[Frontiers In Bioscience, Landmark, 23, 1701-1720, March 1, 2018]

The biological and diagnostic role of mirna's in hepatocellular carcinoma

Kurt Sartorius[1,2,3], Benn Sartorius[1,3], Cheryl Winkler[4], Anil Chuturgoon[5], Julia Makarova[6,7]

[1]Department of Public Health Medicine, School of Nursing and Public Health, University of KwaZulu-Natal, Durban, South Africa, [2]Faculty of Commerce, Law and Management, University of the Witwatersrand, Johannesburg, South Africa,[3]UKZN Gastrointestinal Research Centre, Durban, South Africa, [4]Basic Research Laboratory, Centre for Cancer Research, National Cancer Institute, Leidos Biomedical Research, Inc. Frederick Nat. Lab. for Cancer Research, Frederick, Maryland, USA, [5]Medical Biochemistry, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa, University of KwaZulu-Natal, Durban, South Africa, [6]Hertsen Moscow Oncology Research Institute, Branch of the National Medical Research Radiological Center, Ministry of Health of the Russian Federation, 125284, Moscow, Russian Federation, [7]Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 119991, Moscow, Russian Federation

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1. ABSTRACT

The potential of exploitation of miRNA as diagnostic agents and therapeutic tools will likely only be realized when a complete knowledge of their biology is revealed. Despite more than a decade of research, the use of miRNA as diagnostic and therapeutic tools remains a 'work in progress'. The objective of this review is to explore more recent developments in the role of deregulated miRNAs in hepatocellular carcinoma (HCC). This includes emerging insights involving miRNA biogenesis, their deregulation by cancer and their role in deregulating the principal HCC cancer pathways. Specific attention is directed at the role of deregulated miRNAs in HCC in a developing country context with high hepatitis B/C burden, as well as an examination of the challenges that confront the use of extracellular miRNAs as commercially viable diagnostic tools to detect early stage HCC.

2. INTRODUCTION

Despite the decline of hepatocellular carcinoma (HCC) incidence in traditionally high risk countries, HCC remains the second leading cause of cancer deaths around the world, (1, 2). Factors influencing the high mortality rate of HCC include the dynamic nature of its changing etiology, an incomplete understanding of its molecular biology (3, 4), as well as the problem of early detection. Currently, the early detection of HCC is problematic because it relies almost entirely on imaging techniques and the quantification of serum alpha fetoprotein (AFP) which are unreliable measures in early stage HCC (5). The deployment of serum based markers like microRNA, therefore, offers a unique opportunity to develop an early stage HCC biomarker.

Viral hepatitis continues to account for the majority of HCC cases worldwide but its aetiology

is demonstrating increased linkages with obesity and diabetes (6), the rising incidence of non-alcohol related fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) (4, 7-9). Across the broad spectrum of HCC etiology, common conditions influencing the progression of HCC tumorigenesis include inflammation, fibrosis, oxidative stress and chronic cirrhosis of the liver (10-12). The progression from these conditions to HCC is orchestrated in a number of cancer pathways that become deregulated as a result of both somatic mutations and epigenetic triggers (13). These pathways include deregulation of the RB1-TP53 suppressor networks, the WNT pathway, PI3K/MAPK pathways, and the JAK/STAT pathway (14, 15)

MicroRNAs (miRNAs) act as post-transcriptional gene regulators that collectively silence mRNA thereby playing a homeostatic role that fine tunes the translation of proteins. The ancillary role of miRNAs as mild suppressors has been explained by the inherently stochastic nature of gene transcription and environmental fluctuations (16). In HCC, and its secondary conditions like chronic inflammation and cirrhosis, a wide spectrum of miRNAs become deregulated thus contributing to aberrant messaging in specific cancer pathways that regulate cell proliferation, apoptosis, angiogenesis, DNA repair, invasion and metastasis (17-19). In HCC the expression of many miRNAs is deregulated (upwards or downwards) to adversely influence the expression of their target mRNAs/specific genes (20). In HCC development, the miRNA mediated expression of messenger RNA (mRNA) can have either a direct oncogenic effect or promote a loss of tumor suppressor function in their target genes (19-21).

Despite the early promise of extracellular miRNAs as diagnostic tools, as well as a decade of ongoing investigation, the role of miRNAs is still regarded as a 'work in progress' and mostly restricted to research programs (22, 23) and lack practical translation at present. Simultaneously, the limited diagnostic and treatment options for HCC are reflected in rising HCC mortality rates. Recent literature has demonstrated, however, that certain extracellular miRNA found in body fluids plays an important role in cell to cell messaging (24, 25). The aim of this review is to explore more recent developments in the role of deregulated miRNAs in HCC. This will more specifically include emerging insights involving miRNA biogenesis, their deregulation by cancer (HCC) and their role in deregulating the principal HCC cancer pathways. Specific attention will be directed at the role of deregulated miRNAs in Hepatitis B virus (HBV)/HCC in a developing country context, as well as an examination of the challenges that confront the use of extracellular miRNAs as commercially viable diagnostic tools to detect early stage HCC.

3. miRNA BIOGENESIS, MRNA SILENCING AND EXPORT INTO THE EXTRACELLULAR SPACE

The canonical and alternative biogenesis pathways of miRNAs are well covered in the literature since their discovery in the early 1990s (26). In excess of 2500 human miRNAs have been identified that are under the transcriptional control of ~ 1000 genes (27, 28). MiRNA genes are present either as independent transcription units or located in introns and exons of other genes. Fifty per cent of miRNA coding genes are located within introns and they are frequently transcribed from their own promoters independently of their host genes (29). Briefly, miRNA biogenesis begins with the transcription of pri-miRNAs by RNA polymerase 11. Successive stages begin with the editing of pri-miRs in the nucleus by the DROSHA/DGCR8 endonuclease to produce pre-miRNAs that are then exported out of the nucleus and into the cytoplasm by Exportin5/RanGTP. Pre-miRNAs are then further edited and their biogenesis in the cytoplasm involves a second endonuclease (Dicer/TRBP) that is complemented by helicases that separate the edited miRNA duplex into a single messenger strand. This ≈ 22 nucleotide strand is then incorporated in a protein complex (AGO family) for transportation to intra and extra cellular locations (29-31). In recent review articles, a summary of the role of miRNA-related gene mediation confirms that combinations of miRNAs act as fine tuners of translation in order to maintain homeostasis (16, 24, 25).

3.1. Intracellular mRNA silencing

MiRNAs typically contain a 6 nucleotide (nt) sequence at their 5' end that provides its recognition base to attach to the 3' end of complementary mRNA target (16, 29). MiRNA based RNA silencing complexes (RISCs) repress mRNA translation and promote their destabilization as a result of the degree of complementarity of their sequence specific bases. Mounting evidence indicates that post-transcriptional gene silencing (PTGS) occurs at the surface of the endoplasmic reticulum (ER) because this is the primary location of protein synthesis and RISC loading, however, the exact location of all PTGS locations remains unresolved because both small RNA and Argonaut proteins (AGO1-4) are found in all the organelles of human cells.

