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REVIEW

Micro-RNAs in hepatitis B virus-related chronic liver diseases and hepatocellular carcinoma

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that modulate gene expression at the post-transcriptional level by affecting both the stability and translation of complementary mRNAs. Several studies have shown that miRNAs are important regulators in the conflicting efforts between the virus (to manipulate the host for its successful propagation) and the host (to inhibit the virus), culminating in either the elimination of the virus or its persistence. An increasing number of studies report a role of miRNAs in hepatitis B virus (HBV) replication and pathogenesis. In fact, HBV is able to modulate different host miRNAs, particularly through the transcriptional transactivator HBx protein and, conversely, different cellular miRNAs can regulate HBV gene expression and replication by a direct binding to HBV transcripts or indirectly targeting host factors. The present review will discuss the role of miRNAs in the pathogenesis of HBV-related diseases and their role as a biomarker in the management of patients with HBV-related disease and as therapeutic targets.

Key words: Hepatitis B virus infection; MicroRNAs; Hepatitis B virus pathogenesis; Molecular mechanisms

Core tip: This review article will focus on the emerging puzzle of hepatitis B virus (HBV)-hepatocyte interaction *via* miRNAs, indirectly or directly modulating HBV

replication and pathogenesis, and thus on the role of microRNAs in the natural history of HBV infection. We evaluated the literature on their possible future role as a biomarker in the management of patients with HBV-related disease and as therapeutic targets.

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INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNAs that modulate gene expression at the post-transcriptional level by affecting both the stability and translation of complementary mRNAs[1]. MiRNAs play crucial roles in a variety of physiological processes, such as cell development and differentiation[2,3]. miRNA mutations, dysregulation of their expression or dysfunction of miRNA biogenesis lead to an interference with biological pathways involved in the development and evolution of human diseases, including cancer, cardiovascular diseases and infectious diseases[4-8].

About the host-virus interplay, various studies have shown that miRNAs are important regulators in the conflicting efforts between the virus (to manipulate the host for its successful propagation) and the host (to inhibit the virus), culminating in either the elimination of the virus or its persistence. In fact, different viruses encode miRNAs that modulate not only the viral mRNA expression to regulate its own lifecycle, but also the host mRNA expression to establish a cellular environment resulting favorable to their replication; on the other hand, host cells encode miRNAs counteracting viral replication[8-10]. An increasing number of studies report a role of miRNAs in hepatitis B virus (HBV) replication and pathogenesis. HBV is a non-cytopathic virus belonging to the Hepadnaviridae family. It has a 3.2 kb partially double-stranded DNA, showing 4 known open reading frames (ORFs): The S region, which contains three in-frame initiator codons, codes for the small, medium and large surface antigen (HBsAg) proteins; the C region, with two initiator codons for the core and "e" antigen (HBcAg, HBeAg); the P frame, coding for an RNA-dependent DNA polymerase; and the X region, coding for a protein regulating the transcription of both viral and cellular genes[11].

The present review will discuss the emerging puzzle of HBV-hepatocyte interaction *via* miRNAs, indirectly or directly modulating HBV replication and pathogenesis, and thus will focus on the role of microRNAs in the natural history of HBV infection and on their possible future role as biomarkers in the management of patients with an HBV-related disease and as therapeutic targets.

HBV INFECTION

Despite the universal vaccination campaigns against HBV infection undertaken in many countries over the last two decades, this infection remains a global health problem. In 2015, the WHO estimated that nearly 3.5% of the world population live with chronic HBV infection[12], with about 800000 deaths per year (460000 for complications of liver cirrhosis and 340000 for hepatocellular carcinoma-HCC)[13]. For non-immune/non-infected subjects any parenteral or mucosal exposure to blood, blood products or blood-contaminated material should be considered a risk for acquiring HBV infection[14]. In addition, being present in semen and cervical secretions at infectious concentrations, HBV is also transmitted by sexual and vertical routes[15]. The age at the time of infection strongly modulates the progression to chronicity, which occurs in around 90% of subjects infected at birth, a rate progressively decreasing with the increase in age at infection, up to 2%-5% in the adult population[16].

