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Review

Cancer-related microRNAs and their role as tumor suppressors and oncogenes in hepatocellular carcinoma

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Summary. MicroRNAs (miRNAs) have emerged as key factors involved in several biological processes, including development, differentiation, cell proliferation, and tumorigenesis. In hepatocellular carcinoma (HCC), miRNAs frequently present aberrant expression profiles, which make them potentially attractive for diagnostic or prognostic applications. Currently, accumulating evidence is indicating the role of miRNAs as tumor suppressors or oncogenes in hepatic malignancies. In particular, comprehensive studies have made possible a better understanding of HCC behavior, such as tumor growth, response to therapies, metastatic potential, or recurrence, regarding the altered expression of cancer-related miRNAs. Based on these findings, efforts are under way to define new markers for liver cancer in both invasive (hepatic biopsy or tumor resection) and non-invasive (circulating miRNAs in blood serum) ways. Due to their implication in the control of various cell processes altered in HCC, cancer-related miRNAs also offer encouraging perspectives for the development of innovative cancer therapies. In this article, we review the importance of miRNA deregulation in HCC progression and the role of these small non-coding RNAs as tumor suppressors and oncogenes. The significance of miRNAs in HCC diagnosis and miRNA-based therapeutic strategies is then discussed.

Key words: Hepatocellular carcinoma, MicroRNA, Tumor suppressor, Oncogene

Introduction

Hepatocellular carcinoma (HCC) represents the third cause of death from cancer and the major form of liver malignancy worldwide, as it accounts for almost 90% of primitive hepatic tumors (Farazi and DePinho, 2006). HCC is generally encountered in patients exhibiting an underlying chronic liver disease related to well-known risk factors, including hepatitis B virus (HBV) and/or C virus (HCV) infection, alcohol abuse, genetic diseases (e.g., hemochromatosis), genotoxic intoxication (e.g., aflatoxin B1), and liver steatosis. In the absence of diagnosis and clinical management, chronic hepatitis leads to fibrosis and gradually evolves into cirrhosis. Global studies estimate that approximately 80-90% of all HCCs arise from cirrhotic livers (El-Serag and Rudolph, 2007). To date, surgical resection and liver transplantation remain the only effective therapeutic options. However, a majority of the patients present an unresectable tumor due to late diagnosis, as such a disease tends to remain asymptomatic until late advancement or distant metastasis. In addition, chemotherapy resistance, high metastatic potential and tumor recurrence are generally associated with liver cancer, leading to a low survival rate (less than 10% after five years). Consequently, the discovery of innovative and effective biomarkers ensuring an early diagnosis of the disease in order to maximize the positive response of therapeutics before spreading and metastasizing remains an essential purpose in modern hepatology.

The critical role of microRNAs (miRNAs) has been described in the control of various biological processes frequently altered in cancer. In addition, several reports

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Abbreviations. AFP: alpha-fetoprotein; CDK: cyclin-dependent kinase; CDKI: cyclin-dependent kinase inhibitor; DNMT: DNA methyl-transferase; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; IFN-alpha: interferon-alpha; MET, c-MET, or HGFR: hepatocyte growth factor receptor; miRNA: microRNA; MMP: matrix metalloprotease; mTOR: mammalian target of rapamycin; P-bodies: processing-bodies; pre-miRNA: precursor miRNA; pri-miRNA: primary miRNA; PTEN: phosphatase and tensin homolog; RISC: RNA-induced silencing complex; RT-qPCR: real-time quantitative polymerase chain reaction; TGF-beta: transforming growth factor-beta; TRAIL: TNF-related apoptosis-inducing ligand; 3'-UTR: 3'-untranslated region; 5-FU: 5-fluorouracil

indicate that the altered expression of specific sets of miRNAs can contribute to liver tumorigenesis. Remarkably, even slight changes in the amount of a few miRNAs can substantially modify cellular physiology and contribute to carcinogenesis. It has been shown that more than 50% of miRNA genes are located at fragile sites or in cancer-associated genome regions (Calin et al., 2004). Therefore, following mutation, deletion, translocation, or amplification, miRNAs can be subjected to the same alterations as classic oncogenes or tumor suppressors (Esquela-Kerscher and Slack, 2006). In the last decade miRNA functions have begun to be elucidated, especially in the understanding of their major physiological implications. In mammals, miRNAs are predicted to participate in the regulation of almost all cellular processes, including development, cell differentiation, proliferation, and apoptosis (Bartel, 2009). As the abnormal expression of a number of miRNAs has been reported in a wide range of human cancers, a strong consensus has emerged that these cancer-related miRNAs may function as oncogenes or tumor suppressors (Calin and Croce, 2006; Kent and Mendell, 2006). Regardless of cell origin, a plethora of studies has revealed the overall and recurrent down-regulation of miRNAs in tumor tissues compared with normal tissues (Lu et al., 2005; Lujambio and Lowe, 2012). More recently, the establishment of miRNA signatures is interesting with regard to the management of liver cancer patients from both diagnostic and therapeutic perspectives (Gailhouste et al., 2013). In the present review, we describe the miRNA biogenesis mechanism and focus on miRNA-altered expression in liver cancer. By considering well-defined cases, the role of miRNAs as tumor suppressors and oncogenes is then explored. Lastly, the potential applications of cancer-related miRNAs for diagnosis and their therapeutic value in human HCC are discussed.