Mature miRNAs in the intracellular space can be packaged and transported in a variety of protein and membranous bodies that are directed by the endosomal sorting system. The secretion/excretion of multiple sub-populations of RISCs into the extracellular space, as well as their exact role in messaging systems, remains a controversial issue (24). Interestingly, recent evidence suggests that RISCs can also re-enter the nucleus by binding to facilitating proteins like Importin

8 to either promote or silence gene transcription in the promoter regions of certain genes (29).

3.2. Extracellular mRNA silencing

Extracellular miRNAs are secreted (or excreted) in a range of extracellular vesicles (EVs) including exosomes, micro-vesicles, apoptotic bodies, high-density lipoprotein (HDL) complexes and as solely free-floating complexes with AGO proteins. Solid evidence has emerged that certain of these extracellular RISCs (exosomes) possess paracrine mRNA silencing capability although all exported miRNA may not have this capability. The miRNA sorting system and secretion pathways, however, are not fully understood (29). The extracellular silencing capability of miRNA-containing exosomes has been questioned because of the low volume of miRNA relative to the total number of exosomes and it has been hypothesized that mechanisms (oxidative stress, heat-shock, growth factors) exist to increase the concentration of these EV-miRNA containing bodies that are capable of influencing paracrine or endocrine messaging (23, 32). Evidence in support of cell to cell messaging have been demonstrated in a number of *in vivo* studies. In particular, extracellular miRNA encapsulated in exosomes have demonstrated the ability to convey paracrine messages (33). Evidence suggests, however, that only a small fraction of miRNAs in the extracellular space is capable of PTGS bringing in to question the interpretation of the role of various miRNA sub-populations in body sera (23, 29).

4. DEREGULATION OF miRNA IN CANCER

Dysregulation of miRNA volumes, as a result of genetic and epigenetic triggers, can occur at any stage in miRNA biogenesis from the transcription of pri-miRNAS to the packing of mature miRNAs in RISC complexes (27, 34). The transcription of pri-miRNAs is frequently deregulated by mutations in miRNA genes. For example, the loss of expression of miR-15/-16 is caused by a deletion on chromosome 13q14 (35). In addition, epigenetic changes, caused by hyper-methylation and histone modification, can influence miRNA transcription. The CpG islands found in gene promoters are often hyper-methylated in cancer thus blocking the transcription of miRNAs (35). In addition, changes in the expression levels of cancer pathways can increase or decrease miRNA transcription. The activation of the p53 network, for instance, triggers the production of the miR-34 family that functions as a tumor suppressor by silencing cell proliferation proteins (27, 36).

The miRNA machinery can be dysregulated in both the nucleus and the cytoplasm. In the nucleus the cleavage of pri-miRNAs can be disrupted by deregulation of the DROSHA/DGCR8 microprocessor proteins due to genetic or epigenetic factors. The disruption of this initial endonuclease in the nucleus often results in the decreased production of pre-miRNAs. Pre-miRNAs, in turn, may not be exported from the nucleus due to the downregulation of export proteins like Exportin 5. Similar deficiencies can interrupt the production of mature miRNAs in the cytoplasm due to deregulation of the DROSHA/DICER 1 machinery (27, 34). Finally, conditions of hypoxia, often activate EGFR which interact with the AGO family of proteins to prevent the successful packaging of mature miRNAs in RISCs. The association between EGFR and AGO2 is enhanced by hypoxia, leading to elevated AGO2-Y393 phosphorylation, which in turn reduces the binding of Dicer to AGO2 (27, 37).

In cancer, miRNA volumes can also be deregulated even when the miRNA machinery is not dysregulated as a result of the dysregulation of other Microprocessor RNA binding proteins. The let-7 family of miRNAs, for instance, is often downregulated by expression of LIN28A/B RNA binding proteins (27, 38).

5. miRNA UPREGULATION AND DOWNREGULATION IN HCC

Deregulated miRNAs in HCC (See Tables 1-2) inversely influence the expression of their target mRNAs/genes involved in cell cycle regulation, apoptosis, angiogenesis, DNA repair, invasion and metastasis (20, 39). In HCC development, deregulated miRNA expression can have either oncogenic effects or alternatively, promote a loss of tumor suppressor function (19-21). Although the downregulation of miRNAs is more common in cancer (40), certain upregulated miRNAs are consistently reported in HCC studies, for example, miR-21/-221/-222/-224 (41).

Upregulated miRNAs (see Table 1) typically illustrate an inverse relationship with the expression of tumor suppressor proteins. For example, upregulated miR-21/-148a/-216a/-221/-222/-519d have been cited in HCC as playing a role in the downregulation of the PTEN tumor suppressor gene, a key control point in the P13K/MAPK cancer pathway (42-45). Another miRNA that is consistently upregulated in HCC is miR-224, which targets the apoptosis inhibitor-*5 (API-5*) gene to inhibit its transcript expression (46).

It has been suggested that miR-122a is the most abundant miRNA in hepatocytes (47), and that it is down-regulated in ~70% of HCC (48). The downregulation of miRNAs results in a reduced capacity to modulate (silence) oncogenic proteins involved in cell cycle/proliferation, angiogenesis and metastasis (see Table 2). Downregulated miR-26a/-34a/-122/-138/-195/-497 in HCC, for instance, have a reduced ability to regulate cell cycle proteins like cyclins and cyclin dependent kinases (CDKs) (41, 49,

Table 1. Upregulated miRNAs in HCC

miR	Target gene	cellular process
miR-10a/b	EphA4, CADM1	EMT, metastasis
miR-17-92	c-Myc, E2F	cell cycle, apoptosis
miR-17-5p	p38 pathway	Migration
miR-18a	ERalpha	Proliferation
miR-21	PTEN, PHOB, MAPK2K3, PDCD4, HEPN1, hSulf-1, DCC6	Metastasis
miR-22	Era, IL-IA	Carcinogenesis
miR-23a	PGC-1α, G6PC	Gluconeogenesis
miR-25	Bim, Bid, TGF-β	apoptosis, invasion
miR-30d	GNAI2	invasion, metastasis
miR-106b	Bim, E2F1, Axin1	apoptosis, proliferation, EMT
miR-130a	RUNX3	drug resistance
miR-130b	TP53INPI	cell growth, self-renewal
miR-135	MTSS1, APC, Axin	Metastasis
miR-143	FNDC3B	Metastasis
miR-148a	PTEN	Metastasis, invasion
miR-155	PhoA, TLR, APC, ATIR, AHIPI, C/EBPBeta, SOX6	metastasis, proliferation
miR-181b	TIMP3	Proliferation
miR-182	IGF-IR, MTSSI, TP53INPI, CEBPA, RASAI	proliferation, metastasis, angiogenesis
miR-210	VMP1, AIFM3	migration, invasion
miR-216a	PTEN, SMAD7, TSLC1, Bmf, CDKN1B/p27	Metastasis
miR-221	Bmf, Bid, HDAC6, CDKNIB/p27/kip1, CDKNIC/p57/kip2, PTEN, DDIT4, Amt, Erα, Trps1, RBI	apoptosis, metastasis, angiogenesis
miR-222	AKT, PTEN, p27, p57, PPP2R2A, Trps1	metastasis, angiogenesis
miR-224	Bcl-2, Bcl-w, RKIP, CDC42, CDH1, PAK2, MAPK1, API-5,smad4	Apoptosis
miR-301a	Gax	Metastasis
miR-315	APC, Axin	angiogenesis, invasion
miR-335	RBI	cell cycle
miR-373	PPP6C	Proliferation
miR-490-3p	ERGIC3	EMT
miR-519d	CDKN1A/p21, PTEN, AKT3, TIMP2, MKi67	proliferation, metastasis
miR-550a	CREB4	Metastasis
miR-590-5p	TGF-beta R11	Metastasis
miR-615-5p	IGF-11	cell growth, migration
miR-657	TLE1	Proliferation
miR-1246	Axin2	EMT

Data in the table are derived with permission from (19, 21, 30, 62).

