

# MicroRNAs as Important Players in Host-hepatitis B Virus Interactions

Bouchra Kitab<sup>1</sup>, Hanane Salih Alj<sup>2</sup>, Sayeh Ezzikouri<sup>1</sup> and Soumaya Benjelloun<sup>\*1</sup>

<sup>1</sup>Viral Hepatitis Laboratory, Virology Unit, Institut Pasteur du Maroc, Casablanca, Morocco; <sup>2</sup>Laboratory of Biology and Health, URAC34, Faculty of Sciences Ben M'sik, University Hassan II Casablanca, Morocco

## Abstract

Hepatitis B virus (HBV) infection, a major public health problem, causes acute and chronic hepatitis that is often complicated by liver cirrhosis and hepatocellular carcinoma. The pathogenic mechanisms of HBV-related liver disease are not well understood, and the current licensed therapies are not effective in permanently clearing virus from the circulation. In recent years, the role of micro-ribonucleic acids (miRNAs) in HBV infection has attracted great interest. Cellular miRNAs can influence HBV replication directly by binding to HBV transcripts and indirectly by targeting cellular factors relevant to the HBV life cycle. They are also involved in the regulation of cellular genes and signaling pathways that have critical roles in HBV pathogenesis. HBV infection, in turn, can trigger changes in cellular miRNA expression that are associated with distinctive miRNA expression profiles depending on the phase of liver disease. These alterations in miRNA expression have been linked to disease progression and hepatocarcinogenesis. We provide here an up to date

review regarding the field of miRNAs and HBV interplay and highlight the potential utility of miRNAs as diagnostic biomarkers and therapeutic targets for the management of HBV-related liver disease.

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## Introduction

Hepatitis B virus (HBV) is a prototype member of the *Hepadnaviridae* family and one of most common human pathogens worldwide. Despite the availability of an effective prophylactic vaccine for nearly three decades, HBV remains the cause of a number of important public health problems.<sup>1</sup> HBV is transmitted among humans by contact with the blood or other bodily fluids of an infected person and is commonly acquired through perinatal, horizontal, sexual, and parenteral/percutaneous transmission. The clinical manifestations of HBV infection range from acute or fulminant hepatitis to various forms of chronic infection, which may evolve to liver cirrhosis and hepatocellular carcinoma (HCC).<sup>2</sup> According to World Health Organization estimates, more than 240 million people worldwide have chronic hepatitis B, and more than 780,000 people die annually due to the acute or chronic complications of hepatitis B.<sup>3</sup> At present, two types of antiviral drugs are approved for the treatment of chronic hepatitis B, including conventional or pegylated interferon alpha (IFN- $\alpha$  or PEG-IFN- $\alpha$ ) and nucleos(t)ide analogs.<sup>4</sup> IFN- $\alpha$  or PEG-IFN- $\alpha$  have significant toxicity. Nucleos(t)ide analogs have limited success in achieving sustained virological response and are susceptible to drug resistance.<sup>4,5</sup> Growing evidence has highlighted the importance of investigating the interactions between HBV and the host in order to understand better the mechanisms of HBV pathogenesis and to develop novel and improved anti-HBV therapeutic strategies.<sup>6,7</sup>

Recent findings about the role of micro-ribonucleic acids (miRNAs/miRs) in various aspects of HBV-host interactions have added another dimension to our understanding of HBV pathogenesis. MiRNAs represent a large class of highly conserved noncoding RNAs of ~22 nucleotides (nt) in length, which modulate gene expression at the post-transcriptional level. They bind to the 3'-untranslated region (3'-UTR) of target messenger RNAs (mRNAs), resulting in gene silencing through translational repression or mRNA degradation.<sup>8</sup> First discovered in the nematode *Caenorhabditis elegans*, miRNAs have been identified in all multicellular eukaryotes and some viruses.<sup>9-11</sup> MiRNAs modulate HBV replication and host responses. Therefore, exploring the function of miRNAs and

**Keywords:** MicroRNA; Hepatitis B virus; Host-virus interaction; Gene expression; Biomarker.

**Abbreviations:** 3'-UTR, 3'-untranslated region; AFP, alpha-fetoprotein; AGO2, argonaute 2 protein; ALT, alanine aminotransferase; AUC, area under the curve; cccDNA, covalently closed circular DNA; CCl<sub>4</sub>, carbon tetrachloride; C/EBP, CCAAT enhancer-binding protein; CHORDs, cysteine- and histidine-rich domain; DGC8, DiGeorge syndrome critical region 8; DNMT, DNA methyltransferase; ECM, extracellular matrix; ER- $\alpha$ , estrogen receptor alpha; FNDC3B, fibronectin type III domain containing 3B; FXR $\alpha$ , farnesoid X receptor  $\alpha$ ; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B early antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBx, hepatitis B X protein; HBXIP, HBX-interacting protein; HCC, hepatocellular carcinoma; HDAC4, histone deacetylase-4; HDAd, helper dependent adenoviral vectors; HLA-A, human leukocyte antigen A; HNF, hepatocyte nuclear factor; HO-1, heme oxygenase-1; HSCs, hepatic stellate cells; IFN- $\alpha$ /PEG-IFN- $\alpha$ , pegylated interferon alpha; IL-6, interleukin-6; JAK/STAT, Janus kinase/signal transducer and activator of transcription; LC, liver cirrhosis; LHBsAg, large HBsAg; Mcl-1, myeloid cell leukemia sequence 1; MHBsAg, middle HBsAg; miRNA, microRNA; mTOR, mammalian target of rapamycin; NDRG3, N-myc downstream-regulated gene 3; NFIB, nuclear factor I/B; NF- $\kappa$ B, nuclear factor kappa B; nt, nucleotides; ORF, open reading frames; P-bodies, processing bodies; PI3K, phosphatidylinositol 3-kinase; pgRNA, pregenomic RNA; Pol II, polymerase II; PPAR $\alpha$ , peroxisome proliferator-activated receptor- $\alpha$ ; pre-miRNAs, precursor miRNAs; PTEN, phosphatase and tensin homolog; PTTG1, pituitary tumor-transforming gene 1; RASSF1A, ras-associated domain family member 1A; RISC, RNA-induced silencing complex; RLC, RISC loading complex; ROC, receiver operating characteristic; SHBsAg, small HBsAg; TALENs, transcription activator like effector nucleases; TGF- $\beta$ , transforming growth factor- $\beta$ ; TLR, toll-like receptor; TRBP, transactivation-response RNA-binding protein.

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**\*Correspondence to:** Soumaya Benjelloun, Virology Unit, Institut Pasteur du Maroc, 1 Place Louis Pasteur, Casablanca 20360, Morocco. Tel: +212-527-016-076; +212-522-434-450, Fax: +212-522-260-957, E-mail: soumaya.benjelloun@pasteur.ma

their application in therapeutics and diagnostics has become an emerging area of interest in the field of HBV research. In the current review, we will describe the current knowledge regarding the biological relevance of miRNAs in the context of HBV infection. We will also discuss the potential value of miRNAs as diagnostic biomarkers and therapeutic targets for HBV-related liver disease.

