Table 3. A comparison of BCP/PC mutations between age-, sex-, and HBeAg-status-matched chronic viral hepatitis patients from Turkey with and without LC and/or HCC

	CH (n = 52)	LC/HCC (n = 22)	P-values
Age (years, mean ± SD)	46.6 ± 11	57.1 ± 10.1	NS
Male	38 (73.1)	20 (90.9)	NS
HBeAg	21 (40.4)	7 (31.8)	NS
T1653	3 (5.8)	3 (13.6)	NS
C1727	12 (23.1)	3 (13.6)	NS
C1752	12 (23.1)	3 (13.6)	NS
V1753	14 (26.9)	5 (22.7)	NS
G1757	13 (25)	3 (13.6)	NS
T1762/A1764	19 (36.5)	7 (31.8)	NS
T1764/G1766	7 (13.5)	7 (31.8)	NS (0.065)
C1773	27 (51.9)	6 (27.3)	NS (0.053)
Kozak	8 (15.4)	2 (9.1)	NS
H1862	5 (9.6)	5 (22.7)	NS
A1896	22 (42.3)	16 (72.7)	0.017

NS, not significant.
Numbers in brackets represent percentages (%).
V base contains A, C or G bases. H base contains A, C or T bases. Kozak, Kozak sequence used in translation initiation site of eukaryotic mRNA.

Table 4. A comparison of BCP/PC mutations between age-matched patients with chronic viral hepatitis from Turkey with positive or negative HBeAg status

	HBeAg-positive (n = 47)	HBeAg-negative (n = 50)	P-values
Age (years, mean ± SD)	37.6 ± 14.4	37.6 ± 14.4	Matched
LC/HCC	7 (14.9)	14 (28)	NS
Male	30 (63.8)	37 (74)	NS
T1653	2 (4.3)	4 (8)	NS
C1727	7 (14.9)	10 (20)	NS
C1752	9 (19.1)	9 (18)	NS
V1753	5 (10.6)	16 (32)	0.011
A1757	43 (91.5)	37 (74)	0.024
T1762/A1764	10 (21.3)	17 (34)	NS
T1764/G1766	9 (19.1)	11 (22)	NS
C1773	16 (34)	24 (48)	NS
Kozak	2 (4.3)	8 (16)	NS (0.057)
T1862	7 (14.9)	7 (14)	NS
A1896	6 (12.8)	38 (76)	<0.001

NS, not significant. Numbers in bracket represent percentages (%).
V base contains A, C or G bases. Kozak, Kozak sequence used in translation initiation site of eukaryotic mRNA.

Table 5. Association between T1773 and T1764/G1766 double mutation

	T1773 (n = 26)	C1773 (n = 24)	P-values
LC/HCC	9 (34.6)	5 (20.8)	NS
Male	22 (84.6)	15 (62.5)	NS
Age (years, mean ± SD)	36.9 ± 12.5	38.3 ± 14.3	NS
T1653	0	4 (16.7)	0.030
V1753	7 (25.9)	9 (37.5)	NS
A1757	20 (76.9)	17 (70.8)	NS
T1762/A1764	8 (29.6)	9 (37.5)	NS
T1764/G1766	11 (40.7)	0	< 0.001
Kozak	2 (7.4)	6 (25)	NS (0.095)
T1862	6 (22.2)	1 (4.2)	NS (0.054)
A1896	19 (70.4)	19 (79.2)	NS
A1757 + T1762/A1764	3 (11.1)	4 (16.7)	NS
A1757 + T1764/G1766	11 (40.7)	0	0.001
Al 757 + wild 1762/1764/1766	6 (23.1)	13 (54.2)	0.024
G1757 + T1762/A1764	5 (18.5)	5 (20.8)	NS
Gl 757 + wild 762/1764/1766	1 (3.7)	2 (8.3)	NS
HBV DNA $\log_{10}$ copies/mL	5.4 ± 1.8	4.6 ± 1.3	0.009

NS, not significant. Numbers in brackets represent percentages (%).
V base contains A, C or G bases. Kozak, Kozak sequence used in translation initiation site of eukaryotic mRNA.

experiments indicated that the A1757 and T1764/G1766 mutations are associated with the levels of viral. In the present study, we observed an association between T1773 and T1764/G1766 and a higher viral load in Turkish patients, but identified no clear correlations between mutations in the BCP, PC, and/or core region and disease prognosis. This may have been a result of the uneven group sizes of the samples or a specific pattern of viral mutation that is dependent on geographical area. Further *in vitro* and clinical studies are needed to clarify the role of the 1773 mutation.

In this study, we observed an accumulation of T1773 mutations in CH patients and no statistically significant difference between HBeAg positive and HBeAg negative patients, in contrast to a previous paper on Taiwanese subjects (17). These discrepancies might be related to the different study populations because HBV mutation patterns are dependent on genotype and race. Turkey is a high prevalence area for HBV/D according to nation-wide collection of samples, whereas Taiwan area is known to have a high prevalence of HBV/B and C. As previous studies have reported (21, 22), HBV/D1 has a unique mutation pattern in the BCP/CP region. The T1762/A1764 double mutation frequently occurs in HBV/B and C, whereas the T1764/G1766 double mutation tends to occur in HBV/D1. The amount of HBV-DNA in the A1757/T1764/G1766/T1773 mutation group was higher than that in the non-A1757/T1764/G1766/T1773 group; these findings are in concordance with those of Sendi *et al.* (22). Therefore, the specific mutation pattern of HBV/D1 might provide advantages in viral replication. Detection of coordinated mutations such as A1757/T1764/G1766/T1773 suggests the possibility that a

mechanism such as secondary structure or a distinct transcriptional factor binding in the BCP/CP region of HBV/D1 is having an effect. Computer simulation shows binding of hepatocyte nuclear factor 3 on A1757/T1764/G1766/T1773.

Hepatitis B virus has a compact and constrained genome (23), and correlations between particular mutations in *cis*-acting elements of the virus and different phenotypic features of the virus have been shown clinically (17, 24–26), *in vitro*, and *in vivo* (27–29). In addition to viral factors, environmental factors such as exposure to aflatoxin (30) and the prevalence of co-infections (19, 31), may play important roles in causing regional differences in the clinical manifestation of HBV infections. Recent progress associated with the human genome project indicates the importance of host genetic factors in the outcome of HBV infections (32). There is still much to discover about HBV genotype D infection. We recommend that future work focus on characterizing the disease at a sub-genomic level in different parts of Asia in which genotype D is endemic, and broadening studies to include host factors.

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DISCLOSURE

All authors have no conflicts of interest.

