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The roles and mechanisms of miRNA in HBV-HCC carcinogenesis: Why no therapeutic agents after 30 years?

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Abstract: Hepatitis B-associated hepatocellular carcinoma (HBV-HCC) remains an intractable high-mortality solid tumor cancer that accounted for 42% of global HCC cases in 2019. Despite some developments in systemic therapy, only a small subset of late-stage HCC patients responds positively to recently developed therapeutic innovations. MicroRNAs (miRNAs) act as an ancillary epigenetic system that can regulate genome expression in all cancer pathways including HCC. The molecular mechanisms of miRNA regulation in cancer pathogenesis offered researchers a new approach that was widely hoped would translate into miRNA-based drugs and diagnostics. Thirty years on, miRNA-based diagnostic and therapeutic agents for HCC remain a work-in-progress (WIP) and no current miRNA HCC clinical trial has progressed to Phase 4. The question remains why this is the case after 30 years and what is the way forward. The major findings and contribution of this paper are that it illustrates the complexity of the HBV-miRNA interactome in HBV-HCC in all cellular processes, as well as the ancillary role of miRNA in the epigenetic and immune systems. This is combined with a review of the outcomes and problems of clinical trials, to explain why miRNA therapeutics and diagnostics have not progressed to approved drugs or serum-based diagnostic tests. The way forward suggests a radical rethink might be so that involves the incorporation of AI, bioinformatics, and nanotechnology to solve the problem.

Schematic: The roles and mechanisms of miRNA in HBV-HCC carcinogenesis: Why no therapeutic agents after 30 years?

Introduction

In 2019, hepatitis B-associated hepatocarcinoma (HBV-HCC) still accounted for 42% of global incidence despite the global ASR per 100,000 declining from 3.557 to 2.478 between

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1990 and 2019 [1,2]. Moreover, in the same period, the incidence of HBV-HCC rose significantly in Australasia, Central Asia, Eastern Europe, Asia Pacific, and North America [1]. Surgical resection remains the most effective intervention for early-stage disease while new developments deploying a combination of immunotherapy and targeted therapy (atezolizumab plus bevacizumab) have yielded modest results [3,4]. Despite these developments in systemic therapy, only a small subset of late-stage HCC patients respond positively to these innovations [5]. Recent evidence also suggests postoperative patients (resection) with HBV-HCC have a worse prognosis than non HBV-HCC cases [6]. MicroRNAs (miRNAs), first identified in the 1990s, act as an ancillary epigenetic system that is capable of fine-tuning mRNA translation in all solid tumor cancer pathways including HCC [7]. In the non-coding RNA (ncRNA) HBV-HCC interactome, multiple miRNAs regulate host and viral gene expression in HBV-HCC pathogenesis from early HBV infection in the pre-malignant environment (PME) to the onset of HCC [8]. The miRNA regulatory interactome in HBV-HCC, which regulates host and viral genomic expression, includes its interaction with the mainstream epigenetic machinery, the innate and adaptive immune systems, and another ncRNA like long non-coding RNA (lncRNA) and circular RNA (circRNA) [9–11]. The molecular role of miRNA in cancer pathogenesis offered researchers a new approach based on regulating oncogenic mRNA expression. It was, thus, widely hoped that miRNA-based therapeutics and diagnostics would translate into novel systemic drugs and liquid biopsy based diagnostic biomarkers to detect early-stage HCC [12,13]. Thirty years on, and thousands of research studies later, miRNA-based therapeutics and diagnostics for HBV-HCC remain a work-in-progress (WIP) and a recent systematic review indicates no miRNA HCC clinical trial has progressed to Stage 4 [14]. The question remains why this is the case after 30 years and what is the way forward?

To answer this question, this paper first reviews the roles and molecular mechanisms of miRNA in HBV-HCC pathogenesis to illustrate the complexity of the miRNA interactome. The paper then provides an explanation that contributes to understanding why miRNA-based therapeutics and diagnostics have not translated into approved drugs and diagnostic tests. More specifically, the paper first introduces the regulatory mechanism of miRNAs and how their expression is dysregulated in HBV-HCC pathogenesis. The regulatory role of miRNAs is then outlined in the pre-malignant micro-environment (PME) of chronic hepatitis B infection (CHB), inflammation, and the onset of fibrosis. Next, we review the multiplicity of the Hepatitis B x protein (HBx) dysregulated miRNA, and their molecular mechanisms, in the HCC tumor microenvironment (TME) including cell proliferation, apoptosis, angiogenesis, invasion-migration, and metastasis in their respective HBV-HCC signaling pathways. The complexity of the miRNA interactome is further illustrated by the regulatory role of miRNAs as an ancillary epigenetic system before examining miRNA interaction with immune response in HBV-HCC pathogenesis. The paper then assesses the status quo of miRNA-based therapeutic and diagnostic developments before outlining the problems that explain why this field is still in the work-in-progress phase. A way forward is then discussed and a conclusion is developed highlighting the contributions and limitations of this paper.

The Function and Dysregulation of miRNA

Dynamic combinations of miRNAs act as a homeostatic ancillary epigenetic system that fine-tune mRNA translation in all cellular processes [15–17] and a recent estimate indicated that 2300 classified human miRNAs are under the transcriptional control of ~1000 genes [18]. In order to exert a regulatory role, miRNAs form Argonaut (AG) based RNA-induced silencing complexes (RISCs) that bind to specific mRNA through complementarity sequences that result in either the inhibition of mRNA translation and/or de-adenylation followed by mRNA decay [19]. The degree of silencing and/or degradation of miRNA-mRNA translation is a function of the degree of complementarity of their sequence specific bases in the 5′UTR region with the corresponding target mRNA 3′UTR region [20]. It has been demonstrated that intracellular miRNA silencing often occurs at the surface of the endoplasmic reticulum (ER) because this is the primary location of mRNA protein synthesis and RISC loading. Mature miRNAs in the intracellular space can be packaged and transported in a variety of protein and membranous bodies to regulate mRNA expression in the cytosol [16]. Mature miRNAs can also be exported to the extracellular space (serum) in a range of vesicles including exosomes, micro-vesicles, apoptotic bodies, high-density lipoprotein (HDL) complexes, and other AG complexes [21]. Solid evidence has emerged that these extracellular miRNAs contain paracrine mRNA silencing capability [22], however, the extent of their regulatory ability remains in question [23,24].

The molecular mechanisms of miRNA dysregulation in HBV-HCC pathogenesis include genomic alterations, mainstream epigenetic expression, defects in the miRNA biogenesis machinery, immune response, co-expression with host genes, the presence of carcinogens, viral infection [25–27] and other competing ncRNA like lncRNA and circRNA [28]. HBV expression of viral proteins like HBx, for instance, dysregulates multiple miRNAs in every stage of pathogenesis by inducing host genomic and epigenetic changes from early infection in the PME to the onset of HBV-HCC [8]. Dysregulation can also occur at any stage in the miRNA transcription machinery from the early pri-miRNA stage to the production and packaging of mature miRNAs because of various processing defects [29]. In HBV-HCC pathogenesis, dysregulated miRNAs can potentially regulate both pro and anti-oncogenic expression [30].

The Regulatory Role of miRNA in the Pre-Malignant Micro-Environment (PME)

An understanding of the role of miRNA regulation in every stage of HBV-HCC pathogenesis, from the PME to the

tumor-microenvironment (TME), is especially important because chronic hepatitis B infection (CHB) triggers inflammation, injury, and tissue replacement that collectively dysregulates multiple miRNAs [8,31]. For example, one study indicated >80 dysregulated miRNAs in the PME as a result of CHB-induced inflammation including important miRNAs like miR-195a-5p/-1974/-203a/-21/-22/-210/-34a/-451/-548d-5p/-654/-711/-760/-767-3p [8]. Interestingly, many of these miRNAs are also dysregulated in HBV-HCC serum [32]. MiRNA regulation in the PME is both complex and (often) contradictory and three miRNAs, namely, miR-155/-122/-let-7 illustrate some of these regulatory complexities in early stage CHB and inflammation. In one feedback loop, the HBx protein can upregulate miR-155 expression to subdue HBV replication by repressing CCAAT-enhancer-binding protein (C/EBP) induced enhancement of HBV core promoters [33,34]. Pro-inflammatory cytokines in the PME can also upregulate miR-155 expression which can repress tumor suppressors like PTEN and C/EBPβ to promote pro-oncogenic progress in the PME [33]. HBV infection can downregulate miR-122 which, in turn, can also repress HBV replication by modulating cell cycle controls like cyclin G1 and by binding to HBV mRNA and HBx [35–37]. This important liver miRNA also acts as an anti-inflammatory agent by repressing inflammatory cytokines like Interleukin 6 (IL-6) and Tumor necrosis factor-alpha (TNF-α) [38], however, these inflammatory cytokines can induce C-MYC inhibition of miR-122 expression in CHB [39]. In early-stage HBV infection, all the let-7 family members have been reported as significantly downregulated by the HBx protein resulting in the reduced modulation of multiple pro-inflammatory cytokines, interleukins, activated Kupffer cells, and liver-derived macrophages [40,41] that promote a wide range of cellular responses in injured liver tissue [42,43].