### miRNAs biogenesis and functions

The biogenesis of miRNAs involves numerous critical steps that occur in both the nucleus and cytoplasm. The genes encoding miRNAs can be mono- or polycistronic and are frequently located in intergenic regions of genomes and introns of protein-coding or noncoding genes.<sup>12,13</sup> Generally, miRNA genes are transcribed by RNA polymerase II (Pol II) as large primary transcripts (pri-miRNAs) that contain a stem-loop structure of ~80 nt.<sup>14</sup> Pri-miRNAs are then processed by the RNaseIII-like enzyme Drosha together with its binding partner DiGeorge syndrome critical region 8 (DGCR8), to produce the ~60-70 nt precursor miRNAs (pre-miRNAs).<sup>15</sup> These are transported into the cytoplasm by Exportin-5 via a Ran-GTP-dependent mechanism.<sup>15,16</sup> Once in the cytoplasm, pre-miRNAs are processed by Dicer, another RNaseIII-like enzyme, and transactivation-response RNA-binding protein (TRBP) to generate the ~22 nt duplexes comprising the mature miRNAs and the complementary fragments miRNAs\*.<sup>17</sup> Mature miRNAs are connected to the RNA-induced silencing complex (RISC), whereas miRNAs\* are rapidly degraded.<sup>18</sup> TRBP, Argonaute 2 protein (AGO2), and Dicer are involved in the formation of the RISC loading complex (RLC), which can facilitate the binding of the mature miRNAs to RISC.<sup>19,20</sup> Finally, mature miRNAs guide RISC to complementary sites within the target mRNAs to execute the post-transcriptional regulatory activity. In the case of perfect sequence complementarity between target mRNAs and miRNAs, mRNAs are degraded by RISC, whereas in the case of partial sequence complementarity, a repression of mRNA translation occurs.<sup>21,22</sup> Evidence also indicates that miRNA-bound transcripts are sequestered into processing bodies (P-bodies), where they are maintained in a silenced state.<sup>23</sup> Thus, P-bodies serve as a site of temporary storage before mRNA degradation or translational inhibition.<sup>23</sup>

The human genome encodes 2,565 miRNAs (miRBase ver. 21; <http://www.mirbase.org/>, released in June 2014). Each miRNA can regulate hundreds of different mRNAs, and conversely, a single mRNA can be targeted by several miRNAs. Bioinformatic analyses have indicated that more than 30% of human genes are under miRNA-mediated regulation.<sup>24</sup> Accordingly, cellular miRNAs play an important role in several biological and physiological processes, such as cell differentiation, proliferation, metabolism, apoptosis, developmental timing, and immune responses.<sup>25</sup> Deregulation of their expression and function is also involved in various diseases, especially cancers.<sup>26,27</sup> In viral infections, cellular miRNAs can positively or negatively influence virus replication by regulating the expression of viral genes and/or cellular factors relevant to the course of virus-induced disease.<sup>28</sup> At the same time, viruses can alter cellular miRNA expression. In fact, viruses are equipped with complex machinery to exploit and manipulate the host pathways to establish an environment favorable for their persistence.<sup>29</sup> It is not surprising, therefore, that the miRNA pathway can be used to do this, either

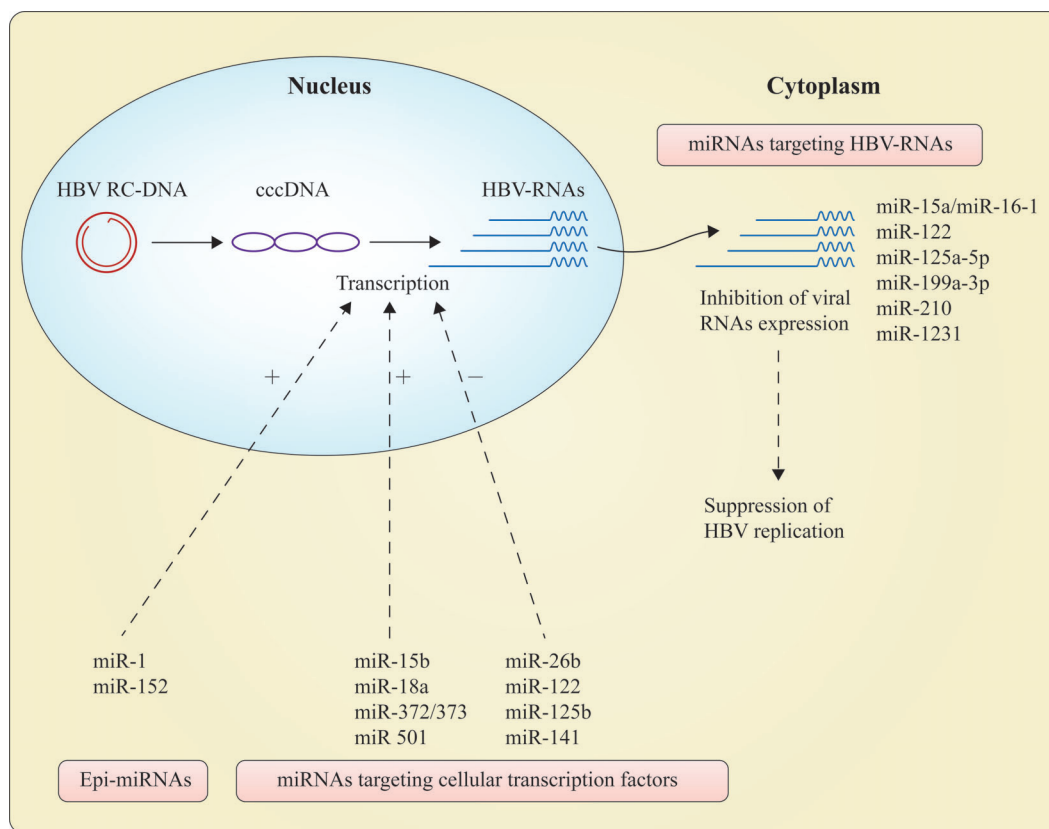
by encoding their own miRNAs or encoding molecules that inhibit or stimulate cellular miRNA expression.<sup>30</sup>

### miRNAs in HBV replication

HBV has a relaxed circular, partially double-stranded (RC-DNA) genome containing four overlapping open reading frames (ORF).<sup>31</sup> The preS/S ORF encodes three viral surface proteins [large hepatitis B surface antigen (LHBsAg), middle HBsAg (MHBsAg), and small HBsAg (SHBsAg)], the preC/C ORF encodes the core protein (hepatitis B core antigen (HBcAg)) and the soluble e protein (hepatitis B early antigen (HBeAg)), the X ORF encodes the hepatitis B X protein (HBx), and the Pol ORF encodes the viral polymerase that possesses DNA polymerase and reverse transcriptase activities.<sup>31</sup> After infecting hepatocytes, the HBV genome is released into the nucleus and converted to covalently closed circular DNA (cccDNA), which serves as a template for the transcription of all viral transcripts, including pregenomic RNA (pgRNA) and subgenomic RNAs (pre-S, S and X mRNAs).<sup>32</sup> The transcription of viral transcripts is regulated by four distinct viral promoters that are under the control of two regulators, designated enhancers I and II.<sup>33</sup> In addition, a variety of liver-enriched transcription factors and nuclear receptors have been shown to bind to HBV promoter/enhancer elements and to be critical for the regulation of HBV transcription.<sup>33,34</sup> Viral miRNAs encoded by HBV have not been identified, but one putative HBV-encoded pre-miRNA was predicted using computational approaches.<sup>35</sup> Nevertheless, numerous cellular miRNAs have emerged that either repress or promote HBV replication by direct interaction with HBV transcripts or indirectly by targeting crucial cellular factors relevant to HBV life cycle (Fig. 1).