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MicroRNAs: New tools to tackle liver cancer progression

Primary hepatic tumours are one of the most aggressive and resistant forms of cancer. Early diagnosis of liver cancer and the development of more accurate markers for biological classification are crucial to improving the clinical management and survival of patients. This article discusses the emerging use of microRNAs for the diagnosis of liver cancer.

by Dr Luc Gailhouste and Dr Takahiro Ochiya

Liver cancer and diagnosis

Primary liver cancer is mainly represented by hepatocellular carcinoma (HCC) and accounts for almost 90% of primitive hepatic malignancies. Statistically, HCC is the third most common cause of death from cancer worldwide [1] and is generally encountered in patients exhibiting an underlying chronic liver disease such as hepatitis B virus (HBV) and/or C virus (HCV) infection, alcohol abuse, or liver steatosis. Chronic hepatitis leads to fibrosis and gradually evolves into cirrhosis. Global studies estimate that approximately 80–90% of all HCCs arise from cirrhotic livers. Despite great advances in the treatment of the disease, hepatic cancer exhibits one of the lowest remission rates (less than 10% after five years), mainly due to its late diagnosis and high resistance to the conventional agents of chemotherapy. Indeed, as such a disease tends to remain asymptomatic, approximately 50% of newly diagnosed patients already exhibit late advancement.

Common HCC diagnostic methods include liver imaging techniques such as triphasic computed tomography scanning, magnetic resonance imaging (MRI), and abdominal ultrasound [2]. A panel of serological biochemical markers, including aminotransferases ALAT and ASAT, has also been used for several decades to monitor liver pathologies in a non-invasive manner.

Until recently, imaging tests were frequently combined with the non-invasive measurement of serum alpha-fetoprotein (AFP). Normally produced by the fetal liver, AFP decreases soon after birth whereas its high level in adults can be correlated with the appearance of malignant hepatic disease. However, the American Association for the Study of Liver Diseases (AASLD), in its practice guidelines, discontinued the use of the blood tumour marker AFP for surveillance and diagnosis due to the limited sensitivity and specificity of the method. When uncertainty regarding the diagnosis persists, a percutaneous biopsy followed by histological examination of the nodule is indicated [3]. This technique remains the gold standard method for determining the degree of underlying fibrosis and shows appreciable sensitivity (more than 80%) for HCC diagnosis.

An important breakthrough in the clinical management of liver cancer would come from the accurate correlation of the alterations of cancer-related genes and the tumour phenotype. Although HCC lesions can be broadly distinguished by histological or immunological assessment, their prognosis and clinical evolution vary greatly from one individual to another. The discovery of innovative and effective biomarkers ensuring an early diagnosis of the disease correlated with the etiology, the pathogenic tendency, and the malignancy of the tumour could significantly enhance the molecular assessment of HCC and its classification in order to maximize the positive response of therapeutics.

MicroRNAs: biogenesis and mechanism of action

MicroRNAs (miRNAs) constitute a group of 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) [4]. These 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 specifically bind to *lin*-14 mRNA and repress its translation.

miRNA biogenesis is a multistep process that has been reviewed extensively [Figure 1]. 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 wide control over a plethora of targets, attesting to the complexity of this mechanism of gene expression regulation. Several reports have described the key role of these post-transcriptional regulators in the control of diverse biological processes such as development, differentiation, cell proliferation, and apoptosis. The alterations of miRNA expression have also been reported in a wide range of human diseases, including cancer [5].

In HCC, the atypical expression of miRNAs frequently contributes to the deregulation of critical genes known to play an essential role in tumorigenesis and cancer progression. The current consensus is that cancer-related miRNAs function as oncogenes or tumour suppressors [6]. As for other malignancies, two situations can occur in HCC: (i) tumour suppressor miRNAs can be downregulated in liver cancer and cause the upregulation of oncogenic target genes repressed in normal hepatic tissues, increasing cell growth, invasion abilities, or drug resistance; (ii) oncogenic miRNAs, also called oncomirs, can be upregulated in HCC and can downregulate their target tumour suppressor genes, potentially leading to hepatocarcinogenesis.

miRNA as a diagnostic tool

As miRNA signatures are believed to serve as accurate molecular biomarkers for the clinical classification of HCC tumours, the availability of consistent technologies that enable the detection of miRNAs has become of interest for both fundamental and clinical purposes. The most current detection methods commonly used are microarray and real-time quantitative polymerase chain reaction (RT-qPCR).

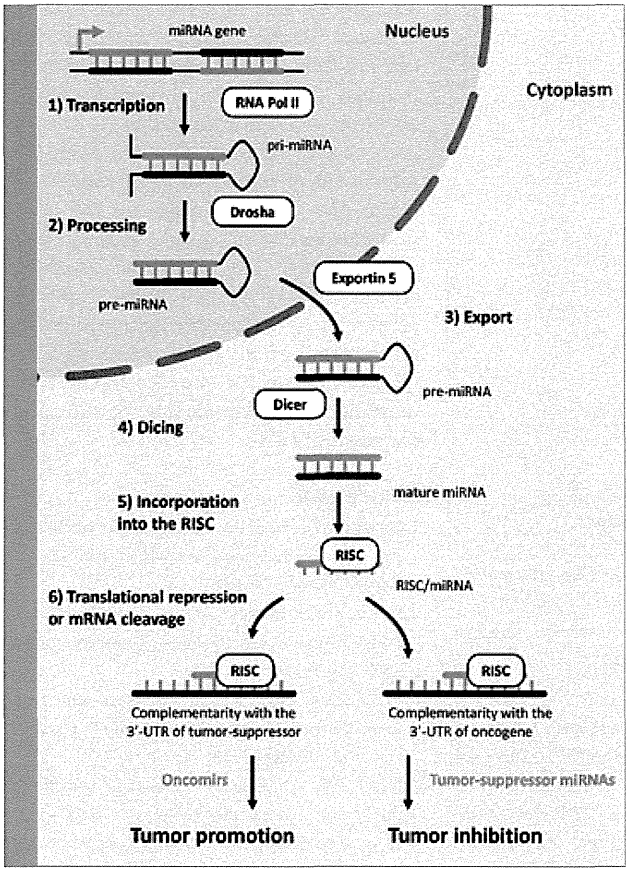

Figure 1. Biogenesis of miRNAs. Transcription from the miRNA genes by the RNA polymerase II occurs in the nucleus to give precursor miRNAs (pri-miRNAs). A pri-miRNA is further cleaved into precursor miRNA (pre-miRNA) by the RNase III enzyme Drosha in association with its co-factor, Pasha. Pre-miRNAs are then exported out of the nucleus, where they undergo a supplemental process to form mature miRNAs. Mature miRNAs are then loaded onto the RNA-induced silencing complex (RISC) and directed to the 3'-untranslated region (3'-UTR) of target mRNAs. Tumour suppressor miRNAs complementarily bind to oncogene coding sequences, potentially repressing tumour progression, whereas oncogenic miRNAs bind to tumour suppressor genes leading to cancer promotion.