A hallmark of HBV-HCC is the presence of fibrosis/cirrhosis that often precedes the onset of HBV-HCC for many years. In the inflammation–fibrosis axis, fibrogenesis is orchestrated by a complex network of common cytokine-mediated signaling pathways that regulate the activation of hepatic stellate cells (HSCs) and downstream extracellular matrix (ECM) proteins (see Fig. 1). These cytokines include Transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF), TNF-α, interferons (IFNα/β), and interleukins (IL-1/6/17) [42,43]. One study showed that >75 dysregulated miRNA could potentially regulate liver fibrogenesis in the PME illustrating a range of miRNA that directly target the TGF-β pathway in fibrogenesis including miR-17-5p/-122-5p/-181b/-21/-23a-5p/-26b/-27a-b/-30/-34a/-148a/-150/-151b/-152/-214-3p/-221-5p/-222/-29a-b/-33a/-942/-192/-200 [8]. Upregulated miRNA can either repress or attenuate fibrogenesis. Upregulated miR-17-5p/-21/33a promote fibrogenesis by repressing the SMAD 7 inhibitor [44–47] and miR-29a-b/-214-5p attenuate fibrogenesis by repressing growth factors and TGF-β signaling [48,49]. Important downregulated miRNA like

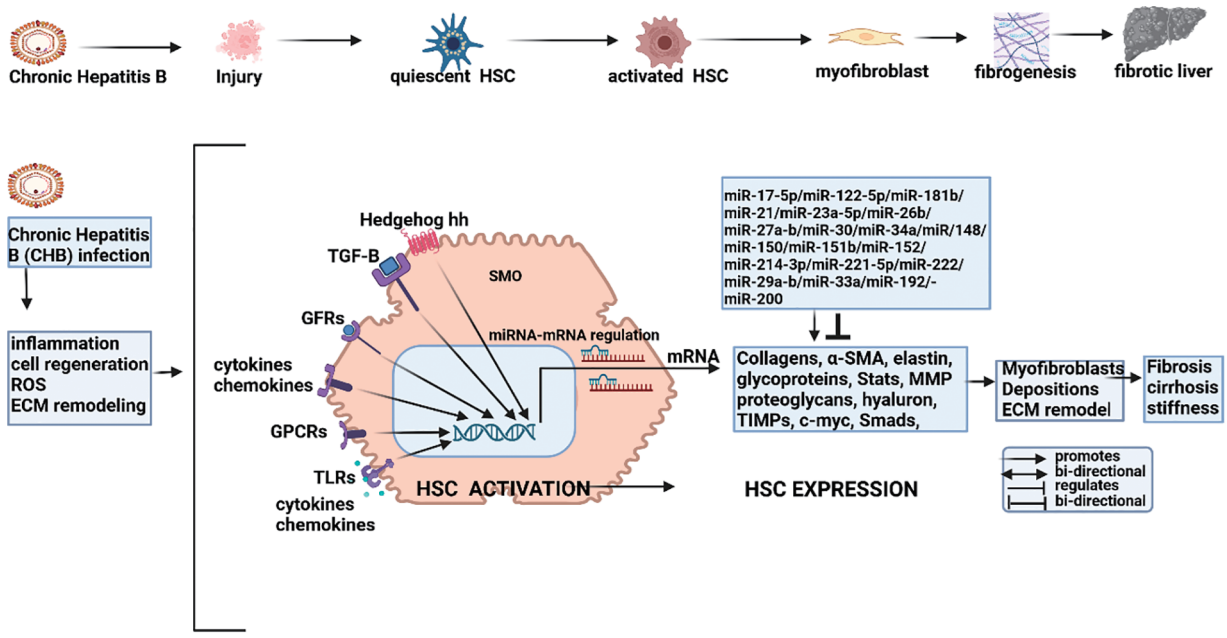

FIGURE 1. miRNA modulation in HBV-induced fibrogenesis. Chronic Hepatitis B infection (CHB) induces inflammation and injury to activate quiescent hepatic stellate cells (HSCs) to promote fibrogenic materials like myofibroblasts and fibrosis. CHB also promotes cell regeneration, oxidative stress (ROS), and ECM remodeling. Collectively, these conditions trigger the activation of HSCs by promoting the expression of growth factors (GFs), protein-coupled receptors (GPCRs), cytokines and chemokines, signaling pathways (e.g., TGF-B and Hedgehog) and toll-like receptors (TLRs). Activated HSCs promote the transcription of a wide range of fibrogenic mRNA including collagens, α-SMA, glycoproteins, metalloproteinases (MMPs), proteoglycans, tissue inhibitors of metalloproteinases (TIMPs) and onco-proteins like β-catenin and C-MYC. These mRNA, in turn, are modulated by ncRNA (miRNA/lncRNA/circ-RNA) that, in turn, are modulated by the mainstream epigenetic machinery including DNA methyl transferases (DNMTs), histone de-acetyltransferases (HDACs), histone acetyltransferases (HATs), histone methyl transferases (HMTs) and histone de-methylation transferases (HDMTs). The transcription of fibrotic depositions and ECM remodeling is thus modulated by a combination of ncRNA and the mainstream epigenic machinery to determine fibrogenesis, ECM remodeling, and eventually advanced fibrosis/cirrhosis (Figures done in Biorender).

miR-29a/-29b/-34a/-122 mostly fail to repress fibrogenesis because they have a reduced ability to repress collagens, growth factors (GFs), and SMAD 4 [40,44,50].

The three important miRNAs, namely, miR-155/-122/let-7a, not only modulate viral expression and inflammation but also play a regulatory role in liver fibrosis. Upregulated miR-155 can invoke pro-inflammatory induced liver fibrogenesis [51] via the Signal transducer and activator of the transcription 3 (STAT3) pathway [52] and promote liver fibrosis by repressing suppressor of cytokine signaling 1 (SOCS1) [53]. miR-122 can promote fibrosis by repressing Insulin-like growth factor 1 receptor (IGF1R), Cyclin G1 (CCNG1) and Prolyl 4-hydroxylase subunit alpha-1(P4HA1) to induce hepatic stellate cell (HSC) activation and the expression of collagen [54] and let-7 family members can regulate TGF-β expression that activates HSCs and the expression of multiple fibrogenic materials [55]. Illustrating the complexity of miRNA regulation in this microenvironment, HBx can suppress p53-led transcription of the miR-192/-200 cluster that then reduces its regulation of Zinc Finger E-Box Binding Homeobox 1/2 (ZEB1/2) resulting in a reduction in E-cadherin in the WNT/β-catenin pathway that is often an early feature of fibrosis/epithelial-mesenchymal transition (EMT) in the PME [56–58].

Molecular Mechanisms of miRNA Regulation in HBV-HCC Pathways

This section illustrates how miRNAs regulate cell proliferation, apoptosis, angiogenesis, migration-invasion-metastasis, and cell cycle in the main HBV-HCC pathways and networks (see Figs. 2 and 3, Table 1). Collectively, miRNAs regulate oncogenic or tumor suppressor expression in multiple pathways and networks in HBV-HCC pathogenesis including the TP53, PI3K/MAPK, JAK/STAT, WNT/ β-catenin, TGF-B/SMAD, SAPK/JNK and NF-κB signaling pathways [59]. In HBV-HCC pathogenesis, moreover, chronic hepatitis B infection dysregulates both host gene expression, as well as miRNAs to (stealthily)

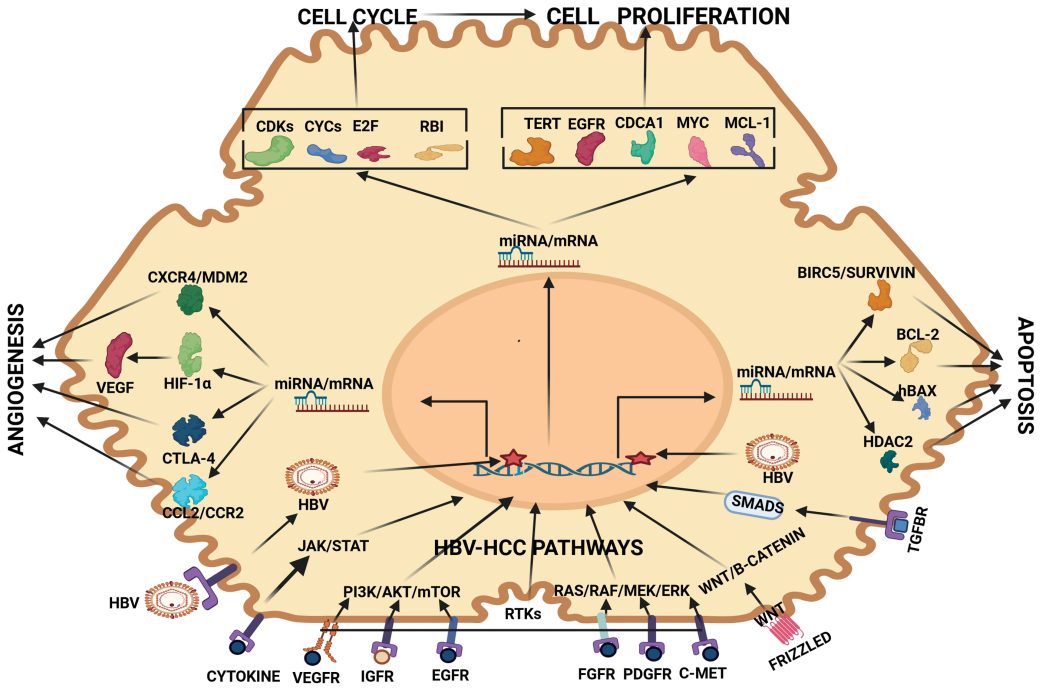

FIGURE 2. miRNA regulation of HBV-HCC pathways. HBV-HCC: The HBV virion enters the host cell and integrates into the host DNA causing genomic changes. In tandem, the TME activates HBV-HCC pathways like JAK/STAT, PI3K/AKT, RAS/RAF, WNT/β-catenin and TGF-SMAD via multiple cell receptors to activate miRNA/mRNA expression to influence cell cycle, proliferation, apoptosis, and angiogenesis. **HBV-HCC Receptors** include VEGFR, IGFR, EGFR, FGFR, PDGFR, C-MET, HGFR, WNT that promote transcription of mRNA/miRNA to influence **Cell Cycle mRNA:** miRNA regulates cell cycle mRNA including CYCs, CDKs, E2F and RB1, **Cell proliferation mRNA:** miRNA regulate mRNA like TERT, EGFR, CDCA1, C-MYC, MCL-1, and ARL4C, **Apoptosis mRNA:** miRNA regulate mRNA like BIRC5/Survivin, BCL-2, hBAX, HDAC2 to influence apoptosis and **Angiogenesis mRNA:** miRNA regulates CXCR4/MDM2, CTLA-4, HIF-1α and VEGF to influence angiogenesis. Abbreviations: **VEGFR:** Vascular endothelial growth factor receptor; **IGFR:** Insulin-like growth factor receptor; **EGFR:** Epidermal growth factor receptor; **FGFR**: Fibroblast growth factor receptor; **PDGFR:** Platelet-derived growth factor receptor; **C-MET:** Receptor tyrosine kinase; **TGF-BR:** Transforming growth factor-beta receptor; **PI3K:** Phosphatidylinositol 3-kinases; **AKT:** Protein kinase B; **mTOR:** mammalian target of rapamycin; **RAS:** Rat sarcoma virus; **RAF:** Rapidly Accelerated Fibrosarcoma; **MEK**: Mitogen-activated protein kinase; **ERK:** Extracellular signal-regulated kinase; **WNT:** Wingless and Int-1 **SMADs:** Suppressor of mother against decapentaplegic; **HBV:** Hepatitis B virus; **HDAC2:** Histone de-acetylase 2; **hBAX:** Bcl-2 Associated X-protein; **BCL-2:** B-cell lymphoma 2; **BIRC-5:** Baculoviral IAP Repeat Containing 5; **MCL-1:** Myeloid cell leukemia-1; **MYC:** Myelocytomatosis oncogene; **CDCA-1:** Cell division associated 1 **TERT:** Telomerase reverse transcriptase; **CDKs**: Cyclin Dependent Kinases; **CYCs:** Cyclins; **E2F:** Eukaryote 2 transcription factor; **RB1:** Retinoblastoma protein 1; **CXCR4**: Chemokine receptor type 4; **MDM2:** Mouse double minute 2 homolog; **HIF-1α:** Hypoxia inducible factor 1α; **CTLA-4:** Cytotoxic T lymphocyte antigen-4; **CCL2:** Chemokine (C-C motif) ligand 2; **CCR2:** CC chemokine receptor 2 (Figures done in Biorender).