The geographical distribution of HBV chronic carriers is highly variable, ranging from 0.7%-1% in developed western countries to 8% or more in some countries in sub Saharan Africa and South-East Asia, depending on environmental factors, earning potential, educational levels and lifestyles. In countries with an intermediate-high level of endemicity, HBV infection is most frequently acquired at birth from an HBeAg-positive mother or through horizontal transmission in early childhood by household contacts (most frequently between siblings); in these cases the rate of progression to chronicity is high, which keeps the prevalence of infection in these geographical areas intermediate or high. Intravenous drug addiction is the major risk factors for acquiring HBV infection in countries with a low HBV endemicity like Western Europe[15] and North America[17], whereas promiscuous unprotected sexual activity is a main risk factor worldwide.

The clinical presentation of chronic HBV infection is variable, ranging from asymptomatic carriage of the virus to liver cirrhosis with or without HCC[18]. Patients with chronic hepatitis develop liver cirrhosis with an incidence of 1-5 per 100 persons/year, with a 5-year cumulative probability of progression ranging from 8% to 20%, depending on the degree of disease activity, HBeAg/anti-HBe status, the HBV load and co-morbidities[19]. The incidence of HCC in patients with HBV-related liver cirrhosis is estimated around 3.7 persons/year[12]. HCC may develop, but with a lower frequency also in patients with chronic HBV infection without cirrhosis, depending on demographic (male sex, older age), viral (higher levels of HBV replication; co-

Table 1 miRNAs involved in hepatitis B virus infection

miRNA	Validated target	Effect on HBV	Ref.
miRNA targeting HBV transcripts			
miR-15a/miR-16-1	HBx	↓	[28,33]
miR-20a/miR-92a-1	Polymerase/HBx	↓	[29]
miR-122	Polymerase/HBc	↓	[27]
miR-125a	HBsAg	↓	[23-26,32]
miR-199a-3p	HBsAg	↓	[22]
miR-205	HBx	↓	[31]
miR-210	HBsAg pre-S1 region	↓	[22]
miR-1231	HBc	↓	[30]
miRNAs targeting HBV regulators			
miR-1	HDAC4	↑	[51-53]
miR-15b	HNF1	↑	[50]
miR-34a	CCL22	↓	[60]
miR-122	Cyclin G1	↑	[43-45]
	HO-1		[45,46]
miR-130a	PGC1 PPAR	↓	[41]
miR-141	PPAR	↓	[24,40]
miR-146a	STAT1	↑	[58]
miR-152	DNMT-1	↑	[54-57]
miR-155	C/EBP	↓	[35-38]
	SOCS1	↓	[39]
miR-370	NFIA	↑	[49]
miR-372/373	NFIB	↑	[48]
miR-501	HBXIP	↑	[42]
miR-548a	IFNλ-1	↑	[59]

MiRNAs are listed according to their increasing number name. ↓: Suppress HBV infection; ↑: Promote HBV infection; HBV: Hepatitis B virus.

infections) and environmental (alcohol abuse) factors[20].

MIRNAS ASSOCIATED WITH HBV INFECTION

So far, there is no experimental evidence confirming the synthesis of miRNAs by HBV, though a computational analysis suggested one HBV pre-miRNA candidate[21]; however, HBV is able to modulate different host miRNAs, particularly through the transcriptional transactivator HBx protein. Conversely, different cellular miRNAs can regulate HBV gene expression and replication by a direct binding to HBV transcripts or indirectly targeting host factors, which in turn modulate viral replication.