Microarray analysis presents 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 the most widely used method.

miRNA RT-qPCR is based on the use of stem–loop primers, which can specifically bind to the mature miRNA during reverse transcription, granting a high degree of accuracy to the method [7]. Analysis of miRNAs by RT-qPCR is a cost-effective technique and, due to its efficiency, a valuable way to validate 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.

A plethora of studies have already reported various miRNA profiles potentially reflecting HCC initiation and progression that could be employed as specific cancer biomarkers [8]. Comparative analysis of bibliographic data provides evidence of the persistent augmentation of miR-21 in cancer, regardless of the tumour origin. In the HCC, miR-21 is also frequently overexpressed where it acts as an oncogenic miRNA. The major overexpression of miR-21 is associated with the inhibition of the tumour suppressor PTEN and the poor differentiation of the tumour. The use of an miRNA-based classification correlated with the etiology and the aggressiveness of the tumour appears very promising, as it could significantly enhance the accuracy of the molecular diagnosis of HCC and its classification, leading to the consideration of more appropriate therapeutic strategies.

In this regard, 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 multinodular or single tumours, including those at an early stage of the disease [9]. Remarkably, the highlighted expression profile showed a similar accuracy regarding patient prognosis when compared to the conventional clinical parameters, suggesting the 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.

Tumour suppressor miRNA: the case of miR-122

The case of miR-122 is of prime interest, first, because it represents by itself more than half of the total amount of miRNAs expressed in the liver [10]. Remarkably, miR-122 is a key host factor required for HCV replication. A phase 2 clinical trial was recently initiated that reported the world's first miRNA-based therapy targeting miR-122 in HCV-infected patients using the locked nucleic acid

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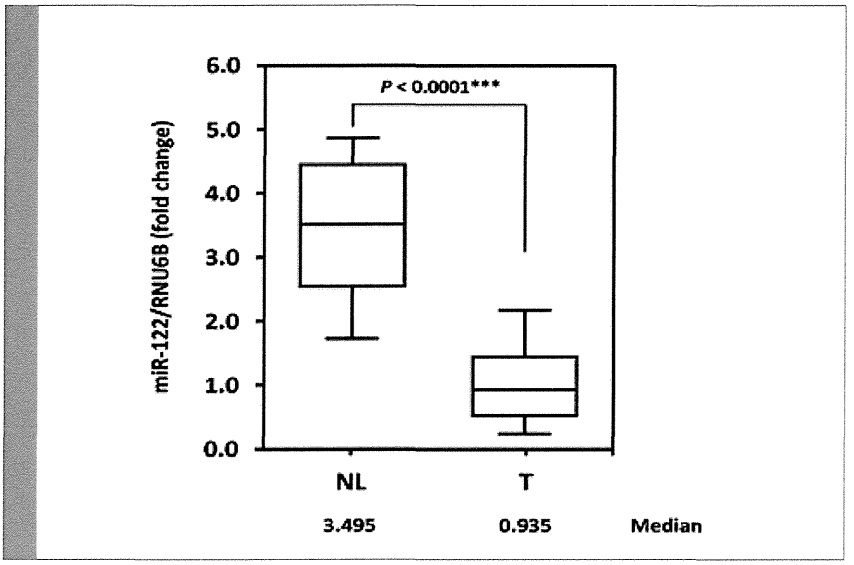

Figure 2. Diagnostic significance of miR-122 in liver cancer. RT-qPCR analysis shows the differential expression of miR-122 in 10 normal livers (NL) and 20 primary hepatic tumours (HCC). All HCCs were related to HBV (n=10) and HCV infection (n=10). The Mann-Whitney U test indicated statistical underexpression of miR-122 in HCC compared to the normal liver group (P<0.0001).

(LNA)-modified antisense oligonucleotide miravirsen [11]. Thus, a four-week miravirsen treatment by subcutaneous injection provided long-lasting antiviral activity and was well tolerated.

However, the experimental silencing of miR-122 resulted in increased expression of hundreds of genes normally repressed in normal hepatocytes. The miR-122 knockout mouse model displays hepatosteatosis, fibrosis, and a high incidence of HCC, suggesting the tumour suppressor role of miR-122 in the liver. In primary liver carcinoma, the existence of an inverse correlation was demonstrated between the expression of miR-122 and cyclin G1, which is highly implicated in cell cycle progression.

Regarding the potential of miR-122 as a diagnostic biomarker in liver cancer, numerous studies have already reported the significant and specific downregulation of miR-122 expression in both human and rodent HCC models. Obviously, miR-122 was shown as downregulated in more than 70% of the samples obtained from HCC patients with underlying cirrhosis as well as in 100% of the HCC-derived cell lines [12].

To illustrate this statement, we analyzed the expression levels of miR-122 in 20 patients who exhibited HCC using RT-qPCR. Following RNA extraction from biopsies with the miRNeasy Mini Kit (Qiagen), 100 ng of total RNA was reverse-transcribed using the Taqman miRNA Reverse Transcription Kit (Applied Biosystems). The expression levels of mature miR-122 were determined in each sample by RT-qPCR with Taqman Universal PCR Master Mix in a 7300 Real-Time PCR System from Applied Biosystems. The expression levels of miRNAs were normalized with respect to the endogenous levels of RNU6B. RT-qPCR data were obtained easily and rapidly by a routinely conventional method used in our laboratory. As a result, miR-122 expression was reduced more than threefold in HCC biopsies relative to the normal liver group (median 0.935 and 3.495, respectively; $P<0.0001$, Mann–Whitney U test) [Figure 2]. These data suggest that cancer-related miRNAs, such as miR-122, which are deregulated in HCC tissues, could be relevant with regard to the development of new diagnostic tools and the clinical management of liver cancer patients.

Conclusions and emerging approaches

The expression profile of specific miRNAs has been found to reflect the biological behaviour of HCC tumours, such as aggressiveness, invasiveness, or drug resistance. As a consequence, miRNA investigations may offer opportunities 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. Recently, tumour-derived miRNAs have been efficiently detected in the serum of patients and characterized as potential non-invasive biomarkers for HCC.

The concept that miRNAs could serve as potential plasma markers for liver diseases is, thus, gaining attention. Due to its frequent deregulation in viral hepatitis, cirrhosis, and cancer as well as its specific and massive expression in the liver, the assessment of serum miR-122 could represent one reliable strategy for the non-invasive diagnosis of chronic liver pathologies. Although the process of assessing serum miRNAs remains under improvement, cancer-related circulating miRNAs represent an exciting and promising field of investigation for the development of more accurate technologies for the early diagnosis of HCC.