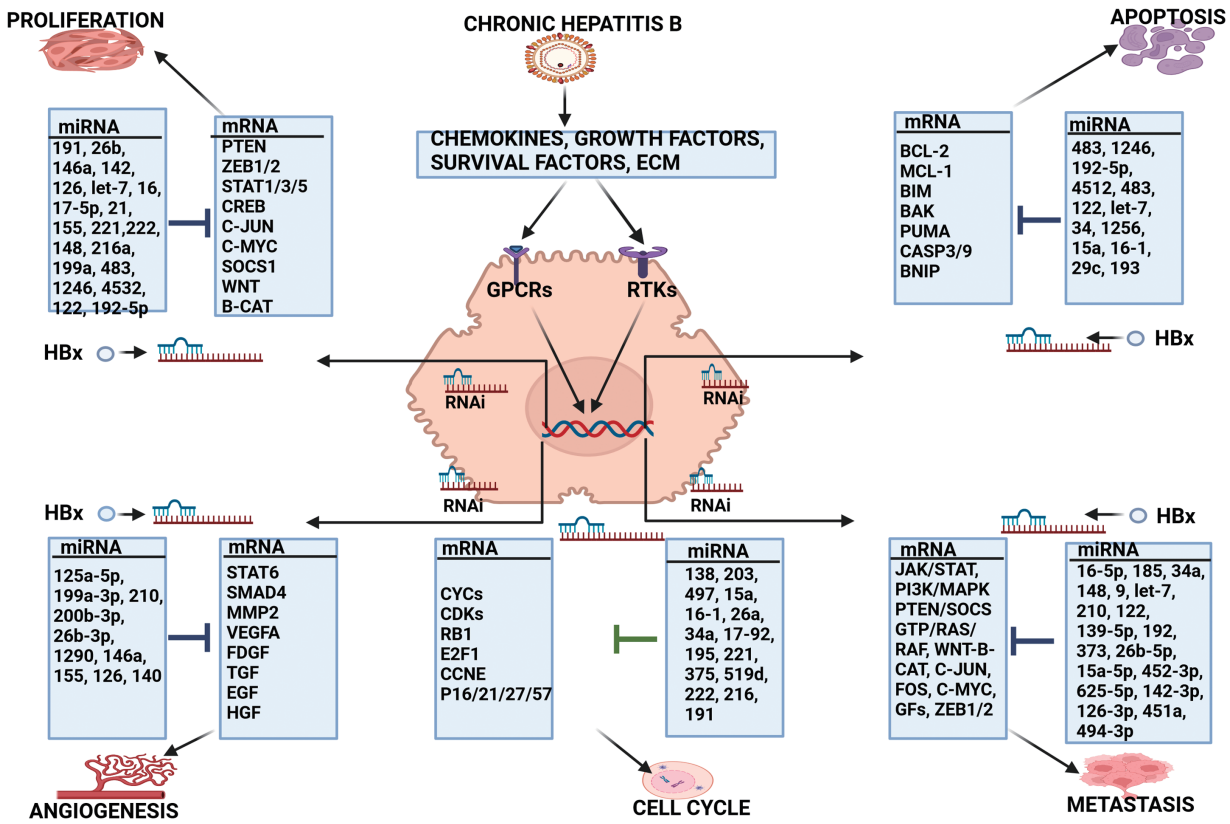

FIGURE 3. miRNA regulation of HBV-HCC pathogenesis. CHB infection promotes the expression of a wide range of chemokines, growth and survival factors and changes in the extra-cellular matrix (ECM) and the HBx protein can dysregulate miRNA expression to influence a wide range of cellular processes. **Cell Proliferation:** HBx dysregulated miR-191/-26b/-146a/-142/-126/let-7/-16/-17-5p/-21/-155/-221/-222/-148/-216a/-199a/-483/-1246/-4532/-122/-192-5p regulate PTEN/ ZEB1-2/STAT1,3,5/CREB/C-JUN/C-MYC/SOCS1/WNT/β-catenin expression to influence proliferation. **Apoptosis:** HBx dysregulated miR-483/-1246/-192-5p/-4512/-122/let-7/-34/-1256/-15a/-16-1/-29c/-193 collectively regulate BCL-2/MCL-1/BIM/BAK/PUMA/CASP3/CASP9/BNIP to influence apoptosis. **Angiogenesis:** HBx dysregulated miR-125a-5p/-199a-3p/-210/-200b-3p/-26b-3p/1290/-146a/-155/-126/-140 can collectively modulate STAT/SMAD/MMP2/VEGF/FDGF/TGF/EGF/HGF to influence angiogenesis. **Cell Cycle:** HBx dysregulated miR-138/-203/-497/-15a/-16-1/-26a/-34a/-17-92/-195/-221/-375/-519d/-222/-216/-191modulate CYCs/CDKs/RB1/E2F1/CCNE/P16/21/27/57. **Metastasis:** HBx dysregulated miR-16-5p/-185/-34a/-148/-9/-let-7/-210/-122/-139-5p/-192/-373/-26b-5p/-15a-5p/-452-3p/-625-5p/-142-3p/-126-3p/-451a/-494-3 that regulate JAK/STAT/PI3K/MAPK/PTEN/SOCS1/GTP/RAS/RAF/WNT/β-catenin/C-JUN/-C-FOS/C-MYC/GFs/ZEB1-2 to influence invasion and metastasis. **Abbreviations:** Extra cellular matrix (ECM), Receptor tyrosine kinase (RTK), G protein coupled receptors (GPCR) (Figures done in Biorender).

balance viral replication with immune evasion [9]. In many cases, miRNAs recorded as dysregulated in the PME remain dysregulated in HBV-HCC pathogenesis, however, their mRNA targets and direction of dysregulation are often different [32].

Cell cycle and miRNA regulation

A key regulator of cell cycle processes is cyclin dependent kinase (CDK) activity and CDKs are activated by specific cyclins (CYCs) that accumulate during different stages of the cell cycle [60]. CDK activity activates cell cycle-regulated transcription to initiate cell cycle entry and its progression through the pre-replicative G1 phase and the post-replicative G2 phase [61]. Commitment to replication initiation and S phase entry is closely linked to the activation of the E2F transcriptional network [62] which, in turn. is regulated by the key tumor suppressor RB1 in HCC [63]. Other transcription repressors in HCC including p16/21/27/57 are closely linked to the p53 protein [64,65]. CHB infection can fundamentally dysregulate cell cycle regulators and the HBx protein, for example, can promote the cell cycle by downregulating TGF-β to promote G1/S to G2/M [66], as well as attenuating cell cycle progression by upregulating p21/27 induced downregulation of CDK activity, and by disrupting E2F1 inhibition by repressing RB1 [67].

Although multiple miRNAs have been linked directly to the regulation of cell cycle control, a bioinformatics approach suggests the five most important miRNA/mRNA hubs in HBV-HCC include miR-195-5p/Cyclin-dependent kinase 1 (CDK1), miR-5589-3p/Cyclin B1 (CCNB1), let-7c-3p/Cyclin-dependent kinase regulatory subunit 2 (CKS2), miR-195-5p/Cyclin E1 (CCNE1) and miR-30c-2-3p/CCNE1 [68]. Other examples in HBV-HCC (see Table 1) include miR-138/-203/-497/-15a/-16-1/-26a/-34a that can all directly regulate cyclins and cyclin-dependent kinase activity including CYCD/CYCE/CDK2-4-6 [69,70]. In addition, miR-17-92/-195/-221/-375 can directly regulate cell cycle tumor suppressors RB1/G1 [71,72], miR-519d/-216/-17-92/-221/-222 can target transcription repressors including p16/21/26/57 [73,74]. Other miRNA regulating cell cycle controls include miR-191/-26b/-146a/-142/-126/let-7/-16-5p that can directly target a wide range of cell cycle machinery including both activators and repressors like CDK1NA/CDK2/CCNE/RB1/E2F1 [32]. Further illustrating the

complexity of molecular mechanisms involving miRNA regulation of cell cycle, various studies show that the HBx can dysregulate multiple miRNA regulating cell cycle controls including downregulating miR-138/-15a/16-1/-17-92/-375/-216/-222/-let-7 expression and upregulating miR-203/-221/-146a [8] (see Fig. 3, Table 1).

