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Regulation of hepatocarcinogenesis by microRNAs

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1. ABSTRACT

Liver cancer (hepatocellular carcinoma, HCC) is a major malignancy worldwide. Etiologically, hepatocarcinogenesis is closely associated with HBV and HCV infections; however, its underlying molecular mechanism is not completely understood. MicroRNAs are a class of small non-coding RNAs that negatively regulate gene expression by interacting with the 3'UTR of protein-coding mRNA. MicroRNAs are implicated in nearly all major biological and cellular events, and recent findings further link microRNA deregulation to human carcinogenesis. In this review, we will focus on the aberrant expression of miRNAs in liver cancer and the pathological implications and molecular functions of some well-characterized oncogenic and tumor suppressive miRNAs. Finally, the clinical prospect of miRNAs as a novel diagnostic and therapeutic intervention will be discussed.

2. INTRODUCTION

Liver cancer is one of the most common malignancies, with an estimated annual incidence of over 500,000 cases worldwide (1). Hepatocellular carcinoma (HCC), a malignant tumor derived from transformed hepatocytes, is the most common type of primary liver cancer and accounts for approximately 80% of liver cancer diagnoses. Etiologically, viral hepatitis (HBV and HCV) infections and the resulting chronic hepatitis are the predominant risk factors for this disease. In fact, liver cancer displays a remarkable geographical distribution, with higher incidence in China, Japan, Southeast Asia, and sub-Saharan Africa. This pattern strictly overlaps with the prevalence of viral hepatitis infections. Although the etiology of liver cancer is relatively clear, its underlying molecular mechanisms remain largely elusive. Numerous efforts have been made by the research commu define the molecular pathogenesis of liver cancer, and nity to

substantial progress has been made in the past decades. The literature contains ample evidence indicating that genomic and epigenetic abnormalities, some of which result in deregulation of proto-oncogenes and tumor suppressor genes, are common in liver cancers and might contribute to the initiation and progression of the disease (2). Due to the traditional one gene-one enzyme hypothesis, most of these studies focused on protein-coding genes. Recently, however, the biological functions of non-coding RNAs have gained much attention due to their ability to regulate the expression of protein-coding genes at both the transcriptional and post-transcriptional levels. Since the initial studies of non-coding RNAs, the pathological significance of these RNAs in human carcinogenesis has been increasingly recognized. Of all the non-coding RNAs, microRNAs are the most well characterized class (3). In this review, we will discuss the findings of recent microRNA studies in liver cancer, including the expression, biological functions, pathological roles, and diagnostic and therapeutic prospects of microRNAs.

3. MICRORNA BIOGENESIS AND FUNCTIONS

MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs approximately 22 nt in length. miRNA genes can be intragenic or intronic (i.e., located within the intronic regions of protein-coding genes) in the human genome. These miRNA genes are either transcribed as primary transcripts known as primary microRNAs (pri-miRNAs) using their own promoter or coexpressed with their host genes under the regulation of host gene promoters. Some miRNAs are tandemly located as a miRNA cluster and transcribed as a polycistronic miRNA transcript (4). The size of pri-miRNAs varies from hundreds to thousands of nucleotides in length and may contain one or multiple miRNA stem-loop structures. After transcription, the miRNA loop of the pri-miRNA is recognized and processed by a microprocessor complex consisting of DGCR8 and Drosha to form a precursor miRNA (pre-miRNA) with a double-stranded hairpin structure usually less than 100 nt in length. Pre-miRNAs are then exported from the nucleus to the cytoplasm via the Exportin-5/Ran-GTPase nucleocytoplasmic shuttle. In the cytoplasm, pre-miRNAs are further processed by Dicer to yield ~22-nt single-stranded small RNAs known as mature miRNAs (5).

Mature miRNAs post-transcriptionally regulate the expression of protein-coding genes by interacting with the 3'UTRs of their target mRNAs (6). In mammalian cells, miRNAs and target mRNA sequences are usually not perfectly complementary; however, the seed sequence consisting of 2-7 nucleotides in the 5' region of the miRNA is essential for target recognition (7). miRNAs exert their biological functions by silencing specific mRNA targets through the inhibition of translation or promotion of mRNA degradation via deadenylation (8-9). According to the record in miRBase (www.mirbase.org), the human genome has been estimated to encode more than 1400 miRNAs, and each of these miRNAs can potentially regulate hundreds of mRNA targets (10). Moreover, a single protein-coding mRNA can be simultaneously regulated by multiple miRNAs (11). Thus, the miRNA:mRNA interaction provides an additional layer of regulation or fine-tuning of the expression of protein-coding genes in mammalian cells.

4. MICRORNA EXPRESSION PROFILES IN LIVER CANCER

Since the discovery of miRNA two decades ago, this class of small non-coding RNAs has been linked to a wide spectrum of biological processes, including cell proliferation, apoptosis, and differentiation. In 2006, Murakami and colleagues performed the first miRNA profiling study on HCC using a miRNA microarray including 180 mature miRNAs and 206 pre-miRNA oligo probes (12). This study found that several miRNAs were aberrantly expressed in HCC samples when compared to adjacent non-tumorous tissues; miR-18, pre-miR-18, and miR-224 were upregulated in HCC samples, whereas miR-199a*, miR-195, miR-199a, miR-200a, and miR-125a were downregulated, suggesting that these miRNAs might have oncogenic or tumor suppressive roles in HCC, respectively. Furthermore, the expression levels of miR-92, miR-20, miR-18 and pre-miR-18 were inversely associated with the differentiation state of HCC tumors, whereas the expression level of miR-99a was positively associated with the tumor differentiation state, implying that these miRNAs might contribute to the dedifferentiation of the tumor (12).

In another study, miRNA expression profiles of HCC and cirrhotic liver samples were analyzed by a genome-wide miRNA microarray consisting of 381 probes for 238 mature and 143 precursor human miRNAs. A panel of 35 miRNAs was found to be significantly deregulated in HCC when compared to cirrhotic liver (13). Among these deregulated miRNAs, some have well-characterized roles in cancer, such as the let-7 family and miR-145, which are frequently downregulated in various human malignancies (14-18). Interestingly, the majority of the deregulated miRNAs are downregulated in HCC tissues, and miR-221 is the only overexpressed miRNA in this panel. Downregulation of miR-122a was observed in nearly 70% of the HCC specimens and in all examined HCC-derived cell lines, implicating it as a tumor suppressor in HCC. Further analysis of these aberrantly expressed miRNAs using SV and PAM algorithms suggests that the miRNA signature identified by Gramantieri *et al.* is sufficient to differentiate most HCC samples from cirrhotic tissues.