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Potential applications of miRNAs as diagnostic and prognostic markers in liver cancer

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1. ABSTRACT

Primary liver tumors are mainly represented by hepatocellular hepatocarcinoma (HCC), one of the most aggressive and resistant forms of cancer. Numerous studies have reported the key role of microRNAs (miRNAs) in development, cell proliferation, apoptosis, and tumor biology. The alteration of cancer-related miRNA expression can be associated with tumorigenesis. In HCC, deregulated miRNAs frequently act as oncogenes or altered tumor suppressors. Distinct subtypes of hepatic cancer can also be related to an aberrant expression of particular miRNAs, arguing for the significance of using miRNAs as tumor biomarkers in order to refine the HCC grading assessment. In this article, we review the latest reports regarding miRNA profiling and the potential of small RNAs in HCC diagnosis. The relevance of cancer-related miRNA signatures for the prognosis and better understanding of liver cancer outcome is then considered.

2. LIVER CANCER AND MiRNA

2.1. The hepatocellular carcinoma (HCC)

Malignant primary tumors in the liver are mainly represented by the hepatocellular carcinoma (HCC), the cholangiocarcinoma, and the hepatic angiocarcinoma. The HCC accounts for almost 90% of the primitive hepatic tumors and is the third leading cause of death from cancer worldwide (1). The development of an HCC is a complex multistep process involving various genetic aberrations that alter hepatocyte proliferation, differentiation, and survival. The scheme observed in HCC development and progression is typical as it generally affects patients exhibiting a chronic liver disease. Chronic hepatitis is mainly caused by underlying pathologies, such as the hepatitis B virus (HBV) and/or C virus (HCV) infection, alcohol abuse, genetic diseases (e.g. hemochromatosis), genotoxic intoxication (e.g. aflatoxin B1), or liver steatosis, which lead to liver fibrosis. Without early diagnosis and

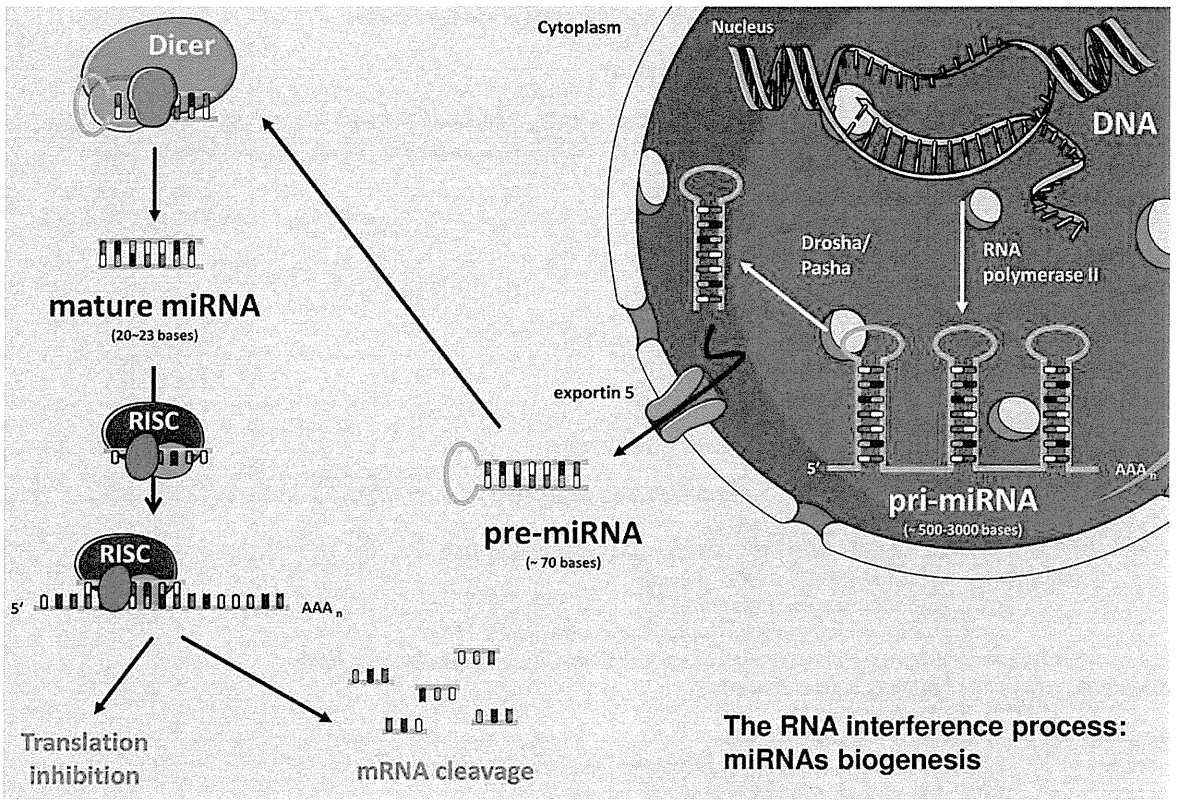

Figure 1. Biogenesis of miRNAs. A miRNA gene consisting of several different miRNAs is transcribed by the RNA polymerase II into primary precursor miRNAs (pri-miRNAs). A pri-miRNA is further cleaved into precursor miRNAs (pre-miR) by the RNase III enzyme Drosha in association with its co-factor Pasha. Pre-miRNAs are then exported out of the nucleus, where they undergo a supplemental process to form mature miRNAs. Mature miRNAs are then loaded onto the RNA-induced silencing complex (RISC) and directed to 3'-untranslated region (3'-UTR) of target mRNAs.

treatment, the fibrosis evolves into cirrhosis after an interval of 15 to 20 years. In the most advanced stages of the disease, a cirrhotic patient will develop an HCC, the achievement of the malignant phenotype occurring with intrahepatic metastasis and distal dissemination (2). It has been reported that 80% of the HCCs develop in cirrhotic liver. During the precancerous stages, the hepatocytes accumulate numerous genetic and epigenetic abnormalities that lead to cell transformation. The progression of an HCC generally involves the deregulation of critical functions essential for cellular homeostasis, such as cell cycle, cell growth, apoptosis, and cell motility (3). Despite great advances in the treatment of the disease, the hepatic cancer exhibits one of the lowest remission rates (less than 10% after five years), mainly due to its high resistance to the conventional agents of chemotherapy and its significant metastatic potential. Therefore, the development of innovative and reliable methods for the early diagnosis and curative management of patients suffering from fibrosis and liver cancer is an essential goal in modern clinical hepatology.