### miRNAs suppressing HBV replication

The first attempt to search for miRNAs that directly target HBV transcripts was made by Zhang *et al.*, using a miRNA-loss-of-function approach.<sup>36</sup> A total of 328 human miRNAs were individually knocked-down in HepG2.2.15 cells that are designed to support the HBV life cycle.<sup>36</sup> Among these, miR-199a-3p and miR-210 efficiently reduced HBsAg expression and HBV replication. Bioinformatics analysis revealed that miR-199a-3p and miR-210 inhibited HBV by directly targeting the HBsAg encoded region and the pre-S1 region of the HBV genome, respectively.<sup>36</sup> The ability to affect HBsAg expression was also demonstrated for miR-125a-5p, which binds to HBsAg mRNA and inhibits its translation.<sup>37</sup> Another study revealed a self-inhibitory feedback loop where HBV, through HBx, increased the expression of miR-125a-5p and subsequently interfered with HBsAg expression.<sup>38</sup> Furthermore, extensive studies reported a remarkable decrease in HBV replication when the expression of miR-122, the most abundant liver-specific miRNA, was upregulated in both cell lines and liver tissues.<sup>39-41</sup> These studies proposed several mechanisms underlying miR-122-mediated suppression of HBV replication. Chen *et al.* found that miR-122 binds to a highly conserved sequence of HBV pgRNA, which is a bicistronic mRNA encoding the viral polymerase and core protein, resulting in a decrease in HBV core-associated DNA level.<sup>39</sup> Qiu *et al.* suggested that miR-122 inhibited HBV replication through increasing expression of cellular heme oxygenase-1 (HO-1), a cytoprotective enzyme that decreases HBV cccDNA levels both *in vitro* and *in vivo*.<sup>40</sup> Another regulatory mechanism of



**Fig. 1. Summary of cellular miRNAs involved in the regulation of HBV replication.** MiRNAs may suppress (–) or promote (+) HBV replication by targeting positive or negative cellular transcription factors, critical for HBV transcription. Epi-miRNAs contribute to increased synthesis of HBV transcripts by regulating epigenetic modifications of HBV covalently closed circular DNA (cccDNA). Other cellular miRNAs directly target HBV-RNAs and suppress viral proteins expression.

miR-122 involved cyclin G1-modulated p53 activity.<sup>41</sup> Cyclin G1 specifically interacted with p53, and this interaction blocks the binding of p53 to HBV enhancers, subsequently abrogating p53-mediated inhibition of HBV transcription.<sup>42</sup> However, cyclin G1 was targeted by miR-122, thereby promoting the anti-HBV activity of p53.<sup>41</sup> Reduced HBV replication has been also observed in presence of high miR-1231 levels in HBV-transfected HepG2 cells.<sup>43</sup> Extensive analysis revealed that miR-1231 targeted HBV core and HBx sequences, resulting in decreased expression of HBV core protein with no reduction in HBx protein.<sup>43</sup> Other viral sequences critical for HBV replication, including the coding region for HBV polymerase and the overlapping region between HBV polymerase and HBx, were shown as potential targets for miR-15a/miR-16-1 cluster.<sup>44</sup> The antiviral effect of miR-15a/miR-16-1 was validated *in vitro*, showing a negative correlation between the expression of this cluster and HBV replication in HBV-transfected HepG2 cells.<sup>44</sup>

Besides miRNAs targeting HBV transcripts, other miRNAs elicit their antiviral activity by targeting a positive regulator of HBV. The peroxisome proliferator-activated receptor- $\alpha$  (PPAR $\alpha$ ), which binds and transactivates HBV promoters, was described as a target of miR-141.<sup>45</sup> Transfection of synthetic miR-141 in HBV-transfected HepG2 cells suppressed PPAR $\alpha$  expression, leading to reduced HBV transcription.<sup>45</sup> The role of the CCAAT enhancer-binding protein (C/EBP) in transactivation of HBV enhancer II, core, and S promoters was abolished by miR-155, which targets and downregulates

C/EBP, thereby repressing HBV replication.<sup>46,47</sup> Recently, it was reported that elevated miR-26a levels in hepatoma cells resulted in a marked decrease in HBV-DNA and protein expression.<sup>48</sup> Subsequent analysis demonstrated that miR-26b inhibited the expression of cysteine- and histidine-rich domain containing 1 (CHORDC1), which functions as a positive regulator of HBV enhancer/promoter activities.<sup>48</sup> Targeted inhibition by miR-125b of another cellular factor, the sodium channel nonvoltage-gated 1 alpha (SCNN1A), repressed HBV protein expression.<sup>49</sup> However, the mechanism by which SCNN1A affects HBV replication is not well-known and needs further investigation.<sup>49</sup>

#### miRNAs promoting HBV replication

For miRNAs that promote HBV replication, it appears they share a common pattern of targeting negative cellular regulators of HBV replication. HBx-interacting protein (HBXIP) was originally isolated as a human protein that binds to HBx protein and may reduce HBV replication by interacting with a domain necessary for HBx transactivation.<sup>50</sup> However, HBXIP was identified as a putative target of miR-501, and its expression was inversely correlated with miR-501 expression in HepG2.2.15 cells.<sup>51</sup> Moreover, downregulation of miR-501 significantly inhibited HBV-DNA replication, and HBXIP knock-down rescued the inhibition of HBV with miR-501 loss in HepG2.2.15 cells, suggesting that miR-501 enhanced HBV replication by targeting HBXIP.<sup>51</sup> A negative correlation was

also identified between miR-372/373 and nuclear factor I/B (NFIB), a transcription factor that downregulates HBV enhancer I and core promoter activities.<sup>52</sup> High expression of miRs-372/373 reduced endogenous NFIB levels in HepG2 cells. Further analysis of the 3'-UTR of NFIB mRNA revealed two predicted sites for miR-372/373.<sup>52</sup> In addition, knock-down of NFIB in HepG2 cells resulted in increased HBV gene expression, whereas enhanced expression of miR-372/373 stimulated the production of HBV proteins.<sup>52</sup> Taken together, these findings indicated that miR-372/373 can promote HBV expression through a pathway involving NFIB. Another negative regulator of HBV enhancer I activity, the hepatocyte nuclear factor 1 $\alpha$  (HNF1 $\alpha$ ), was recently described as a mediator in miRNA promotion of HBV replication.<sup>53</sup> HNF1 $\alpha$ -mRNA was directly downregulated by miR-15b, resulting in transactivation of HBV Enhancer I.<sup>53</sup> However, it has been shown that increased expression of HBV proteins, especially HBx, resulted in downregulation of miR-15b expression in both HBV-producing cells and an HBV transgenic mouse model.<sup>53</sup> This reciprocal regulation between miR-15b and HBV may help to control the level of HBV replication. Moreover, dysregulation of the estrogen pathway by miRNAs may be an important mechanism underlying the regulation of HBV replication. The estrogen pathway can reduce HBV replication by enhancing the expression of estrogen receptor alpha (ER- $\alpha$ ), which interacts with hepatocyte nuclear factor 4 alpha (HNF-4 $\alpha$ ), and preventing HNF-4 $\alpha$  binding to the HBV enhancer I and activation of HBV transcription.<sup>54</sup> For instance, miR-18a has been shown to repress ER $\alpha$  translation by binding to its mRNA, thus promoting HBV transcription.<sup>55</sup>

Epigenetic modifications of HBV cccDNA, such as DNA methylation and deacetylation, play a pivotal role in the control of HBV replication by suppressing cccDNA transcription to viral mRNAs.<sup>56</sup> Epi-miRNAs, including miR-1 and miR-152, have been recently implicated in the regulation of epigenetic modifications of HBV cccDNA. In a study by Zhang *et al.*, miR-1 was shown to directly target histone deacetylases-4 (HDAC4), and transfection of miR-1 in HepG2.2.15 cells led to markedly reduced expression of histone deacetylases-4 (HDAC4) and enhanced HBV transcription.<sup>57</sup> Furthermore, cotransfection of an HDAC4 expression vector with miR-1 attenuated HBV mRNAs levels. These findings indicated that HDAC4 plays a significant role in the action of miR-1 on HBV replication.<sup>57</sup> The modulation of HDAC4 expression by miR-1 may lead also to enhanced expression of farnesoid X receptor  $\alpha$  (FXR $\alpha$ ), resulting in a marked increase in HBV-DNA and proteins levels by enhancing transcriptional activity of the HBV core promoter.<sup>57,58</sup> Regarding miR-152, its expression was shown to be inversely correlated with DNA methyltransferase (DNMT1) expression in liver cell lines. In silico predictions defined DNMT1 as a potential inhibitory target of miR-152.<sup>59</sup> Consequently, the downregulation of DNMT1 resulted in a decrease of cccDNA methylation, subsequently enhancing HBV replication.<sup>59</sup>