Cellular miRNAs directly targeting HBV transcripts

The first miRNAs found to bind viral transcripts and repress HBV gene expression and replication were miR-210, miR-199-3p and miR-125a-5p. They were identified by two different experimental approaches (Table 1). In one procedure, Zhang *et al*[22] systematically screened for cellular miRNAs affecting HBV replication by a loss-of-function approach: Antagomirs targeting 328 miRNAs were transfected into a HepG2.2.15 cell model supporting full HBV replication, and then HBV surface antigen (HBsAg) expression was measured. Among the six miRNAs whose antagomirs caused an increase in HBsAg expression, miR-199a-3p and miR-210 were predicted to bind the HBsAg coding region and the HBV pre-S1 region, respectively; the direct effect of miRNAs on viral transcripts were further validated by GFP reporter assay[22]. In a different approach, Potenza *et al*[23] first predicted the potential targets of human hepatic miRNAs in different HBV sequence subtypes. The most promising targets were then subjected to a validation test based on cultured hepatic cells and luciferase reporter genes, demonstrating that miR-125a-5p was able to bind viral sequences. In particular, miR-125a was shown to be able to interfere with the HBsAg expression, since the transfection of miR-125a mimic or inhibitor into PLC/PRF/5 cell line that secretes HBsAg induced a marked decrease or enhancement in the amount of secreted HBsAg, respectively[23]. Two independent studies then confirmed the ability of miR-125a to inhibit HBsAg translation: in a screening of HBV replication-related miRNAs, a pri-miR-125a expression vector could repress HBsAg synthesis in HepG2 cells[24]; again, miR-125a mimic and inhibitor transfection in HepG2.2.15 cells resulted in an increase or decrease in HBV replication, respectively. The expression analysis of a panel of 814 miRNAs revealed that iron or TGF-α treatments, which increased or decreased HBV replication, respectively, had opposite effects on the expression of miR-125a, supporting its ability to interfere with HBV replication[25,26].

Later, another study reported an inhibitory effect of a cellular miRNA on HBV replication: Chen *et al*[27] focused on the liver-specific microRNA, miR-122, first demonstrating its inhibitory effect on HBV gene expression and replication in cultured cells and then

validating its target sequence located at the coding region of the mRNA for the viral polymerase and the 3' untranslated region of the mRNA for the core protein[27]. In a similar approach, two other studies found that miR-15a and miR-16-1 (differing by only one base outside the seed region) target HBx transcript and miR-20a/miR-92a-1 (belonging to miR-17-92 polycistron) and may inhibit HBV replication by targeting the viral transcripts[28,29].

More recently, miR-1231 has also been shown to suppress HBV replication by targeting the HBV core (HBc) protein. In HBV-transfected HepG2 cells, overexpression of hsa-miR-1231 resulted in the suppression of HBV replication by targeting the HBV core protein[30]. Also miR-205 was found to target a viral transcript, in particular HBx mRNA[31]. miR-125a, miR-205 and miR-15/miR-16-1 expression were found to be modulated by HBx protein, resulting in an upregulation for miR-125a and downregulation for the others[31-33]. These regulatory feedback loops may have an impact on the development of liver disease progressing to HCC, given the crucial role of HBx in hepatocarcinogenesis[34].

Cellular miRNAs targeting regulators of HBV infection

HBV transcripts are under the control of four promoters and two enhancers (enhancer Ⅰ and Ⅱ), interacting with cellular factors that regulate HBV gene expression. Thus, miRNA regulation of these factors results in the modulation of HBV transcription and replication. Some miRNAs suppress HBV replication by targeting positive regulators of HBV (Table 1). One example is miR-155, which suppresses HBV transcription and replication, given its ability to target CAAT enhancer-binding protein (C/EBP), which is a positive regulator of HBV transcription through its binding to HBV enhancer Ⅱ, core promoter and S promoter[35-38]. miR-155 suppresses HBV infection also by modulating the host immune system (see below)[39]. Also miR-141 is able to suppress HBV replication, since it represses at both the transcriptional and translational levels the peroxisome proliferator-activated receptor alpha (PPARα), a transactivator of HBV promoters, with a critical role in HBV replication[24,40]. Similarly, miR-130a reduced HBV replication by targeting two major metabolic regulators PGC1α and PPARγ, both of which can potently stimulate HBV replication[41].