MiRNA biogenesis and mechanism of action

MiRNAs are evolutionary conserved small non-coding RNAs of approximately 22 nucleotides that accurately regulate gene expression by complementary base pairing with the 3'-untranslated regions (3'-UTRs) of messenger RNAs (mRNAs) (Bartel, 2004). These fine post-transcriptional regulators were first evidenced in *C. elegans* by Ambros and co-workers, who discovered that *lin-4*, a gene known to control the timing of nematode larval development, did not code for a protein but produced small RNAs that can specifically bind to *lin-14* mRNA and repress its translation (Lee et al., 1993; Wightman et al., 1993). Then, miRNAs have been reported in a variety of organisms ranging from virus to mammalians. In order to facilitate miRNA-based investigations, a miRNA registry (miRBase) has been established and is currently maintained by the University of Manchester (Griffiths-Jones, 2004). So far, 1921 mature miRNA sequences have been registered in the miRBase database (http://www.mirbase.org, release 18, November 2011). It was estimated that more than 25% of all mature human miRNAs belong to a family comprising 2 or more members (based on a 7-seed sequence homology). As attested by computational studies, more than 30% of the protein-coding regions may be directly targeted and modulated by miRNAs (Lewis et al., 2005). An essential feature of miRNAs is that a single miRNA can recognize numerous mRNAs, and, conversely, one mRNA can be recognized by several miRNAs. These pleiotropic properties enable miRNAs to exert a wide control on a plethora of targets, attesting to the complexity of this mechanism of gene expression regulation. In addition, recent studies have demonstrated that certain miRNAs exhibit a tissue-specific distribution in rodent endoderm-derived tissues (Gao et al., 2011), although other miRNAs can be expressed ubiquitously (Lagos-Quintana et al., 2002). Thus, endodermal-derived hepatic and pancreatic tissues display differential miRNA enrichment in comparison to other organs. Typically, miR-122, miR-21, miR-101, miR-192, and miR-221 expression progressively increases during liver morphogenesis to become predominant in the adult hepatic tissue. Among these miRNAs highly expressed in hepatocytes, the liver-specific miR-122 represents 70% of the total amount of miRNAs in the organ. Intriguingly, the pri- and pre-miR-122 are regulated in a circadian manner (Gatfield et al., 2009). In addition, the turnover of mature miR-122 appears to be relatively long compared to other miRNAs, as its half-life may reach several weeks.

MiRNA biogenesis is a multistep process that has been reviewed extensively (Fig. 1). Briefly, miRNAs are produced by the RNA polymerase II into transcriptional precursors of hundreds of nucleotides called primary miRNAs (pri-miRNAs). These long primary precursor transcripts exhibit several stem-loop structures of approximately 80 nucleotides. In the nucleus, pri-miRNAs undergo processing by the nuclear endonuclease Drosha and the double-stranded RNA-binding protein Pasha to be cleaved into precursor miRNAs (pre-miRNAs). Pre-miRNAs are then exported to the cytoplasm by exportin-5, where they undergo further processing by the RNase III endonuclease Dicer. Dicer cleaves the pre-miRNA loop to produce an imperfect duplex consisting of a mature miRNA and a complementary fragment of a similar size (miRNAs*). A mature miRNA measures 20 to 23 nucleotides in length, which can be incorporated into the RNA-induced silencing complex (RISC), whereas the complementary miRNA* separates from the duplex and is generally degraded. Functional target sites within the mRNA usually consist of a 6-7-nucleotide-long sequence, the so-called miRNA "seed sequence.'' The silencing complex binds complementarily the 3'-UTR of the target sequences and negatively regulates gene expression either through the endonucleolytic cleavage of the mRNA or inhibition of its translation (Bartel, 2004). Lastly, miRNAs and their target mRNAs will be localized in the cytoplasmic processing-bodies (P-

bodies) where they will be degraded (Liu et al., 2005). The turnover of miRNAs is still a largely unexplored area. However, RNA degradation enzymes might target not only mature miRNAs but also the precursor pri- and pre- miRNAs. In humans, the decrease of mRNA levels related to miRNA activity has been shown to precede protein diminution in 84% of cases (Bartel, 2009).

Cancer-related miRNAs and their altered expression in HCC

MiRNA expression can be regulated at various levels from sequence identity, processing, stability and mRNA binding. Thus, all these steps are susceptible to be altered in cancer cells, impacting the global production of miRNAs. In general, miRNAs display a globally repressed expression profile regardless of tumor origin (Lu et al., 2005). However, the oncogenic properties of a number of miRNAs and their over-expression in several types of cancers have also been reported (Calin and Croce, 2007).

The progression of HCC generally involves various genetic and epigenetic aberrations (Aravalli et al., 2008). Genetic alterations usually result from chromosomal abnormalities that can lead to the depletion, amplification, or translocation of miRNAs. Approximately 50% of all annotated human miRNA genes are located at fragile sites of the genome that are associated with cancer (Calin et al., 2004). For instance, the gene that codes for miR-21 is located in a region at chromosome 17q23.2 frequently amplified in various types of solid tumors (Volinia et al., 2006). Epigenetic mechanisms and DNA methylation are also critical for miRNA regulation (Sato et al., 2011). Thus, it has been demonstrated that the down-regulation of several tumor suppressor miRNAs observed in cancer cells is mediated by the hyper-methylation of their promoting sequences (Lujambio and Esteller, 2007; Datta et al., 2008). For example, Furata and colleagues showed that the silencing of the tumor-suppressive miR-124 and miR-203 through CpG-island methylation represents a major event in hepatocarcinogenesis (Furata et al., 2010). This aberrant methylation is frequently due to the increased expression of DNA methyltransferase (DNMT) enzymes and observed in a number of human cancers, including HCC (Kondo et al., 2000). The case of miR-148a and miR-152 is of interest as the inactivation of these two miRNAs by DNA methylation frequently occurs in malignancies, such as gastric tumors (Zhu et al., 2011), pancreatic cancers (Hanoun et al., 2010), and cholangiocarcinoma (Braconi et al., 2010).

In addition to genomic and epigenetic alterations, miRNA processing itself is frequently altered in liver cancer. First, the transcription of pri-miRNAs can be regulated and modified by several transcription factors. The oncogenic transcription factor c-Myc binds, for instance, the upstream of let-7 and miR-26a, repressing the transcription of these two miRNAs and contributing to tumorigenesis (Chang et al., 2008). Another crucial point is the altered expression of DICER. In most cancer types, the down-regulation of DICER has been shown to be related to the global deregulation of miRNA expression (Faggad et al., 2010; Wu et al., 2011a-c). In the liver, Sekine and co-workers tested the consequence of DICER1 depletion by performing conditional knockout in hepatocytes (Sekine et al., 2009). Remarkably, the hepatocytes exhibiting DICER1-specific depletion displayed a gene expression profile indicative of cell growth and de-differentiation into liver progenitors. At one year of age, approximately 60% of the mutant mice spontaneously developed HCC derived