### miRNAs in HBV-related liver disease

The clinical course of hepatitis B is dynamic and varies widely between patients from self-limited infection after acute hepatitis to chronic infection with a clinical presentation ranging from asymptomatic carrier state to severe expressions of disease, including active chronic hepatitis, liver cirrhosis, and HCC.<sup>60</sup> Approximately 90–95% of immunocompetent individuals who acquire HBV infection in adulthood will clear the virus

spontaneously, whereas the remaining (5–10%) develop chronic hepatitis. The rate of chronicity is higher in perinatal and early childhood infections (over 90% and 30%, respectively).<sup>60</sup> Approximately 15–40% of HBV chronic carriers develop liver cirrhosis and HCC.<sup>61</sup> Several host and viral mechanisms have been described to explain these diverse liver disease outcomes.<sup>62,63</sup> There is accumulating evidence detailing the functions of miRNAs in HBV pathogenesis and identifying differentially expressed miRNAs and target genes in the different phases of liver disease. Some miRNAs have been shown to be altered by HBV, especially liver-specific miRNAs, contributing to liver damage and HCC development.

### miRNAs in the chronicity of HBV infection

It is widely believed that ineffective immune responses play an important role in HBV persistence and disease chronicity.<sup>63</sup> MiRNAs have been found to function as regulators of the immune responses against HBV (Table 1). Su *et al.* demonstrated that miR-155 enhances the expression of several IFN-inducible antiviral genes against HBV through direct suppression of the expression of suppressor of cytokine signaling 1 (SOCS1), and subsequently enhancing the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway.<sup>64</sup> In contrast, miR-548 downregulates host antiviral responses by direct targeting IFN- $\lambda$ 1.<sup>65</sup> Interestingly, endogenous miR-548 levels are reduced during HBV infection, which may be a host mechanism to defend against HBV.<sup>65</sup> In turn, HBV infection may modulate the expression of many miRNAs involved in immune responses. By comparing the expression of 1,008 miRNAs between HepG2.2.15 cells and parenteral hepatoma cells HepG2, Jiang *et al.* observed a differential expression of miRNAs related to toll-like receptor (TLR) pathways.<sup>66</sup> The expression of seven miRNAs (miR-200b-3p, miR-148a-3p, miR-145-5p, miR-146b-5p, miR-200c-3p, miR-455-3p and miR-455-5p) was upregulated by 5-fold or greater in HepG2.2.15 cells compared to HepG2 cells, whereas the expression of eight miRNAs (from let-7 family) was downregulated by 5-fold or greater in HepG2.2.15 cells compared to HepG2 cells.<sup>66</sup> Most upregulated miRNAs have been demonstrated to modify the efficiency of TLR signaling. In addition, repression of the let-7 family of miRNAs relieved interleukin (IL)-6 and IL-10 mRNAs from negative posttranscriptional control in the TLR4 signaling pathway. This induced an inhibition of immune responses directed against HBV.<sup>66</sup> These findings indicated that alteration of miRNAs related to the TLR pathway during HBV infection could play an important role in the prevention of HBV elimination by host innate immunity. Furthermore, it has been reported that HBV infection promoted the transcriptional activity of miR-146a in HepG2.2.15 cells, which represses the production of type I interferon-induced antiviral factors, particularly STAT1, and results in interferon resistance.<sup>67</sup> These observations revealed an important role for miR-146a in HBV immunopathogenesis.<sup>67</sup> Another study analyzing the modified expression profiles of miRNAs in a stable HBV-expressing cell line revealed that upregulation of miR-181a might participate in HBV persistence through inhibition of the human leukocyte antigen A (HLA-A)-dependent HBV antigen presentation.<sup>68</sup> Recently, Momeni *et al.* showed that expression of miR-1, miR-21, and miR-125a in peripheral blood immune cells was significantly increased in patients with chronic HBV infection compared to healthy controls and that ectopic expression of these miRNAs was responsible for



**Table 1. Summary of miRNAs involved in the chronicity of HBV infection and liver cirrhosis**

miRNA	Expression <sup>a</sup>	Target gene (s)	Functions
miRNAs involved in the chronicity of HBV infection			
Let-7 family	Down	IL-6, IL-10, TLR4	Inhibits the expression of proinflammatory cytokine IL-6, anti-inflammatory cytokine IL-10, and TLR4, thereby influencing the control of immune responses and inflammation <sup>66</sup>
miR-146a	Up	STAT1	Inhibits the production of type I interferon-induced antiviral factors <sup>67</sup>
miR-155	Up	SOSC1	Upregulates the expression of IFN-inducible antiviral genes by suppressing SOSC1 and promoting JAK/STAT signaling pathway <sup>64</sup>
miR-181a	Up	HLA-A	Downregulates the expression of HLA-A and inhibits the HLA-A dependant HBV antigen presentation <sup>68</sup>
miR-548	Down	IFN- $\lambda$ 1	Inhibits expression of IFN- $\lambda$ 1 and downregulates immune responses <sup>65</sup>
miR-145-5p, miR-146b-5p, miR-148a, miR-200b-3p, -200c-3p, miR-455	Up	SOCS7, IRAK1, TRAF6, CaMKII, MyD88, E2F1	Dowregulates the efficiency of TLR signaling pathways, thereby affecting host innate defenses against HBV <sup>66</sup>
miR-1, miR-21, and miR-125	Up	Unknown	Induces impaired immune responses against HBV and facilitates HBV's evasion of the host immune system <sup>69</sup>
miRNAs involved in HBV-related cirrhosis			
miR-19b	Down	TGF- $\beta$	Inhibits TGF- $\beta$ -induced HSCs activation and prevents fibrosis <sup>72</sup>
miR-29	Down	COL1A1, COL4A5, COL5A3	Inhibits the expression of collagen in HSCs and prevents fibrosis <sup>74</sup>
miR-33a	Up	Smad7	Stimulates TGF- $\beta$ -induced HSCs activation and promotes fibrosis <sup>75</sup>
miR-150	Down	c-myb	Inhibits collagen-I expression and HSCs activation <sup>73</sup>
miR-194	Down	rac1	Inhibits collagen-I expression and HSCs activation <sup>73</sup>
miR-615-3p	Up	Unknown	Promotes hypersplenism in HBV-related cirrhosis <sup>76</sup>

<sup>a</sup>Denotes endogenous expression in HBV-infected liver tissues or HBV-infected cell lines.

Down: downregulate; Up: upregulate; IL-6: interleukin 6; IL-10: interleukin 10; TLR4: toll-like receptor 4; STAT1: signal transducer and activator of transcription 1; SOSC1: suppressor of cytokine signaling 1; HLA-A: human leukocyte antigen A; IFN- $\lambda$ 1: Interferon  $\lambda$ 1; SOCS7: suppressor of cytokine signaling 7; IRAK1: interleukin-1 receptor-associated kinase 1; TRAF6: TNF receptor-associated factor 6; CaMKII: Ca<sup>2+</sup>/calmodulin-dependent protein kinase II; MyD88: myeloid differentiation primary response gene 88; E2F1: E2F transcription factor 1; TGF- $\beta$ : transforming growth factor beta; COL1A1: collagen type 1 alpha 1; COL4A5: collagen type 4 alpha 5; Smad7: SMAD family member 7.

impaired immune responses in chronic HBV carriers.<sup>69</sup> However, the role of these miRNAs in persistent HBV infection remains elusive and still awaits further investigation.<sup>69</sup>