Other miRNAs promote HBV replication by targeting negative regulators of HBV activity or by enhancing a positive regulator. miR-501 promotes HBV replication by targeting HBXIP, a negative modulator of HBV replication, because of its binding to the transactivation domain of HBx protein[42]. A positive effect on HBV replication has also been described for miR-122, in contrast with that reported above. In particular, miR-122 can promote HBV replication in two ways: It prevents cyclin G1 from interacting with p53, which has a suppressive effect on HBV replication, and it targets heme oxygenase-1 (HO-1), an anti-HBV enzyme[43,44]; miR-122 targets heme oxygenase-1 (HO-1), whose anti-HBV activity has been shown in HBV-transfected hepatoma cells and in persistently HBV replicating transgenic mice. HO-1 acts by decreasing stability of HBV core protein, thus blocking refill of nuclear HBV covalently closed circular (ccc)DNA[45,46]. According to these mechanisms, the liver-rich miR-122 may have a similar role in stimulating the replication and gene expression of the two hepatotropic viruses, HCV and HBV[47]. microRNA-372/373 promote the expression of HBV by targeting the nuclear factor I/B (NFIB), a transcription factor able to reduce viral HBsAg and HBeAg protein levels and viral core-associated DNA levels, due to its binding to enhancer Ⅰ and core promoter of HBV[48]. A similar mechanism has been described for miR-370, which suppresses HBV transcription and replication by targeting nuclear factor IA (NFIA)[49]. Also miR-15b is able to target a negative regulator of HBV Enhancer Ⅰ, *i.e.*, hepatocyte nuclear factor 1α (HNF1α) mRNA, thus resulting in the transactivation of HBV Enhancer Ⅰ, in turn causing the enhancement of HBV transcription and replication[50].

Another two miRNAs have a role in HBV transcription and replication by modulating epigenetic modifications such as histone modification and methylation. In particular, miR-1, by targeting histone deacetylase 4 (HDAC4) changes the expression of different genes, including an upregulation of farnesoid X receptor a, which enhances HBV transcription and replication by binding to the HBV core promoter[51-53]. miR-152 has been shown to target DNA methyltransferase (DNMT-1), eventually resulting in a reduced methylation of covalently closed circular DNA (cccDNA), with an impact on the replicative activity of HBV[54,55]. Consistently, miR-152 expression was also shown to be downregulated in the livers of HBx transgenic mice and inversely correlated with DNMT1 expression in HBV-related HCC patients[56,57].

Some miRNAs may also have an indirect effect on HBV infection because of their role in the modulation of the host immune system. From the point of view of HBV, they represent a strategy to suppress antiviral immune responses, thus facilitating viral replication. This is the case of miR-146a and miR-548a, which promote HBV infection by suppressing T cell function through targeting Stat1 and by binding the 3'UTR of IFN-λ1, respectively[58,59]. Conversely, miR-34a and miR-155 suppress HBV infection by inhibiting Treg cell recruitment *via* chemokine CCL22 and augmenting the IFN signaling pathway, respectively[39,60].

Overall, it is clear that the host-virus interaction in HBV infection is mediated not only by immune responses but also by miRNAs; during the co-evolution and adaptation between HBV and humans, complex miRNA-based networks have been established; this complexity may partly explain the opposing effects on HBV transcription and replication reported for some miRNAs (*e.g.*, miR-122).

MICRORNA DYSREGULATION IN HBV-RELATED HEPATOCELLULAR CARCINOMA

An aberrant expression of miRNAs has a causative effect on several pathological conditions, including cancer[5]. In this field, several studies indicate that miRNAs can act as either tumor suppressors by downregulating the expression of oncogenes, or tumor promoters (oncomirs) by limiting the expression of oncosuppressor proteins[61-63]. Cancer cell downregulation of Dicer, an enzyme playing a critical role in the biosynthesis of miRNAs, or mutations in its structure suppress miRNA biogenesis, leading to increased tumor progression[64-67]. This implies that the oncosuppressive effect of miRNAs generally overcomes their oncogenic potential. It is also known that cell differentiation is often accompanied by increased Dicer expression[68-70].