TABLE 1

HBx dysregulated MiRNA targets and pathways in cell processes

HBx dysregulated miRNA	Target	Pathway	Ref.
Cell cycle			
miR-138/-203/-497/-15a/-16-1/-26a/-34a	CYCD/CYCE/CDK2-4-6	TP53	[69,70]
miR-17-92/-195/-221/-375	RB1/G1	TP53	[71,72]
miR-519d/-216/-17-92/-221/-222	P21/P27/P16/P57	TP53	[73,74]
miR-191/-26b/-146a/-142/-126/let-7/-16-5p	CDK1NA/CDK2/CCNE/RB1/E2F1	TP53	[32]
Apoptosis			
miR-122/-let-7/-34/-29c/-125b/-15a/-16-1	BCL2/BCL-XL/MCL1/CASP9-CASP3	TP53	[75,76]
miR-101-29c/-193/-125b/-let-7	CL-XL/MCL1/CASP9	TP53	[77,78]
miR-483	PUMA/BCL2/BCL-XL/MCL1/CASP9/3	TP53	[79]
miR-483/-1246/-192-5p/-4532/-122	CASP3	TP53	[32]
miR-199a/-let-7/-125b/-29c/-7/-34/-26a-c	BCL2	PI3K/MAPK	[32]
miR-483/-1246/-192-5p/-4532/-122	PTEN	PI3K/MAPK	[32]
miR-483/-1246/-192-5p/-4532/-122	MM9/BCL2	NF-κB	[32]
Proliferation			
miR-1/-23b/-34a/-299-3b/-26a-c	HGFR/MET	PI3K/MAPK	[80]
miR-17-5p/-21/-155/-221/-222/-148/-216a	PTEN/TRPS1/ZEB1/ZEB2/E-CAD	PI3K/MAPK	[81,82]
miR-199a/-let-7/-125b/-29c	AKT/MTOR	PI3K/MAPK	[83]
miR-483/-1246/-192-5p/-4532/-122	SRC/RAS/MAPK/CREB/STAT3	PI3K/MAPK	[32]
let-7	GTP/RAS/RAF/ERK/C-JUN/FOS/CYC	PI3K/MAPK	[84]
miR-191/-26b/-146a/-142/-126/let-7/-16	MAP2K1/MAPK1/MYC	PI3k/MAPK	[32]
miR-155/221	SOCS1/SOCS3	JAK/STAT	[85,86]
miR-637/let-7	STAT3	JAK/STAT	[87,88]
miR-483/-1246/-192-5p/-4532/-122	JAK1/STAT3/5	JAK/STAT	[32]
miR-21	DCC6/WNT/PDCD4/SNAIL1/E-CAD	WNT/β-catenin	[80,89]
miR-122/-148	β-catenin/C-MYC/C-JUN/CYCD	WNT/β-catenin	[90,91]
miR-30/-148	SNAIL1/α-catenin	WNT/β-catenin	[92,93]
miR-135/-106b/-155/-200/-34a	APC/GSK3/AXIN 1-2/B-CATENIN/ZEB1	WNT/β-catenin	[33,80]
miR-1246/-315/-106b	ROR-α/C-MYC/-C-JUN/CYCD	WNT/β-catenin	[94,95]
miR-320a/-145/-214	β-catenin	WNT/β-catenin	[96,97]
miR-200/-205/-101/-34a	ZEB1/ZEB2/E-CAD	WNT/β-catenin	[98]
let-7	STAT3	JAK/STAT	[87]
miR-191/-26b/-146a/-142/-126/let-7/-16	KRAS/MAP3K/MAPK3/JUN/SMAD4	SAPK/JNK	[32]
miR-191/-26b/-146a/-142/-126/let-7/-16	JAK1/STAT1/3	JAK/STAT	[32]
miR-191/-26b/-146a/-142/-126/let-7/-16	ARAF/MAP2K1/MAPK1/MYC	MAPK	[32]
Angiogenesis			
miR-125a-5p	VEGFA	HIF-VEGF	[99]
miR-199a-3p	VEGFA/VEGFR1/2/MMP2/HGF	HIF-VEGF	[100]
miR-210	STAT6/SMAD4	Multiple	[101]
miR-200b-3p	ERG	Multiple	[102]
miR-26b-3p	E-CADHEREN/SNAIL/MMP2	WNT/β-catenin	[103]

(Continued)

Table 1 (continued)

HBx dysregulated miRNA	Target	Pathway	Ref.
miR-1290	SMEK1	Multiple	[104]
miR-146a	PDGFRA	Multiple	[105]
miR-155	VEGF/HIF-1α	HIF-VEGF	[106]
miR-126	EGFL7	Multiple	[107]
miR-140	VEG-A	HIF-VEGF	[108]
migration/Invasion/metastasis			
miR-16-5p	IGF1R	Multiple	[109]
miR-191/-26b/-146a/-142/-126/let-7/-16-5p	AKT2/CHUK/NFκB1/BCL2	PI3K/AKT	[32]
miR-185	KCNN3/COL1A1	TGF-B/SMAD	[110,111]
miR-34a	YY1/SPTBN2/E2F3/DLL1/C-MET	Multiple	[112,113]
miR-148a/b	GTF2H1/PSCD3/MET/SNAIL/B-CAT	WNT/β-catenin	[91,92]
miR-9	RAB8A/SLC20A2/E-CAD/KLF17	WNT/β-catenin	[114,115]
let-7g	COL1A1/PSCD3/KRAS/NRAS	MAPK/other	[116,117]
miR-210-3p/5p	HIF-1	EMT	[118]
miR-122	β-catenin/ADAM17	WNT/β-catenin	[58,119]
miR-139-5p	ZEB1/ZEB2/ROCK1	WNT/β-catenin/EMT	[120,121]
miR-192	G1-G2//SLC39A6/SNAIL	Multiple	[122,123]
miR-373	E-CADHEREN	WNT/β-cateninT	[124]
miR-26b-5p/miR-15a-5p	SMAD1	TGF-B/SMAD	[125]
miR-15a-5p/-15b-5p	GL12/E2F3	HH/cell cycle	[126,127]
miR-452-5p	EPB41L3/COLEC10	Not identified	[128,129]
miR-625-5p	PDLIM5/E2F1	MAPK/cell cycle	[130,131]
miR-142-3p	HMGβ1/ZEB1	WNT/β-catenin/ANO	[132,133]
miR-126-3p	LRP6/PIK3R2	WNT/β-catenin/AKT	[134]
miR-451a	YWHAZ/ADAM10	EMT/PI3K/AKT	[135,136]
miR-494-3p	EZH2/BMAL1	Epigenetic/TP53	[137]

Cell proliferation and miRNA regulation

Cell proliferation is regulated by multiple miRNAs that are dysregulated by the HBx protein in various HBV-HCC pathways including the PI3K/MAPK, JAK/STAT, WNT/β-catenin, SAPK/JNK/TGF-B/SMAD and TP53 suppressor network (see Table 1). Cell proliferation in HBV-HCC pathogenesis is invariably increased by upregulated onco-protein expression, downregulated tumor suppressor genes, the dysregulation of cell cycle controls, and a range of HBV proteins [138,139]. Simultaneously, multiple miRNAs also become dysregulated often failing to regulate onco-protein mRNA or alternatively by repressing tumor suppressor expression [8]. Onco-expression and proliferation in HCC pathways are typically precipitated by a wide range of growth factors (GFs) including TGF-B/FGF/EGF/HGF/IGF, as well as multiple onco-proteins including JAKs/SMADs/C-MYC/C-JUN/C-FOS/RAS/MTOR/KRAS and CTNN1B amongst many more [140]. Other tumor suppressor targets include p21/p27/p16/E-CAD/AXIN1-2/APC/SOCS1-3/KLF6 and PTEN [141]. The HBx protein can both stimulate and repress proliferation by targeting growth factors like CTGF [142], as well as by dysregulating C-MYC, TGF-α, FAS, and RAS/RAF pathway expression [8,67]. Examples of HBx dysregulated miRNA regulation in cell proliferation include let-7/miR-191/-26b/-146a/-142/-126/-16 that target RAS/RAF/ERK/C-JUN/C-FO S/C-MYC in the PI3K/MAPK pathway [32,84] and miR-637/-483/-1246/-192-5p/-4532/-122/-let-7 that target JAK1/STAT3/5 in the JAK/STAT signaling pathway [32,87,88]. In the WNT/β-catenin pathway, cell proliferation can be regulated by miR-122/-148/-30/-320a/-145/-214 that target onco-protein expression of B-CATENIN/C-MYC/C-JUN [90,91,96,97]. Examples of tumor suppressor regulation include miR-155/221 which targets SOCS1/3 in the JAK/STAT pathway [85,86,143], and miR-17-5p/-21/-155/-221/-222/-148 which can target the tumor suppressor PTEN expression in the PI3K/MAPK pathway [81,82]. Other examples of miRNA repression of tumor suppressors include miR-135/-106b/-155/-200/-34 which represses the tumor suppressor APC [80] and miR-21 regulates Programmed cell death protein 4 (PDCD4) in the WNT/β-catenin pathway [80,89]. Further illustrating the complex HBx-miRNA-HCC interactome, many cell proliferation miRNA are also dysregulated by the HBx protein including miR-192-5p/-122/-let-7/-146a/-148/-30c/-145/-155/-221/-222/-17-5p/-21 [8] (see Fig. 3, Table 1).

Apoptosis and miRNA regulation

HBV-HCC pathogenesis typically involves the upregulation of anti-apoptotic proteins including NF-κB/BCL-2/BCL-XL and MCL-1, however, the TGF-β pathway can be stimulated at the cirrhosis stage to promote apoptosis by activating SMAD3 mediated BCL-2 downregulation [138]. Typically HBx dysregulated miRNA like miR-122/-let-7/-34/-29c/-125b/-15a/-16-1 target multiple anti-apoptotic targets like BCL2/BCL-XL/MCL1/CASP9/CASP3 [75,76] but all of these miRNA are reported as frequently downregulated by the HBx protein [8]. Illustrating some of the complexities in HCC pathogenesis, pro-apoptotic proteins like BAX/BCL-XS can be both downregulated [144] and upregulated while simultaneously being regulated by dysregulated miRNA [145]. In the TP53 network, miR-483/-145/-122/-519d/-221 can regulate apoptosis by regulating targets like PUMA/MDM2/P21, as well as their downstream targets like B-cell lymphoma 2 (BCL2)/B-cell lymphoma-extra large (BCL-XL)/Myeloid cell leukemia-1 (MCL1)/CASP9/CASP3. Simultaneously, miR-221/-519d can regulate PTEN expression in the PI3K/MAPK pathways to influence apoptosis [146]. In this microenvironment, the HBx protein can also promote both anti and pro-apoptotic expression. The HBx protein, for example, can promote MCL-1/BCL-2 expression to repress pro-apoptotic BAX activation of CASP9/3 [147], as well as induce the pro-apoptotic expression of TRAIL-R2 (DR5) [148] (see Fig. 3, Table 1).

Angiogenesis and miRNAs

HBV-HCC is sometimes characterized as a highly angiogenic cancer that is characterized by hypoxia and the overexpression of VEGF [149] and the HBx protein is capable of promoting pro-angiogenic expression by upregulating IL-6/COX2 [150]. VEGF expression is regulated by oncogenic gene mutations, hormones, cytokines, and various signaling molecules like nitric oxide and MAPKs [67]. Moreover, VEGF may be released by stromal cells and from the ECM via MMP-9-mediated proteolysis [151,152]. Multiple pro-angiogenic growth factors like PDGF/FGF/TGF-α and TGF-β/HGF/EGF/VEGF are triggered in HCC pathogenesis, as well as other pro-angiogenic factors including Angiopoietin-2 (ANG2)/IL-4/IL-6 and IL-8 [153]. Simultaneously, both anti-angiogenic signaling-inducing angiostatins, endostatins, and thrombostatins can be orchestrated in the NOTCH pathway while pro-angiogenic signaling can be induced in the PI3K/AKT and WNT-B-CAT pathways [154–156]. Multiple HBx dysregulated miRNAs including miR-125a-5p/-199a-3p/-210/-200b-3p/-26b-3p/-1290/-146a/-155/-126/-140 modulate angiogenic growth factors and their receptors like VEGF/HGF/PDGF/EGF (see Table 1). In addition, in HBV-HCC pathogenesis, many of these miRNA are simultaneously dysregulated by the HBx protein including mIR-125a-5p/-199a-3p/-200b-3p/-146a/-155 [8] suggesting an increased or reduced ability to influence pro or anti-angiogenic mRNA expression (see Fig. 3, Table 1).