Liu *et al.* elucidated the molecular mechanism underlying the global regulation of miRNA in HBV-associated HCC in a similar miRNA expression profiling study including 94 pairs of tumor and adjacent non-tumor tissues from Chinese HBV-associated HCC patients. In this study, Liu *et al.* demonstrated that the aberrant expression of some miRNAs, such as miR-30d, miR-151, miR-21 and miR-34a, is highly correlated with the amplification or deletion of their corresponding chromosomal regions. The expression of other miRNAs, such as miR-18a, miR-93, miR-106a, miR-106b, miR-222, miR-301 and miR-324-5p, is positively correlated with DROSHA levels, suggesting that aberrant expression of genes involved in miRNA biosynthesis may impact miRNA expression levels in HCC (19).

Table 1. Oncogenic and tumor suppressive miRNAs in human HCCs

Oncogenic miRNAs		
MicroRNA	Target mRNA	References
miR-151	RhoGDIA	[21]
miR-17-92	HSP27	[112]
miR-21	PTEN	[72]
miR-221	CDKN1B/1C, DDIT4, BMF	[28, 35, 81]
miR-222	PP2RA	[102]
miR-224	API-5	[96]
miR-30d	GNAI2	[114]
miR-373	PPP6C	[103]
Tumor suppressive miRNAs		
MicroRNA	Target mRNA	References
Let-7g	COL1A2, c-myc	[47, 57]
miR-101	Mcl-1, FOS	[64, 86]
miR-122	CyclinG1, CULT1	[29, 33, 106]
miR-124	ROCK2, EZH2	[118]
miR-125b	LIN28B	[67]
miR-139	ROCK2	[100]
miR-198	c-Met	[90]
miR-199a-3p	mTOR, c-Met, CD44	[30, 41]
miR-29	Mcl-1, BCL2	[105]
miR-29b	MMP-2	[25]
miR-29c	TNFIAP3	[93]
miR-375	YAP	[68]
miR-519d	MKi67	[43]
miR-99a	IGF-1R, mTOR	[62]

In another study, Jiang *et al.* used real-time PCR to examine the expression of more than 200 precursor and mature miRNAs in clinical HCC specimens, adjacent non-tumorous tissues positive for cirrhosis and hepatitis infection and normal liver tissues with no cirrhosis or viral infection. A total of 16 miRNAs were differentially expressed in the HCC samples when compared to adjacent non-cancerous tissues (20). Of these, the deregulation of miR-199a, miR-199a* and miR-18 were reported by both Murakami and Jiang *et al.*, supporting their determining role in hepatocarcinogenesis (12, 20). Moreover, some miRNAs with well-characterized associations with solid tumors, including miR-21, which is associated with pancreatic and thyroid cancer (17, 21), and miR-221, which is associated with glioblastomas, breast cancer and pancreatic cancer (21-23), were also reported to be upregulated in HCC tumors by Jiang *et al.* More importantly, this group also characterized a panel of 19 cell cycle-related miRNAs as putative prognostic markers of HCC, as the expression of these miRNAs is positively correlated with survival in HCC patients (20).

Recently, a more comprehensive miRNA profiling study was performed in a larger cohort including 104 HCC samples, 90 adjacent cirrhotic liver samples and 21 normal liver samples. To identify miRNAs potentially important for the transition from normal liver to cirrhosis and from cirrhosis to HCC, Pineau and coworkers analyzed these three miRNA expression profiles by performing pairwise comparisons: cirrhosis vs. normal liver, HCC vs. normal liver, and HCC vs. non-cancerous cirrhotic tissues. The miRNAs consistently deregulated in each comparison were further validated in 35 HCC-derived cell lines. Using this strategy, the authors identified a set of 12 miRNAs (miR-106b, miR-21, miR-210, miR-221/222, miR-224, miR-34a, miR-425, miR-519a, miR-93, miR-96 and let-7c) that define the signature of hepatocarcinogenesis from the normal liver to cirrhosis to HCC (24).

Although much effort has been made to investigate cancer-specific miRNA signatures through a profiling approach, few studies have combined existing miRNA profiles to identify more promising miRNA signatures. A recent comprehensive meta-analysis of 33 miRNA expression datasets from 28 published cancer studies allowed the stringent identification of a panel of miRNAs differentially expressed in tumors and corresponding non-tumorous tissues. Based on the results of this meta-analysis, a common miRNA signature associated with human tumors was defined. This signature consists of 52 miRNA genes that are deregulated in various human tumors. By comparing the miRNA expression profiles of five well-studied human cancers, including breast, colon, prostate, liver and lung cancers, a tissue-specific miRNA deregulation pattern was also revealed. Interestingly, more than 60% of the differentially expressed miRNAs in HCC were not shared by the other 4 solid tumors. This finding suggests the existence of an HCC-specific miRNA signature that might reflect some unique characteristics of HCC tumors. Due to its enhanced statistical power, the meta-analysis was thought to have an advantage over previous studies in identifying novel cancer-related miRNAs. Using this approach, miR-154, which was not recognized in any previous HCC miRNA expression profiling studies, was identified as a potential tumor-suppressive miRNA in HCC. Further molecular and functional analysis revealed that miR-154 affected HCC cell proliferation by regulating cell cycle progression (25).