50). Downregulated miR-125a/-126/-195 are less able to modulate angiogenesis (51, 52) and the let-7 family has a reduced ability to block oncogenic targets that prevent apoptosis and/or promoting metastasis (53, 54). One of the most consistently under-expressed miRNAs in HCC, namely, miR-199 plays a role in failing to modulate the mTOR pathway thus contributing to cell proliferation and invasion (55).

Interestingly, miR-148a has been reported as upregulated in HBV/HCC (56), as well as downregulated in HCC resulting in diminished suppression of Snail1 which suppresses E-Cadherin thus facilitating the progression to EMT in HCC (57, 58).

6. miRNA IN HCC CANCER PATHWAYS

A full understanding of the molecular etiology of HCC remains incomplete (3). Evidence to date shows that HCC induces a range of changed expression in the PI3K /MAPK pathways, the p53 network, as well as increased WNT/B-Catenin activity and inactivation of key tumor suppressors (SOCSI) in the JAK/STAT network (14, 15). Alterations of RB1, p53 and WNT

Table 2. Downregulated miRNAs in HCC

miR	Target gene	cellular process
miR-1	ET-1, ets1	metastasis, proliferation
miR-7	PIK3CD, mTOR, p70S6K, CCNE1	metastasis, cell cycle
miR-15a/16	Bcl-2, Bcl-w	Apoptosis
miR-22*	HDAC4	cell proliferation
miR-26a	CDK6, cyclin D1, PIK3C2α, c-MET, cyclin E1	cell cycle, metastasis, angiogenesis
miR-29	Bcl-2, Bcl-w, Ras, matrix, MMP-2, SPARC	apoptosis, angiogenesis, metastasis
miR-30	Snail	EMT
miR-34a	Cyclin D1, CDK2/4, c-Met, CCL22	cell cycle, apoptosis, metastasis
miR-99a	PLKI, IGF-IR, mTOR	Cell proliferation
miR-100	PLKI, Angpt2 (mTOR), Rac1, ICMT	Invasion, metastasis
miR-101	ZEB1, Rab5a, DNMT3A, SOX9, FOS, EZH2, NLK, STMN1, ATG4D, Mcl-1, EED	EMT, proliferation
miR-122	CycG, Bcl-w, Bcl-2, Mcl-1, Dxl4, Rho, N-Myc, Adam17, Wnt1, Tace	apoptosis, angiogenesis, metastasis
miR-124	ROCK2, EZH2, PIK3CA, STAT3, Cyclin D	EMT, cell cycle
miR-125a	MMP11, VEGF-A, SIRT7	proliferation, metastasis, metabolism
miR-125b	Bcl-2, Bcl-w, LIN28B2, PIGF, Mcl-1, IL6R, SUV39H1, elf5A2, SIRT7	apoptosis, development, metastasis
miR-126	VEGF, VCAM-1, LRP6, PIK3R2,	angiogenesis, metastasis
miR-138	Cyclin D3,	cell-cycle
miR-139	TCF-4, FOS, ROCK2	proliferation, metastasis
miR-140-5p	TGFβRI, FIF9, DNMTI, Pin1	Proliferation, metastasis
miR-141	DLC-1, Tiam1	Invasion, migration
miR-145	IRS1, IRS2, Oct4, B-Catenin, IGF-IR	Proliferation
miR-146a	HAb18G	Metastasis
miR-148a	c-Met, HRIP, c-Myc, Wnt1, Snail1, DNMT1	Metastasis, EMT
miR-152	DNMT1, GSTP1, CDHI	Metastasis
miR-195	CDK6, cyclin D1, CBX4, Wnt3a, VEGF, VAV2, CDC42, E2F3, LATS2	cell cycle, apoptosis, metastasis, angiogenesis
miR-198	c-Met	Metastasis
miR-199a	mTOR, PAK4, HIF1A, E2F3, DDR1, ATG7, caveolin 2	cell growth, apoptosis, invasion
miR-200fam	ZEB1, ZEB2, HNF-3β, Rho/ROCK, ASB4,HDAC4	EMT, metastasis
miR-203	Survivin, Cyclin D	Proliferation
miR-205	ZEB1, ZEB2	EMT, cell adhesion
miR-214	HDGF, B-Catenin	Angiogenesis
miR-218	E2F2, RET, HoxA10	apoptosis, growth arrest
miR-219-5p	GPC3	Proliferation
miR-223	STMN1	Proliferation
miR-363-3p	c-Myc	Cell proliferation
miR-375	ATG7	Autophagy
miR-376a	PIK3R1	apoptosis, proliferation
miR-429	Rab18,	Metastasis
miR-449	c-Met, SIRT1	Metastasis
miR-450a	DNMT3	Proliferation
miR-497*	CDK 4, CDK 6, Cyclin D	cell cycle
miR-520d	MEKK2, cyclin D1	cell cycle, proliferation
miR-520e	NIK	Proliferation
miR-612	AKT2, EpCAM	EMT, metastasis
miR-637	STAT3	Cell growth, apoptosis
miR-718*	HOXB8	proliferation, invasion
miR-1271	GLP3, Foxk2	Cell growth, invasion
let-7 fam	Bcl-xl, Stat3, c-Myc, COLIA2, Mcl-1, HMGA2, Caspase 3, p16INK	apoptosis, metastasis

Data in the table are derived with permission from (19, 21, 30, 62).

pathways in hepatocellular carcinomas are frequently associated with hepatitis C, hepatitis B and alcoholic liver cirrhosis (59). In addition, the homeostatic role of miRNAs is deregulated in all these pathways (16).

6.1. miRNA in pi3k/mapk pathways

Activation of the PI3K/Akt/mammalian target of rapamycin (mTOR) and MAPK (Raf/MEK/ERK) pathways is a key feature of HCC (51, 60) where angiogenesis, precipitated in Raf/MEK/ERK pathway, has been reported as playing an important role in the development of HCC (61). Deregulated miRNAs in the PI3K/MAPK pathways play a key role in facilitating translation, proliferation and cell cycle progression (see Figure 1). The upregulation of both the P13K and MAPK pathways is also facilitated by the reduced silencing of hepatocyte growth factors and their receptor proteins (HGFR). Downregulated miR-1/-23b/-34a/-299-3b in HCC fail to provide a suppressive effect in both pathways, thus promoting higher levels of protein synthesis and cell proliferation. In the MAPK /MEK/ ERK pathway the downregulation of the let-7 family contributes to the increased activation of GTP/RAS (62).