Migration/Invasion/metastasis and miRNA

It is difficult to isolate consistent hub miRNA-mRNA patterns in advanced HBV-HCC because of the elevated level of miRNA dysregulation and aberrant expression in multiple cancer pathways including PI3K/MAPK, JAK/STAT, WNT/B-CATENIN, SAPK/JNK, TGF-B/SMAD, NOTCH, Hedgehog (HH) and TP53 networks [157]. Understandably, because of the multiplicity of miRNA/mRNA dysregulation, no meta-studies illustrate a comprehensive list of miRNAs specifically related to the advanced stages of HBV-HCC pathogenesis. However, multiple studies have identified HBx dysregulated miRNA and its targets in the migration/invasion and metastasis phases of HBV-HCC (see Fig. 3, Table 1). The accumulation of β-catenin has been widely associated with multiple cancers including HCC [157] and the dysregulation of miRNA expression targeting key mRNA in the WNT/B-CATENIN pathway appears to be a common feature in advanced stages of HBV-HCC oncogenesis [158]. Various studies identify important miRNA like miR-148a-b/-122/-139-5p/-192/-373 that directly target SNAIL/β-catenin/ZEB 1-2/E-CADHEREN and LRP6 (see Table 1). Interestingly, the ubiquitous role of the HBx protein is also illustrated in this pathway. HBx, for instance, can activate the WNT/β-catenin pathway via modulation of WNT1 to activate SRC kinase to promote β-catenin [159]. HBx can also bind to APC from interacting with it and obstruct its regulation of the APC/AXIN/GSK3b complex to promote β-catenin expression [160].

In HBV-HCC related migration and invasion, HBx dysregulated miRNAs like downregulated miR-16-5p have a reduced ability to regulate IGF1R mRNA which plays an important role in this aspect of HCC pathogenesis [109]. Invasion and migration can also be increased by upregulated miRNA like miR-34a-5p which can attenuate the expression of transcription factor YY1 to mediate MYCT1 [112]. Interestingly, hypoxia-induced migration and EMT can also be influenced by miR-210-5p/-3p [118]. On the other hand, repressed miR-26b-5p expression can fail to modulate EMT, migration, and invasion as a result of an inability to repress SMAD1 [125]. Other examples of studies illustrating miRNA regulation of migration and invasion in HBV-HCC pathogenesis include HBx dysregulated miR-452-5p [128,129], miR-625-3p [130,131], miR-15a-5p [126,127].

Multiple miRNAs modulate HBV-HCC induced metastasis and the following examples are merely illustrations rather than purporting to provide a comprehensive list (see Table 1). Downregulated miR-142-3p in HBV-HCC, for instance, fails to repress HMGβ1 resulting in the reduced attenuation of metastasis [132]. Similarly, downregulated miR-126-3p also fails to repress LRP6 and PIK3R2 expression to promote metastasis and angiogenesis [134]. Similarly, repressed expression of miR-451a fails to regulate YWHAZ and ADAM10 thus promoting metastasis and EMT [135,136] while upregulated miR-494-3p augments enhance HCC metastasis by targeting BMAL1 [137]. Additional example of miRNAs influencing metastasis in HCC pathogenesis include miR-96-5p [161–164], miR-1246 [95,165–168] and miR-210-3p [101,118,169,170].

miRNA as an Ancillary Epigenetic System

MicroRNAs can be classified as an ancillary epigenetic system because they are able to regulate gene expression at a post-

transcriptional level, as well as because they are directly connected to the mainstream epigenetic machinery via upstream and downstream regulatory loops [171,172]. In this regard, miRNAs regulate gene expression, rather than silence it, by attaching themselves to complementary 5′ mRNA to repress a small percentage of mRNA translation [20]. It differs, therefore, from the mainstream epigenetic machinery in terms of the degree of silencing, as well as the molecular mechanism employed [173]. In all cancers, the mainstream epigenetic machinery is either activated or de-activated by multiple clinical, environmental or genomic changes that interact with miRNA expression [174]. In HBV-HCC pathogenesis, the HBx protein can manipulate the expression of DNMTs, HMTs, HDMTs, HATs and HDACs [175]. The important role of the HBx protein in HBV-HCC pathogenesis is illustrated by its ability to upregulate DNMTs like DNMT1, DNMT3A1, and DNMT3A2, as well as selectively promote regional hypermethylation of specific tumor suppressor genes [176]. Similarly, the HBx can promote the silencing of mRNA expression by inducing histone deacetylases (HDACs) which are made up of zinc-dependent HDACs (HDAC1-11) and non-zinc-dependent HDACs or sirtuins (Sirt1-7) [177]. Conversely, HBx can upregulate mRNA expression by promoting HATs which include four families including GNATs (Gcn5, PCAF, Hat1, Elp3 and Hpa2), p300/CBP (p300 and CBP), MYST (Esa1, MOF, Sas2, Sas3, MORF, Tip60 and Hbo1) and Rtt10 [178]. Similar to HATs and HDACs, the HBx protein can promote histone methyl transferases (HMTs) and histone dimethyl transferases (HDMs) that are responsible for the cycling of methyl groups in histone tails [179]. In particular, the HBx protein can interfere with the Polycomb Repressive Complex 2 (PRC2) to induce H3K27me3-mediated gene expression silencing by targeting PRC2 proteins like embroyonic ectoderm development (EED), enhancer of Zeste 1/2 (EZH1/2) and suppressor of Zeste 12 (SUZ12) [180].

In many cases, HBV infection promotes epigenetic/miRNA feedback loops that include upstream epigenetic manipulation of miRNA expression to target downstream epigenetic targets [171,181] (see Table 2). HBx-dysregulated miRNAs, therefore, can be regarded as ancillary epigenetic regulators in HBV-HCC pathogenesis that interact with the mainstream epigenetic machinery. Examples of the interaction of the mainstream epigenetic machinery with miRNA regulation in HBV-HCC includes the dysregulation of miR-17-92 family members by the HDAC SAHA who can regulate downstream DNMT activity [182] while miR-221 can be significantly increased by hypermethylation of HDAC 6 expression [183,184]. Two important epi-miRNAs, miR-29a/b, can be upregulated by HBx induced HATs to repress downstream DNMT1/3A to reduce PTEN silencing and exert an anti-oncogenic influence [185–189]. Other examples of HBV induced upstream epigenetic regulation of miRNA expression includes the HBx recruitment of the epigenetic protein EZH2 to downregulate miR-139-5p [190]. Illustrating the complexity of these interactions, HBx dysregulated miRNA can repress multiple mRNA targets that include proto-oncogenes, tumor suppressors and epigenetic proteins (see Table 2).

DNA methylation in HBV-HCC pathogenesis, for instance, can downregulate miR-1/-122/-124/-132/-/148/-200/-205 (see Fig. 2). Conversely, HATs or HDAC inhibitors can upregulate miR-224/-29/-155/-17-92, and HMTs can downregulate let-7c/miR-101/-125b/-139-5p (see Fig. 2). In many cases, these same miRNAs regulate downstream epigenetic targets. HBx induced HMT, for instance, can attenuate let-7c expression that fails to regulate downstream epigenetic proteins like EED/EZH1-2/-SUZ12 that promote HMT [190–192]. HBx induced DNMT1 can

TABLE 2

miRNA interactome with epigenetic machinery in HBV-HCC (HBx)*

HBx upstream	miRNA#	Epi target	HBV-HCC target	Epi-Ref.
HMT/EZH2 EED/SUZ12	Let-7c	SUZ12/EED/ EZH1/2	COL1A2/NGF/BCL-XL/BCL-2/MCL-1/CNKD1/SFRP5/B-CAT/STAT3/RAS/MYC/IL-6-10/TLR-4/	[190–192]
DNMT	miR-1	HDAC4	PI3K/AKT/EDN1/ METFOXP1	[187,193,194]
HMT/EZH2/ EED/SUZ12	miR-101	DNMT3A/ EZH2/ EED/SUZ12	MTOR/SOX9/DNMT3A/FOS/RAP1B/VEGF GSTP1/FOS/ MCL-1/RASSF1A/CNKD1/MCL-1/ROCK2/	[190,192,195]
DNMT	miR-122		ADAM10/CCNG1/Igf1R/ADAM 17/BCL-W/CTNNB1/ CCNG1/p53/GLD2/NDRG3/GALNT10	[196–197]
DNMT1	miR-124	EZH2	CYCD/CDK6/E2F6/STAT3/PIK3CA/ROCK2/STAT3	[190,198,199]
	miR-125a-5p	SIRT7	VEGF-A/ERBB2/MMP11/HBsAg	[200,201]
EZH1/2/HMT/ SUZI2/EED	miR-125b	SUZI2/ EED/ EZH1/2	SMAD2/4/Sirt7/LIN28 B/PIGF/BCL-2/MCL-1/CNKD1/ PRICKLE/B-CAT/PIGF/MMP2/MMP9/	[192,198,202,203]
DNMT3	miR-132	p300	AKT/GSK3/WNT-BCAT	[204,205]
HMT/EED/ SUZ12/EZH2	miR-139-5p	EED/SUZ12/ EZH1/2	PRICKLE/ZEB1/2/CNKD1/SFRP5/B-CAT	[190,192]

(Continued)

Table 2 (continued)

HBx upstream	miRNA[#]	Epi target	HBV-HCC target	Epi-Ref.
DNMT1	miR-148a	DNMT1	/ERBB3/BCL-2/MTOR/MET/SNAIL/IGF-IR/CDC25B	[206,207]
	miR-152	DNMT1/3A	GSTP/CDH1/KIT/AKT/ERK/FOXO4	[187,204,208]
HDAC-I/EZH2/ ZHX2	miR-155	PRC2	SOX6/ZHX2/PTEN/SOCS1	[34,203,209]
HDAC-I	miR-17-92	DNMT	PTEN/p21/p27/p57/E2F1, Cyclin G1/cccDNA	[182,210]
DNMT	miR-200a	HDAC4	Rho/ROCK/ZEB1/2/HNF-3β/ASB4	[211,212]
EED/SUZ12/ EZH1/2	miR-200b	EED/SUZ12/ EZH1/2	PRICKLE/SFRP5CNKD1/B-CAT	[192,212]
DNMT	miR-205		ACSL4/E2F1/ZEB1/2	[213]
DNMT	miR-221	HDAC6	BMF/p27 p57/PTEN/ERα/DDIT4/p21/SOCS3	[183,184]
DNMT	Mir-222		PTEN/PPP2R2A/P27Kip1//p57/p21	[184]
HAT	miR-224		inhibitor-5/SMAD4/PAK4/MMP9	[214,215]
	miR-26a	EZH2	Cyclin D2/Cyclin E2/IL-6/IFN/ER/c-JUN/CDK4/6	[216–218]
	miR-29c	DNMT3B	MCL-1/BCL-2/TNFA1P3	[185,186,219]
H3K4ac	miR-29a-b	DNMT1/ DNMT3A	PI3K/AKT/MMP-2/PTEN	[185–189]
DNMT1/3A	miR-34a	SIRTI	CCND1/CDK2/4/6/CCL22/MAP4K4/MET/C-JUN	[220]