In 2011, Hou *et al*[71] performed an extensive study of the miRNomes of healthy human liver and HCC. In this paragraph, their work will be discussed in some detail since it provides a good example of the experimental procedures currently employed to study the miRNAs with oncogenic or oncosuppressive roles. The authors used a next-generation sequencing (NGS) technique to analyze human liver miRNome and found that 9 miRNAs accounted for about 90% of the liver miRNA content, with miR-122 being the most represented (52%). Other highly expressed miRNAs included miR-192 (16.9%), miR-199a/b-3p (4.9%), miR-101 (3.7%), let-7a (3.3%), miR-99a (2.2%), let-7c (2.1%), let-7b (1.7%), and let-7f (1.5%). MicroRNAs -199a-3p and -199b-3p have an identical nucleotide sequence but are transcribed from three genes, a-1, a-2, and b, with a2 being the most expressed in the liver. The authors then analyzed liver biopsies from patients with hepatocellular carcinoma by comparing tumor samples with adjacent non-cancer tissues. In HBV-related HCCs, a remarkable decrease in 199a-3p was observed. This result was then validated by qRT-PCR in a cohort of 40 HBV-related HCCs. This analysis showed that the miRNA was downregulated in 100% of the patients, with a mean decrease of 8.3-fold. Other miRNAs markedly downregulated were miR-99a and miR-125b, both decreased by about 7-fold in the majority of the patients. MicroRNA-122 and miR-125a were also downregulated (Table 2). On the other hand, miR-21 was upregulated by 4.8-fold in half of the tumor samples. In the same study, genomic analyses of DNA samples from liver biopsies indicated that miR-199a-3p downregulation was not due to gene deletion or promoter DNA methylation but to histone modification and subsequent repression of transcription. Biological assays were then performed showing that transfection of a synthetic miR-199a-3p mimic in cultured HCC cell lines repressed cell proliferation and induced apoptosis, thus suggesting a tumor suppressive role *in vitro*. Experiments *in vivo* were then performed by monitoring human HCC cell growth in nude mice. Intra-tumoral injection of cholesterol-conjugated miR-199a-3p or its over-expression with a recombinant adeno-associated virus system markedly decreased tumor growth, further supporting its tumor suppressive role. The mechanism of action of miR-199a-3p was then studied. A computational analysis with TargetScan was used to identify the human genes whose mRNA sequence may allow binding of miR-199a-3p, possibly leading to gene silencing. Most of them were associated with the mitogen-activated protein kinase (MAPK) pathway, thus providing a possible explanation for the anti-proliferative activity of the miRNA. Among them, PAK4 was downregulated by miR-199a-3p transfection in HCC cells, and this effect was found to be due to a direct interaction with the gene transcript using a luciferase-based reporter assay. Finally, measurement of the miR-199a-3p content in the liver biopsies from two other cohorts of 142 and 152 patients showed that a markedly decreased HCC level of the miRNA correlated with poor survival. It should be noted that a downregulation of miR-199a-3p in HCC had already been observed in 2006 by Murakami *et al*[72] employing much less powerful miRNA detection techniques based on microarrays and Northern blotting analyses. The same study had also shown a tumor downregulation of miR-125a. Since then, other studies have confirmed the tumor-suppressive role of miR-199a-3p in HCC and identified CD44, CD51, c-MET, mTOR, YAP1, and ZHX1 as other direct miRNA targets contributing to its anti-proliferative and pro-apoptotic effects[73-78] (Table 2). The ability of miR-199a-3p to downregulate several target proteins should not surprise given the pleiotropic effects of microRNAs that are able to interact with several mRNAs provided with the same or similar binding sites located in their 3'-UTR. Therefore, a single microRNA often silences several genes with related functions, thus showing a marked effect on the cell physiology.

Other studies conducted with similar experimental approaches, profiling microRNAs by qPCR-arrays on multi-well plates, have confirmed the HCC downregulation of miR-99a, -122, -125a, -125b, and the upregulation of miR-21, also identifying their molecular targets (Table 2). Other miRNAs consistently downregulated in HCC included the let-7 family of microRNAs and miR-29; other examples of upregulated miRNAs were miR-155 and -221 (Table 2). These data have raised substantial interest in microRNAs in the field of molecular and cellular oncology, leading to the publication of several interesting reviews[79-84].