Further downstream in the P13K pathway, downregulated let-7 miRNAs and miR-199a also fails to regulate the mTOR pathway (63, 64). The simultaneous suppression of the PTEN tumor suppressor network, as well as a failure to modulate oncogenic proteins thus promotes increased protein synthesis and cell proliferation. The progression of tumours to promote angiogenesis, as well as invade surrounding tissue, are modulated by miRNA expression. Overexpression of MET in the P13K/MAPK pathways is found in between 40-70% of all HCC (62). MiRNAs that regulate this axis include miR-1, miR-23b, miR-34a and miR-199-3p which are downregulated in HCC and thus fail to act as a control of downstream tyrosine kinase activity that promotes hepatocyte proliferation, migration, survival and angiogenesis. The targeting of the PTEN tumour suppressor by upregulated miR-21 and miR-221 in HCC further facilitates invasion and metastasis whereas the downregulation of the miR-199/214 clusters results in the diminished modulation of the mTOR pathway (62).

6.2. miRNA in JAK/STAT pathway

The promotion of abnormal expression in the JAK/STAT pathway in HCC is influenced by deregulated miRNAs (see Figure 2). Methylation of the CpG island of the SOCS-1 gene is a common feature in HCC and its silencing demonstrates its important tumour suppressor role in the JAK/STAT pathway (65). Suppressor of cytokine signalling (SOCS-1) switches this signalling 'off' by means of its direct interaction with Janus kinase (JAK). The further deregulation of the SOCS1 growth suppressor in this pathway by upregulated miR-155 is a common feature in HCC (56, 66). Acting in tandem with upregulated miR-155, downregulated miR-637 provides a reduced suppressive effect on the STAT proteins (19, 67).

6.3. miRNA in TP53 pathway

The deregulation of multiple p53 pathways is reported in the development and progression of HCC and mutations along the p53-MDM2 network are a frequent occurrence in HCC (68). The INK4alpha/ARF locus that encodes p14 (ARF) and p16 (INK4alpha), that function to arrest the cell cycle through the p53 and RB pathways, is frequently disrupted in HCC (69). The primary TP53 networks include cell cycle controls (p21, GADD45), the induction of apoptosis (FAD, BAX), the inhibition of angiogenesis (BAI), inhibition of the p13k/AKT pathway (PTEN), inhibition of the p53 feedback and the transcription of miR-34 family of tumour suppressors (16, 62). Deregulated miRNA play a role in all these pathways (see Figure 3).

6.3.1. Cell cycle and proliferation control

Cell cycle and proliferation regulators include the RB1 tumor suppressor, cyclin-dependent kinases (CDKs), Cyclins and CDK inhibitors. Upregulated miRNAs largely play a role in silencing of cell cycle tumor suppressor check points while downregulated miRNAs mostly fail to modulate oncogenic proteins in the GI cycle phase. Upregulated miR-221, miR-222, and miR-519d, for instance, suppress the p21, p27, and p57 tumor suppressor proteins that modulate (block) CDK2 and Cyclin E (43). The deregulation of the p21 tumour suppressor protein is also influenced in HCC by upregulated miR-17/92 and miR-519d (63, 70). In turn, p21 acts as a block to the production of cyclins and cyclin dependent kinases in cell cycle progression (62).

Upregulated miRNAs, e.g.miR-17-92, miR-221, and miR-335 also downregulate the RB1 tumor suppressor control network that modulates the initiation of the G1 cell cycle phase (62, 70).

Conversely, a wide range of miRNAs, that target cyclins and CDKs in the G1 cell cycle phase, are downregulated in HCC and therefore fail to provide modulation of these cell cycle proteins resulting in increased cell cycle activity. Typically, an inverse relationship exists between the expression of cyclins and the volume of miRNAs that target them (48, 62).

Downregulated miRNAs that target Cyclin D, E and CDK 2, 4 and 6 include miR-26a/b, miR-34a, mIR-520d, miR-138, 497 and miR-195 (50, 71-73) (see Figure 3).

6.3.2. The role of miRNA and apoptosis

Typically, anti-apoptotic molecules, like Bcl-2, Bcl-w, Bcl-xl and Mcl-1, are modulated by miRNAs like