Note: *[171]; [#]red = upregulated, blue = downregulated.

downregulate miR-124 which reduces its repression of the PRC2 protein, EZH2 which promotes HMT [190,198,199]. Conversely, HBV infection can promote hypermethylation of tumour suppressor ZHX2 that upregulates miR-155 that increases its modulation of downstream PRC2 epigenetic proteins [34] (see Fig. 4).

miRNA Interaction with Immune Response in HBV-HCC Pathogenesis

The immune system is influenced by an interactome of genes whose expression is modulated by extracellular signaling, the mainstream epigenetic machinery, multiple transcription/ splicing factors, translational protein modifiers, and miRNAs [9]. MiRNA regulation of both the innate and adaptive immune system in HBV-HCC has been well documented. In the innate immune system, for instance, granulopoiesis can be modulated by miR-155/-21/-223/-21/-196b/130 [221] while miRNA like miR-181a/-150 and Let-7 can regulate natural killer (NK) cell expression [221]. Macrophage output, for example, can be modulated by miR-155/-146a/-124/-125b/-21/-9 and let-7e [221] while dendritic cell (DC) update and differentiation can be modulated by miR-155 [222] and miR-21/-34a, respectively [223]. In the adaptive immune system, miR-17-92/-181a can have a regulatory role in T-cell development [224,225] while B-cell development can be modulated by miR-181/-150/-212/-132/-17-92/-34a/-21/-148/-125b/146a/155 [221]. To provide a more detailed explanation of miRNA immune system regulation than the simplistic examples above, four key HBx dysregulated miRNAs, namely, miR-21/-181a/17-92/miR-155 illustrate the respective molecular mechanisms (see Fig. 5).

HBx induced miR-21 in HBV-HCC can influence immuno-expression in a number of ways: In the innate immune system HBx upregulated miR-21 can exert both pro and anti-inflammatory influences due to its modulation of the transcription inhibitor PDCD4 which influences macrophage activity. First, miR-21 repression of transcription inhibitor PDCD4 can promote pro-inflammatory NF-κB led signaling, and conversely, upregulated miR-21 repression of PDCD4 augments the promotion of anti-inflammatory IL-10 expression [226,227]. HBx upregulated miR-21 can also regulate monocyte-derived dendritic cell (MDDC) differentiation by attenuating JAG1 and WNT1 [223]. In the adaptive immune system, HBx upregulated miR-21 can promote Th17 differentiation by representing SMAD-7, an inhibitor of TGF-β signaling [228]. This miRNA also suppresses SMAD7 to promote TGF-β led promotion of Th17 differentiation [228], as well as represses IL-12, which can induce Th1 responses to attenuate IFNγ production resulting in a diminuation of the Th1:Th2 ratio in T-cell production [229]. This HBx upregulated miRNA can also attenuate IFN by repressing MYD88/IRAK to influence HBV replication [230]. Finally, miR-21 can promote NFκB activation and TNF-α and IFNγ production in activated T-cells to promote inflammation in the HBV TME [231]. These examples illustrate that miR-21 expression can exert multiple pro- and anti-inflammatory responses [227] in both the innate and adaptive immune systems.

In the innate immune system, HBx upregulated miR-181 can regulate inflammatory responses by repressing IL-1a to exert an anti-inflammatory response influencing the expression of monocytes and macrophages [232]. This miRNA can also repress the inflammatory response in dendritic cells (DCs) cells by targeting FOS [233], as well as

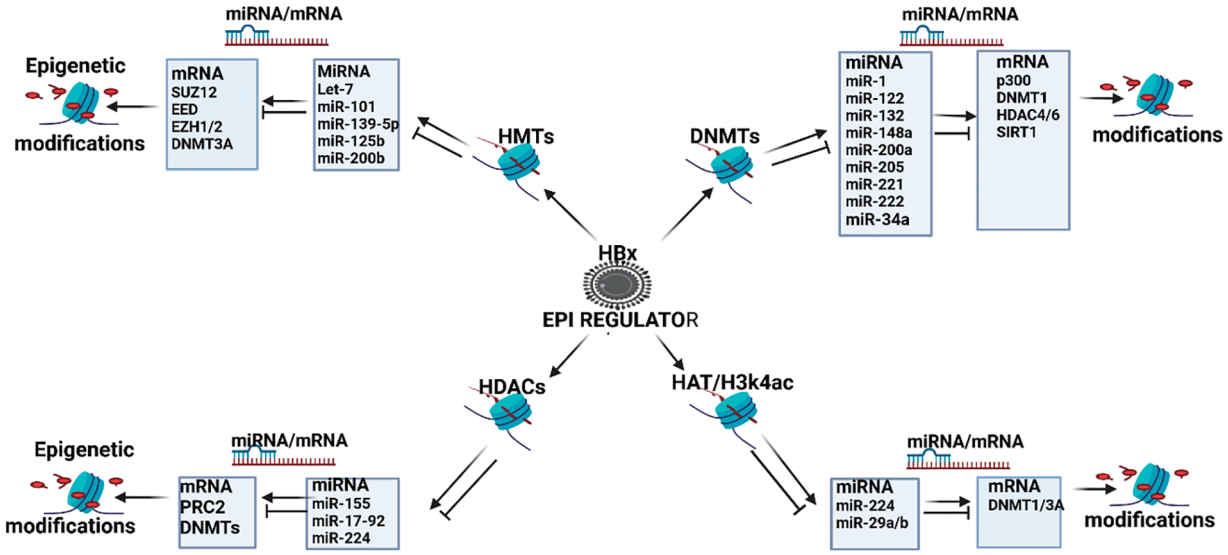

FIGURE 4. HBx promoted epigenetic regulation of miRNAs in HBV-HCC. The HBx protein can modulate the mainstream epigenetic machinery including **Histone methyl transferases (HMTs)** that can dysregulate miRNA like let-7/-miR-101/-125b/-139-5p/-200b that, in turn, modulate downstream epigenetic proteins like EED, EZH1/2 and SUZ12 to influence a range of downstream HMTs, as well as DNMT3a to activate/silence gene expression in all the HBV-HCC pathways. **DNA Methyl Transferases (DNMTs):** The HBx protein can regulate DNMTs that dysregulate miRNA like miR-1/-122/-132/-148a/-200a/-2 05/-221/-222/-34a that regulate downstream epigenetic proteins including the HAT E1A-binding protein (p300), DNMT1, HDAC4/6, and SIRT1. **Histone deacetylases (HDACs):** The HBx protein can also influence a range of HDACs that can dysregulate miRNA like miR-155/-17-92/-224 that modulate downstream PRC2 proteins, as well as DNMT expression. **Histone acetylases (HATs):** The HBx protein can influence HATs that can dysregulate miRNA like miR-224/-29a/-29b that regulate downstream DNMT1/3A silencing (Figures done in Biorender).

modulate an anti-inflammatory response by targeting IL-6 and TNFα [234,235]. HBx upregulated miR-181a can also promote natural killer (NK) cell output by upregulating NOTCH signaling as a result of repressing NLK [236]. In the adaptive immune system, miR-181 can stimulate T-cell receptors (TCRs) by repressing DUSP5/DUSP6/SHP2/PTPN22. Conversely, this upregulated miRNA can also repress T-cell expression by repressing CD69 [225]. The overexpression of miR-181 can also skew haematopoiesis towards the development of B-cells at the expense of T-cells by repressing DUSP5/6/SHP2 and PTPN22 [237].

In a further example, the HBx upregulated miR-17-92 family members also play a prominent role in modulating immune response in HBV-HCC pathogenesis. This miRNA can reduce monocyte production by repressing RUNX1 which then fails to promote Granulocyte colony stimulating factor receptor (G-CSFR) expression. Conversely, RUNX1 can also repress miR-17-92 expression and promote monocyte differentiation [238]. CSFR stimulation is linked to increased macrophage activity, inflammation, tissue remodeling, and HCC [239,240]. In the adaptive immune system, the upregulated expression of miR-17-92 can repress the tumour suppressor PTEN to promote Th1 response *vs.* Treg generation [241]. In addition, miR-17-92 family members can also target CD69 resulting in reduced T-cell output [242]. Upregulated miR-17-92 can also suppress BIM to promote B-cell development [243] and miR-17-92 family members can modulate the migration of $CD4^+$ T cells into B cell follicles by repressing PHLPP2 to promote T-follicular helper (T_{FH}) cell differentiation [244].

The miR-155 family is a crucially important multifunctional regulator of immune responses including inflammation, hematopoietic lineage differentiation, and the onset of carcinogenesis [245]. This important miRNA is expressed by macrophages, dendritic cells (DCs), B cells, T cells, and progenitor/stem cell populations. In the presence of healthy cases, the expression of miR-155 in immune cells is low until their activation by antigens, Toll-like receptor (TLR) ligands, and inflammatory cytokines, which rapidly promote their expression [246,247]. In summary, this important miRNA plays a major role in regulating cytokine production, inflammation, and myeloid and lymphoid differentiation and has a unique ability to modulate the transcriptome of activated myeloid and lymphoid cells [248].