### miRNAs in HBV-related liver fibrosis and cirrhosis

Long term persistence of HBV infection can result in the development of liver inflammation and injury. HBV itself is considered to be a noncytopathic virus, and it is usually accepted that the process of destruction of HBV-infected cells is due to cell-mediated immune responses.<sup>63</sup> Persistent inflammation has been shown to be the driving force leading to liver fibrosis, generated by an imbalance between production of extracellular matrix (ECM) by hepatic stellate cells (HSCs).<sup>70</sup> This imbalance results in an excessive accumulation of ECM and fibrogenesis.<sup>70</sup> A number of studies have

focused on the role of miRNAs in HBV-associated liver fibrosis/cirrhosis and unveiled the miRNA expression profiles during this phase (Table 1). Guo *et al.* identified several miRNAs that were differentially expressed during HSCs activation and showed that different signaling pathways, already reported to take part in HSCs activation, were modulated by miRNAs.<sup>71</sup> Lakner *et al.* reported that miR-19b inhibited HSC-mediated fibrogenesis by regulating transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling in activated HSCs.<sup>72</sup> In addition, Venugopal *et al.* showed that liver fibrosis may cause downregulation of miR-150 and miR-194 in HSCs and that their overexpression could repress HSCs activation.<sup>73</sup> These miRNAs were observed to be downregulated in a feedback mechanism that occurs during the early phases of liver regeneration.<sup>73</sup> Roderburg *et al.* applied a systematic approach to identify miRNAs involved in liver fibrosis using the well-established model of carbon tetrachloride (CCl<sub>4</sub>) treatment for hepatic

fibrogenesis in mice.<sup>74</sup> They identified a panel of miRNAs that was specifically regulated in fibrotic-livers from mice treated with CCl<sub>4</sub> compared to livers from control mice. Within those, the miR-29-family members (miR-29a/b/c) were significantly downregulated in CCl<sub>4</sub>-treated mice.<sup>74</sup> Interestingly, decreased expression of miR-29 in these animals was significantly correlated with the degree of liver fibrosis. The same study showed that miR-29b was downregulated during the activation of HSCs in a TGF- $\beta$  and lipopolysaccharide/nuclear factor kappa B (NF- $\kappa$ B)-dependent manner, whereas overexpression of miR-29b in murine HSCs resulted in downregulation of collagen expression.<sup>74</sup> Another study reported that miR-33a levels in liver tissue from chronic HBV-infected patients increased significantly in a fibrosis progression manner.<sup>75</sup> Interestingly, stimulation of HSCs with TGF- $\beta$ 1 led to a critical increase of miR-33a.<sup>75</sup>

Hypersplenism is common in portal hypertension in patients with liver cirrhosis and results mostly from the increased phagocytosis and destruction of blood cells by splenic macrophages. To determine whether miRNA expression is altered in hypersplenism during HBV-related cirrhosis, Li *et al.* analyzed the entire miRNAome in macrophages from normal and portal hypertensive spleen samples.<sup>76</sup> Compared to normal spleen samples, 99 miRNAs were found to be differentially expressed in splenic macrophages associated to portal hypertension, with a remarkable overexpression of miR-615-3p.<sup>76</sup> These findings suggest that miRNAs could be novel regulators in hypersplenism in patients with HBV-related cirrhosis.

### miRNAs in HBV-related HCC

Chronic HBV infection remains a major etiological factor of HCC worldwide, increasing the risk for HCC development by 100-fold compared to uninfected individuals.<sup>62</sup> Many reports have shown that HCC develops through aberrant activation of various signaling pathways involved in cell proliferation, differentiation, and angiogenesis.<sup>77</sup> However, the precise mechanisms underlying HBV related-HCC development remain unclear.<sup>77</sup> It is now well-established that miRNAs play a critical role in HBV-associated hepatocarcinogenesis, as they can function as oncogenes or tumor suppressor genes depending on the cellular function of the target genes (Table 2).<sup>78</sup> A study performed by Ura *et al.* showed that HBV infection induced specific sets of miRNAs during hepatocarcinogenesis, and their targets included genes involved in pathways related to cell proliferation, apoptosis, DNA damage, and signal transduction.<sup>79</sup> Several research groups found that the liver-specific miR-122 was repressed in HBV-expressing HCC cell lines and in clinical tumor specimens.<sup>80–82</sup> Suppression of miR-122 led to upregulation of its target N-myc downstream-regulated gene 3 (NDRG3), a tumor promoter, contributing to a malignant phenotype.<sup>80</sup> In another study, low miR-122 levels promoted an overexpression of pituitary tumor-transforming gene 1 (PTTG1) binding factor (PBF), leading to increased transcriptional activity of PTTG and HCC cell growth and invasion.<sup>81</sup> Furthermore, decreased miR-122 expression was linked to upregulation of cyclin G1, which promoted dephosphorylation of Mdm-2, a repressor protein of p53, and led to HCC with unrestricted growth.<sup>82</sup> A recent study showed that decreased miR-122 led to increased expression of its target UDP-N-acetyl- $\alpha$ -D-galactosamine polypeptide N-acetylgalactosaminyltransferase-10 (GALNT10), conferring a malignant phenotype to HCC cells by modifying

EGFR O-glycosylation and activating the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway.<sup>83</sup> Given the functions of miR-122, investigators sought to explain its low expression in HBV-infected livers and found that miR-122 was downregulated by HBx.<sup>83,84</sup> HBx is a multifunctional HBV-encoded protein that acts as a pleiotropic transactivator and modulates host gene expression by interacting with various cellular transcription factors and signaling pathways.<sup>77</sup> Besides miR-122, several tumor suppressor-like miRNAs were shown to be suppressed by HBx. Decreased expression of miR-101 by HBx resulted in overexpression of DNA methyltransferase 3A (DNMT3A), promoting aberrant DNA hypermethylation of several tumor suppressor genes.<sup>85</sup> In addition, under-expression of miR-101 contributed to increased levels of myeloid cell leukemia sequence 1 (Mcl-1), an antiapoptotic member of the Bcl-2 family.<sup>86</sup> Antitumorigenic function of miR-148a was repressed by HBx via inhibition of p53-mediated activation of miR-148a.<sup>87</sup> This led to increased expression of hematopoietic pre-B cell leukemia transcription factor-interacting protein (HPIP) and subsequent activation of the mammalian target of rapamycin (mTOR) pathway, which plays a critical role in cancer cell growth.<sup>87</sup> HBx also downregulated the let-7 family of miRNAs, widely viewed as potential growth suppressor miRNAs in many cancers.<sup>88</sup> Particularly important is the ability of the HBx protein and its RNA to act synergistically to downregulate another tumor suppressor pathway involving the miR-15a/miR-16-1 cluster, which contributes to the activation of the proto-oncogene c-Myc.<sup>44</sup> Epigenetic modulation of miRNA expression could be also an important mechanism underlying HBV-mediated hepatocarcinogenesis. For example, HBx induces epigenetic repression of two tumor-suppressors, miR-132 and miR-205, through DNA methylation of its promoters.<sup>89,90</sup> Chen *et al.* found in HBV-HCC tissues that increased miR-129-2 methylation was associated with subsequent miR-129-2 suppression.<sup>91</sup> This suppression may be involved in HCC development through enhancing oncogenic SOX4 expression.<sup>91</sup> Moreover, miR-152 was shown to be targeted and downregulated by HBx, causing an upregulation of DNMT1 activity and consequent DNA methylation of promoters of many tumor suppressor genes.<sup>99</sup> Subsequent studies reported that miRNAs involved in the inhibition of cell proliferation (miR-15b, miR-22, miR-26a/b, miR-29c, miR-145, and miR199-a-3p) were downregulated in HBV-related HCC.<sup>92–98</sup>