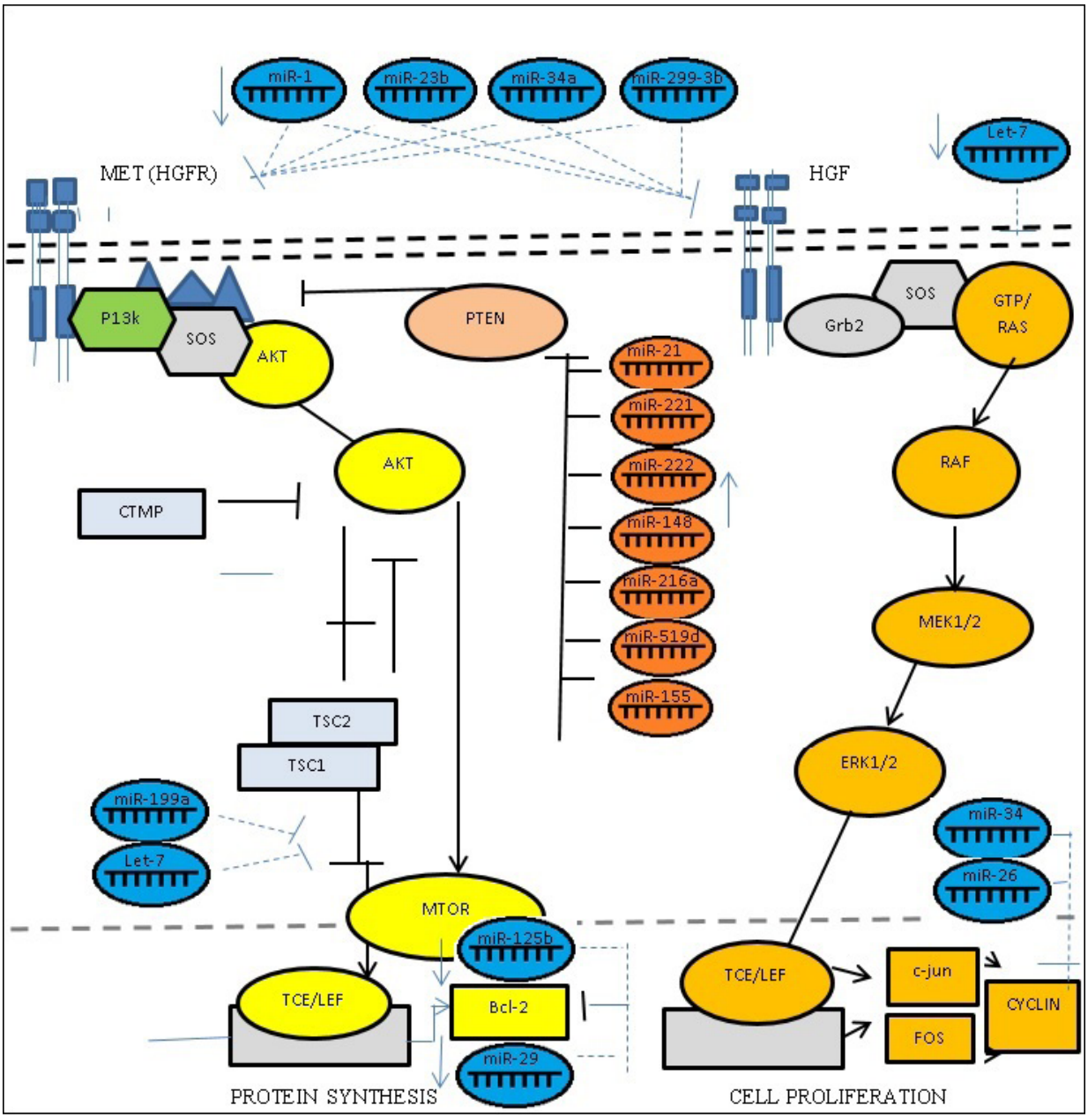

Figure 1. Examples of deregulated miRNA in P13K/MAPK (Blue miRNAs are downregulated and Orange miRNAs are upregulated).

miR-29, miR-15a/16-1, miR-101, miR-122 and the let-7 family. In HCC, these miRNAs are down-regulated and thus fail to modulate the effect of these anti-apoptotic molecules. (30, 74, 75). The up-regulation of miR-106b-25, in turn has a suppressive effect on the pro-apoptotic gene Bim expression (30) while the upregulation of miR-224, the most significantly up-regulated miRNA in many HCC patients, increases apoptotic cell death, as well as proliferation by targeting the apoptosis inhibitor-5 (*API*-5) gene to inhibit its transcription (46). In addition, pro-apoptotic proteins like BMF and BID and the TGF-β pathway are inhibited by overexpressed miRNAs like miR-25 (BMF, BID, TGF-β) and miR-221 (BMF, BID). The disruption of apoptosis in these paths is a critical step in metastasis in HCC (62). The inhibition of apoptosis in HCC is also facilitated by the downregulation of miR-203 as a result of DNA methylation which results in the reduced control of the anti-apoptotic protein, Survivin (62). The silencing of the PTEN and PDCD4 tumour suppressors by up-regulated miR-21 in HCC also contributes strongly to silencing the p53 pathway that activates apoptosis (62).

The suppression of apoptosis in the p53 BAX/NoXa/Puma pathway in HCC is also influenced by downregulated miRNAs that fail to suppress anti-

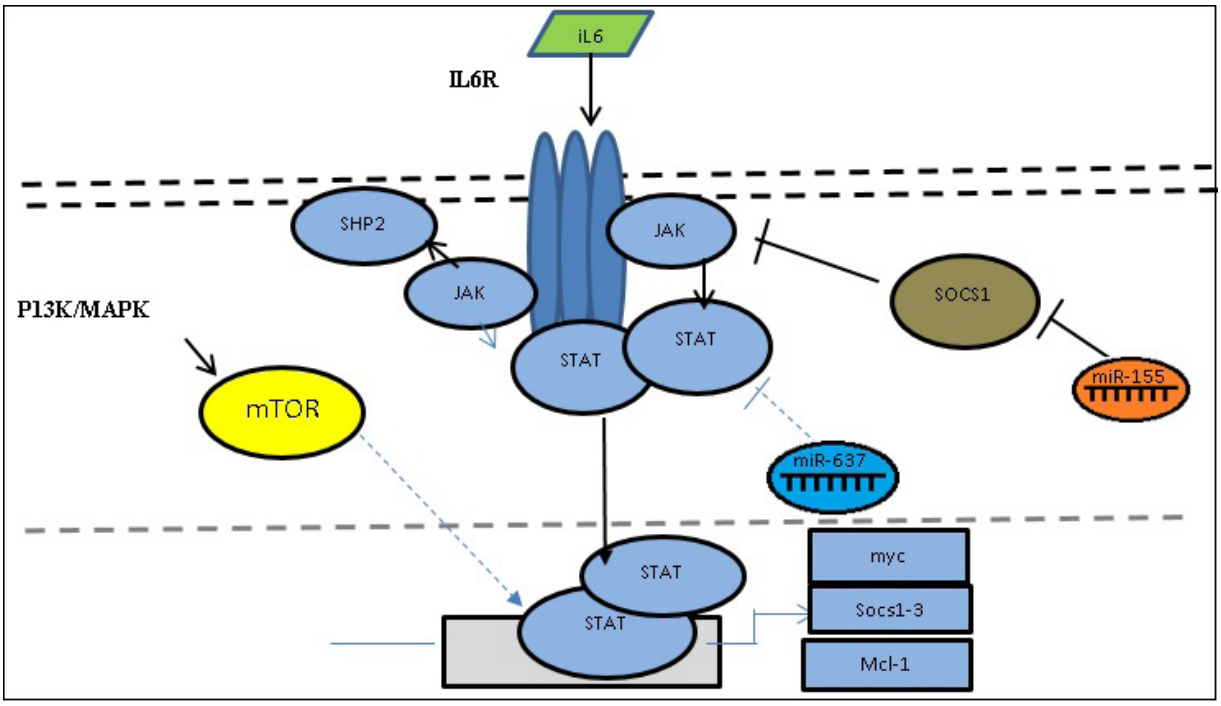

Figure 2. Examples of deregulated miRNA in JAK/STAT pathway in HCC.

apoptotic proteins that block the CASP3 stage in this pathway (see Figure 3). The anti-apoptotic proteins include Bcl-2, Bcl-xl and Mcl1 that act as a suppressors of the CASP3 stage. The downregulated miRNAs miR-29/-34/-122/-125b/let-7 provide reduced suppression of Bcl-2 and downregulated miR-29/-101/-125b/-193/let-7 have a reduced ability to modulate the expression of proteins like Mcl1 (62).

6.4. The role of miRNA in WNT/β-catenin pathway and epithelial-mesenchymal transition (EMT)

The upregulation of the WNT/β-catenin pathway is a frequent event in early HCC (76). It yields an aggressive phenotype in this pathway that is implicated in proliferation, migration, invasion and survival of cancer cells (3). Figure 4 illustrates some examples of deregulated miRNAs and their target genes.

Increased expression in the WNT/β-catenin pathway can be influenced by the upregulation of miR-21 that targets the tumor suppressor protein DCC6 that suppresses WNT (62). The upregulation of miR-106b/-135/-315 also collectively suppresses the APC/Axin1/GSK3 core that binds to β-Catenin thus resulting in the release of activated β-Catenin that promotes the transcription of oncogenic proteins like c-Myc, c-jun and Cyclin D (62, 77).