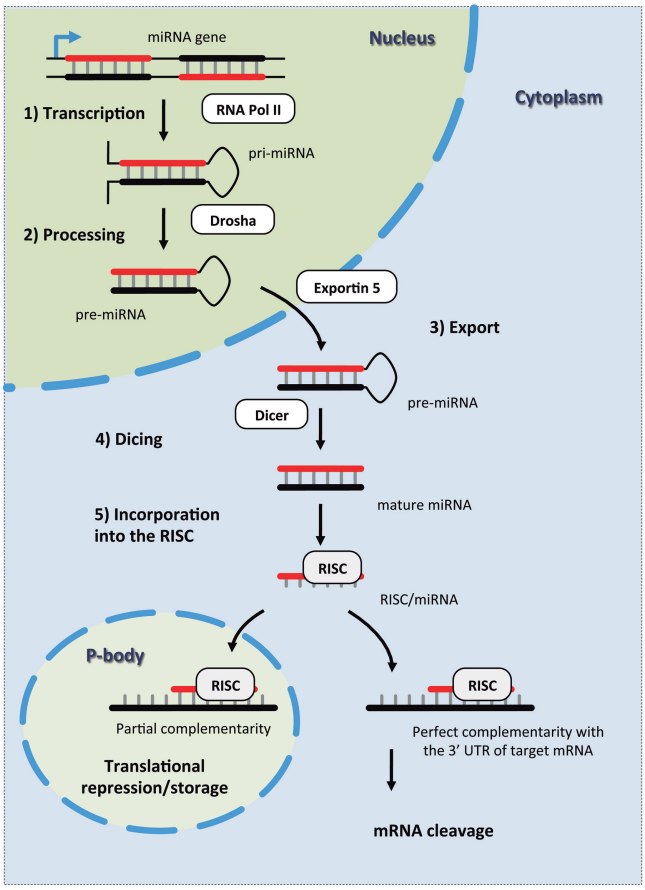

Fig. 1. The RNA interference process: biogenesis and regulation of miRNAs. Transcription from the miRNA genes by the RNA polymerase II occurs in the nucleus. The primary precursor miRNAs (pri-miRNAs) are then cleaved by the RNase III enzyme Drosha, producing precursor molecules (pre-miRNAs). With the help of the Exportin-5, the pre-miRNAs are exported into the cytoplasm, where they undergo further process by the ribonucleases Dicer to generate mature miRNAs. Mature single-stranded miRNAs are incorporated onto the RNA-induced silencing complex (RISC) to carry out their silencing function. The regulation mechanism is dependent on the degree of complementarity between the 3'-UTR region of the target mRNA and the seed region in the 5'-end of the miRNA. In the case of perfect complementarity, the mRNA is cleaved by RISC. If the complementarity is partial, the regulation is carried out by repression of the translation in the P-body.

from the DICER1-deficient hepatocytes. In another study, TARBP2 (TAR RNA-binding protein 2), encoding an integral component of a DICER1-containing complex, has been described as important for maintaining DICER1 stability, its mutation leading to DICER1 alteration and the global down-regulation of miRNAs (Melo et al., 2009). Lastly, and on the mRNA side, oncogenes acquire mutations that remove miRNA-binding sites in tumor cells. This phenomenon has been described in lipoma development, where the disruption of the pairing between let-7 and HMGA2 enhanced oncogenic transformation (Mayr et al., 2007).

MiRNA as a diagnostic tool

In recent years, several groups have reported the over-expression or the down-regulation of a number of miRNAs in a large variety of cancers. MiRNA signatures are believed to serve as accurate molecular biomarkers for the clinical classification of tumors as well as for the development of innovative therapeutic strategies. Therefore, the availability of consistent technologies that enable the detection of miRNAs has become of interest for both fundamental and clinical purposes. The current detection methods commonly used are microarray, real-time quantitative polymerase chain reaction (RT-qPCR), next generation sequencing (NGS), and, in a less routine way, Northern blot or *in situ* hybridization. Microarray analyses present the advantage of offering a high speed of screening by employing various miRNA probes within a single microchip. However, the technique has lower sensitivity and specificity than RT-qPCR, which is still the gold standard for miRNA analyses. MiRNA RT-qPCR is based on the use of stem-loop primers, which can specifically bind to the mature miRNA during reverse transcription, conferring a high degree of accuracy to the method (Chen et al., 2005). Analysis of miRNAs by RT-qPCR is a cost-effective technique and, due to its efficiency, a valuable way for the validation of miRNA signatures. Moreover, the development of RT-qPCR protocols has improved the sensitivity of miRNA detection down to a few nanograms of total RNAs. This amount can be easily and routinely obtained by extracting total RNAs from a small fragment of a hepatic percutaneous biopsy. However, the procedures employed for the normalization of miRNA data still remain a major point of discordance that requires attention. Indeed, distinct miRNA signatures have been emphasized despite the fact that miRNA profiling assays were carried out under similar conditions (the same type of tumor and the same screening technique). This dispersion might be caused by variations in patient population, such as ethnicity, gender, difference in diagnostic classification, as well as the standardization methods employed or the references used (adjacent non-tumor tissue or hepatic samples from healthy donors). Obviously, the disparity observed between miRNA profiles established in the same type of cancer may also reflect different underlying causes of the diseases as well as diverse HCC outcomes.

The aberrant expression of cancer-related miRNAs in HCC frequently contributes to the deregulation of a tumor suppressor and/or oncogene pathways, indicating the direct and crucial role of miRNAs in liver carcinogenesis (Gramantieri et al., 2008; Mott, 2009; Gailhouste et al., 2013). Different miRNA signatures have also been related to chronic hepatic infections (Calin and Croce, 2006), cirrhosis, and steatosis (Cheung et al., 2008). Using microarray technologies, Murakami and colleagues were one of the first groups to report a pattern of 7 mature miRNAs that exhibit differential expression between HCC and adjacent non-tumor samples (Murakami et al., 2006). In the 25 pairs analyzed, 5 miRNAs appeared to be significantly down-regulated (miR-199a, miR-199a*, miR-195, miR-200a, and miR-125a), whereas 2 miRNAs displayed a higher expression in HCC samples (miR-18 and miR-224). Ladeiro and co-workers also identified specific miRNA expression profiles that can unambiguously differentiate between benign and malignant HCCs as well as between several subtypes of HCC tumors (Ladeiro et al., 2008). In this study, HCC tumors exhibited a redundant over-expression of miR-224 regardless of the underlying disease. Moreover, the case of the hepatospecific miR-122 may also be of prime interest. Krutzfeldt and colleagues demonstrated that the silencing of miR-122 by using antagomir resulted in an increased expression of hundreds of genes known to be putatively targeted by miR-122 and normally repressed in normal hepatocytes (Krutzfeldt et al., 2005). This argues for the involvement of miR-122 in maintaining the "adult-liver" phenotype by suppressing the expression of several non-hepatic genes. Furthermore, the replication of HCV is known to be related to the expression of miR-122 in infected cells. Thus, HCV viral RNA can replicate in the Huh-7 cell line, which expresses miR-122, but not in HepG2 cells, which do not express miR-122. In addition, the experimental sequestration of miR-122 in cells leads to a marked loss of HCV RNA replication (Jopling et al., 2005). Importantly, miR-122 was highlighted as down-regulated in more than 70% of the samples from HCC patients with underlying cirrhosis, as well as in 100% of the HCC-derived cell lines analyzed (Gramantieri et al., 2007). Finally, miR-122 knock-out mice display hepatosteatosis, fibrosis, and a high incidence of HCC, suggesting the tumor suppressor role of miR-122 in the liver (Hsu et al., 2012).