5. ONCOGENIC MIRNAS IN HUMAN HCC

miR-21. miR-21 is a well characterized oncogenic miRNA overexpressed in many types of solid tumors, including lung, breast, stomach, colon, prostate, head and neck, esophagus, pancreas, and brain tumors (16, 23, 26). The importance of miR-21 in hepatocarcinogenesis was first described in miRNA profiling studies conducted

by Henson and colleagues that indicated that miR-21 is overexpressed in primary HCC tumors and cell lines (27). *In vitro* inhibition of miR-21 in hepatoma cell lines reduces the phenotypic traits of cancer cells, including cell proliferation, migration and invasion; in contrast, introduction of miR-21 precursor enhances these traits *in vitro*. Further analysis revealed that miR-21 exerts its oncogenic activity in HCC mainly by modulating the expression of the well-established tumor suppressor PTEN. By repressing the expression of PTEN, miR-21 increases tyrosine phosphorylation of FAK (a well-known downstream effector of PTEN involved in the regulation of cell-cycle progression, cell survival and cell migration), which in turn upregulates the expression of MMP-2 and MMP-9 (27). This miR-21-dependent modulation of the PTEN-mediated pathway might contribute to the enhanced aggressiveness of HCC tumors and thus result in poor prognosis in HCC patients. Correlation analysis of miR-21 expression and patients' survival is warranted to evaluate the prognostic value of this miRNA.

miR-221. Interest in studying miR-221 in HCC has arisen from studies linking overexpression of this miRNA to carcinogenesis in several human malignancies (17, 28-30). Indeed, miR-221 is another oncogenic miRNA noted to be frequently upregulated in human HCC (24, 31-32). miR-221 overexpression caused a significant increase in cell number, particularly in the S-phase population, whereas inhibition of miR-221 by antimiR-221 showed the opposite effect, suggesting that miR-221 plays an important role in HCC cell proliferation. A spectrum of functional targets of miR-221 has been validated in human HCC by different research groups. Fornari *et al.* provided experimental evidence that miR-221 targets the cell cycle inhibitors CDKN1B/p27 and CDKN1C/p57 in HCC cells (31). Both CDKN1B/p27 and CDKN1C/p57 were characterized as biomarkers of poor prognosis in HCC, and their involvement in the development of HCC has been well described (33-35). Of note, the loss of CDKN1C/p57 protein expression in HCC has been reported to be associated with enhanced aggressiveness of tumors and poor clinical outcome (36). miR-221 was upregulated in 71% of HCC, and CDKN1B/p27 and CDKN1C/p57 proteins were downregulated in 77% of clinical specimens; thus, a significant inverse correlation has been reported between miR-221 and both CDKN1B/p27 and CDKN1C/p57 in human HCC tissues, suggesting that miR-221 promotes cancer cell growth mainly by targeting these two cell cycle inhibitors during hepatocarcinogenesis (31). The involvement of miR-221 in liver tumorigenesis was further characterized by Pineau and coworkers using a mosaic mouse model of liver cancer. The miR-221-overexpressing mice recapitulated the growth-stimulatory features of miR-221 observed *in vitro*, providing further support for the role of miR-221 in HCC. Furthermore, DNA damage-inducible transcript 4 (DDIT4) was identified as a direct functional target of miR-221 (24). DDIT4 is an essential regulator of the mTOR kinase through stimulation of the tuberous sclerosis tumor suppressor TSC1/2 complex, and as such, is considered a putative tumor suppressor (37). This finding thus provides evidence linking miR-221 to the PTEN-PI3K-AKT-mTOR pathway in hepatocarcinogenesis. In addition to promoting cell proliferation, miR-221 has been demonstrated to function as an anti-apoptotic miRNA, and its silencing resulted in increased apoptotic cell death in HCC. Using luciferase reporter assays and western blot analysis, miR-221 was demonstrated to regulate BMF at the post-transcriptional level by directly binding to its 3'UTR (32). BMF is generally characterized as one of the pro-apoptotic BH3-only members of the Bcl-2 family. Inhibition of endogenous miR-221 in HCC cells led to an increase in BMF expression and caspase-3 cleavage, and knocking down BMF attenuated the increase in miR-221-stimulated caspase-3 cleavage. Therefore, Gramantieri *et al.* concluded that BMF modulates the susceptibility of HCC cells to apoptotic stimuli through a caspase-3-dependent pathway. The clinical significance of the miR-221/BMF interaction was further revealed by correlation analysis. Expression analysis of miR-221 and BMF in HCC clinical specimens indicated an inverse relationship, suggesting that BMF is regulated by miR-221 in HCC patients. More importantly, BMF has also been shown to play a pro-apoptotic role *in vivo*, as expression of BMF was reported to be significantly correlated with the proportion of caspase-3 that was cleaved. Although miR-221 levels were not correlated with most clinicopathologic features, miR-221 expression was elevated in multifocal HCC and was found to be associated with post-operative tumor recurrence (32). Based on these data, it is reasonable to believe that by targeting BMF, miR-221 favors anti-apoptotic pathways, leading to the generation of multifocal tumors in HCC, which might represent a population of more aggressive cancer cells. In summary, the current knowledge of miR-221 suggests it contributes to liver carcinogenesis by simultaneously affecting multiple oncogenic pathways that favor cell proliferation and survival.

miR-222. The miRNA profiling studies conducted by Pineasu and colleagues demonstrated that miR-221 and miR-222 are the most highly upregulated miRNAs in HCC tumor samples, suggesting that both miR-221 and miR-222 function as oncogenic miRNAs (24). To further investigate the role of miR-222 in HCC, Wong *et al.* performed a detailed analysis of miR-222 expression in a cohort of 99 primary HCC tumors and 94 tumor-adjacent cirrhotic livers and reported a stepwise increase in miR-222 expression from cirrhotic livers to early HCC and advanced HCC, demonstrating the contribution of miR-222 to hepatocarcinogenesis (37). More importantly, they also showed that increased expression of miR-222 is associated with shorter disease-free survival in HCC patients. Functional analysis illustrated an oncogenic role for miR-222 in promoting metastatic phenotypes. *In vitro* inhibition of miR-222 significantly reduces cell migration, invasion and motility in Hep3B and HKCI-9 cells but does not affect cell proliferation, suggesting that miR-222 is a metastatic-related miRNA. Mechanistically, reduced AKT phosphorylation was also observed following miR-222 inhibition, suggesting that AKT signaling is regulated by miR-222. AKT signaling is one of the major determinants of the regulation of HCC metastasis, and its activation is correlated with venous invasion and intrahepatic metastasis