As regards the reasons for deregulated miRNA expression in HCC, it should be noted that the viral HBx protein plays a primary role in HBV cancerogenesis[85]. It is a transcriptional trans-activator lacking a DNA-binding domain but able to interact with several transcription factors[86,87]. Therefore, it is not surprising that HBx can modify the hepatic miRNA expression[88]. It is noteworthy that HBx downregulates the expression of

Table 2 MicroRNAs playing oncogenic or oncosuppressive roles in hepatocellular carcinoma

MicroRNA	Expression in HCC	Cellular effects	Direct targets in HCC	Ref.
let-7 family	Downregulated	Proliferation (-) migration (-) apoptosis (+)	Bcl-xL, c-Myc, collagen type 1α2, RAS, STAT3	[89,132-136]
miR-21	Upregulated	Proliferation (+) migration (+) apoptosis (-)	IL-12, HBP1, PDCD4, PTEN, RECK, TIMP-3	[72,137-141]
miR-29	Downregulated	Proliferation (-) apoptosis (+)	Bcl-2, lncRNA MEG3, Mcl-1, SIRT1	[80,142,143]
miR-99a	Downregulated	Proliferation (-)	AGO2, IGF1R, mTOR	[144-147]
miR-122	Downregulated	Proliferation (-) migration (-)	ADAM17, c-Myc, CUTL1, CCNG1, WNT1	[145,148-153]
miR-125a	Downregulated	Proliferation (-) migration (-) angiogenesis (-)	c-RAF, LIN28B, MMP11, SIRT7, VEGFA, Zbtb7a	[72,123,145,154-159]
miR-125b	Downregulated	Proliferation (-) apoptosis (+)	Bcl-2, LIN28B, SIRT7	[72,145,157,160-162]
miR-155	Upregulated	Proliferation (+) migration (+)	ARID2, C/EBPbeta, PTEN, SOCS1, SOX6	[38,163-165]
miR-199a-3p	Downregulated	Proliferation (-) apoptosis (+)	CD44, CD51, c-MET, mTOR, PAK4, YAP1, ZHX1	[71-78,154]
miR-221	Upregulated	Proliferation (+) apoptosis (-)	BMF, Caspase-3, CDKN1B, CDKN1C, DDIT4	[166-169]

ADAM17: Disintegrin and metalloprotease 17; AGO2: Argonaute-2 protein; Bcl-2: B-cell lymphoma 2 protein; ARID2: AT-rich interactive domain 2; BMF: Bcl-2-modifying factor; C/EBPbeta: CCAT/enhancer binding protein beta; CCNG1: Cyclin-G1; CDKN: Cyclin-dependent kinase inhibitor; DDIT4: DNA-damage inducible transcript 4; HBP1: HMG-box transcription factor 1; IGF1R: Insulin-like growth factor 1 receptor; IL-12: Interleukin-12; Mcl-1: Induced myeloid leukemia cell differentiation protein 1; PAK4: Serine/threonine-protein kinase 4; PDCD4: Programmed cell death protein 4; PTEN: Phosphatase and tensin homolog; RECK: Reversion-inducing-cysteine-rich protein with kazal motifs; SIRT: Sirtuin; SOCS1: Suppressor of cytokine signaling 1; STAT3: Signal transducer and activator of transcription 3; TIMP3: Metalloproteinase inhibitor 3; VEGFA: Vascular endothelial growth factor A; YAP1: Yes-associated protein 1; Zbtb7a: Zinc finger and BTB domain-containing protein 7A; ZHX1: Zinc-fingers and homeoboxes-1.

oncosuppressive miRNAs let-7 and miR-122, whereas it upregulates oncomirs -21 and -221. MicroRNA-125a is induced by HBx[32,89] but is downregulated by its carboxyl-terminal truncated variant that is frequently found in HBV-related HCC[90]. These data indicate that the tumorigenic effect of HBx is partially mediated by microRNAs. Besides HBx, dysregulation of microRNAs in cancer cells may be determined by other genetic or epigenetic factors[91]. Chromosomal abnormalities, deletions, and mutations can downregulate cellular miRNA expression[92,93], and several miRNA genes associated with CpG islands are also transcriptionally repressed by promoter DNA methylation[94].