A plethora of studies have reported various miRNA profiles potentially reflecting HCC initiation and progression which could be employed as specific cancer biomarkers (Chen, 2009; Ji and Wang, 2009). To generalize, miR-21, miR-221, miR-222, and miR-224 were widely reported as up-regulated in HCC, whereas miR-122, miR-199, miR-223, and the let-7 family members were frequently found to be down-regulated in most studies. Considering these data, the establishment of accurate miRNA-based signatures could be of prime interest in the development of new tools for the

diagnosis and advancement staging of liver cancer. However, elucidating the functional implication of the hepatic cancer-related miRNAs and the consequence of their deregulation in HCC progression also remains of prime interest from the perspective of a miRNA-based curative management.

Circulating miRNAs

To date, the development of consistent and reproducible methods to improve the diagnosis of HCC at an early stage in a non-invasive way represents a real necessity in clinical hepatology. HCC tissues secrete various tumor-related compounds into the blood, and these may serve as circulating biomarkers for the diagnosis of liver cancer. It was recently proposed that miRNAs can be conveyed in blood serum, participating in intercellular communication or conditioning the tumor environment (Kosaka et al., 2010, 2012). The concept that miRNAs could serve as potential plasma markers for liver diseases is, thus, gaining attention. Moreover, the American Association for the Study of Liver Diseases (AASLD), in its practice guideline, recently discontinued the use of the blood tumor marker alpha-fetoprotein (AFP) for surveillance and diagnosis, increasing the need for novel HCC biomarkers in blood tests.

Tumor-derived miRNAs have been efficiently detected in the serum of liver disease patients and characterized as potential biomarkers for HCC (Borel et al., 2012). In a relevant manner, the levels of 3 miRNAs (miR-21, miR-122, and miR-223) have been found to be significantly elevated in the serum of the patients exhibiting HBV infection or HBV-related HCC (Xu et al., 2011). A study carried out by Li and collaborators highlighted a specific set of miRNAs significantly up-regulated in HBV-positive HCC samples (Li et al., 2010a,b). Among them, miR-122 was highly increased in the serum of HBV patients but not in those of HCV patients. By employing a combination of characterized miRNAs, the authors could finally discriminate HCC cases from the controls or the infected non-HCC patients. Another study also emphasized the prognostic significance of serum miR-221, which represents another miRNA frequently over-expressed in HCC. In this report, the high expression of circulating miR-221 was correlated with the size of the tumor and the advancement of the disease (Li et al., 2011). Furthermore, the overall survival rate of patients exhibiting high levels of serum miR-221 was significantly lower than that of patients with low miR-221 rates. Remarkably, miR-500 has also been detected in increased amounts in the serum of HCC patients and found to be significantly reduced after the surgical resection of their tumor (Yamamoto et al. 2009). Conversely, Shigoka and collaborators highlighted the low level of circulating miR-92a in HCC, whereas tumor resection was followed by the drastic augmentation of this miRNA in blood serum (Shigoka et al., 2010). More recently, Tomimaru and collaborators evaluated the significance of plasma miRNAs as biochemical markers for HCC and demonstrated the relevance of assessing circulating miR-21 for the non-invasive diagnosis of liver cancer (Tomimaru et al., 2012).

To validate the clinical relevance of serum miRNAs, further studies will be required, with a special focus on the standardization methods or the choice of the most appropriate miRNAs for internal references. Despite the availability of good endogenous normalizers in liver tissues, no circulating miRNAs have been identified and validated as a standard reference. Although the process of assessing serum miRNAs remains under improvement and will require procedures for validations, cancer-related circulating miRNAs represent an exciting and promising field of investigation in order to develop more accurate technologies for the non-invasive diagnosis of HCC.

Tumor suppressive miRNAs and oncomirs

Under physiological conditions, the mechanisms for DNA repair, cell proliferation, motility, and programmed cell death are tightly regulated in order to maintain tissue homeostasis. Alteration of critical genes that modulate these cellular processes may tilt this balance, predisposing cells to transformation. MiRNAs have been closely associated with a number of these critical genes and found to exert an essential role in conditioning tumorigenesis and cancer progression. The current consensus is that cancer-related miRNAs function as oncogenes or tumor suppressors (Calin and Croce, 2006; Gailhouste et al., 2013). As for other malignancies, two situations can occur in HCC: i) tumor suppressor miRNAs can be down-regulated in HCC and cause the up-regulation of oncogenic target genes, repressed in normal hepatic tissues, increasing cell growth, migration, or invasion, and potentially leading to hepatocarcinogenesis (Table 1); ii) oncogenic miRNAs can be up-regulated in HCC and lead to the down-regulation of target tumor suppressor genes, participating in the development of the cancer phenotype (Table 2). Importantly, the expression profile of certain miRNAs has been found to reflect the biological behavior of HCC tumors, such as aggressiveness, invasiveness, or drug resistance. As a consequence, miRNA investigations may offer the opportunity to determine miRNA signatures that would provide valuable information to stratify and refine HCC diagnosis in terms of prognosis, response to treatment, and disease relapse. However, the gap between bench and bedside has not been bridged, and a better understanding of the cellular mechanisms that are altered in HCC through miRNA deregulation will be required to identify the miRNAs that would serve as relevant diagnostic makers and therapeutic targets for clinical practice.