(39-40). In human HCC, the AKT protein phosphatase-2A subunit B (PPP2R2A) is a direct functional target of miR-222 (37). Identification of PP2RA as a bona fide target of miR-222 provides evidence that the metastatic-promoting effect of miR-222 may result from its ability to activate AKT signaling in HCC.

miR-17-92. Overexpression of the miR-17-92 cluster has been demonstrated in various human malignancies, including lung cancer, B-cell lymphoma, HCC, and stomach solid tumors (16, 41-43). A study performed by Connolly *et al.* demonstrated that this miRNA cluster was overexpressed in all of the examined primary human and woodchuck HCC samples. Using a miRNA knockdown strategy, the authors found that *in vitro* silencing of this miRNA cluster resulted in a 50% reduction in both hepatocyte proliferation and anchorage-independent growth (44). The miR-17-92 cluster is composed of miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92-1. miR-17-5p, an important member of the miR-17-92 cluster, was recently reported to be overexpressed in HCC. The oncogenic role of this miRNA was supported by both *in vitro* and *in vivo* experimental evidence, illustrating that overexpression of miR-17-5p enhances the migration and proliferation of HCC cells, whereas inhibition of miR-17-5p expression attenuates the migration capacity of HCC cells. Using the two-dimensional differential in-gel electrophoresis (DIGE) approach and other proteomic techniques, heat shock protein 27 (HSP27) was identified as one of the major effectors activated by miR-17-5p. As p38 MAPK signaling is a well-known stimulus for HSP27 activation, the authors also suggested that miR-17-5p might activate HSP27 through the p38 MAPK pathway in human HCC cells, resulting in increased metastatic potential (45).

miR-30d. Interest in miR-30d has arisen from miRNA profiling studies performed to identify miRNAs with diagnostic and prognostic significance in human HCC. Of the 69 deregulated miRNAs identified in human HCC, overexpression of miR-30d was found to be prevalent in metastatic HCC tumors, suggesting its association with tumor metastatic potential (46-47). The role of miR-30d in regulating metastasis was further investigated using *in vitro* approaches, which showed that ectopic expression of miR-30d enhanced HCC cell migration and invasion. Similarly, the capacity of HCC cells to seed intrahepatic and lung metastases was also remarkably enhanced in cells stably expressing miR-30d. In silico analysis and cDNA expression arrays identified Galphai2 (GNAI2) as a direct functional target of miR-30d, and restoring the expression of this target gene in miR-30d-overexpressing cells inhibited miR-30d-mediated HCC cell migration and invasion. Given that GNAI2 is often repressed in HCC and that its expression is inversely correlated with that of miR-30d, the recently identified miR-30d/GNAI2 axis plays a critical role in hepatocarcinogenesis and represents a novel pathway contributing to tumor invasion and metastasis in HCC (47).

miR-151. Recently, Ding *et al.* performed a comprehensive analysis of the chromosomal regions that commonly display aberrations in HCC to screen for aberrantly expressed miRNAs associated with common chromosomal abnormalities in human HCC. The authors of this study identified 22 miRNAs that displayed genomic copy gains or losses in HCC. Of these 22 miRNAs, the most frequently amplified gene was miR-151. Located within the frequently amplified region of chromosome 8q24.3, miR-151 resides within intron 22 of its host gene, FAK, with which it functions synergistically to enhance cell motility and spreading in HCC. Ectopic expression of miR-151-5p significantly enhanced the *in vitro* migratory and invasive capacity of HCC cells but did not enhance cell proliferation, suggesting that miR-151-5p is a metastatic regulator in HCC. Moreover, the pro-metastatic effect of miR-151-5p was confirmed in an *in vivo* orthotopic implantation model, as demonstrated by the increased number of intrahepatic metastatic nodules in mice overexpressing miR-151. Using a combination of bioinformatics and molecular biology approaches, RhoGDIA was demonstrated to be a direct and functional target of miR-151-5p. RhoGDIA is a well-characterized metastatic suppressor that is frequently downregulated in other human cancers (48-49). In primary HCC, the expression levels of miR-151-5p and RhoGDIA are inversely correlated. By targeting RhoGDIA, overexpression of miR-151 may result in activation of Rho GTPase proteins such as Rac1 and Cdc42, which in turn facilitates HCC cell migration and invasion (50).

6. TUMOR SUPPRESSIVE MIRNAS IN HUMAN HCC

miR-122. The liver-specific miRNA miR-122 is the most abundantly expressed miRNA in the liver, accounting for approximately 70% of the total liver miRNA population (51-53). Due to its high abundance and tissue specificity, investigators have evaluated the diagnostic and prognostic values of miR-122 in HCC. Interestingly, Coulouarn *et al.* found that expression of miR-122 in HCC is etiology-dependent, as downregulation of miR-122 was observed mainly in HCC arising in HBV-infected livers (38). Further evaluation of miR-122 expression also indicated that miR-122 repression is associated with large, poorly differentiated tumors and poor overall survival in HCC patients. Together, these observations suggest that the loss of miR-122 is a marker of poor prognosis in HCC (38).

Several independent studies have demonstrated that miR-122 is frequently downregulated in primary HCC tissues and in most hepatoma cell lines, suggesting that miR-122 is a tumor-suppressive miRNA in liver carcinoma (42, 51, 54). Indeed, *in vitro* experiments using HCC cell lines revealed that miR-122 appears to play an important role in the regulation of metastatic characteristics such as cell migration and invasion (38).