Conversely, the downregulation of miR-122 fails to provide a control for the regulation of Wnt1 (78). Downregulated miRNAs, like miR-145/-214, also fail to provide a suppressive effect to β-Catenin activity (79, 80). In addition, downregulated miRNAs like miR-101/-141/-200 family/203/205 /429 fail to provide a suppressive effect to transcription factors ZEB1/2 thus promoting the loss of E-Cadherin which acts an addition control to modulate β-Catenin expression (30, 81).

The loss of E-Cadherin is associated with the progression of HCC because it reduces cell-cell adhesion and promotes epithelial mesenchymal transition (EMT) thus facilitating the invasion of neighboring tissue with tumor cells (14). MiRNAs that modulate EMT include miR-221/-222 which downregulate Trps-1, a repressor of ZEB1/2 (21). Various miRNAs like miR-30 and miR-148a, which are downregulated in HCC, fail to modulate the snail 1 suppressor of E-Cadherin thus further contributing to the loss of E-cadherin (57, 82, 83).

Interestingly, it has been suggested that aflatoxin (AFB1) might down-regulate the *WNT/β-catenin* signalling pathway in HepG2 cells by up-regulating *miR-34a* which acts as a control checkpoint to suppress c-MET, cyclins and cyclin dependent kinases in the G1 cell cycle phase (84, 85). Conversely, the upregulation of miR-24 has been associated with increased cell proliferation in the presence of AFB1 mediated HCC (86).

6.4.1. The role of miRNA in angiogenesis, metastasis and invasion

Nineteen deregulated miRNAs and their targets (see Table 3) have been specifically linked to

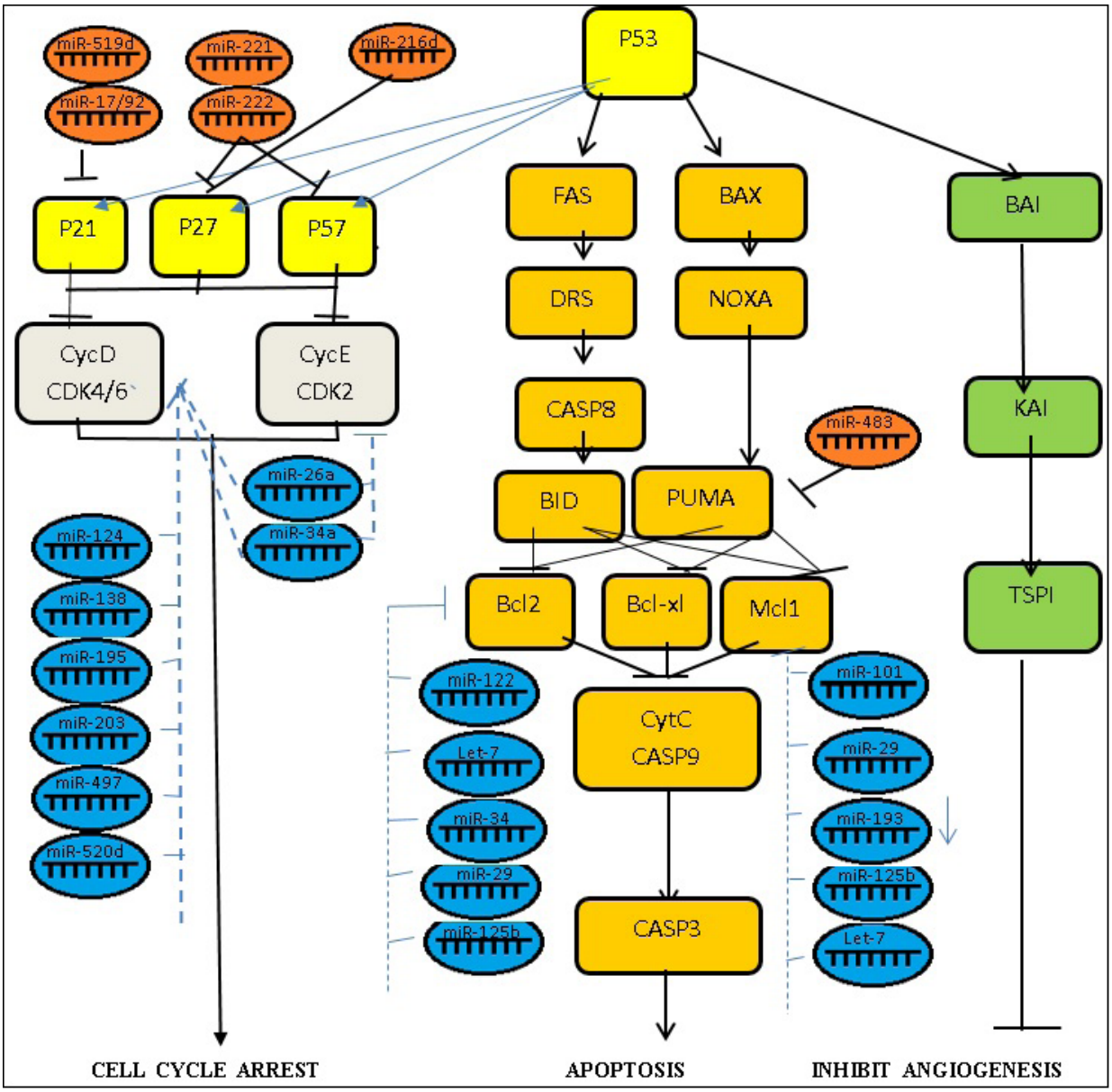

Figure 3. Examples of deregulated miRNA in p53 pathways in HCC.

metastasis in HCC (87). The downregulation of miR-122, for example, which is found in 70 per cent of HCC, enhances cell migration, invasion and metastasis as a result of its role in the p53 pathway to control cell cycle (88). The downregulation *miR-122* also results in the reduced ability to modulate ADAM17 which is a key protein involved in metastasis (89). Loss of miR-122 can also promote angiogenesis and intrahepatic metastasis by failing to suppress the expression of the tumour necrosis factor-α- converting enzyme (TACE) (21). Other deregulated miRNAs that facilitate invasion and metastasis include miR-139 and miR-151. In the HNE-*TACE* signalling *pathway*, invasion and metastasis is increased by reduced levels of miR-139 which target ROCK2 (90). Conversely, the upregulation of miR-151 in HCC targets RhoGDIA, which is a suppressor of metastasis (62, 91).