Tumor suppressor miRNAs

Table 1. Key tumor suppressor miRNAs down-regulated in HCC.

Function	miRNAs	Target(s)	References
Cell growth/Proliferation (-)			
	Let-7g	c-Myc	Lan et al. (2011)
	miR-1	FoxP1, c-MET, HDAC4	Datta et al. (2008)
	miR-7	PIK3CD, mTOR, p70S6K	Fang et al. (2012)
	miR-22	HDAC4	Zhang et al. (2010)
	miR-26a	Cyclin D2, Cyclin E2	Kota et al. (2009)
	miR-29a	PPM1D	Meng et al. (2011)
	miR-34a	MACF1	Cheng et al. (2010)
	miR-99a	IGF-1R, mTOR	Li et al. (2011)
	miR-122	Cyclin G1 SRF IGF1R PTPN1, SEPT2, SEPT9 CUTL1	Gramantieri et al. (2007); Fornari et al. (2009) Bai et al. (2009) Bai et al. (2009); Zeng et al. (2010) Boutz et al. (2011) Xu et al. (2010)
	miR-124	CDK6	Furuta et al. (2010)
	miR-125b	Not determined	Liang et al. (2010)
	miR-185	Six1	Imam et al. (2010)
	miR-193b	Cyclin D1, ETS1	Xu et al. (2010)
	miR-195	Cyclin D1, E2F3, CDK6	Xu et al. (2009)
	miR-199a-3p	mTOR	Fornari et al. (2010)
	miR-219-5p	GPC3	Huang et al. (2012)
	miR-223	STMN1	Wong et al. (2008)
	miR-375	YAP AEG-1	Liu et al. (2010) He et al. (2012)
	miR-449	c-MET	Buurman et al. (2012)
	miR-519d	MKi67	Hou et al. (2011)
	miR-637	LIF	Zhang et al. (2011)
Cell motility/Invasion (-)			
	let-7g	COL1A2	Ji et al. (2010)
	miR-1	c-MET	Datta et al. (2008)
	miR-7	PIK3CD, mTOR, p70S6K	Fang et al. (2012)
	miR-23b	c-MET, uPA	Salvi et al. (2009)
	miR-34a	c-MET LMNA, GFAP, MACF1, ALDH2	Li et al. (2009) Cheng et al. (2010)
	miR-122	ADAM10 ADAM17 MMP7, PXN	Bai et al. (2009) Tsai et al. (2009) Boutz et al. (2011)
	miR-124	ROCK2, EZH2	Zheng et al. (2012)
	miR-125b	LIN28B2	Liang et al. (2010)
	miR-139	ROCK2	Wong et al. (2011)
	miR-142-3p	RAC1	Wu et al. (2011)
	miR-181a	OPN	Bhattacharya et al. (2010)
	miR-185	Six1	Imam et al. (2010)
	miR-193b	Cyclin D1, ETS1	Xu et al. (2010)
	miR-199a-3p	c-MET, mTOR	Fornari et al. (2010)
	miR-199a-5p	DDR1	Shen et al. (2010)
	miR-375	AEG-1	He et al. (2012)
Cell viability (-)/apoptosis (+)			
	Let-7c Let-7g	Bcl-xL	Shimizu et al. (2010)
	miR-15b	Bcl-w	Chung et al. (2010)
	miR-29	Bcl-2, Mlc-1	Xiong et al. (2010)
	miR-101	Mlc-1	Su et al. (2009)
	miR-122	Bcl-w	Lin et al. (2008)
	miR-199a-3p	c-MET, mTOR	Fornari et al. (2010)
	miR-223	Stathmin 1	Wong et al. (2008)
	miR-375	AEG-1	He et al. (2012)
	miR-449	c-MET	Buurman et al. (2012)
	miR-637	LIF	Zhang et al. (2011)

Cell proliferation/tumor growth

In several studies, the role of specific miRNAs has been reported in the regulation of the proliferation signaling pathways by a direct interaction with critical cell cycle regulators. Among those miRNAs are tumor suppressors targeting cyclin-cyclin-dependent-kinase (Cyclin-CDK) complexes, a class of positive modulators of the cell cycle. One of the miRNAs identified as a major regulator of cyclin-CDK complexes in liver cancer is miR-26a. MiR-26a has been shown to induce cell cycle arrest by directly targeting cyclin D2 and E2 (Kota et al., 2009). Moreover, miR-195 exerts a tumor suppressor activity by targeting CDK6 (Xu et al., 2009). This miRNA can also inhibit the G1/S transition by repressing cyclin D1. In addition to proving the overall inhibition of miR-122 in liver cancer, Gramantieri and colleagues also demonstrated the existence of an inverse correlation between miR-122 and cyclin G1 expression

Table 2. Key oncomirs over-expressed in HCC.

Function	miRNAs	Target(s)	References
Cell growth/Proliferation (+)			
	miR-18a	ESR1	Liu et al. (2009)
	miR-21	PTEN	Meng et al. (2007)
	miR-106b	p21	Ivanovska et al. (2008)
	miR-130b	TP53INP1	Ma et al. (2010)
	miR-141	DLC-1	Banaudha et al. (2011)
	miR-155	SOX6	Xie et al. (2012)
	miR-191	SOX4, IL1A, TMC7	Elyakim et al. (2010)
	miR-216	TSLC1	Chen et al. (2012)
	miR-221	DDIT4 PTEN p27 p57	Pineau et al. (2010) Garofalo et al. (2009) Le Sage et al. (2007); Fornari et al. (2008); Pineau et al. (2010) Fornari et al. (2008)
	miR-222	PTEN p27	Garofalo et al. (2009) Le Sage et al. (2007); Pineau et al. (2010)
	miR-373	PPP6C	Wu et al. (2011)
	miR-423	p21Cip1/Waf1	Lin et al. (2011)
	miR-517a	Not determined	Toffanin et al. (2011)
Cell motility/Invasion (+)			
	miR-17-5p	Not determined	Yang et al. (2010)
	miR-21	RHOB PTEN	Connolly et al. (2010) Meng et al. (2007)
	miR-30d	GNAI2	Yao et al. (2010)
	miR-143	FNDC3B	Zhang et al. (2009)
	miR-151	RhoGDIA	Ding et al. (2010)
	miR-181b	TIMP3	Wang et al. (2010)
	miR-210	VMP1	Ying et al. (2011)
	miR-216	TSLC1	Chen et al. (2012)
	miR-221	PTEN, TIMP3	Garofalo et al. (2009)
	miR-222	PTEN, TIMP3 PPP2R2A	Garofalo et al. (2009) Wong et al. (2010)
	miR-517a	Not determined	Toffanin et al. (2011)
Cell viability (+)/apoptosis (-)			
	miR-15b	Bcl-w	Chung et al. (2010)
	miR-21	PTEN	Meng et al. (2007)
	miR-25	Bim	Li et al. (2009)
	miR-183	PDCD4	Li et al. (2010)
	miR-221	Bmf	Gramantieri et al. (2009)
	miR-221 miR-222	PTEN	Garofalo et al. (2009)
	miR-224	API-5	Wang et al. (2008)
	miR-602	RASSF1A	Yang et al. (2010)