Early studies of miR-122 in HCC demonstrated that it directly modulates cyclin G1 expression (51, 55). Despite being a transcriptional target of p53, cyclin G1 also negatively regulates p53 by recruiting PP2A to desphosphorylate Mdm-2 (56). Hence, further investigation indicated that the miR-122/cyclin G1 interaction increases

p53 stability and consequently affects the sensitivity of HCC cells to doxorubicin-dependent apoptosis. In a subset of HCC patients who had undergone surgical tumor resection, the time to recurrence was shorter in patients with lower miR-122 expression levels, whereas overall survival was shorter in patients with higher cyclin G1 expression levels. (55). Together, these observations suggest that miR-122 might modulate p53 activity to sensitize HCC cells to apoptosis through inhibition of cyclin G1. As a versatile miRNA, miR-122 also targets CULT1, which is a conserved transcriptional repressor of genes specifying terminal differentiation during development. By silencing the protein expression of CULT1 in the liver, miR-122 regulates the balance between terminal cell division and differentiation of hepatocytes during development. Therefore, downregulation of miR-122 in HCC shifts the balance toward excessive cell proliferation, and conversely, restoration of miR-122 expression in HCC cells significantly suppresses their proliferation (57).

miR-122 regulates numerous cancer network components and liver disease-related genes. In a recent study, a large set of miR-122 targets was mapped using high-throughput approaches. These target genes were enriched for genes related to liver metabolism, liver diseases and HCC. For instance, genes involved in liver metabolism, such as ALDOA, PKM2, GYS1 and citrate synthase, are direct targets of miR-122. Furthermore, miR-122 can also directly target multiple genes involved in oncogenic transformation and tumor metastasis, such as PTPN1, Septins, Vimentin, Paxillin and MMP7 (58). In addition, miR-122 has also been reported to be a key regulator of the mitochondrial metabolic gene network in HCC. For instance, Burchard *et al.* have demonstrated that the elevation of miR-122-seed-matched genes resulting from the downregulation of miR-122 can contribute to the loss of mitochondrial metabolic function, which interferes with the critical functions of the liver and eventually leads to morbidity and mortality in HCC patients (59).

Given the biological and clinical significance of miR-122 in the liver, investigators sought to identify the factors governing the expression of this miRNA. Accumulating evidence suggests that miR-122 is under the transcriptional control of numerous liver-enriched transcription factors, such as hepatocyte nuclear factor (HNF) 1a, HNF3a, and HNF3B, and downregulation of miR-122 in HCC might result from aberrant expression of these transcription factors (54, 57). In addition to HNF, further characterization of the miR-122 promoter demonstrated that CCAAT/enhancer-binding protein alpha (C/EBPα) acts as another transactivator of miR-122 transcription under the regulation of glycogen synthase kinase 3 beta (GSK-3b); however, the same research group also showed that miR-122 could sustain GSK-3b activity through direct repression of IGF-1R expression (60). This GSK-3b-C/EBPa-miR-122-IGF-1R regulatory circuitry might restrain hepatocyte proliferation, and perturbations of this pathway might lead to the development of HCC.

Although miR-122 is a well-characterized tumor suppressive miRNA in HCC, Jopling *et al.* have demonstrated that interaction between miR-122 and the 5' noncoding region of the HCV genome can facilitate its replication (61). Lanford *et al.* have suggested blocking miR-122 using LNA-modified oligonucleotides as a possible antiviral therapeutic strategy (62); however, in these studies, the risks of HCC development after prolonged exposure to miR-122 antagonists have not been assessed.

miR-101. In two independent studies, miR-101 was consistently shown to be underexpressed in hepatoma cell lines and in HCC tumor tissues (63-64). The tumor-suppressive role of miR-101 was first established by Su and colleagues, who found that forced expression of miR-101 clearly suppressed colony formation *in vitro* and tumor formation *in vivo* (64). Luciferase reporter assays and western blot analysis identified myeloid cell leukemia sequence 1 (Mcl-1) as a bona fide target of miR-101. Given that Mcl-1 is an anti-apoptotic member of the Bcl-2 family, the researchers went on to investigate the potential pro-apoptotic role of miR-101. Interestingly, miR-101 overexpression sensitized HCC cells to apoptosis induced by serum starvation or chemotherapeutic drugs. Notably, silencing of Mcl-1 mimicked the apoptosis-promoting effect of miR-101, whereas overexpression of Mcl-1 lacking the 3'UTR sequence completely abolished the pro-apoptotic effect of miR-101, providing direct experimental evidence that miR-101 promotes apoptosis through regulation of Mcl-1 expression. Another study demonstrated that miR-101 suppresses the expression of v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS) at the post-transcriptional level by binding to the 3'UTR of FOS (63). FOS is an oncogene that plays an important role in hepatocarcinogenesis and that is highly expressed in several cohorts of HCC clinical specimens (65-67). Ectopic expression of miR-101 in hepatoma cell lines inhibited HGF-induced cell invasion and migration, and this inhibition was attributed to the repression of FOS expression (63). Together, these studies indicate that miR-101 acts as a tumor suppressor in HCC, and its downregulation may promote tumorigenesis by modulating the invasiveness and apoptosis of tumor tissues.

miR-29. Investigation of miR-29 began with a miRNA profiling study, which revealed a significant downregulation of miR-29 family members (miR-29a/b/c) in HCC (68). In addition, given that the underexpression of miR-29b is closely associated with poor disease-free survival of HCC patients, this miRNA was suggested to be an independent prognostic factor in human HCC (69). Several research groups have identified a spectrum of apoptosis-related targets of the miR-29 family, such as TCL1, Mcl-1, CDC42, and PIK3R1, linking the miR-29 family to the modulation of apoptosis in human cancers. In line with these observations, Mcl-1 and Bcl-2 were shown to be functional targets of miR-29 in HCC (39). Enhanced expression of miR-29b suppressed the endogenous expression of Mcl-1 and Bcl-2, resulting in the activation of the mitochondrial pathway. Remarkably, ectopic expression of Bcl-2 and Mcl-1 in miR-29-overexpressing

HepG2 cells abrogated the apoptosis triggered by serum starvation, hypoxia and even chemotherapeutic drugs, confirming that both Bcl-2 and Mcl-1 are involved in miR-29-sensitized apoptosis. Consistent with these findings, ectopic expression of miR-29 significantly reduced the *in vivo* tumorigenicity of HCC cells. Based on these data, the authors concluded that downregulation of miR-29 and the resultant increase in Mcl-1 and Bcl-2 levels might activate the mitochondrial pathway, rendering HCC cells resistant to apoptosis and favoring tumor progression (39).