7. HCC, VIRAL INFECTION AND CIRCULATING miRNA

Viruses encode their own sets of miRNA which are used to control the expression of their host's genes (92). The ability of a virus to package its own miRNAs into exosomes and transport them to non-infected cells was first demonstrated by the EBV virus (93). Both viral transcripts and proteins can affect host miRNA expression, which in turn can modulate viral and/or host protein expression (94). MiRNAs can bind to viral transcripts and regulate viral infection and, conversely,

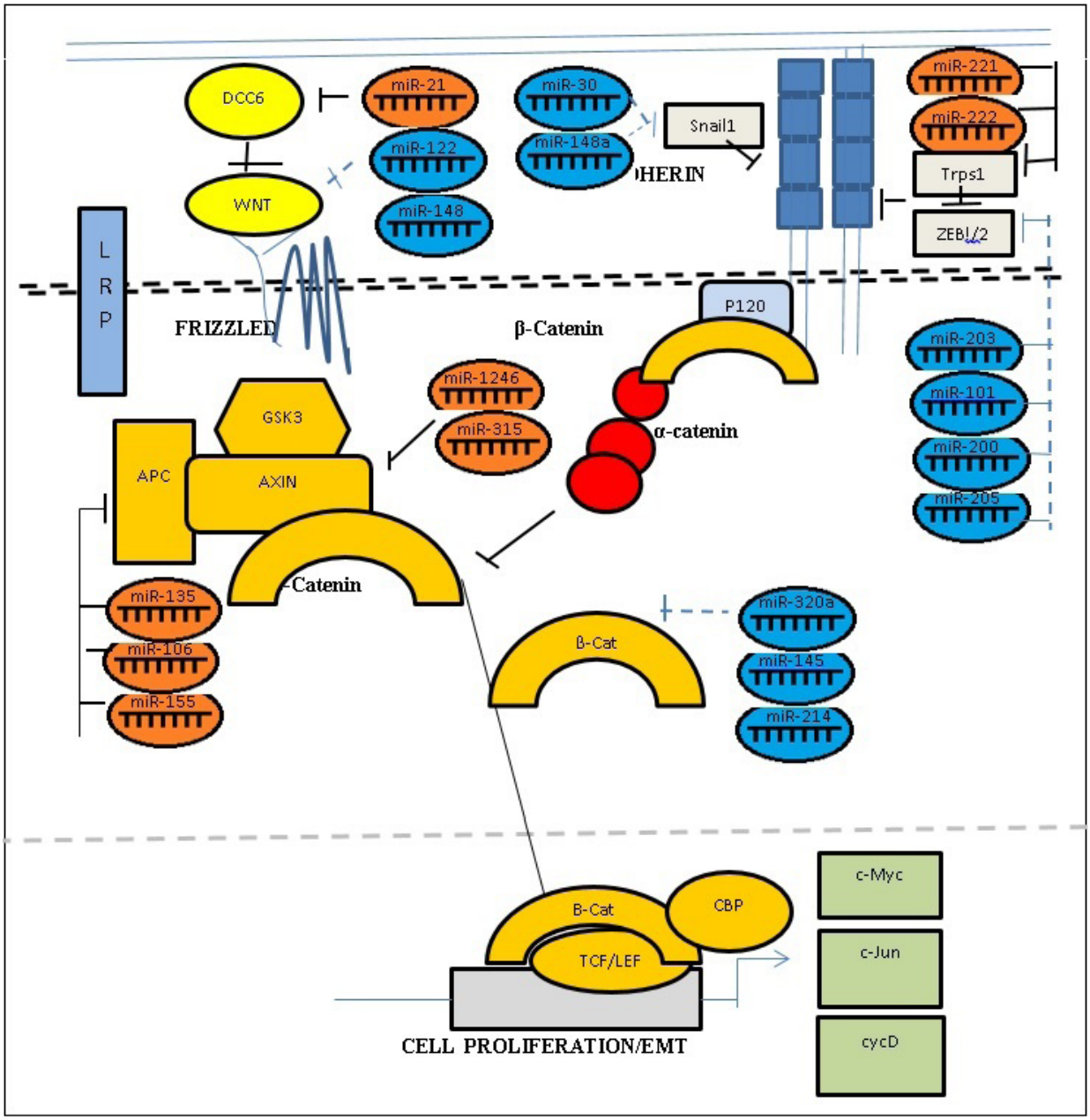

Figure 4. Examples of deregulated miRNA in WNT pathway in HCC.

viral infection (e.g. HIV/HCV) can modulate host-cell microRNA machinery (95). The downregulation of miR-15a/16 in HBV infected patients, for instance, is caused by a complementary HBV mRNA binding site that acts as a sponge for these miRNAs (96). In turn, miR-15b has been shown to modulate HBV replication by targeting the hepatocyte nuclear factor 1α (HNF1α) (97). MiR-122 also down-regulates HBV replication by binding to the viral target sequence (98) and, conversely, binds to the HCV genome to increase its expression (99-101). In other examples, miR-199a and miR-210 bind to different sites on mRNA coding of HBsAg, reducing HBsAg expression in HepG2 2.2.1.5. cells (102).

Two review papers, summarizing a wide range of studies (20, 103), identified a marker group of seven circulating miRNAs, including miR-122, miR-192, miR-223, miR-21, miR-26a, miR-27a, miR-801 that were able to distinguish between HCC, HBV, cirrhosis and healthy controls, as well as identify HCC tumor stages. A further study concluded that eight deregulated miRNAs could distinguish between HBV/HCC patients and healthy controls but not between HBV/HCC patients and those with cirrhosis. These miRNAs included miR-206/-141-3p/--433-3p/-1228-5p/-122-5p/-192-5p/-260-5p (28). Others have shown that serum levels of miR-10a and miR-125b were lower

Table 3. Deregulated miRNAs in HCC metastasis

miRNA	deregulation	Target gene
miR-338	Up	IFR2
miR-219	Up	ADD2
miR-207	Up	n.a.
miR-185	Up	KCNN3
miR-30c	Down	KIAA0063
miR-1-2	Down	G3BP2, GCLC, HAND2, TMSBAX, HDAC4, GJA1, KCNJ2
miR-34a	Down	SPTBN2, E2F3, DLL1, NOTCH1
miR-19a	Down	PTEN
miR-148a	Down	GTF2H1, PSCD3
miR-9-2	Down	VAMP3, MTPN, MAPK14
miR-122a	Down	GTF2H1, PSCD3
miR-125b	Down	GYS1, CAT-1
miR-194	Down	ITGA9, YES1, LIN28
miR-30a	Down	HBEGF
miR-126	Down	KIAA0063, VERATIN, TMEM2, THB51, SLC7A6, PRO2730, TUBA3, CYR61, CDK6
let-7g	Down	n.a.
miR-15a	Down	ASPH, SLC20A2, SPTBN2, BCL2, DMTF1
miR-30e	Down	KIAA0063

Data in the table are derived with permission from (87).

in HBV infected HCC patients than in chronic hepatitis B patients and that a triplet of circulating miRNAs (namely miR-375, miR-25, miR-Let-7f) were able to diagnose HCC with ~98% accuracy (103). Circulating miR-21 was also higher in HCC than chronic hepatitis patients and healthy controls; furthermore, its levels correlated with miR-21 expressed in HCC tumor tissue and it had better diagnostic sensitivity than alpha fetoprotein (AFP) (20, 103). In another study, it was found that serum miR-21/-122/-223 were higher in HCC and chronic hepatitis (CH) compared to healthy controls, whereas miR-122 and miR-21 were higher for CH than HCC (104).