Several oncogene-like miRNAs have been found to be upregulated in HBV-related HCC. Connolly *et al.* found that miR-17-92 cluster and miR-21 were significantly increased in human HBV-positive HCC tissues and woodchuck hepatitis virus-positive HCCs.<sup>99</sup> They also demonstrated that miR-17-92 and miR-21 contributed to a malignant phenotype by promoting cell proliferation and anchorage-independent growth.<sup>99</sup> Oncogenic properties of the miR-17-92 cluster was shown to be related to a complex interaction network involving HBx protein and the transcription factors c-Myc and E2F1. Indeed, HBx transactivation of c-Myc led to induction of miR-17-92 expression by binding to its promoter.<sup>100</sup> In turn, miR-17-92 inhibited E2F1 expression, causing defects in cell cycle control and HCC formation.<sup>100</sup> Regarding miR-21, its expression was mediated by HBx-activation of the IL-6 pathway followed by activation of STAT3, a positive regulator of the miR-21 promoter.<sup>101</sup> MiR-21 enhanced cell proliferation by targeting tumor suppressor genes, including phosphatase and tensin homolog (PTEN) and programmed cell death protein-4 (PDCD4).<sup>102,103</sup> Suppression of PTEN activity has been also

**Table 2. Summary of oncogene- and tumor suppressor-like miRNAs involved in HBV-related HCC**

miRNA	Expression <sup>a</sup>	Target gene (s)	Functions
Tumor suppressor-like miRNAs			
Let-7	Down	STAT3	Inhibits cell proliferation <sup>88</sup>
miR-15a/miR-16-1	Down	Bcl-2, Cyclin D1	Inhibits cell proliferation <sup>44</sup>
miR-15b	Down	Fut2	Inhibits cell proliferation <sup>92</sup>
miR-22	Down	CDKN1A	Inhibits cell proliferation <sup>93</sup>
miR-26a/c	Down	IL-6	Inhibits cell proliferation <sup>94</sup>
miR-29c	Down	TNFAIP3	Inhibits cell proliferation and promotes apoptosis <sup>95</sup>
miR-101	Down	DNMT3A	Downregulates DNA hypermethylation <sup>85</sup>
miR-122	Down	NDRG3, GALNT10 Cyclin G1, PTTG1	Promotes cell apoptosis, inhibits cell proliferation and invasion <sup>80-83</sup>
miR-129-2	Down	SOX4	Reduces cell proliferation and clonogenicity <sup>91</sup>
miR-132	Down	Akt	Inhibits cell proliferation <sup>90</sup>
miR-145	Down	HDAC2, ADAM17	Inhibits cell proliferation and cell invasion <sup>96,97</sup>
miR-148a	Down	HPIP, mTOR	Inhibits cell growth, epithelial-to-mesenchymal transition, invasion, and metastasis <sup>87</sup>
miR-152	Down	DNMT1	Reduces global DNA hypermethylation <sup>59</sup>
miR-199-a-3p	Down	mTOR, c-Met	Blocks G1-S transition of the cell cycle, thereby inhibiting cell growth, induces cell apoptosis, impairs invasion capability <sup>98</sup>
miR-205	Down	E2F1	Inhibits cell proliferation <sup>89</sup>
Oncogene- like miRNAs			
miR-17-92	Up	E2F1	Enhances cell growth and anchorage-independant growth <sup>99,100</sup>
miR-18a	Up	ESR1	Suppresses ER $\alpha$ synthesis and stimulates cell proliferation <sup>55</sup>
miR-21	Up	PTEN, PDCD4	Promotes cell growth and anchorage-independant growth <sup>102,103</sup>
miR-27a	Up	Unknown	Enhances cell proliferation, activates cell cycling, Promotes migration and invasion <sup>110</sup>
miR-29a	Up	PTEN	Promotes cell invasion/migration <sup>102</sup>
miR-143	Up	FNDC3B	Promotes cell invasion and tumor metastasis <sup>105</sup>
miR-155	Up	SOX6	Promotes cell growth <sup>111</sup>
miR-181	Up	Fas, E2F5	Inhibits cell apoptosis and promotes tumor cell growth <sup>108,109</sup>
miR-222	Up	p27 (kip1), PTEN	Promotes cell growth and migration <sup>102</sup>
miR-224	Up	SMAD4	Promotes cell growth, migration and invasion <sup>96,107</sup>
miR-602	Up	RASSF1A	Inhibits cell apoptosis and promotes cell growth <sup>106</sup>

<sup>a</sup>Denotes endogenous expression in HBV-infected liver tissues or HBV-infected cell lines.

Down: downregulate; Up: upregulate; STAT1: signal transducer and activator of transcription 1; Fut2: fucosyltransferase 2; CDKN1A: cyclin-dependent kinase inhibitor 1A; IL-6: interleukin 6; TNFAIP3: tumor necrosis factor alpha-induced protein 3; DNMT3A: DNA methyltransferase 3A; NDRG3: N-myc downstream-regulated gene 3; GALNT10: UDP-N-acetyl- $\alpha$ -D-galactosamine polypeptide N-acetylgalactosaminyltransferase-10; SOX4: sex-determining region Y box 4; HDAC2: histone deacetylase 2; IGF1R: insulin-like growth factor 1 receptor; ADAM17: ADAM metalloproteinase domain 17; HPIP: pre-B cell leukemia transcription factor-interacting protein; mTOR: mechanistic target of rapamycin; DNMT1: DNA methyltransferase 1; E2F1: E2F transcription factor 1; ESR1: Estrogen receptor 1; PTEN: phosphatase and tensin homolog; PDCD4: programmed cell death 4; FNDC3B: fibronectin type III domain containing 3B; SOCS1: suppressor of cytokine signaling 1; SOX6: Sex-determining region Y box 6; E2F5: E2F transcription factor 5; SMAD4: SMAD family member 4; RASSF1A: Ras-associated domain family member 1A.

attributed to miR-29a and miR-222, which are upregulated by HBx to promote HCC cell growth and migration.<sup>102,104</sup> Another relevant miR-222 target is the p27 (kip1) protein, a cell cycle inhibitor and tumor suppressor.<sup>102</sup> In addition, HBx can induce the expression of nuclear factor-kappa B (NF- $\kappa$ B), an activator of miR-143 expression in HBV-related HCC. Overexpression of miR-143 inhibited the expression of fibronectin type III domain containing 3B (FNDC3B), a

regulator of cell motility, thus stimulating HCC metastasis.<sup>105</sup> Another HBx-upregulated miRNA was miR-602, which was shown to inhibit the tumor suppressive function of Ras-associated domain family member 1A (RASSF1A).<sup>106</sup> Notably, in chronic HBV hepatitis, liver cirrhosis to HCC, there is a progressive trend where an increase in miR-602 expression is correlated with a decrease in RASSF1A level, suggesting that miR-602 may promote HCC in HBV carriers at a very

early stage.<sup>106</sup> Similarly, a gradual increase in the expression of miR-224 was observed from premalignant dysplastic nodules to HCC.<sup>96</sup> High miR-224 levels were found to be correlated with downregulation of its target gene Smad4, a tumor suppressor in liver tumorigenesis.<sup>107</sup> Epigenetic modifications in miRNA promoters represent another mechanism whereby HBx affects onco-miRNA expression. MiR-181a was identified as an epigenetic target of HBx, which upregulates its expression in both HCC cell lines and specimens.<sup>108</sup> MiR-181a promoted cell growth *in vitro* and tumor formation *in vivo* by targeting the transcription factor E2F5, a key regulator of cell growth.<sup>108</sup> Another study reported that miR-181a decreased apoptosis by inhibiting expression of the TNF receptor superfamily member 6 (Fas), an important inducer of apoptosis.<sup>109</sup> The ability to promote cell growth was also demonstrated for miR-27a, which was upregulated in both HBV-positive HCC tissues and hepatoma cell lines.<sup>110</sup> MiR-27a may also enhance migration and invasion of HCC cells.<sup>110</sup> Moreover, a serial cohort study performed by Liu *et al.* reported an important miRNA-mediated regulatory mechanism that may promote malignant transformation in hepatocytes by affecting the estrogen receptor  $\alpha$  (ER $\alpha$ ) signaling pathways.<sup>55</sup> Estrogen plays an important role in protecting against the development of HBV-related HCC, which partially explains the sex disparity characteristic in HCC. However, it has been found that miR-18a directly suppress ER $\alpha$  mRNA translation, thereby blocking the tumor-protective activity of estrogens in women.<sup>55</sup> In correlation to these data, increased levels of miR-18a in female HCC tissues correlated with reduced ER $\alpha$  expression. Similar results were also found in cell lines where overexpression of miR-18a not only decreased ER $\alpha$  levels but also stimulated cell proliferation.<sup>55</sup> Additionally, aberrant expression of miRNAs involved in the modulation of immune responses during HBV infection plays a significant role in the development of immune-mediated liver damage, thereby promoting HCC. MiR-155 is well-known for its role as an enhancer of antiviral immunity against HBV.<sup>64</sup> However, Wang *et al.* showed that overexpression of miR-155 can lead to prolonged exposure to inflammatory responses, which contribute to progressive liver damage and HCC.<sup>46</sup> Another study reported that miR-155 may promote HCC by targeting sex-determining region Y box 6 (SOX6), a transcriptional regulator that inhibits cell growth by stimulating the p21 growth regulator in a p53-dependent manner.<sup>111</sup>