MICRORNAS AS BIOMARKERS OF HBV-RELATED LIVER DISEASES

Biomarkers of liver damage and treatment response in chronic hepatitis B

As mentioned above, chronic HBV infection is associated with a wide spectrum of clinical manifestations: An inactive carrier state, chronic hepatitis of different grade of activity and liver cirrhosis in different stages of compensation, with or without HCC. The mechanisms of virus/host interactions leading to different outcomes have been only partially clarified and a substantial contribution to their knowledge is expected from the studies on the expression profile of microRNAs and their role in liver fibrogenesis. To this regard, one of the most interesting microRNAs is miR-122, which accounts for 50%-70% of all miRNAs expressed in the human liver. Several investigations[90-97] have shown a higher miR-122 serum concentration in patients with chronic HBV infection than in normal subjects and a correlation between its serum levels and HBV load, HBsAg titers and liver biochemistry. However, the interpretation of these data as a consequence of an upregulation of microRNA requires the exclusion of the possibility that they could be a consequence of an increased release of miR-122 in the blood due to concomitant hepatic cytolysis. To this regard, Wang *et al*[44] showed that the miR-122 expression in the liver was significantly downregulated in 41 Chinese patients with HBV infection compared with 10 healthy controls, and that the miR-122 levels negatively correlated with the intrahepatic viral load and with the degree of necroinflammation, confirming *in vivo* the inhibitory activity of miR-122 on HBV replication.

Interesting information on the correlation between microRNAs and HBV-related liver damage comes from some investigations on miR-29. A study[98] performed on serum samples of 91 HBV-infected patients and 12 healthy controls demonstrated a downregulation of this miRNA in patients with more advanced liver fibrosis. In addition, the serum levels of three microRNAs (miR-29a, miR-143, miR-223) predicted the progression of liver fibrosis better than APRI or FIB-4 tests in 123 Chinese patients with chronic HBV infection[99].

In 2013, we identified the miR-125a-5p as an independent predictor of more severe liver lesions (necroinflammation and fibrosis) in a cohort of 27 treat-

ment-naïve patients with HBeAg-negative chronic hepatitis B[100], findings confirmed by the data of a study by Zheng *et al*[101] on 91 HBV-infected patients. More recently, a Chinese study performed on 211 patients with chronic hepatitis B demonstrated that serum concentrations of miR-125b, a microRNA classified in the same family, correlate with the histological activity and HBV load[102].

The clinical use of microRNAs in chronic HBV infection may go beyond the assessment of liver damage. For example, Brunetto *et al*[103] reported that the use of a serum six miRNAs signature (MiR-B-Index) correctly discriminated 61 HBV subjects in a naturally inactive stage and 84 in a stage of treatment-induced immune-control. More recently, pre-treatment serum levels of two microRNAs predicted the off-treatment biochemical and virological response after a 48-wk combination therapy with Peg-IFN and adefovir, the miR-301a-3p in 41 HBeAg-positive patients and the miR-145-5p in 45 HBeAg-negative patients[104].

Biomarkers and therapeutic targets in HBV-related HCC

HCC is the fifth most common cancer in men and the ninth in women, with respectively 554000 and 228000 new cases per year worldwide[105]. In addition, HCC is the second most common cause of death for cancer worldwide, responsible for nearly 750000 deaths per year, half of which in HBV-infected patients. Despite the great efforts of the scientific communities and Healthcare Authorities, the mortality rate of HCC has not significantly decreased in the last decade, mainly because the diagnosis is very late in most cases. In fact, the level of serum alpha-fetoprotein (α-FP) lacks sensitivity and is no longer indicated for screening[106,107] and the imaging diagnostic techniques require quality of equipment and considerable experience by the radiologists. In addition, the use of sorafenib, the only treatment shown to improve the overall survival of patients in the advanced stages[108] is limited by the high rates of adverse reactions and treatment failures[109].

In this context, many microRNAs have been found dysregulated in serum and liver of HCC patients and therefore considered as possible diagnostic biomarkers and therapeutic targets[84,110-113]. In 2011, Zhou *et al*[111] screened for 723 microRNAs the serum samples of 934 Chinese patients with HBV-related chronic hepatitis, cirrhosis or HCC and identified and validated a panel of 7 miRNAs providing a high diagnostic accuracy for HCC, regardless of cancer stage. Subsequently, the serum level of miR-21 was proposed as a novel biomarker of HCC[112]. The diagnostic accuracy of miR-21 serum level has been further investigated in several subsequent studies[113-115] and in a meta-analysis[116] including 677 patients of different etiologies, with 81.2% sensitivity and 84.8% specificity in the diagnosis of HCC.