2.2. The miRNAs: Small RNAs regulating gene expression

The identification of small non-coding RNAs has led to the development of new research strategies in the field of RNomics. Several classes of non-coding RNAs have been discovered in mammalian cells, including small interfering RNAs (siRNAs), small nucleolar RNAs (snRNAs), and microRNAs (miRNAs). miRNAs constitute a group of evolutionary conserved small non-coding RNAs that accurately regulate gene expression by complementary base pairing with the 3'-untranslated regions (3'-UTRs) of target messenger RNAs (mRNAs) (4). Several reports have described the key role of these post-transcriptional regulators in the control of diverse biological processes such as development, differentiation, cell proliferation, and apoptosis. Computational studies have shown that miRNAs may directly target more than 30% of the protein-coding genes and modulate their expression (5). In the early 1990s, miRNAs were first evidenced in *C. elegans* when Ambros and colleagues discovered that *lin-4*, a gene known to control the timing of nematode larval development, does not code for a protein but instead produces a pair of small RNAs that can specifically bind to *lin-14* mRNA and repress its translation (6,7). In Human, 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 (Figure 1). 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 the 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 that can be incorporated into the RNA-induced silencing complex (RISC), whereas the complementary miRNA* separates from the duplex and is generally degraded. Finally, the silencing complex binds complementarily the 3'-UTR of target sequences and negatively regulates gene expression either through the endonucleolytic cleavage of the mRNA or the inhibition of its translation (4). One miRNA can recognize numerous mRNAs, and, conversely, one mRNA can be recognized by several miRNAs, attesting to the complexity of this mechanism of gene expression regulation.

2.3. MicroRNA and cancer

In the last decade, the miRNA functions began to be elucidated especially in the understanding of their major physiological implications. Meanwhile, the alterations of miRNA expression have been reported in a wide range of human diseases, including cancer (8), and a strong consensus has emerged that miRNAs can function as oncogenes or tumor suppressors during tumor development or progression (9). Indeed, it has been reported that more than 50% of miRNA genes are located at fragile sites or in cancer-associated genome regions (10). Thus, following mutation, deletion, translocation, or amplification, miRNAs can be subjected to the same alterations as classic oncogenes or tumor suppressors which then lead to tumor formation (11).

The first definitive demonstration that altered miRNA expression could play a causative role in tumorigenesis came shortly after miRNAs were recognized as a broad class of gene regulators and has been shown in B cell lymphocytic leukemia (CCL). CCL is highly associated with the loss of chromosomal 13q14, which contains two clusters of miRNAs, miR-15a and miR-16-1. By targeting the anti-apoptotic protein Bcl-2, miR-15a and miR-16-1 normally exhibit tumor suppressive properties. In CLL, the expression of these miRNAs is reduced or lost in more than 65% of the cases (12,13). Thereby, the inhibition of miR-15a/16-1 contributes to abnormal Bcl-2 expression and promotes cell survival (14). Generally, miRNA expression is globally repressed in tumor tissues (15). However, the oncogenic properties of a number of miRNAs and their over-expression in several types of tumors have also been reported (16). The only miRNA found to be over-expressed regardless of solid tumor origin is miR-21. In fact, the gene that codes for miR-21 is located in a region at chromosome 17q23.2 frequently amplified in various types of cancer (17). miR-21 is known to exert its oncogenic activity at least by targeting the phosphatase and tensin homolog (PTEN) that normally acts as a tumor suppressor by inhibiting the survival and cell growth promoted by the phosphatidylinositol tri-phosphate (PI3K) signaling pathway (18). The knock-down of miR-21 in glioblastoma cell lines can induce a caspase-mediated apoptosis (19). In recent years, several groups have reported over-expression and down-regulation of a number of miRNAs in a large variety of cancers. Using microarray screening analysis in a significant number of human tumors, the establishment of miRNA expression profiles between tumor and non-tumor tissues has also been achieved (15,20). Importantly, a plethora of comprehensive studies have aimed to define specific miRNA expression schemes characterizing the subtype of the tumor (phenotype or genotype) as well as its prognostic feature (15). MicroRNA expression profiles are believed to serve not only as accurate signatures to determine the advancement of a lesion (diagnosis) but also as valuable molecular biomarkers for the classification and prognosis of a large panel of tumors (21) as well as for the development of innovative therapeutic strategies (16).

In this review, we report the latest advances regarding the miRNA profiling data collected and their potential application in HCC diagnosis. In addition, we discuss the relevance of the cancer-related miRNAs for the prognostic consideration of liver cancer.

3. THE SIGNIFICANCE OF MiRNAs IN HCC DIAGNOSIS

3.1. Conventional diagnostic methods for HCC

In advanced fibrosis and cirrhosis, regenerative nodules can give rise to dysplastic nodules (DN) (22) as well as tumor-like nodules leading to early HCC (23-25). The outcome of patients who develop this neoplasm is generally poor due to the asymptomatic evolution of chronic hepatitis and early HCC (26,27). Consequently, the HCC is generally diagnosed at an advanced stage of the disease.

Common HCC diagnostic methods include liver imaging techniques such as triphasic computed tomography (CT) scanning, magnetic resonance imaging (MRI), and abdominal ultrasound (28). These procedures help to assess the size of the tumor, its location, the invasion of the hepatic vasculature, and the existence of metastatic foci outside the liver. Studies aimed at determining the most accurate imaging method between CT and MRI for the diagnosis of HCC concluded that MRI is slightly more sensitive than a CT scan for detecting smaller lesions. Nevertheless, the techniques are comparable in terms of tumor lesion staging (29). Imaging tests are also frequently combined with the measurement of serum alpha-fetoprotein (AFP). Normally produced by the fetal liver, AFP decreases soon after birth. AFP is generally considered to be a significant marker for HCC, as its high level in adults can be correlated with the appearance of malignant hepatic disease (30). In general, the diagnostic accuracy is significant in patients exhibiting AFP levels higher than 500ng/dl and a liver mass consistent with an HCC (CT or MRI images). In this case, the non-invasive data are considered adequate for the HCC diagnosis, leading to clinical treatment without histological evaluation (27). However, approximately 30% of HCCs are not related with AFP production, suggesting the limited relevance of this biomarker (31). When uncertainty regarding the diagnosis persists, a percutaneous

biopsy followed by the histological examination of the nodule is indicated (32). This invasive technique shows appreciable sensitivity (more than 80%), but it can be associated with clinical complications, such as internal bleeding (33).

3.2. Aberrantly expressed miRNAs in chronic liver diseases

3.2.1. HBV and HCV

HBV and HCV infections are the main causes of hepatic diseases worldwide. Chronic hepatitis represents a major risk factor for developing liver fibrosis that can evolve into cirrhosis and HCC (34,35). Although both types of viral infection can potentially promote HCC emergence, a differential miRNA expression pattern has been reported in each case. A study carried out by Ura *et al.* in liver tissues obtained from patients exhibiting HBV and HCV infection revealed that 19 miRNAs were differentially expressed between the two cohorts. In the same study, the authors showed that down-regulated miRNAs in HBV-infected livers targeted the signaling pathways related to cell death, DNA damage, recombination, and signal transduction, whereas down-regulated miRNAs in the HCV-group were related to immune response, antigen presentation, cell cycle, proteasome, and lipid metabolism (36). In addition, miR-143 has been demonstrated to be dramatically increased in HBV-related HCC, conferring metastatic potential in both p21-HBx transgenic mice and HCC patients by repressing fibronectin type III domain containing 3B (FNDC3B) (37). A study addressing the role of miRNAs in HBV-related tumor formation demonstrated that miR-602 can promote hepatocarcinogenesis which suggests the potential of this miRNA as an early diagnostic marker for HBV-mediated HCC (38). (Table 1).