### miRNAs as diagnostic biomarkers of HBV-related liver disease

One of the major challenges in HBV research is identifying effective noninvasive biomarkers of HBV-related liver disease. Such markers can allow for rapid intervention and improved patient outcomes after curative treatment. The discovery of cell-free circulating miRNAs, which were able to mirror pathophysiological conditions in human bodily fluids, has opened new avenues for application of miRNAs as biomarkers for liver disease.<sup>112</sup> Moreover, their accessibility and high stability in the circulatory system make them ideal biomarkers for the diagnosis of disease at an early, presymptomatic stage.<sup>112</sup> Several studies have analyzed miRNA profiles in sera from HBV carriers in order to identify useful biomarkers (Table 3). Zhang *et al.* selected miRNA biomarkers from patients with HBV infection, patients with skeletal muscle disease, and healthy subjects.<sup>113</sup> They showed that increased serum miR-122 levels correlated with

histopathologic alterations and appeared earlier compared with alanine aminotransferase (ALT) levels, which remained within the reference intervals.<sup>113</sup> Also, increased serum miRNA-122 levels were more specific for liver injury than for other organ damage.<sup>113</sup> Other studies showed that serum miR-122 may allow for discrimination of HCC patients from healthy subjects but was not useful for distinguishing HCC cases from HBV carriers.<sup>114,115</sup> Serum miR-223 together with miR-125b-5p have been reported as promising biomarkers of very early HBV-positive HCC, even in advanced stages of liver disease due to chronic hepatitis B.<sup>116</sup> Other miRNAs have been suggested to have great diagnostic value. Serum miR-124 levels have been found to be significantly higher in HBV-infected patients with considerable liver necroinflammation than HBV-patients without or with mild necroinflammation.<sup>117</sup> In addition, after antiviral therapy, serum miR-124 levels considerably declined, in association with histological improvement.<sup>117</sup> Huang *et al.* showed that serum miR-29 levels were significantly higher in patients with minimal fibrosis than patients with liver cirrhosis (LC), suggesting that patients with minimal liver inflammation tended to express higher serum miR-29 levels than those with advanced inflammation.<sup>118</sup> MiR-18a has been shown to have a significant diagnostic value for HBV-related HCC screening.<sup>119</sup> MiR-18a yielded an area under the curve (AUC) of receiver operating characteristic (ROC) of 0.88 (specificity: 75%; sensitivity: 86%) in discriminating HBV-related HCC from healthy controls, and an AUC of ROC of 0.78 (specificity: 70%; sensitivity: 77%) in discriminating HBV-related HCC from chronic hepatitis B or HBV-related LC.<sup>119</sup> A recent study by Xie *et al.* reported that miR-101 can serve as a potential biomarker to differentiate HBV-HCC from HBV-LC.<sup>120</sup> Serum miR-101 is downregulated in HBV-HCC patients compared with HBV-LC patients. ROC analysis of serum miR-101 yielded an AUC of 0.98 (sensitivity: 96%; specificity: 90%) when differentiating between HBV-HCC and HBV-LC.<sup>120</sup> Interestingly, serum miR-101 was superior to alpha-fetoprotein (AFP) for diagnosing HBV-HCC derived from HBV-LC. Combining serum miR-101 with AFP had no advantage over serum miR-101 alone for detecting HBV-HCC derived from HBV-LC.<sup>120</sup> The frequent finding of dysregulated miRNAs in HBV-related HCC has sparked several research groups to conduct searches to identify a panel of serum miRNAs that may be potentially useful in the diagnosis of HBV-related HCC. Zou *et al.* identified a serum miRNA panel (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, and miR-801) that provided a high diagnostic accuracy of HBV-related HCC and was able to successfully distinguish HCC patients from chronic hepatitis and liver cirrhosis patients and healthy cases.<sup>121</sup> Furthermore, Li *et al.* successfully identified 13 HBV-specific miRNAs that can clearly discriminate not only HBV cases from healthy controls and HCV cases but also HBV-positive HCC cases from controls and HBV cases.<sup>122</sup> Six of these miRNAs (miR-1, miR-25, miR-92a, miR-206, miR-375, and let-7f) were significantly elevated in HCC cases, and two miRNAs, miR-375 and miR-92a, were previously identified as HBV-specific. In addition, the use of three of these miRNAs (let-7f, miR-25, and miR-375) as biomarkers, clearly separated HCC cases from controls, and miR-375 alone had an AUC of ROC of 0.96 (specificity: 96%; sensitivity: 100%) for HCC diagnosis.<sup>122</sup>

Interestingly, a study by Novellino *et al.* provided evidence that HBV subviral particles carry hepatocellular miRNAs to be released from HBV-infected cells into the blood.<sup>123</sup> The experimental strategy consisted on the isolation of HBV subviral



**Table 3. Clinical relevance of miRNAs in the diagnosis of HBV-related liver disease**

miRNA	Expression	Clinical relevance
miR-18a	Up <sup>a</sup>	Biomarker to detect HCC patients from healthy subjects and patients with chronic hepatitis or liver cirrhosis <sup>119</sup>
miR-29	Down <sup>b</sup>	Biomarker of severe liver necroinflammation and cirrhosis <sup>118</sup> Negative correlation with the severity of liver injuries <sup>118</sup>
miR-101	Up <sup>a</sup>	Biomarker to differentiate HBV-HCC from HBV-liver cirrhosis <sup>120</sup> Screening HCC patients from healthy subjects <sup>120</sup> Increased serum miR-101 levels correlated with tumor size <sup>120</sup>
miR-122	Up <sup>a,b</sup>	Biomarker of severe liver necroinflammation <sup>113,114</sup> Positive correlation with the severity of liver injuries <sup>113,114</sup> Potential biomarker for detection of HCC in healthy subjects <sup>115</sup>
miR-124	Up <sup>b</sup>	Biomarker for considerable liver necroinflammation in patients with chronic hepatitis B, particularly in those with normal or mildly increased ALT level <sup>117</sup> Declined serum miRNA-124 after antiviral treatment was a promising biomarker of histological remission <sup>117</sup>
miR-125b-5p and miR-223-3p	Up <sup>a</sup> Down <sup>a</sup>	Biomarkers of HBV-positive HCC in very early, even at chronic hepatitis B stage of liver disease <sup>116</sup>
miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, and miR-801	Up <sup>a</sup>	Biomarkers to detect HCC patients from those with chronic hepatitis, liver cirrhosis and healthy cases <sup>121</sup>
let-7f, miR-25, and miR-375	Up <sup>a</sup>	Biomarkers for HBV infection and HBV-related HCC <sup>122</sup> High sensitivity to detect HBV-positive cases from HCV-positive cases and healthy subjects <sup>122</sup> High sensitivity to detect HBV-positive HCC cases from those with chronic hepatitis B and healthy cases <sup>122</sup>

<sup>a</sup>Denotes serum miRNA level in patients with HBV-related HCC compared to patients with chronic hepatitis, liver cirrhosis or healthy controls.

