Akt and Erk signaling pathways [192]. Recently, interaction of Pak with Wnt/β-catenin has been unveiled in numerous studies [193–195]. Pak1 has been found in β-catenin phosphorylation at S-663 and S-675 sites, and resulted in promotion and its re-localization to nucleus to up-regulate Cyclin D1 and MYC expression. Pak4 and Pak5 show similar effects, but all members do not behave in a similar fashion. As in breast epithelial cells, no effect on phosphorylation and expression of β-catenin was observed due to depletion of Pak2 [193]. Down-regulation of miR-199a-3p has been observed in HCC and other malignancies [196]. In another study, consistently decreased expression of miR-199a/b-3p was correlated with poor HCC patients' survival. Additionally, Pak4 was found as a target site of miR-199a/b-3p and suppressed HCC by inhibiting Pak4/Raf/MEK/ERK pathway [197]. miR-199a/b-3p is down-regulated in HBV to cause HCC-metastasis [111]. Still, no data is available on the direct modulation of components of Pak signaling by HBV for the activation of Wnt pathway to cause HCC. However, the above discussion suggests that HBV can manipulate Wnt/β-catenin and Pak pathways, after down regulating the miR-199a/b-3p, which is a negative regulator of Pak signaling pathway and can develop HCC. A current study sheds a light on the role of miR-199a/b-3p in inhibiting the proliferation of gastric cancer cells, by down-regulating the expression of Pak4/MEK/ERK signaling pathway [198]. Another recent study, reveals the antitumor potential of miR-199a-3p in a transgenic mouse model with HCC by modulating the Pak4 and MTOR [199]. Therefore, miR-199a/b-3p is a promising therapeutic future candidate to treat HCC or HBV-Wnt/β-catenin and Pak associated HCC. From above discussion, it can be figure out that HBV can modulate the Wnt/β-catenin pathway through Pak pathway by down-regulation of miR-199a/b-3p, which is a negative regulator of Pak signaling pathway. This is a possible mechanism which can be modulated by HBV to cause HCC.

Conclusion

HBV has plagued humankind for the decades, and is a serious health problem in terms of HCC.

HBV disturb the host pathways genes controlling the apoptosis, proliferation and cell cycle leading it to end stage HCC. Understanding the cross talk of HBV proteins with host cellular factors like Wnt signaling may help in designing the better therapeutics against HBV. HBV not only modulate Wnt signaling in a direct way but also has an indirect influence through the altered expression of different cellular miRNAs. This review unveiled the possible communication between HBV and some crucial cellular miRNAs for the induction of Wnt/β-catenin pathway to cause HCC. Researchers have established the role of ncRNAs alterations in HBV infection, which assist in development of HCC to EMT and metastasis. But the erudite knowledge is needed to explore their mechanisms for making them possible diagnostic and therapeutic options in HBV related HCC interventions. By restoring the miRNAs, down-regulated by HBV, like miR-26a, miR-16 family members (miR-15a-b and miR-16), miR-148a, miR-132, miR-199a/b/3p (A member of miR-199 family) miR-34a and miR-122, may prove a significant path towards HCC prevention caused by aberrant activation of Wnt β-catenin signaling pathway. Similarly, the anti-miR approach for miRNAs, which are up-regulated by HBV like miR-21,-29a and miR-222 and regulate abnormal activation of Wnt β-catenin cascade, would be a crucial tool to treat HBV-Wnt-HCC. Currently, an approach has been utilized to develop a drug under the name of Miravirsen. This drug has entered into clinical trials to treat HCV, and showed promising results

in Phase 2a clinical trials. Miravirsen sequesters miR-122 upon binding because of its antisense oligonucleotide character. Miravirsen holds a promise to cope up HCV viral load [200]. The similar technique in future may be helpful to resolve HBV infection. However, still no single miRNA has been declared as an absolute regulator of HBV infection. It is a need of time to identify the specific miRNA regulating the HBV infection, and take it into account to treat HBV infection. The above discussed HBV mediated miRNAs involved in direct or indirect modulation of Wnt β-catenin pathway to cause HCC, may be a potential weapon in the battle of treating HBV-Wnt associated HCC.

Disclosure of interest

The authors declare that they have no competing interest.

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