In 2010, Li *et al*[117] identified a panel of 13 miRNAs differentially present in serum samples of 120 HBV-related HCC, 135 HBV-infected patients and 210 healthy controls. Using a panel with miR-25, miR-375, and let-7f, they obtained a 97.9% sensitivity and 99.1% specificity in HCC prediction. Furthermore, the miR-375 proved to be of high diagnostic accuracy in two prospective Chinese cohorts[118].

The tissue expression of several miRNAs in HCC tissue has been investigated to identify therapeutic targets. Gao *et al*[119] analyzed the expression profile of 7 miRNAs in 24 dysplastic nodules, 29 HCC tissues and 40 non-tumoral liver tissues surrounding HCC from HBV-infected patients and found a downregulation of miR-145 and miR-199b and an upregulation of miR-224. They also demonstrated that the restoration of miR-145 in both HepG2 and Hep3B HCC cells significantly inhibited cell proliferation and reduced cell migration and invasion. As mentioned above, miR-122 is downregulated in liver tissue of patients with chronic hepatitis B, and its concentration is inversely correlated with the degree of liver fibrosis. This downregulation was reported also in 19 HBV-related HCC tissues by Li *et al*[120] in 2013; they also demonstrated that the pituitary tumor-transforming gene 1 (PTTG1) binding factor (PBF), a validated molecular target of miR-122, enhances the proliferation and invasion of HCC cells, while its silencing induced a significant reduction in tumor growth in a murine HCC model. It has also been demonstrated that the deletion of mouse Mir122 resulted in hepatosteatosis, hepatitis, and the development of tumors resembling HCC[121], while its re-expression reduced disease manifestations and tumor incidence[122]. We recently reported a lower expression of miR-125a-5p in HCC tissues compared with non-tumor tissue in 55 patients with hepatocellular cancer of different etiologies[123]. In addition, we found a significant upregulation of three oncogenes in HCC tissue, MMP-11, c-Raf and Sirt-7, already validated as molecular targets of miR-125a-5p, an observation that provides an explanation for the tumor suppressor activity exerted by this microRNA.

FUTURE PERSPECTIVES: RNA-INTERFERENCE IN THE TREATMENT OF CHRONIC HEPATITIS B AND HEPATOCELLULAR CARCINOMA

Some literature data suggest that microRNA-interference might be useful in the treatment of HBV-related chronic hepatitis and HCC. The RNA-interference directed to inhibit HBV replication has been investigated in several animal models and, more recently, in a clinical study[124]. In a phase 2 clinical trial enrolling entecavir- naïve or -exposed HBsAg-positive patients with chronic hepatitis[125], ARC-520, a mixture of small-interfering RNAs (siRNAs) targeting all viral transcripts, induced HBsAg reduction up to 1.5 log10 in HBeAg-positive and up to 0.5 log10 in HBeAg-negative subjects with a single intravenous administration of up to 4 mg/kg. The reasons for the limited efficacy in HBeAg-

negative patients have been more recently investigated in a preclinical study on chimpanzees[126]. The authors demonstrated a lack of target sites for the siRNAs in the HBV DNA integrated in the host genome, which represents the dominant source of viral transcripts in HBeAg-negative patients. These findings highlight a novel issue that should be addressed by future research on HBV treatment. Other two RNAi-based therapies (TKM-HBV and ALN-HBV) are currently investigated in chimpanzees and mice with promising preliminary results[127].

There is some evidence of the efficacy of miRNAs mimics or inhibitors both in preclinical studies and in a recent phase I clinical trial regarding the treatment of hepatocellular cancer. In an HCC murine model, the systemic administration of miR-26a using an adeno-associated virus resulted in an inhibition of cancer cell proliferation, induction of tumor-specific apoptosis, and protection from disease progression[127-129]; a cholesterol-modified isoform of anti-miR-221 can reduce tumor cell proliferation and increase the tumor doubling time and the survival in mice with hepatocellular cancer. An miR-375 mimic delivered in gold nanoparticles has shown therapeutic efficacy without significant toxicity in primary and xenograft tumor mouse models[130]. Finally, MRX34, a liposomal miR-34a mimic, showed anti-tumor activity in a phase I clinical trial enrolling patients with refractory advanced primary liver cancers or other solid neoplasms[131], but this trial has been stopped because of serious adverse events.

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