<sup>b</sup>Denotes serum miRNA level in patients with moderate-to-severe liver necroinflammation compared to those with no or mild necroinflammation.

Down: downregulate; Up: upregulate

particles from sera of 11 HBV-infected patients using selective immunoprecipitation and screening of human miRNAs in each sample by real-time quantitative polymerase chain reaction (PCR).<sup>123</sup> Thirty-nine miRNAs were identified to be significantly associated with the immunoprecipitated HBV subviral particles. Most of them were liver-specific (miR-27a, miR-30b, miR-122, miR-126, and miR-145) and belonged to the previously described repertoires of miRNAs differentially expressed in liver development, disease, fibrosis, and/or regeneration (miR-122, miR-27a, miR-29a, miR-30a, miR-30b, miR-126, miR-145, and miR-223).<sup>123</sup> These findings suggested that HBV subviral particles provide a new noninvasive tool for liver-specific miRNA profiling, allowing for the study of dynamic changes in hepatocellular miRNA expression during HBV infection in serum samples.

### miRNA-based therapy for HBV-related liver disease

The goal of hepatitis B treatment is to improve patients' quality of life and increase survival by preventing progression to liver cirrhosis and HCC.<sup>4</sup> Achieving this goal is done by sustained suppression of HBV replication, which is accompanied by reduced histological activity of chronic hepatitis, leading to decreased risk of disease progression.<sup>4</sup> The current anti-HBV drugs, including IFN $\alpha$  and nucleos(t)ide analogues, may effectively suppress HBV replication and induce remission of liver disease. However, the side effects of IFN $\alpha$  and the emergence of viral resistance during long-term therapy with nucleos(t)ide analogues make these therapeutic approaches

far from satisfactory.<sup>4</sup> Furthermore, after treatment withdrawal, most patients do not manifest durable control of infection and viral reactivation occurs.<sup>4</sup> The persistence of intrahepatic cccDNA, the template of viral transcription, is the main cause of viral reactivation, given that IFN $\alpha$  and nucleos(t)ide analogues are unable to eradicate cccDNA from the liver.<sup>4,56</sup> Therefore, developing novel antivirals that improve viral clearance with minimal side effects and potentially eradicate cccDNA is required for a cure or durable control of HBV infection.

The close relationship between miRNA expression and HBV-related liver disease has fueled an interest in exploiting miRNAs as therapeutic targets for the control and eradication of hepatitis B. Ely *et al.* have taken advantage of the natural RNA polymerase II-mediated transcriptional control of cellular miRNAs and designed RNA polymerase II promoter cassettes that transcribe anti-HBV primary miRNA (pri-miR)-122 and pri-miR-31 shuttles.<sup>124</sup> MiR-122 and miR-31 backbones were selected because these sequences were predicted to favor intrahepatic processing of adapted anti-HBV shuttles. In addition, miR-122 is liver-specific and pri-miR-31 is efficiently processed by Drosha. They found that anti-HBV miRs generated an intended guide strand of ~21 nt, which confirmed that these sequences functioned as pri-miR mimics and were processed in a manner similar to those of natural pri-miRs.<sup>124</sup> Studies on effects of these anti-HBV miRs shuttles *in vitro* and *in vivo* demonstrated a potent silencing of markers of HBV replication with no evidence of toxicity or disruption of independent miR-mediated silencing.<sup>124</sup> In a

similar strategy, Chattopadhyay *et al.* developed linear expression cassettes that produced anti-HBV miRs shuttles. Silencing of HBV markers of replication was efficient (>75%) in cultured cells and *in vivo*.<sup>125</sup> Furthermore, a knock down of approximately 95% HBV replication was attained in a hydrodynamic infection model.<sup>125</sup> Together, these findings suggest that employing miRs shuttles is potentially valuable for treating HBV infection. However, besides antiviral efficiency, improvement of liver expression and efficient delivery of miRs shuttles is potentially important to limit nonspecific effects. In this context, Mowa *et al.* investigated the utility of the liver-specific murine transthyretin receptor (MTTR) Pol II promoter for expression of anti-HBV pri-miRs by incorporating MTTR promoter into the helper dependent adenoviral vectors (HDAds) carrying pri-miR expression cassettes.<sup>126</sup> The results revealed that MTTR-expressed pri-miRs induced a knockdown of up to 94% of HBV replication in HBV transgenic mouse model and did not result in hepatotoxicity.<sup>126</sup> Notably, HD Ads affected efficient delivery of pri-miRs targeting HBV. Another important property of HD Ads is their capacity for accommodating large transgene sequences.<sup>126</sup> Therefore, as well as miRs activating cassettes, other anti-HBV elements may be incorporated into HD Ads to augment their therapeutic efficacy. Recent advances regarding the role of engineered gene-modifying enzymes such as transcription activator like effector nucleases (TALENs) and zinc finger nucleases in cleavage of HBV cccDNA have been impressive.<sup>127</sup> These enzymes may be used in combination with HBV-targeting miRNAs activators to efficiently control or eradicate HBV infection. Another improvement in miRNA therapy is the use of lentiviral miRNA-based system (LVshHBS) expressing siRNAs targeting the HBsAg gene of HBV genome.<sup>128</sup> LVshHBS significantly inhibited the HBsAg mRNA and protein levels in HepG2.2.15 cells, while HBsAg secretion into the culture supernatant was decreased by 70%.<sup>128</sup> Also, in LVshHBS-transduced HepG2.2.15 cells, expression of genes involved in cell cycle and oncogenesis was downregulated, indicating that LVshHBS not only inhibited HBV replication but also inhibited the growth of HCC.<sup>128</sup> Although these experimental studies have confirmed the capacity of miRNA-based antivirals to treat hepatitis B, adequate evaluation of their efficacy is required and further research is needed to determine their impact alone or in combination to other antiviral elements on HBV eradication.

## Conclusions

In summary, this review adds to a growing body of evidence concerning the complex interactions between host-encoded miRNAs and HBV. MiRNAs are considered as important cellular regulatory molecules that profoundly modulate HBV genes expression. Their functions are mainly mediated by interacting with HBV transcripts, cellular genes, and signaling pathways that have a critical role in viral replication and pathogenesis. In turn, HBV modulates the expression of miRNAs in HBV-infected cells and promotes the development of liver disease and HCC. It is true that these findings regarding HBV-miRNA interactions have contributed to important progress in understanding HBV pathogenesis. However, further research is needed to clarify the mechanisms by which host miRNA expression is altered during HBV infection and the role of miRNA alterations in cellular functions and liver disease processes. Regarding clinical applications, altered expression profiles of cell-free circulating miRNAs in

serum/plasma between the different stages of HBV-mediated liver disease have opened new insights into the utility of miRNAs as noninvasive biomarkers. This is particularly true in the diagnosis of liver inflammation and HCC at an early stage and to predict the risk of disease progression. Although a large number of miRNAs have been identified as promising biomarkers, it is only practical to use the most sensitive and specific ones with complementary functions in clinical settings. Additionally, it is very important to elucidate the mechanisms of how cell-free circulating miRNAs are changed in the serum/plasma of HBV-infected patients and whether these circulating miRNAs influence HBV pathogenesis. Understanding the role of altered miRNA expression either in serum or liver provides a strong basis for targeting miRNAs in the treatment of hepatitis B, and it is likely that miRNA-based therapies will be part of the therapeutic armamentarium against HBV in the future.

## Conflict of interest

None

## Author contributions

Review design (BK, SB), collection of data (BK), writing the manuscript (BK, SB), revising the article for important intellectual content (SB, HSA, SE).

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