Let-7g. The let-7 miRNA family has recently received much attention from oncologists and is now considered one of the most important tumor-suppressive miRNAs in many human malignancies (14-15, 70-71). The let-7 family consists of 11 closely related homologs. Let-7g, located on chromosome 3p21.1, was reported to be significantly downregulated in human HCC specimens and is closely associated with patients' survival (72-73). Let-7g has recently been characterized as a tumor-suppressive miRNA in HCC, as analysis of miRNA signatures associated with HCC metastasis indicated that let-7g expression levels were significantly lower in metastatic HCC samples than in metastasis-free HCC samples (40). The functionally relevant direct target of let-7g in HCC metastasis appears to be type 1 collagen A2 (COL1A2), as the abundance of COL1A2 is inversely correlated with let-7g levels in HCC clinical specimens. As expected, ectopic expression of let-7g inhibits HCC cell migration, and this inhibitory effect is entirely reversed upon addition of COL1A2 (72). Furthermore, a study by Lan and co-workers highlighted the anti-proliferative role of let-7g. Upon transfection of HepG2 cells with let7-g mimics, cell proliferation was significantly inhibited via downregulation of the oncogene c-Myc. The downregulation of c-Myc appeared to occur at both the transcriptional and post-transcriptional levels, as both mRNA and protein levels of c-Myc were reduced dramatically after the transfection of HepG2 cells with let-7g mimics. In contrast, introduction of a let-7g inhibitor showed the reverse effect. Intriguingly, Lan and co-workers also reported that overexpression of let-7g resulted in the upregulation of p16INK4A; this effect may be mediated through its direct regulation of c-Myc in the c-Myc-Bmi-1-p16 regulatory circuit (73). Together, these results suggest that let-7g might function as an inhibitor of HCC cell proliferation through direct repression of c-Myc, which may lead to re-expression of the tumor suppressor p16INK4A.

miR-125b. miR-125b is deregulated in multiple types of human malignancies, including breast (22), oral (75), bladder (76) and anaplastic thyroid carcinomas (77). The prognostic significance of miR-125b in HCC was first emphasized in the miRNA profiling studies conducted by Li and coworkers, which demonstrated that miR-125b is the only deregulated miRNA associated with patient survival (46). Similarly, in a separate patient cohort, miR-125b was shown to be underexpressed in approximately 70% of primary HCCs and was inversely correlated with the expression of the cellular proliferation marker Ki-67 (78). Functional analysis clearly showed that ectopic expression of miR-125b suppressed the migration and invasion of HCC cells; furthermore, miR-125b expression inhibited cellular proliferation by inducing cell cycle arrest at the G1/S transition, suggesting a tumor suppressive role for miR-125b in HCC. The downstream targets of miR-125b are still under intensive investigation. The oncogene LIN28B is an experimentally validated downstream target of miR-125b in HCC. In a study performed by Liang *et al.*, both loss-of-function and gain-of-function experiments indicated that miR-125b targets LIN28B, which in turn might regulate p21Cip1/Waf1 and E-cadherin to suppress HCC cell proliferation and metastasis (78). Another possible mechanism by which the miR-125b-LIN28B pathway exerts its tumor-suppressive functions is through let-7. LIN28B is often overexpressed in poorly differentiated human HCC specimens (77, 79). A large-scale real-time PCR array containing 380 miRNA targets revealed that the let-7 family is the only target of LIN28B in HCC (80). The authors of this study investigated the effect of LIN28B on let-7 in hepatocarcinogenesis and demonstrated that forced expression of LIN28B in an HCC cell line enhanced the expression of known let-7 targets including c-myc, HMGA2, and IGFIR. Furthermore, this study showed that expression of let-7 was upregulated when LIN28B levels were reduced by miR-125b overexpression (78). These findings suggest that the miR-125b-LIN28B-let-7 pathway might function in HCC and play a pivotal role in tumor invasion through the coordinated activation of multiple oncogenic pathways.

miR-139. The importance of miR-139 was first suggested by a miRNA profiling study conducted by Wong and coworkers. Of the 666 unique miRNAs examined using an array of real-time qPCR assays, miR-139 was the most significantly downregulated miRNA in advanced HCC tumor tissues when compared to matched non-tumorous tissues (81). Detailed expression analysis revealed that miR-139 was progressively downregulated throughout the multistep process of hepatocarcinogenesis. Clinically, downregulation of miR-139 in HCC patients was significantly associated with metastatic features of the tumors, such as venous invasion and tumor microsatellite formation. Notably, expression of miR-139 was reduced further in metastatic HCC samples when compared to primary HCC samples, suggesting that miR-139 might play a crucial role in the regulation of metastasis. The pathophysiological significance of miR-139 expression was further investigated using HCC cell lines and an orthotopic implantation mouse model. Introduction of miR-139 to SMMC-7721 and BEL7402 cells suppressed cell migration and invasion *in vitro*. In line with these observations, the incidence of distal pulmonary metastasis was dramatically reduced in nude mice implanted with miR-139-overexpressing xenografts when compared to those implanted with vector-derived xenografts. Wong *et al.* further elucidated the molecular mechanism by which miR-139 suppressed metastasis in HCC by experimentally validating a potential binding site for miR-139 in the 3'UTR of ROCK2 using both luciferase reporter assays and western blot analysis (81). As ROCK2 is an important metastatic gene frequently overexpressed in human HCC (82), miR-139 might exert tumor-suppressive functions through the negative regulation of ROCK2. In HCC,

downregulation of this miRNA may enhance the metastatic potential of the tumors and result in poor patient prognosis. Moreover, the inverse relationship between miR-139 expression and ROCK2 protein abundance in primary HCC samples further supports this notion (81).

7. MIRNA SEQUENCE VARIANTS

Genetic abnormalities such as chromosome gains and losses are known to be associated with aberrant miRNA expression. Well-characterized examples include the deletion of miR-15 and miR-16 in human leukemia (83) and the amplification of miR-151 at chromosome 8q24.3 in primary HCC samples (50). Despite these findings, the presence of somatic mutations in miRNA genes has not been reported in human HCC. Recently, Yang *et al.* examined the genomic sequences of miRNA precursors for natural genetic variations and suggested that mutation might be a rare event in HCC (41). It is still conceivable, however, that some sequence variations in miRNA associated with hepatocarcinogenesis might be concealed in the study of Yang *et al.*, as the authors focused only on a subset of 59 miRNAs in 96 HCC patients. Moreover, sequence variants could be present in a form other than somatic mutations. Single nucleotide polymorphisms (SNPs) are the most common type of genetic polymorphism in the human genome, and several large-scale studies have clearly demonstrated that SNPs within protein-coding genes or non-coding regions could predispose humans to carcinogenesis. Although SNPs in miRNA genes or in the 3'UTRs of their target mRNAs could have important functional consequences, very few cancer association studies concerning SNPs in miRNA genes have been reported.