Liu *et al.* demonstrated that HBV replication is able to modulate the expression of host cellular miRNAs. They compared the miRNA expression profile of a stable HBV-expressing cell line, HepG2.2.15, with its parent cell line, HepG2, and showed that 11 miRNAs were differentially up-regulated in the HBV-expressing cell line, while 7 miRNAs were down-regulated (39). In HCV-infected human livers, a computational study of miRNA-mRNA regulatory modules identified a set of down-regulated miRNAs (miR-122, miR-320, and miR-191) and up-regulated miRNAs (miR-215, miR-16, miR-26, miR-130, miR-199, and miR-155) that target genes involved in chemokine, PTEN, IL-6, MAPK, B cell receptor, and the JAK/STAT signaling pathway, suggesting a critical role of miRNAs in the replication, propagation, and latency of viruses in host cells (40). In HCV patients, other studies have demonstrated that miR-122 expression is inversely correlated with both functional and histo-pathological HCV-related liver damage (41,42). More recently, miR-141, up-regulated in HCV-infected human hepatocytes, was shown to be capable of enhancing cell proliferation by targeting the tumor suppressor gene DLC-1 (a Rho GTPase-activating protein), which is frequently deleted in HCC (43).

3.2.2. Alcohol and non-alcohol fatty liver diseases (AFLD and NAFLD)

Alcoholic and non-alcoholic fatty liver pathologies represent two other causes of chronic liver disease. The etiology is different in each case: AFLD is due to alcohol abuse, whereas NAFLD occurs as a result of metabolic syndromes, such as obesity or type 2 diabetes (44-46). However, both diseases share common pathophysiological mechanisms characterized by an abnormal metabolism that can be related to steatosis, subsequent inflammation (steatohepatitis), and fibrosis (47). Regarding the expression of miRNAs, Dolganiuc *et al.* carried out a comparative study between AFLD liver samples from mice fed an ethanol-containing diet (Lieber-DeCarli) and NAFLD livers from mice fed a methionine-choline-deficient (MCD) diet. Compared to the corresponding controls, both the Lieber-deCarli and the MCD diet were related to the over-expression or the down-regulation of numerous miRNAs. Among these altered miRNAs, miR-705 and miR-1224 were up-regulated in both groups whereas miR-182, miR-183, and miR-199a-3p were down-regulated in the AFLD group and up-regulated in the NAFLD livers (47). Obviously, these data demonstrate the etiologic-specific changes of miRNA profiles occurring in AFLD and NAFLD.

Another study developed by Li and colleagues has related some specific miRNAs with aberrant energy metabolic status and the pathogenesis of NAFLD. Compared to normal C57BL/6 mice, the fatty livers of ob/ob mice, considered to be a natural model of NAFLD (48,49), showed the up-regulation of eight miRNAs (miR-34a, miR-31, miR-103, miR-107, miR-194, miR-335-5p, miR-221, and miR-200a) and the down-regulation of three miRNAs (miR-29c, miR-451, miR-21) (50). In the NAFLD rat model, Alisi *et al.* recently identified a set of altered miRNAs. Three miRNAs showed significant down-regulation (miR-122, miR-451, and miR-27), whereas three other miRNAs (miR-200a, miR-200b, and miR-429) were more expressed in injured livers than in the control samples (51). Another experimental model, using NAFLD C57/BL6 mice fed a choline-deficient and amino acid (CDAA)-defined diet, highlighted a significant up-regulation of miR-181b and miR-181d 32 weeks after initiation of the treatment and was related to HCC emergence after 84 weeks (52). Furthermore, the expression of the tumor suppressor TIMP3, validated as a miR-181 target, was markedly suppressed in the livers of mice fed a CDAA diet. To finish, miR-155 has been reported to be associated with alcoholic liver disease, as demonstrated by Bala and colleagues in the AFLD mouse model, where miR-155 expression was significantly increased in macrophages (53).

3.2.3. Liver fibrosis and cirrhosis

Chronic liver diseases generally lead to liver fibrosis which is characterized by an excessive accumulation of extracellular matrix proteins. Produced by hepatic stellate cells (HSCs), fibrotic compounds alter the hepatic architecture by forming scars and nodules of regenerating hepatocytes (54,55). Without treatment of the

Table 1. MicroRNA altered expression in chronic liver diseases

Pathology	Model	Up-regulated miRNAs	Down-regulated miRNAs	References
HBV-HCC	Human P21-HBx Transgenic mice	miR-143		Zhang *et al.* (2009) [37]
	Human	miR-602		Yang *et al.* (2010) [38]
HCV-HCC	Human	miR-122, miR-100, miR-10a	miR-198, miR-145	Varnholt *et al.* (2008) [71]
HBV	Human	miR-193, miR-211, miR-190, miR-151, miR-134, miR-20	miR-23a, miR-27a, miR-34c, miR-124b, miR-142-5p, let-7a	Ura *et al.* (2009) [36]
	HepG2-HBV	miR-181a, miR-181b, miR-200b, miR-146a	miR-15a	Liu *et al.* (2009) [39]
	Serum of HBV patients	miR-375, miR-92a		Li *et al.* (2010) [94]
	Serum of HBV patients	miR-122		Zhang *et al.* (2010) [93]
HCV	Human	miR-23a, miR-27a, miR-34c, miR-124b, miR-142-5p, let-7a	miR-193, miR-211, miR-190, miR-151, miR-134, miR-20	Ura *et al.* (2009) [36]
	Human	miR-141		Banaudha *et al.* (2011) [43]
	Human	miR-215, miR-16, miR-26, miR-130, miR-199, miR-155	miR-122, miR-320, miR-191	Peng *et al.* (2009) [40]
	Human	miR-21	miR-122	Marquez *et al.* (2010) [41] Morita *et al.* (2011) [62]
NAFLD	Mouse model	miR-705, miR-1224, miR-182, miR-183, miR-199a-3p		Dolganiuc *et al.* (2009) [47]
	Mouse model (ob/ob mice)	miR-34a, miR-31, miR-103, miR-107, miR-194, miR-221, miR-335-5p, miR-200a	miR-29c, miR-451, miR-21	Li *et al.* (2009) [50]
	Rat model	miR-200a, miR-200b, miR-429	miR-122, miR-451, miR-27	Alisi *et al.* (2011) [51]
	Mouse model	miR-34a, miR-155, miR200b, miR-221	miR-29c, miR-122, miR-192, miR-203	Pogribny *et al.* (2010) [162]
	Mouse model	miR-181b, miR-181d		Wang *et al.* (2010) [52]
	Cell model	miR-10b		Zheng *et al.* (2010) [105]
AFLD	Mouse model	miR-705, miR-1224	miR-182, miR-183, miR-199a-3p	Dolganiuc *et al.* (2009) [47]
	Mouse model Plasma	miR-122		Zhang *et al.* (2010) [93]
	Mouse model	miR-155		Bala *et al.* (2011) [53]
Fibrosis	Rat model	miR-874, miR-29c*, miR-501, miR-349, miR-325-5p, miR-328, miR-138, miR-143, miR-207, miR-872, miR-140, miR-193	miR-341, miR-20b-3p, miR-15b, miR-16, miR-375, miR-122, miR-146a, miR-92b, miR-126	Guo *et al.* (2009) [57]
	Culture-activated rat HSCs	miR-27a, miR-27b		Ji *et al.* (2009) [58]
	Mouse model	miR-21	miR-122	Marquez *et al.* (2010) [41]
	Mouse model Human fibrosis		miR-29	Roderburg *et al.* (2011) [59]
	Rat model	miR-34	miR-878	Li *et al.* (2011) [163]
	Rat model	miR-199a, miR-199a*, miR-200a, miR-200b		Murakami *et al.* (2011) [60]