and reported that miR-122 exerts its tumor suppressor ability by inhibiting HCC cell growth through the targeting of cyclin G1 (Gramantieri et al., 2007). Notably, miR-122 controls other factors involved in cell cycle progression, such as serum response factor (SRF), insulin-like growth factor 1 receptor (IGF1R), tyrosine-protein phosphatase non-receptor type 1 (PTPN1), or the members of the septin family SEPT2 and SEPT9 (Bai et al., 2009; Zeng et al., 2010a,b; Boutz et al., 2011). Furthermore, miR-122 may be involved in the regulation of the balance between proliferation and differentiation in hepatocytes by indirectly activating the expression of hepatic functional genes, such as cholesterol-7-alpha hydroxylase gene (CYP7A1) through the repression of the transcription repressor CUTL1, which is known to promote proliferation and suppress differentiation (Xu et al., 2010a,b).

MiRNAs can modulate cell proliferation by targeting a variety of other key factors involved in the control of cell cycle progression. For instance, miR-1 is frequently silenced in HCC through CpG-island methylation and capable of directly targeting and inhibiting of fork head box transcription factor (FoxP1), hepatocyte growth factor receptor (MET, c-MET, or HGFR), and histone deacetylase 4 (HDAC4) (Datta et al., 2008). Another example is miR-223, which down-regulates Stathmin 1 (STMN1), a key microtubule regulatory protein (Wong et al., 2008). Finally, let-7g may act as a tumor suppressor gene that inhibits HCC cell proliferation by down-regulating oncogene c-Myc and up-regulating the tumor suppressor gene p16 (INK4A) (Lan et al., 2011).

Metastasis

Recent studies suggest an important role for miRNAs in metastatic formation. Accordingly, anti-metastatic-related miRNAs are frequently silenced in HCC. The MET signaling pathway plays an important role in the promotion of HCC invasion mechanisms. As reported above, the tumor suppressor miR-1, which is down-regulated in HCC, is a negative regulator of MET (Datta et al., 2008). By direct targeting of MET and inhibition of the MET-induced phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), miR-34a was also reported to suppress tumor invasion and migration in HCC patients (Li et al., 2009a,b). MiR-199a-3p, another miRNA with MET as a direct target, also inhibits the mammalian target of rapamycin (mTOR), leading to G1-phase arrest and invasiveness decline (Fornari et al., 2010). In addition, miR-23b is capable of decreasing the migration and proliferation abilities of HCC cells by targeting MET and the urokinase-type plasminogen activator (uPA) (Salvi et al., 2009). MiR-122 also exerts an anti-metastatic role in the liver, as reported by Boutz and colleagues, who demonstrated the correlation between miR-122, the matrix metalloproteinase (MMP) 7, and paxillin (PXN) (Boutz et al., 2011). Furthermore, miR-122 has been described as a negative regulator of ADAM10 and ADAM17 (a disintegrin and metalloprotease family 10 and 17), both obviously involved in metastasis (Bai et al., 2009; Tsai et al., 2009).

LIN28B, a key RNA-binding protein highly expressed in HCC which regulates tumor formation and invasion through the coordinated repression of the let-7/miR-98 family and the induction of multiple oncogenic pathways in HCC, has also been reported to be inhibited by miR-125b (Liang et al., 2010). Recently, miR-139 was demonstrated to interact with the pro-invasion factor Rho-kinase 2 (ROCK2) (Wong et al., 2011). Accordingly, miR-139 is frequently down-regulated in HCC and associated with a poor prognosis and high metastatic features. The abnormal expression of the oncogene Six1 is generally associated with aggressive forms of cancers. Six1 has been shown to be repressed by miR-185, impeding anchorage-independent growth and cell migration in several kinds of tumors, including HCC (Imam et al., 2010). Another tumor suppressor miRNA that represses the metastatic characteristics of hepatocarcinoma cells is miR-193b. MiR-193b was reported to inhibit the invasion and migration of HCC cells by targeting cyclin D1 and the oncogene ETS1 (Xu et al., 2010a,b). More recently, miR-142-3p has been included in the anti-metastatic miRNA group because of its ability to target RAC1, a factor that regulates diverse cellular events, including migration and invasion (Wu et al., 2011a-c).

Cell viability/apoptosis

Typically, anti-apoptotic factors are known to be frequently the target of tumor suppressor miRNAs. For instance, let-7c or let-7g negatively regulates the expression of the anti-apoptotic Bcl-xL by targeting its 3'-UTR in both Huh-7 and HepG2 cell lines (Shimizu et al., 2010). This suggests that the low expression of let-7 may contribute to the augmentation of Bcl-xL commonly observed in liver cancer. Interestingly, the over-expression of let-7-c significantly enhances the apoptosis of HCC cells induced by Sorafenib. In a similar manner, it has been demonstrated that the forced expression of miR-101, normally down-regulated in HCC cell lines and HCC tumors, can exert a pro-apoptotic action by targeting Mcl-1 (Su et al., 2009).

The hepatospecific miR-122 has also been identified to be capable of directly targeting other anti-apoptotic Bcl-2 family members and subsequently to reduce HCC cell viability (Lin et al., 2008). In a therapeutic perspective, Fornari and co-workers evaluated the effect of restoring miR-122 expression on triggering chemotherapy-induced apoptosis using doxorubicin and demonstrated that miR-122 increased sensitivity to the treatment (Fornari et al., 2009). Moreover, the patients who underwent a surgical resection and displayed lower levels of miR-122 were associated with a shorter time to recurrence. Xiong and co-workers also highlighted a reduced expression of miR-29 in HCC tissues that was related with poor survival rates (Xiong et al., 2010). In

this study, the enhancement of miR-29 expression dramatically increased HCC cell sensitivity to various apoptotic signals through the direct targeting of both the anti-apoptotic Bcl-2 and Mcl-1.