In 2008, Xu *et al.* uncovered the first SNP in a miRNA gene clinically associated with HCC susceptibility. Based on a cancer association study involving a cohort of 479 HCC and 504 control subjects, the authors identified a G>C polymorphism (rs2910164) in the miR-146a gene and demonstrated that male individuals with GG genotypes were 2-fold more susceptible to HCC than those with CC genotypes. This polymorphism affects the maturation of the miR-146a precursor and hence the abundance of mature miR-146a whose overexpression confers oncogenic phenotypes, such as the promotion of cell proliferation and colony formation (85). In another study, Qi *et al.* characterized a T>C polymorphism (rs11614913) in miR-196a-2 as a functional SNP that is associated with susceptibility to HCC. In Chinese male patients with HBV-related HCC, these authors observed that the variant genotype CC of miR-196a-2 (rs11614913) was significantly associated with increased risk of HCC (86). This SNP might affect the biogenesis of mature miR-196a and its interaction with target mRNAs, thereby increasing the risk of HCC development. Moreover, a previous study demonstrating the association between this miR-196a variant and congenital heart disease further supports this notion (87); however, mutation of miRNA genes may not necessarily have clinical significance in all types of human cancers. For example, in chronic lymphoma leukemia (CLL), the miR-16-1 (C>T) + 7 substitution resulted in reduced expression of mature miR-16-1 and contributed to malignant transformation *in vitro* and *in vivo*. In contrast, in a cohort of HCC patients, this association was not detectable by means of TaqMan SNP genotyping assay, suggesting that the miR-16-1 (C>T) + 7 polymorphism might not be important in hepatocarcinogenesis.

Not only do genetic polymorphisms within the functional seed region of miRNAs confer susceptibility to HCC risk, but SNPs in the promoter region of pri-miRNAs might also contribute to cancer risk by altering the abundance of the mature miRNAs. In a case-control study, a potential SNP, rs4938923 (T>C), was identified in the CpG islands of pri-miR-34b/c from Chinese patients with primary HCC. When compared to the wild type TT genotype, the variant genotypes of miR-34b/c rs4938723 TC/CC were significantly associated with increased HCC risk (88).

Given that miRNAs exert their biological functions by binding to the 3'UTR of an mRNA transcript, genetic polymorphisms within the 3'UTR could affect the abundance of the target mRNA by modulating the binding of miRNAs, resulting in increased susceptibility to HCC. A study by Gao *et al.* provides both *in vivo* and *in vitro* evidence that an insertion/deletion polymorphism (rs3783553) is associated with interleukin (IL)-1α expression and that the allele containing a TTCA insertion at the binding site for miR-122 and miR-378 causes upregulation of (IL)-1α expression by attenuating miRNA binding (89). The association of insertion/deletion polymorphisms with HCC risk was also made evident by Chen *et al.* who investigated the role of βTrCP in HCC pathogenesis (90-91). βTrCP is a key cell cycle regulator, and its overexpression is prevalent in multiple human cancers. In a cohort of the Chinese population, increased βTrCP expression was observed in tumors from patients with a homozygous 9N ins/ins genotype when compared to those from patients with 9N ins/del and 9N del/del genotypes. These authors demonstrated that a 9-bp insertion/deletion polymorphism (rs16405) residing in the 3'UTR of βTrCP affects the binding of miR-920, which in

8. DIAGNOSTIC AND PROGNOSTIC VALUES OF MIRNA IN HCC

HCC is one of the most lethal cancers worldwide. Due to its asymptomatic nature, patients suffering from HCC often present at an advanced stage, which is usually associated with poor prognosis or even metastasis. Moreover, current therapeutic options for advanced HCC are very limited and often ineffective, making tumor recurrence inevitable. Currently, early diagnosis of HCC and efficient disease management remain the most promising strategy for combating HCC; therefore, the development of reliable diagnostic and prognostic markers is urgently needed. miRNA expression profiling represents a better method for classifying cancer subtypes than mRNA profiling, as miRNAs display distinct expression patterns in cancers and enhanced stability against ribonuclease activity. These qualities make miRNAs an ideal candidate molecular marker for cancer diagnosis and prognosis.

By comparing the miRNA expression profiles of 241 pairs of HCC and adjacent non-cancerous clinical specimens, Budhu *et al.* recently identified a distinct metastatic signature of HCC consisting of 20 miRNAs including miR-122a, miR-125b and let-7g. This signature was shown to predict the metastatic or non-metastatic status of primary HCC with an overall accuracy of 76%, and the presence of this signature also displayed a strong association with patient survival. In addition, the authors also validated the results using an independent cohort consisting of 110 HCC cases. Similarly, the miRNA signatures showed an overall accuracy of 72% in predicting the metastatic status of primary HCC and a strong correlation with patient survival (40).

In another study, Sato *et al.* used a prediction model constructed by multivariate CoxPH models with the leave-one-out cross-validation method to identify signatures containing 13 and 56 recurrence-related miRNAs in tumor and non-tumor tissues, respectively. The results demonstrated that recurrence-related miRNAs were mostly downregulated in tumors and tended to predict early recurrence better, whereas recurrence-related miRNAs were mostly upregulated in non-tumor tissues and performed better in predicting late recurrence (42). These findings support the concept that early HCC recurrence originates from the dissemination of the primary tumor, whereas late HCC recurrence originates from accumulated genomic abnormalities in non-tumor tissues.