The Status quo of miRNA Therapeutics and Diagnostics: Problems and the Way Forward

In this section, we review the status quo of clinical trials for miRNA deployed as therapeutic and diagnostic agents before outlining some of the problems confronting this field of research and making suggestions for the way forward (see Table 3). In the previous sections of this paper, it is clear after 30 years of miRNA research in HCC pathogenesis, that we have a detailed understanding of the molecular mechanisms of miRNA as a regulatory agent. This detailed understanding includes the molecular mechanisms of miRNA biogenesis, function, and dysregulation, as well as their regulatory targets and role in HBV-HCC pathogenesis

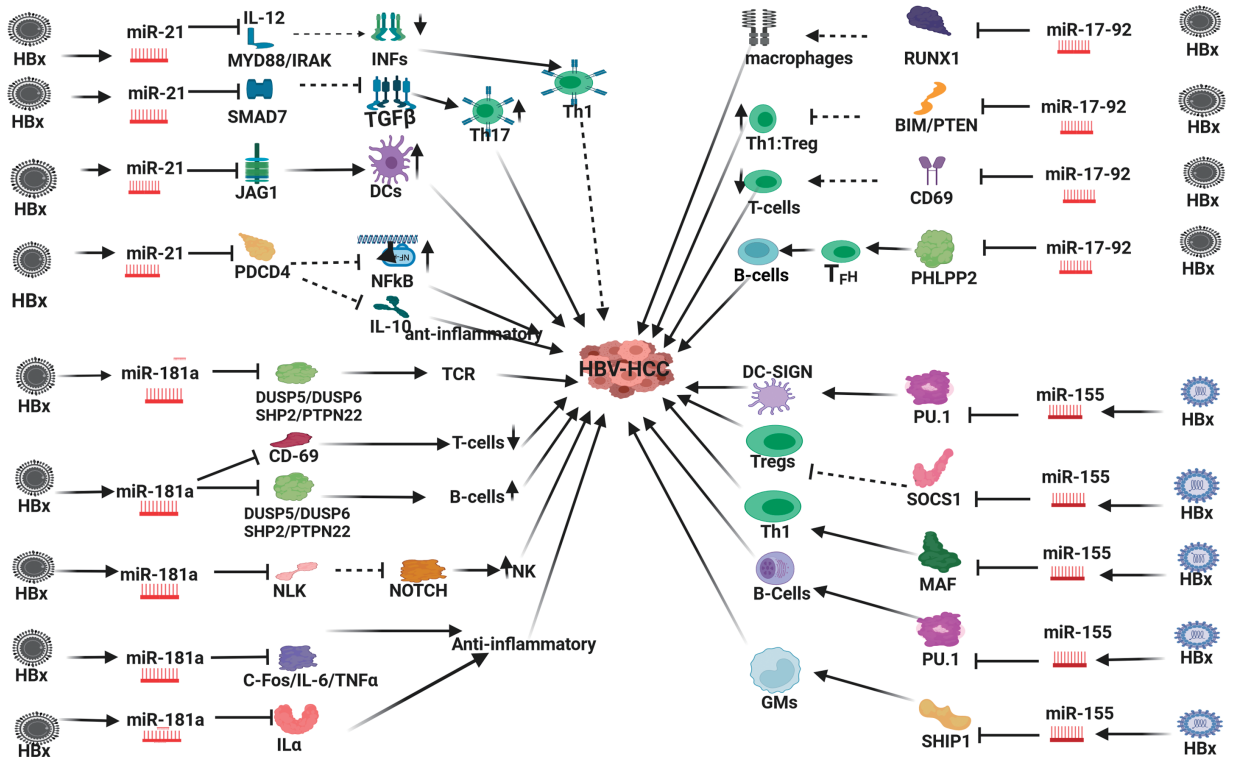

FIGURE 5. HBx dysregulated miRNA and immune response in HBV-HCC. In HBV-HCC pathogenesis the HBx virion dysregulates miR-21/-181a/-17-92 family/-155 which influences immune expression in both the innate and adaptive immune systems: **HBx dysregulated miR-21:** can reduce Th1:Th2 ratio by targeting MYD88/IL-12 induction of INFs to promote Th1; it can also promote Th17 expression by suppressing SMAD7 which is a negative regulator of TGFβ; miR-21 can also increase DC output by repressing JAG1; in T-cells, miR-21 can also influence macrophage output by repressing PDCD4 to reduce its repression of pro-inflammatory NF-κB ledsignalingg; miR-21 repression of PDCD4 can also upregulate of the anti-inflammatory IL-10. **HBx dysregulated miR-181a:** miR-181a can repress DUSP5/6/SHP2/PTPN22 to promote T-cell receptor expression and B-cell output; miR-181a can repress CD-69 to attenuate T-cell production; miR-181a can repress NLK which fails to modulate NOTCH induced NKs; miR-181a can repress C-FOS/IL-6/TNFα/Interleukin 1α (ILα) to promote an anti-inflammatory response influencing monocyte and DC expression. **HBx dysregulated miR-17-92:** miR-17-92 can repress RUNX1 to attenuate macrophage expression; miR-17-92 can repress PTEN/BIM to influence Th1:Treg ratio; miR-17-92 can repress CD-69 to attenuate T-cell production; miR-17-92 can repress PHLPP2 to promote T_{FH} promotion of B-cell output. **HBx upregulated miR-155:** miR-155 can repress PU.1 to promote DCs; miR-155 can repress SOCS1 that fails to repress Treg expression; miR-155 can repress MAF mRNA to promote Th1 differentiation; miR-155 can repress PU.1 to promote B-cell expression; miR-155 can repress phosphoinositide phosphatase 1 (SHIP1) to promote granulocyte-macrophage (GM) population (Figures done in Biorender).

from the PME to the TME. This understanding shows precisely how miRNA regulates mRNA in cell proliferation, apoptosis, angiogenesis, migration, invasion, and metastasis. There is also a detailed understanding of their role as an ancillary epigenetic agent, as well as their interactive regulatory role in the innate and adaptive immune systems. The question remains, if we have this detailed understanding of miRNA regulation in HCC pathogenesis, why after 30 years have we not been able to develop miRNA drugs or biomarkers outside of a laboratory setting? To date, no approved miRNA-based drug or commercially adapted biomarker has been developed. An evaluation of miRNA clinical trials for HCC suggests a much higher proportion were established to find diagnostic biomarkers than to develop miRNA drugs. The following HCC miRNA clinical trials highlight some of the problems (see Table 3).

HCC clinical trials testing miRNA as therapeutic agents

This section examines the results of clinical trials for miR-34a/-193-5p/-221/-222/-155. A Phase 1 clinical trial using a miR-34a mimic (NCT01829971) developed a therapeutic agent (MRX34) that used a synthetic double-stranded (ds) miR-34a mimic that was designed to be encapsulated in a liposomal nanoparticle in order to translate this drug to be used in a clinical application. This miRNA has been extensively tested as a therapeutic agent for HCC in multiple experiments involving animal and cell-line studies [249,250]. The importance of this miRNA in HCC pathogenesis is illustrated in this study where it is cited as playing a regulatory role in the PME (fibrogenesis), cell cycle, proliferation, apoptosis, metastasis, as well as interacting with the mainstream epigenetic machinery (see Tables 1 and 2). The Phase 1 clinical trial of MRX34 was the first-in-human clinical trial of miRNA therapy that was deployed for a range of cancers including primary liver cancer. The clinical trial was abandoned as a result of toxic immune mediated events and not as a result of delivery issues [251]. Although the tumor suppressor miR-34a mimic in this trial successfully reduced expression in a range of onco-targets, it elicited an unintended adverse immune response [252]. From a translational perspective, this drug is designed upregulate the expression of targets like MAP4K4

TABLE 3

Therapeutic and diagnostic miRNA clinical trials

MiRNA	Clinical trial*	Description (last post date)	Status
Therapeutic			
miR-34a	NCT01829971	MRX34 Ds-mimic (liposomal nanoparticle)	1 (terminated)
miR-193-5p	NCT04677596	INT-1B3 mimic (liposomal nanoparticle)	1 (terminated)
mR-221/222	NCT02928627	Evaluation of potential targets, potential (2017)	1 (unknown)
miR-155	NCT03713320	MRG-106 miR-inhibitor (LNA-oligonucleotide)	2 (terminated)
Diagnostic			
Candidate-miR	NCT 05148572	serum miR-profile: response to resection pre/post	1 (recruiting)
Candidate-miR	NCT 04965259	serum miR-profile: diagnostic tool for high-risk cases	1 (recruiting)
Candidate-miR	NCT 06342414	serum miR-profile: HCC *vs.* HC differential expression	1 (recruiting)
Candidate-miR	NCT 02507882	serum miR-profile: IL-28 polymorph in HCV-HCC (2015)	1 (unknown)
Candidate-miR	NCT 02412579	serum miR-profile: pre/post-transplant (2022)	1 (completed)
Candidate-miR	NCT 04720430	serum miR-profile: predict response to treatment (2023)	1 (completed)
Candidate-miR	NCT 05431621	serum miR-profile: Diagnostic tool for HCC (2024)	1 (completed)
Candidate-miR	NCT 05449847	serum miR-21/-125: Diagnostic tool for HCV-HCC (2022)	1 (completed)
Candidate-miR	NCT 03429530	serum miR-profile: Diagnostic tool for HCV-HCC (2018)	1 (unknown)
Candidate-miR	NCT 03227510	serum miR-profile: diagnostic tool for HCC (2017)	1 (unknown)
Candidate-miR	NCT 01247506	tissue miR-profile: Predict post-surgical survival (2010)	1 (unknown)
Candidate-miR	NCt02448056	serum miR-profile: HCC biomarker (2015)	0 (not started)

Note: *ClinicalTrials.gov identifier.

and CCL22 mRNA in HBV-HCC to repress tumor growth in a microenvironment where the HBx protein represses miR-34a [250,253]. A second (more promising) ongoing clinical trial for HCC in Phase 1, developed a drug INT-1B3 that packaged a miR-193-5p miRNA mimic in a lipid nanoparticle (NCT046775996). To date, this drug has demonstrated that it can regulate cell proliferation and from a translational perspective is designed to target CDK2 controls and be delivered directly to tumors *in vivo* [254] but the ClinicalTrial.gov website now lists this trial as terminated. Other trials involving miRNA specifically for HCC appear inconclusive. One trial deploying miR-221/-222 intends to measure circulating miRNA expression in healthy controls *vs.* HCC cases to evaluate their therapeutic potential (NCT02928627) but the last comment was posted on 25/10/2017, no publications have been recorded and the status is unknown. An interesting clinical trial (NCT 03713320), designed for solid tumors in general (not specific to HCC), involved the drug MRG-106 which successfully deployed a miR-155 inhibitor using an LNA-modified oligonucleotide. This trial progressed to Phase 2 when the trial was abandoned due to business related reasons and appears to have been successfully tolerated by patients [255]. Interestingly, it has been argued that deploying miRNA inhibitors results in fewer unintended consequences than miRNA mimics because the targeted mRNA can be made more specific [252]. Although this clinical trial was not specifically targeted for HCC, this oncogenic miRNA plays an important role in HCC pathogenesis (see Tables 1, 2), especially regarding its regulatory role in both the innate and adaptive immune system (see Fig. 5). In summation, therefore, no miRNA clinical trial has progressed to stage 4 and therefore miRNA therapeutics remain in the work-in-progress phase (https://classic.clinicaltrials.gov/ct2/results) (accessed on 10 September 2024).