Oncomirs

Proliferation

Oncogenic miRNAs are generally ubiquitously expressed in normal tissues but are highly enriched in tumors. Obviously, miR-221 and miR-21 appeared as relevant oncomirs responsible for the promotion of HCC growth. First, these miRNAs present high expression levels in liver tumors and HCC cell lines and exhibit the property of inhibiting the tumor suppressor phosphatase and tensin homolog (PTEN), contributing to HCC growth and spread (Meng et al., 2007; Garofalo et al., 2009). Experimental inhibition of miR-21 increases the expression of PTEN and abolishes HCC cell line proliferation and invasiveness. Furthermore, miR-221 can also modulate the cell cycle by directly targeting two cyclin-dependent kinase inhibitors (CDKI) CDKN1B/p27 and CDKN1C/p57 (Fornari et al., 2008). Thus, the up-regulation of miR-221 observed in cancer cells leads to the promotion of HCC growth by increasing the entry in the S-phase through the control of these two CDKIs. Next, miR-221 was identified as a negative regulator of the DNA damage-inducible transcript 4 (DDIT4), a modulator of the mTOR pathway (Pineau et al., 2010). More recently, miR-373 was found to be up-regulated in human HCC tissues but not in adjacent normal tissues. This miRNA exerts its oncogenic activity by promoting cell proliferation through the inhibition of the protein phosphatase 6 catalytic subunit (PPP6C), a negative cell cycle regulator that regulates the G1-S phase transition (Wu et al., 2011a-c).

Metastasis

The most representative miRNA globally exhibiting a persistent over-expression in all solid tumors, where it acts as an oncogene, is miR-21. As reported above, miR-21 mainly exerts its oncogenic activity in HCC by modulating PTEN expression and the PTEN-dependent pathways, mediating the phenotypic characteristics of cancer cells, such as proliferation, migration, and invasion (Meng et al., 2007). The augmentation of miR-21 expression is often associated with the poor differentiation of the tumor. The ability to target PTEN and to inhibit its expression is not exclusive to miR-21, as miR-221 and miR-222 can also significantly repress the expression of this major tumor suppressor. In addition, miR-221 and miR-222 can regulate the expression of the protein phosphatase 2A subunit B (PPP2R2A) and TIMP3, an inhibitor of metalloproteases. Thus, miR-221 and miR-222 over-expression enhances cellular migration through the activation of the AKT pathway and metalloprotease expression (Garofalo et al., 2009; Wong et al., 2010). The induction of the enzymes responsible for the degradation of the extracellular matrix represents a key event for promoting the invasive process. Wang and colleagues demonstrated the induction of miR-181b by transforming growth factor (TGF)-beta and the enhancement of the activity of MMP2 and MMP9 through the decrease of TIMP3, thus promoting HCC cell invasiveness (Wang et al., 2010).

Other pathways or cellular processes responsible for cancer cell invasiveness can be modulated by the pro-metastatic miRNAs. First, the member of the oncogenic miR-106b family, miR-17-5p, has been reported to be over-expressed in liver cancer, leading to the enhancement of HCC cell migration and proliferation through a mechanism that involves the activation of the p38 mitogen-activated protein kinase MAPK pathway and an increased phosphorylation of heat shock protein 27 (HSP27) (Yang et al., 2010a,b). A study performed using a metastatic HBV-related HCC cell model demonstrated that nuclear factor NF-kappaB mediated the increased expression of miR-143, a miRNA that favors the invasive and metastatic behavior of liver tumor cells by repressing FNDC3B (Zhang et al., 2009). The pro-metastatic miR-151, frequently amplified on 8q24.3 and co-expressed with the host gene FAK, significantly increases tumor invasion and metastasis by directly targeting RhoGDIA, a putative metastasis suppressor in HCC, leading to the activation of Rac1, Cdc42, and Rho GTPases. MiR-151 also functions synergistically with FAK to enhance HCC cell motility and spreading (Ding et al., 2010). Recently, miR-517a has been identified as an over-expressed miRNA in liver cancer that promotes tumorigenesis and metastatic dissemination (Toffanin et al., 2011).

Cell viability/apoptosis

As previously exposed, tumor suppressor miRNAs exert a pro-apoptotic activity by targeting anti-apoptotic factors such as Bcl-xL or Mcl-1. Conversely, oncomirs participate in tumorigenesis and carry anti-apoptotic effects through the negative modulation of the pro-apoptotic members of the Bcl-2 family. Gramantieri and collaborators revealed that HCC tissues exhibit an inverse correlation between miR-221 and the expression of Bmf, as well as a direct correlation between Bmf and the activated caspase-3 (Gramantieri et al., 2009). *In vitro*, the enforced expression of miR-221 causes the down-regulation of Bmf, whereas miR-221 silencing induces Bmf up-regulation and leads to an increase of apoptotic cell death. MiR-25, a member of the miR-106b-25 cluster that is over-expressed in HCC, was also evidenced to exert an anti-apoptotic effect by targeting and inhibiting the pro-apoptotic factor Bim (Li et al., 2009a,b). On the other hand, miRNAs can also regulate programmed cell death by targeting other apoptosis-related genes. For example, miR-183 was identified to be frequently up-regulated in HCC tissue samples and targeting programmed cell death 4 (PDCD4), a pro-apoptotic molecule involved in the TGF-beta1-induced

apoptosis (Li et al., 2010a,b).

A number of studies have reported the dramatic role of aberrantly expressed miRNAs in HCC drug-resistance mechanisms. Importantly, Tonimaru and colleagues demonstrated that miR-21 over-expression increases the interferon (IFN)-alpha/5-fluorouracil (5-FU) drug resistance of HCC cells, whereas the use of miR-21 inhibitors renders the cells sensitive to the treatment (Tomimaru et al., 2010). Consequently, a moderated expression of miR-21 in HCC tissues was associated with a favorable response to the IFN-alpha/5-FU combination therapy and a better survival prognosis. Garofalo and colleagues further demonstrated that miR-221 and miR-222 are commonly over-expressed in HCC cells and, by targeting PTEN and TIMP3 tumor suppressors, induce TNF-related apoptosis-inducing ligand (TRAIL) resistance and enhance cellular migration through the activation the AKT pathway and metalloproteases (Garofalo et al., 2009). In the same study, the authors showed that the MET oncogene is implicated in miR-221/222 expression through its action on the c-Jun transcription factor.