underlying pathology, fibrosis evolves into cirrhosis, which finally produces hepatocellular dysfunction. In the quiescent state, HSCs are lipid-storing cells located in the perisinusoidal endothelium. When stimulated by fibrogenic stimuli, HSCs undergo myofibroblastic transdifferentiation, also known as activation, which represents the key event in liver fibrosis (56). According to a study of Guo *et al.* using a fibrosis-induced rat model, this activation is associated with a differential expression of several miRNAs that leads to the activation or the repression of numerous major signaling pathways (57).

miR-27a and miR-27b are two specific miRNAs reported to be over-expressed in activated HSCs. Conversely, their down-regulation allows activated rat HSCs to return to a more quiescent HSC phenotype with restored cytoplasmic lipid droplets and decreased cell proliferation (58). Using the carbon tetrachloride (CCl_4) fibrosis-induced model in mouse, Marquez *et al.* emphasized the drastic down-regulation of miR-122, whereas miR-21 expression appeared positively correlated with the fibrotic stage. The regulatory role of miR-21 in fibrogenesis was proposed to occur through a mechanism involving the enhancement of the transforming growth factor-beta (TGF-beta) signaling

by the direct targeting of its negative regulator, SMAD7 (41). Roderburg and co-workers recently showed that CCl_4-treated mice as well as patients with advanced liver fibrosis exhibited a significant down-regulation of the miR-29 family related with the subsequent up-regulation of extracellular matrix genes after TGF-beta treatment in synergy with lipopolysaccharide and nuclear factor-kappa B (NF-kappaB). Consequently, miR-29 was proposed to be a novel target for the development of therapeutic strategies as well as a potential biomarker for liver fibrosis diagnosis (59). Finally, Murakami and colleagues revealed a significant increase of 11 miRNAs in CCL_4 fibrotic livers relative to that in corresponding controls. Remarkably, the human samples analyzed in this study also showed an important deregulation in the expression of several miRNAs in correlation with the advancement of fibrosis (60). In both models, the expression of 4 miRNAs (miR-199a, 199a*, 200a, and 200b) was positively and significantly correlated to the severity of the disease, suggesting the participation of these 4 miRNAs in the progression of chronic liver pathologies.

3.3. The expression of numerous miRNAs is deregulated in human HCC

Investigations carried out in human liver cancer and HCC-derived cell lines have demonstrated the aberrant expression of numerous miRNAs in hepatic cancer cells. The atypical expression of cancer-related miRNAs in HCC frequently contributes to the deregulation of tumor suppressor and/or oncogene pathways, indicating the direct and crucial role of miRNAs in liver carcinogenesis (61,62). Using microarray technologies, Murakami and colleagues first reported the miRNA expression profiles obtained with 25 pairs of HCC and adjacent non-tumor tissues (NT) as well as 9 chronic hepatitis livers (63). The authors highlighted 7 mature miRNAs and one precursor miRNA that exhibited significantly differential expression patterns between HCC and NT samples. Whereas 3 miRNAs (miR-18, pre miR-18, and miR-224) displayed a higher expression in HCC samples, 5 miRNAs appeared significantly down-regulated (miR-199a*, miR-199a, miR-195, miR-200a, and miR-125a). The classification of the samples (HCC versus NT) using a support vector machine algorithm based on these data provided a prediction accuracy of 97.8%. Moreover, a correlation between 4 miRNAs (miR-92, miR-20, miR-18, and pre-miR-18) and the degree of HCC differentiation was found, suggesting the involvement of specific miRNAs in the progression of the disease. To finish, the altered expression of some miRNAs was associated with distinct risk factors such as HBV infection (miR-96) and alcohol abuse (miR-126*).

Global transcriptomic analysis has tried to propose a molecular classification of the HCC reflecting with more accuracy the clinical and genetic characteristics of the tumor (64). In recent years, a plethora of studies also suggested that miRNAs could be used as specific signatures traducing HCC initiation and progression, which could be exploited as potential cancer biomarkers (65,66). Briefly, miR-21, miR-221, and miR-222 were widely reported to be up-regulated in HCC, whereas miR-122, miR-199, and the let-7 family members were found to be down-regulated in most studies (Table 2). Considering these data, the establishment of specific miRNA profiles could be of prime interest in the development of new diagnostic tools in liver cancer. The major reports dealing with this purpose are discussed in the following section.

3.4. miRNA expression profiling for HCC diagnosis

3.4.1. Over-expressed miRNAs in HCC

Comparative analysis of bibliographic data provides evidence of the persistent augmentation of miR-21 in liver cancer. As previously reported, miR-21 is frequently over-expressed in several types of tumors, where it acts as an oncomiR (67). In the HCC, the major over-expression of miR-21 is associated with the inhibition of the tumor suppressor PTEN and the poor differentiation of the tumor (68). In the HCC Fisher rat model, comparative studies of the miRNA profiles obtained by microarray revealed an altered expression of several miRNAs, such as miR-21, miR-130, miR-190, or let-7a, that are up-regulated in hepatoma (69). In human, the array-based profiling performed by Wong and colleagues on 18 HCC lines derived from HBV, HCV, and non-viral patients highlighted the up-regulation of miR-221, miR-222, miR-182, and miR-31 (miR-222 > miR-221 > miR-31 > miR-182). Obviously, the high deregulation of miR-221 and miR-222 was confirmed in liver samples from patients and permitted to distinguish HCC from adjacent non-tumor tissue regardless of viral association (70). In their study, Varnholt and co-workers demonstrated that miR-10a and miR-100 are significantly over-expressed in HCC patients exhibiting HCV infection and cirrhosis (71). Interestingly, the authors first validated the accessibility and the qualitative adequacy of miRNAs from formalin-fixed paraffin-embedded (FFPE) liver tissues by real-time polymerase chain reaction (RT-qPCR) regardless of the length of sample storage. These data are essential because they show that miRNA expression analyses can be performed reliably using not only fresh tissue but also routinely processed and FFPE stored liver tissues, which represent the main source of biological samples from HCC patients.