8. UNRESOLVED ISSUES

Despite the abundance of literature on the promise of miRNAs as biomarkers and therapeutic agents, this field of study remains a work in progress. Multiple miRNAs target the same genes and PTGS silencing of translation is a collective effort. Even then it is likely miRNAs only exert a mild secondary influence on mRNA stability and translation in response to the stochastic nature of gene expression and changing environmental influences (16). Our knowledge of the role of miRNAs is also compromised many earlier studies that used non-standardized research protocols, as well as a limited overlap of studies investigating the same cancer (105). In the case of 32 publications that identified 143 deregulated miRNAs in the same cancer, 100 of these deregulated miRNAs were supported by only one reference (23). Another problem is that miRNA biomarkers are largely non-specific and most likely triggered by widespread conditions that are associated with carcinogenesis (23). HCC, for example, is often precede by viral infection, fibrosis, chronic inflammation and cirrhosis which remain secondary conditions of this disease (106). Upregulated miRNAs in both fibrosis and HCC includes miR-17-5p/-21/-130a/b/-181b/-221/-222 while common downregulated miRNAs aremiR-16/-29/-101/-122/-125b/-126/-146a/-152/-195/-200fam/-214 (107, 108). Inflammation miRNAs in ulcerative colitis like miR-21/-23a/-126/-195/-375 are also deregulated in HCC and miR-21 is deregulated in HCC, chronic colitis, psoriasis and many other inflammatory conditions (109). Furthermore, small tumors (< 0.5.cm) would be unable by themselves to alter the level of extracellular miRNAs in body sera and the explanation for deregulated miRNAs in early stage carcinogenesis would likely be due to general immune responses (23).

The standardization of protocols will also need to consider the selection of extracellular sub-populations of extracellular vesicles that facilitate small RNA messaging. Emerging research also indicates that only certain types of encapsulated miRNA play a role in cell-cell signaling and others may not. Exosomes, for instance, appear to transport miRNA that promote

Table 4. miRNA biomarkers for early stage HCC

miRNA	Serum/plasma	Sensitivity (%)	Specificity (%)
miR-21	Serum, plasma	86.8.	79.5.
miR-122*	serum	68.0.	73.3.
miR-15b*	serum	98.3.	15.3.
miR-18a	serum	86.1.	75.0.
miR-26a-5p	serum	68.9.	74.4.
miR-29b	serum	75.9.	89.5.
miR-106b	plasma	76.6.	80.6.
miR-130b	serum	87.7.	81.4.
miR-141-3p	serum	68.1.	83.3.
miR-143	serum	73.0.	83.0.
miR-183	serum	57.9.	69.5.
miR-192-5p	serum	71.9.	75.6.
miR-199a-5p	serum	59.3.	66.7.
miR-206	serum	48.1.	78.8.
miR-215	serum	80.0.	91.0.
miR-223	serum	80.0.	76.5.
miR-433-5p	serum	79.3.	64.4.
miR-483-5p	plasma	55.7.	85.7.
miR-1228-5p	serum	79.3.	27.8.
let-7b*	serum	84.8.	50.0.

*HBV/HCC Data in the table are derived with permission from (111, 112).

paracrine communication while other bodies (e.g. apoptotic bodies) may not. It has been estimated that only 1 per cent of miRNA are membrane bound and that 99 per cent are membrane free (24, 25, 32). In addition, the role and presence of miRNAs in other body sera like lymph fluid maybe more promising than their role in blood serum. The high level of miRNA expression in blood cells may be a further issue that distorts the interpretation of miRNA levels in blood serum and another reason to investigate other body sera (23, 110).

9. DIAGNOSTIC POTENTIAL OF EXTRACELLULAR MIRNA FOR EARLY STAGE HCC

The quantification of serum AFP and imaging techniques, remain the most widely used diagnostic tools for HCC, despite the poor sensitivity and specificity of these methods These shortfalls are illustrated by the fact that elevation in AFP levels is not present in 80% of early (small) HCCs (5). As a result of these deficiencies, as well as the potential of extracellular miRNAs, widespread research in this field has proposed multiple deregulated serum miRNAs as candidate biomarkers. The use of a single miRNA as either a diagnostic or therapeutic tool, however, is questionable because the repression of gene expression is a collective effort of multiple miRNAs (16).

A meta-analysis of 50 studies identified 19 circulating miRNAs (See Table 4) as particularly promising candidates as biomarkers of early stage HCC.

The sensitivity and specificity of miR-21 was 86.6.% and 79.5.% respectively, while that of miR-122 was 68.0.% and 73.3.% respectively (111). In another study, serum levels of miR-16, miR-122, miR-221, let-7b and miR-15b were significantly lower in patients with high grade dysplasia than in early stage HBV/HCC (112). When the dysplasia progressed to overt HCC, serum miR-122, miR-let-7b and miR-15b levels increased significantly ($p = 0.046.$, 0.043, and 0.044, respectively). As a single marker, α-fetoprotein (AFP) and miR-122 as well as let-7b had a similar ability to differentiate HCC from high grade dysplasia. As limited to subjects with normal AFP, let-7b resulted in a sensitivity of 84.8.% and a specificity of 50% in separating HCC and dysplasia with a cut-off value of 3.5. ($p = 0.0.0.1.$). In conclusion, miR-122 and let-7b, which are upregulated in the serum of early-HCC patients, can be useful markers for differentiating early HCC from in chronic hepatitis B patients (112). In another recent study, 16 circulating miRNAs were able to differentiate between patients with cirrhotic livers and early stage HCC. These included miR-15a, miR-21, miR-29a, miR-30c, miR-486-3p, Let-7g, miR-122,

miR-18a, MiR-338-3p, miR-126, miR-222, miR-223, miR-26a, miR-192, miR-27a and miR-124 (113).

Advances in technology to classify and quantify RNA in sera are likely to improve the cost benefit of using deregulated miRNAs as biomarkers of HCC. What is increasingly emerging, however, is that panels of deregulated miRNAs in human sera might provide far greater diagnostic proficiency.

10. CONCLUSIONS

The potential of miRNA exploitation as diagnostic agents and therapeutic tools will likely only be realized when a complete knowledge of their biology is revealed. New technologies that can reduce the cost of small miRNA quantification and replication will also facilitate their use as commercially viable diagnostic and therapeutic tools. Extracellular miRNA have clearly demonstrated that they possess high levels of sensitivity and specificity as diagnostic tools yet remain mostly in use in research programs. In terms of their use as therapeutic agents it should be remembered that miRNA only collectively act as a mild homeostatic agent in terms of modulating mRNA translation. This implies that their use as therapeutic agents is likely to support other targeted mainline therapies.

11. ACKNOWLEDGEMENTS

We are grateful for the support received for this study from the National Research Foundation (NRF) of South Africa and the Russian Science Foundation (Grant 16-15-00290).

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Key Words: Micro RNA, miRNA, Deregulation, Hepatocellular Carcinoma, Biomarkers, Early Diagnosis, Review

Send correspondence to: Kurt Sartorius, University of the Witwatersrand, Private Bag 3, Wits 2050, South Africa, Tel: 27072 -326-0629; Fax: 270117178223, E-mail: Kurt.Sartorius@wits.ac.za