Problems and research gaps

In general, a list of challenges in developing successful miRNA drugs include degradation by *in vivo* nucleases, poor cell membrane penetration or getting trapped in the endosome, poor binding affinity for complementary sequences, poor delivery to desired target tissues, off-target and unwanted toxicities and activation of unintended immune responses [256]. To a large extent, delivery problems are less of a problem and, despite a detailed understanding of how miRNA regulate single gene targets, a research gap exists with respect to unintended consequences of off-target regulation and resultant toxic effects [252]. Simultaneously, a central problem of developing systemic therapy for HCC, especially in advanced stages, is that a multiplicity of driver genes, genes, cancer pathways, and miRNA become simultaneously dysregulated [32,257,258]. Collectively, this illustrates that single miRNA mimics could be simplistic tools given that their regulatory role can be categorized as an ancillary epigenetic tool that finetunes mRNA expression [171]. Simultaneously, one miRNA can have >100 mRNA targets making the problems of unintended consequences difficult to avoid [252]. In addition, miRNA regulation in HBV-HCC pathogenesis involves a dynamic orchestra of miRNAs, rather than any single miRNA, that is simultaneously regulated by viral proteins, the mainstream epigenetic machinery, other ncRNA like lncRNA and

circRNA, and the innate and adaptive immune systems [28,259]. A further research gap that needs to be better understood is the reasons for the degree of heterogeneity between miRNA expression in tumor tissue vs. serum. For instance, one HCC study demonstrated that miR-100-5p/-99a-5p/-455-3p/-10a-5p/-30b-5p/204/5p/let-7a-3p were upregulated in plasma and downregulated in tumor tissue suggesting that miRNA can be selectively released from the TME into serum [260]. The differential miRNA expression between tissue and serum is illustrated in multiple cancers including breast and colorectal cancers [261,262] and this needs to be better understood when selecting candidate miRNA for therapeutic or diagnostic purposes. Simultaneously, in HBV-HCC pathogenesis, it is possible that the interests of the hepatitis B virus contradict other aspects of pathogenesis in both the PME and the TME [8]. A research gap indicates that the role of the HBV virion and its resultant expression need to be extended to a more detailed understanding of how this virus interacts with miRNA in HBV-HCC pathogenesis from the PME to the onset of the tumor environment. In conclusion, the multiplicity of interactions, targets, and mechanisms, illustrated by this paper, highlights a central problem confronting miRNA therapeutics, namely, unintended mRNA regulation because a single miRNA can regulate multiple mRNA targets [263]. These problems appear to have influenced current miRNA clinical trials to focus on the potential of miRNA as diagnostic agents rather than their therapeutic potential. In this regard, siRNA therapeutics appear to have progressed further than miRNA and typically target mRNA in a more efficient way than miRNA [264]. Although both ncRNA silence mRNA, one miRNA can regulate multiple different mRNA targets to varying degrees depending on the complementarity of their binding RNA sequences whereas siRNA is designed to bind to a single mRNA target with perfect complementarity [264,265].

HCC diagnostic clinical trials

Currently registered miRNA HCC clinical trials indicate 12 out of 13 trials are for diagnostic purposes and only one for a therapeutic outcome (https://clinicaltrials.gov/search) (accessed on 10 September 2024). These clinical trials mostly focus on the detection of early stage HCC, evaluating the implications of therapeutic interventions like resections/transplants or as a prognostic tool [252]. Of the 12 registered clinical trials, three are in the recruiting phase and nine have been completed or their status is unknown. In cases that are still recruiting, NCT 05148572 will develop circulating miRNA profiles pre *vs*. post resection patients to evaluate the efficacy of this therapy. NCT 04965259 will determine serum miRNA expression profiles for high-risk *vs*. healthy patients to develop an early-stage biomarker and NCT06342414 will also attempt to develop a liquid biopsy based on suitable circulating miRNA candidates as an early-stage biomarker. The outcomes of the other nine clinical trials appear to have produced indeterminate results with no publications generated. The following Phase 1 clinical trials and the last date an update was posted (in brackets) are all listed as completed with no publications including NCT 02412579 (2022), NCT 04720430 (2023), NCT 05431621 (2024) and NCT 05449847 (2022). NCT 02412579 investigated serum miRNA profile to test response to liver transplant, NCT04720430 investigated serum miRNA profile to determine response to pre-transplant bridging treatment, NCT 05431621 attempted to develop serum based biomarker for early stage gastric cancers (including HCC), and NCT 05449847 tested miR-21/-125 as biomarker for early stage HCV-HCC. Multiple clinical trials indicate unknown status including with date of last posted comment in brackets NCT 02507882 (2015), NCT 03429530 (2018), NCT 03227510 (2017), and NCT 01247506 (2010). NCT 02507882 was designed to test serum miRNA profiles of IL-28B polymorphism in HCV-HCC, NCT 03429530 was designed as a serum based tool to diagnose HCV-HCC, NCT 03227510 was designed as a serum based miRNA biomarker for HCC and NCT 01247506 involved tissue based miRNA profiling to determine HCC progression. None of these trials developed any publications.

In summary, no commercially adopted miRNA based diagnostic tools have been adopted. In a meta study involving HBV-HCC cases, miR-125b was listed as the most consistently identified miRNA with high levels of specificity and accuracy [266] while let-7 and miR-122 family members are consistently listed as having high Area under Curves (AUCs), specificity and sensitivity in China (AUC = 0.905) [267], South Africa (AUC = 0.9420) [32] and India [268]. Interestingly, let-7a which is listed as an important regulator of HBV-HCC (see Table 1) is currently being tested in a clinical trial to diagnose non-Hodgkin's lymphoma and acute leukemia (ClinicalTrials.gov identifier: NCT05477667) [252]. Similarly to miRNA clinical trials for therapeutic outcomes, no HCC miRNA clinical trial has progressed to stage 4 concerning its use as a commercially used biomarker (https://classic.clinicaltrials.gov/ct2/results) and this field of research also remains in the work-in-progress phase.

Problems and research gaps

Despite multiple very similar studies, there is limited overlap between these concerning candidate miRNA or miRNA panels. In 32 breast cancer studies, for example, 143 dysregulated miRNAs were identified as potential biomarkers. Of these, 100 miRNAs were only identified in a single study, and in 10 studies only miR-21/-155 was reported as upregulated in three and two studies respectively even though seven of the ten studies used the same method of normalization while other studies showed these two miRNA as downregulated [269]. Furthermore, many candidate miRNA with high levels of specificity are deregulated in multiple cancers. For example, a promising candidate like miR-141 is dysregulated in prostate, breast, colon, HCC, and lupus and, incidentally, has been reported as upregulated during pregnancy [24]. This led researchers to question whether common factors in carcinogenesis like inflammation or injured tissue are the primary triggers of differential miRNA expression in these studies leading to the question are miRNAs potential biomarkers of neoplasms or general disease [270]. A central research gap is evident, namely, in identifying biomarker miRNA that is specific to HBV-HCC and only to HBV-HCC.

Another central problem of overlap between miRNA-based diagnostics is the dynamic nature of miRNA expression that is constantly fluctuating and highly sensitive to any change in the tumor microenvironment, as well as the differential nature of the patient profile including circadian rhymes, diet, and exercise [271]. The differential patient profile including sex, smoking status, and patient specific physiological conditions (diet, active exercise, comorbidities) that intimately affect miRNA expression profiles with potential errors that can be compounded by the modality of sample preparation, RNA extraction, and differences in outcomes from purchased kits, as well as choice of statistical methods used to normalize miRNA expression [271]. Other examples causing limited overlap between similar studies include using miRNA reads *vs.* using unique molecular index (UMI) counts that are different [272]. A further issue is the differential expression of miRNA of circulating miRNA compared to tissue-based miRNA expression that is not fully understood [261]. A research gap, therefore, will be to stabilize miRNA expression across overlap studies to control for multiple confounding variables.

The way forward

Although miRNA research is yet to be translated into tangible therapeutic and diagnostic outcomes, it remains an important opportunity to better detect and manage HCC. Future research is vital to ensure the development of dynamic miRNA panels tailored for patient specific characteristics. To do this, it is not inconceivable that future research might need to incorporate AI based approaches to develop algorithms for predicting miRNA targets that can work alongside current miRNA bioinformatics software [273,274]. Given the lack of control with respect to the unintended consequences of miRNA mimics, tailored delivery sites for miRNA based drugs could reduce systemic unintended consequences [252,254]. Alternatively, tailored packages of upregulated miRNAs can be acutely targeted by miRNA inhibitors or possibly technology to shield selected target tumor suppressor mRNA from overexpressed onco-miRNAs [275]. A new approach is needed for the development of miRNA-based therapeutics given the current depressing status quo illustrated by a lack of promising clinical trials. Refining the targeted ability of miRNA is underlined by siRNA based therapeutics that appear to have progressed to two approved drugs and a number of stage 111 clinical trials including one for breast cancer [263]. Similarly, future research on miRNA as diagnostic agents will need to be able to accommodate the heterogenous nature of patient characteristics, especially in the PME and early-stage HCC pathogenesis. Early detection is vital to provide the most promising prognosis for HCC and miRNA diagnostics in this stage have not been developed beyond Alpha-fetoprotein (AFP) and ultrasound testing which has many limitations remains widely used [276]. The molecular mechanisms resulting in the production and release of miRNA into plasma/serum by tumor cells need to be better understood to determine whether cancer cells selectively release miRNA into serum [277].

Conclusion

HBV-HCC incidence still accounts for a large proportion of global HCC incidence and its idiosyncratic features demand differential therapeutic innovations due to the role of the HBV virion. CHB infection influences HBV-HCC pathogenesis from early-stage infection, inflammation, and fibrosis in the PME to the onset of HBV-HCC. CHB infection simultaneously dysregulates multiple cellular processes, mRNA and miRNA expression to influence cell proliferation, angiogenesis, apoptosis, invasion, and metastasis in a wide range of cancer pathways.

This paper makes the following contributions. First, it summarizes key miRNA aspects of its molecular mechanisms in HBV-HCC pathogenesis including its role in cellular processes, its role as an ancillary epigenetic system, and its interaction with the immune system, importantly it covers an important gap that illustrates miRNA regulation in the PME as a result of CHB infection. This offers an opportunity in HBV-HCC pathogenesis to potentially deploy miRNA in a microenvironment where genomic instability is less pronounced and/or specific aspects of pathogenesis like inflammation or fibrosis can be targeted. Second, the review illustrates the complexity of the HBV-miRNA interactome to attempt to answer the question as to why miRNA therapeutics and diagnostics remain a work-in-progress. Importantly, this is supported by evidence of a lack of successful clinical trials. The paper, therefore, promotes the idea of a rethink as to how this problem can be solved because it becomes clear that the multiplicity of dysregulated gene expression in HBV-HCC is unlikely to be regulated by the deployment of individual miRNA.

We acknowledge there are many limitations in this paper. First, the paper only really provides simplistic snapshots of the complexity of the miRNA interactome, and bioinformatics diagrams of the interaction of a single miRNA can involve 100s of gene targets that are not captured in this paper. Second, this paper does not unpack its interaction with a wide range of other ncRNA like lncRNA and circRNA. In the miRNA interactome with epigenetic machinery, for instance, multiple ncRNA regulate each other, as well as downstream epigenetic targets. Adding to this complexity, upstream epigenetic machinery can modulate downstream ncRNA and all of these players can be regulated by a range of HBV proteins (HBx) that are simultaneously attempting to balance the virus's replication and evading host immune response.

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