HCC recurrence

Highly active drug-metabolizing pathways and multi-drug resistance transporter proteins are known to diminish the efficiency of current chemotherapeutic treatments. In addition, HCC recurrence after surgical resection of the primary tumor represents one of the characteristics leading to the low survival rate associated with liver cancer. Specific miRNA signatures have been linked to the increased risk of tumor recurrence and poor prognosis. The expression profiling of apoptosis-associated and metastasis-related miRNAs may provide clues for each patient to predict drug resistance and invasiveness of HCC that condition the recurrence of their disease. Fornari and colleagues demonstrated that miR-199a-3p repression observed in HCC leads to the over-expression of mTOR and MET, whereas the experimental restoration of miR-199a-3p reduces the growth and invasive properties of HCC cells and increases the apoptosis induced by doxorubicin (Fornari et al., 2010). Thus, an inverse correlation was revealed between miR-199a-3p and mTOR, as well as a shorter time to recurrence after tumor resection, in the patients with lower miR-199a-3p. Another study showed that low expression levels of miR-26 are well correlated with a better response to IFN-based treatment in patients with HCC but are associated with short survival (Ji et al., 2009).

The accurate assessment of cancer-related miRNA expression may predict the risk of relapse and represent an attractive prognostic tool. In particular, the high expression of miR-15b is associated with a low risk of tumor recurrence following surgical resection, as shown by Chung and colleagues who reported a negative correlation between miR-15b expression and the reappearance of HCC (Chung et al., 2010). Experimentally, targeting miR-15b with antagonists increased HCC cell proliferation and inhibited TRAIL-induced apoptosis *in vitro*, while the miR-15b precursor transfection decreased proliferation and enhanced apoptosis by repressing the anti-apoptotic Bcl-w. In addition to their prognostic significance, modulating the expression of specific drug resistance-related miRNAs may clearly represent a valuable method to improve apoptosis-sensitizing strategies for HCC treatment and avoid the recurrence of the tumor.

The “miRNA perspective” in liver cancer

The discovery of miRNAs has considerably modified and complexified conventional concepts regarding gene regulation. Concerning cancer biology, understanding the molecular mechanisms by which miRNAs promote carcinogenesis may lead to novel concepts in the diagnosis and treatment of a large number of malignancies. In addition to the deregulation of cancer-related miRNAs observed in HCC, an association has also been found between miRNA expression and the clinicopathological outcome of liver cancer (tumor growth, response to treatment, metastatic potential, and recurrence). Therefore, the use of a miRNA-based classification correlated with the etiology and the aggressiveness of the tumor could significantly enhance the molecular diagnosis accuracy of HCC and its classification, leading to the consideration of more appropriate therapeutic strategies. In this regard, several teams have reported particular miRNA expression profiles that could be considered as valuable HCC prognostic indicators (Villanueva et al., 2010). Budhu and collaborators defined a combination of 20 miRNAs as an HCC metastasis signature and showed that this 20 miRNA-based profile was capable of predicting the survival and recurrence of HCC in patients with multi-nodular or single tumors, including those at an early stage of the disease (Budhu et al., 2008). Remarkably, the highlighted expression profile showed a similar accuracy regarding patient prognosis when compared to the conventional clinical parameters, suggesting the clinical relevance of this miRNA signature. Consequently, the profiling of aberrantly expressed cancer-related miRNAs might establish the basis for the development of a rational system of classification in order to refine the diagnosis and the prediction of HCC evolution.

The potential implication of miRNAs as oncogenes or tumor suppressors supports the interest paid to cancer-related miRNAs in the past decade for the development of new curative approaches. MiRNAs represent relevant candidates as therapeutic targets, and several strategies have been reported to amend the altered expression of cancer-related miRNAs in the liver (Wang et al., 2012). First, miRNA replacement therapies use short RNA duplexes that mimic down-regulated miRNAs. On the other hand, miRNA inhibitors are chemically modified single-stranded oligonucleotides that antagonize the miRNAs over-expressed in cancer. In combination with the latest developments, which render miRNA delivery

safer and more efficient, the use of RNA interference (RNAi) therapeutic strategies will pave the way to innovative perspectives in the clinical management of HCC. Pertinent studies have already argued that miRNA-based therapy may represent an attractive approach to target hepatic primary tumors. For example, Kota and collaborators showed that a systemic administration of miR-26a in rodents led to a dramatic slow-down of HCC progression without notification of toxicity (Kota et al., 2009). Thus, the delivery of tumor suppressor miRNAs, which are typically highly expressed in the liver, but altered in HCC, may provide a valuable curative approach. However, miRNAs-based therapeutics are still in an early stage of development and more work will be required to identify relevant cancer-related miRNAs and understand the complex implication of these small non-coding RNAs in early or late HCC. In addition, as one miRNA can substantially affect the expression of several down-stream targets, precautions are necessary to avoid undesirable off-target effects. Finally, the safety of the reagents used to deliver miRNA mimics and antagomirs needs to be validated for future clinical applications.

Conclusion

Increasing evidence has highlighted the frequent alteration of miRNA expression in liver cancer, as well as the critical role of these small RNAs in tumorigenesis. Collectively, the investigative studies performed to date have resulted in a better understanding of cancer-related miRNA functions and their role as tumor suppressors and oncogenes. Given the implication of a large number of miRNAs in the control of key tumor suppressors and oncogenes, the deregulation of specific miRNAs has been shown to greatly influence HCC growth, invasiveness, treatment response, and liver tumor curability. From a diagnostic point of view, miRNA profiling (from hepatic tissues and sera) may be beneficial, as it offers additional information that could be used in combination with the conventional methods available for the clinical assessment of liver cancer. In addition, a better understanding of the processes leading to the deregulation of miRNA expression in HCC will yield further insight into the molecular mechanisms of tumorigenesis and provide a promising perspective regarding the development of new curative approaches.

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