More importantly, miRNAs are detectable in serum or plasma, as initially reported by Lawrie *et al.* in diffuse large B-cell lymphoma (94). Indeed, serum miRNAs were shown to have much higher stability than other RNA species. For instance, Chen *et al.* found that miRNAs remain very stable even when they are subjected to harsh conditions (95). These properties highlight the potential use of miRNAs as circulatory biomarkers for cancer diagnosis. Recently, Sukata *et al.* also observed aberrant fluctuations in circulating miRNA levels using a chemically induced hepatocarcinogenesis model in rats. In this study, the authors demonstrated that the levels of 35 miRNAs were elevated not only in HCA- and HCC-bearing rats, but also in rats bearing preneoplastic lesions. Moreover, during hepatocarcinogenesis in rats, the levels of some miRNAs, including let-7a, let-7f, miR-98, miR-338, miR-34a, miR-331 and miR-652, showed a gradual increase during the progression from preneoplastic to neoplastic lesions. In particular, the levels of let-7a, let-7f and miR-98 in serum were significantly elevated in rats bearing preneoplastic lesions when compared to normal rats, suggesting that circulating miRNAs are promising circulatory biomarkers for the early detection of hepatocarcinogenesis (43).

Yamamoto *et al.* were the first to report the aberrant expression of circulating miRNAs in HCC patient sera. In this study, the authors showed that miR-500, which is abundantly expressed in HCC cell lines and tissues, was also elevated in the circulation of HCC patients. Interestingly, circulating miR-500 returned to normal levels after surgical tumor resection in HCC patients (97). In another study, Qu *et al.* demonstrated that the levels of miR-16 and miR-199a were significantly lower in the sera of HCC patients than in the sera of patients with chronic liver diseases or of normal individuals. Moreover, combining miR-16 with conventional serum markers including AFP, AFP-L3% and DCP yielded an optimal sensitivity of 92.4% and a specificity of 78.5% in HCC diagnosis (44). Thus, miR-16 was suggested as a second-line HCC marker to be used in addition to conventional markers to improve early HCC diagnosis. Meanwhile, other studies have demonstrated the use of circulating miRNA markers such as miR-21, miR-122 and miR-375 to discriminate HCC or chronic hepatitis patients from healthy controls (99-100). In particular, miR-375 alone could effectively discriminate HBV-positive HCC cases from HBV cases with a specificity of 96% and a sensitivity of 100% (99).

9. THERAPEUTIC POTENTIAL OF MIRNAS

Current therapeutic options for HCC remain very limited, and the mortality rate remains high; therefore, efficient therapies against HCC are urgently needed. Recently, the therapeutic potentials of miRNAs have been intensively explored, as a growing body of evidence suggests that miRNAs may act as critical players in cancer initiation and progression through their regulation of multiple cancer-related genes and cancer pathways. Numerous strategies have been developed for targeting oncogenic miRNAs and restoring the expression of tumor-suppressive miRNAs. For example, strategies such as anti-miRNA oligonucleotides (AMOs) (45), miRNA sponges (46) and small molecule miRNA inhibitors (47) have been widely employed to target and reduce the levels of oncogenic miRNAs in cancer. Alternatively, restoration of tumor-suppressive miRNAs has been achieved through the use of lentiviral vectors (70), adeno-associated viral vectors (104) and liposomes (105) to target tumors *in vitro* and *in vivo*.

Recently, Kota *et al.* have developed a viral vector system to deliver tumor-suppressive miRNAs in an *in vivo* HCC model. In their study, *in vitro* re-expression of miR-26 downregulated cyclins D1 and E2 and eventually led to the induction of G1 cell-cycle arrest in human HCC cells. Subsequently, systemic adeno-associated virus (AAV) vector-mediated administration of miR-26a *in vivo* resulted in significant hindrance of tumor proliferation and progression and induction of tumor-specific apoptosis (104). Other groups have attempted to target oncogenic miRNAs in HCC tumors *in vivo* using anti-miRNA oligonucleotides. For instance, Elyakim *et al.* demonstrated the targeting of the oncogenic miR-191 in an orthotopic liver xenograft mouse model. In their study, an anti-oligonucleotide targeting miR-191 was injected interperitoneally into tumor-bearing mice, after which the tumors were harvested for analysis. Animals treated with anti-oligonucleotide showed a significant reduction in miR-191 expression levels in the tumors and a significant reduction in tumor size (106).

In addition to acting as a therapeutic agent or therapeutic target, miRNAs are capable of sensitizing tumors to chemotherapeutic drugs and maximizing their effects in treating HCC. For instance, Fornari *et al.* have demonstrated that restoration of the expression of liver-specific miR-122 and miR-199a-3p sensitized HCC tumors to the effect of doxorubicin and increased doxorubicin-induced apoptosis by modulating the expression of downstream oncogenes such as cyclin G1, mTOR and c-Met (55, 107). Moreover, Bai *et al.* also showed that the use of liver-specific miR-122 in combination with the multi-kinase inhibitor sorafenib greatly reduced cell proliferation and clonogenic survival of HCC cells, illustrating the potential use of miRNAs to enhance the effect of anti-cancer drugs (108).

10. SUMMARY AND PERSPECTIVES

The recent advancements in miRNA studies have provided novel insights and enhanced the understanding of molecular hepatocarcinogenesis. The pathological importance of miRNAs has been increasingly recognized. High-throughput profiling studies have clearly demonstrated that deregulation of miRNAs is indeed a hallmark of liver carcinogenesis; however, little is known about the underlying molecular mechanisms contributing to the aberrant miRNA expression frequently found in human HCC. Several frequently deregulated miRNAs have been demonstrated to possess oncogenic or tumor suppressive functions in liver carcinogenesis, and detailed studies have characterized the functional mRNA targets of these miRNAs. Nevertheless, due to the versatile function of miRNAs in regulating multiple target genes, the phenotypic outcome exerted by miRNAs is likely a result of a combination of downstream targets. Further investigations are required to fully elucidate the detailed mechanisms underlying miRNA-mediated modulation of cancer-associated genes. Although preclinical studies have emphasized the potential of miRNAs as a novel diagnostic tool and therapeutic intervention, further experiments in larger scale studies are needed to test these findings. It is anticipated that further studies of miRNAs will shed light onto a novel strategy for the clinical management of liver cancer.

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Abbreviations: HCC: Hepatocelluar Carcinoma; miRNA: MicroRNA; HBV: Hepatitis B virus; SNP: Single nucleotide polymorphism

Key Words: MicroRNA, Hepatocellular carcinoma, Carcinogenesis, Pathology, Review

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