One major interest of miRNA profiling is certainly to contribute to the identification of putative HCC subtypes (etiology, genotype, and phenotype) that could accurately reflect the evolution of the lesion. One example is the miRNA signature associated with the hepatic stem cell-like HCC (HpSC-HCC) subtype. Using microarray-based profiling, Ji *et al.* established a miRNA model allowing the discrimination between the HpSC-HCC subtype and the mature hepatocyte-related HCC (72). In this study, the miR-181 conserved family was reported to be highly expressed in epithelial cell adhesion molecule (EpCAM)-positive cancer stem cells (CSCs) as well as in HpSC-HCC tumors. Remarkably, the members of this family of miRNAs appeared to be relevant markers for the assessment of hepatic CSCs and the diagnosis of the HpSC-HCC subtype (73). Ladeiro and co-workers have also identified specific miRNA expression patterns than can unambiguously differentiate between benign and malignant HCCs as well as between several subtypes of HCC tumors according to specific risk factors, oncogene and tumor suppressor gene mutations, or clinical features (74). This study firstly focused on miR-224 over-expression in HCC tumors

regardless of the underlying disease. Interestingly, miR-96 was more expressed in HBV-related tumors than other kinds of HCCs. Conversely, two miRNAs (miR-122a and miR-422b) have been found to be significantly down-regulated in both benign and malignant tumors, whereas miR-200c and miR-203 were under-expressed only in benign tumors. Finally, miR-126* appeared to be specifically down-regulated in alcohol-related HCC samples.

3.4.2. Down-regulated miRNAs in HCC, the case of miR-122

The primary liver cancer displays numerous genomic alterations, including chromosomal instability, CpG hyper-methylation, DNA rearrangements associated with HBV integration, DNA hypo-methylation, and, to a lesser degree, microsatellite instability (3). It has been demonstrated that the down-regulation and/or silencing of several tumor suppressor miRNAs observed in cancer cell lines can be mediated by the hyper-methylation of their promoter regions (75,76). As an example, the silencing of miR-127 can be reversed by using a combination of a DNA demethylation agent (5-aza-2'-deoxycytidine) and a histone deacetylase inhibitor (4-phenylbutyric acid), suggesting the correlation between the DNA methylome and the miRNAome.

A cumulative analysis highlighted the diagnostic value of commonly down-regulated miRNAs in the primary liver cancer (Table 2). In HCC patients exhibiting underlying HCV infection and cirrhosis, Varnolt *et al.* reported that miR-198 and miR-145 are up to five-fold down-regulated in hepatic tumors relative to the normal liver (71). The array-based profiling performed by Wong and colleagues on HCC cells derived from HBV, HCV, and non-viral patients also emphasized the inhibition of miR-126, miR-223, miR-321, and miR-122 regardless of the viral status (70). The case of miR-122 is of prime interest, first, because it represents by itself more than 70% of the total amount of miRNAs expressed in the liver. By using distinct silencing protocols, the overall importance of miR-122 in the regulation of the metabolism and the hepatic homeostasis has been demonstrated (77). To target miR-122 *in vivo*, Krutzfeldt and colleagues proposed an innovative anti-sense strategy based on cholesterol-conjugated 2'-OMe oligonucleotide and referred to as "antagomir" (78). Silencing miR-122 resulted in an increased expression of hundreds of genes known to be possible targeted by miR-122 and normally repressed in normal hepatocytes. These results strongly argue for the involvement of miR-122 in maintaining the "adult-liver" phenotype by suppressing the expression of several non-hepatic genes (79). Furthermore, the observation made by Jopling *et al.* showed that the replication of the HCV could be related to the expression of miR-122 in infected cells. Remarkably, 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. By intra-cellular sequestration of miR-122, the authors reported a marked loss of HCV RNA replication (80). To finish, a genetic interaction between miR-122 and the 5' non-coding region of the HCV genome was also revealed, suggesting that this miRNA may represent an attractive target for the development of antiviral treatment.

Regarding the potential of miR-122 as a diagnostic biomarker in liver cancer, numerous studies have already reported the significant and specific down-regulation of miR-122 expression in both human and rodent HCC models (69,81). Obviously, miR-122 was shown as down-regulated in more than 70% of the samples obtained from HCC patients with underlying cirrhosis as well as in 100% of the HCC-derived cell lines analyzed (82). However, the aberrant expression of miR-122 in HCC could be correlated with the viral etiology of the tumor, as suggested by a study of Varnholt and co-workers, where miR-122 was significantly and unexpectedly described to be over-expressed in HCC patients exhibiting HCV infection (71). In their study, Gramantieri and colleagues also demonstrated the existence of an inverse correlation between the expression of miR-122 and cyclin G1 in primary liver carcinomas (82). miR-122 is known to target the cyclin G1, which is highly implicated in cell cycle progression. In opposition, cyclin G1 over-expression enhances cancer cell growth, whereas its silencing suppresses cell proliferation (83). In the mouse model, the absence of cyclin G1 is associated with a lower susceptibility to develop liver tumors through the increase of p53 tumor suppressor activity (84). Therefore, the converse expression of cyclin G1 consequent to miR-122 down-expression in human HCC may lead to p53 down-regulation and hepatocarcinogenesis promotion. In fact, the down-expression of miR-122 observed in the early stages of the HCC argues for its implication in early hepatocarcinogenesis (74). Due to its frequent deregulation in viral hepatitis, cirrhosis, and cancer as well as its specific and massive expression in the liver, the assessment of serum miR-122 could at last represent a reliable strategy for the non-invasive diagnosis of liver chronic pathologies.

3.5. Circulating miRNAs: Potential biomarkers for the non-invasive diagnosis of HCC

Clinically, a panel of serological biochemical markers, including aminotransferases (ALAT/ASAT) and AFP, have been used for several decades to monitor liver pathologies in a non-invasive manner. However, these methods may present some restrictions such as limited sensitivity and specificity, particularly with regard to the insidious progress of the HCC. Recently, it has been proposed that miRNAs can circulate and be conveyed in blood serum (85,86). Although serum contains ribonucleases, the existence of circulating miRNAs suggests that these molecules can resist RNase digestion. In fact, plasma miRNAs are probably protected from RNase degradation via their inclusion into lipid or lipoprotein complexes. An increasing number of reports have described this original process that is believed to involve several kinds of micro-particles (85): microvesicles (0.1-1 μm), prostasomes (50-500 nm), exosomes (10-100 nm), and apoptotic bodies (0.5-2 μm). Thereby, strong evidence argues for the existence of an inter-cellular communication involving the exosomal transfer of miRNAs (Figure 2). Numerous tumor-derived miRNAs, such as miR-21 in B-cell lymphoma (87), miR-141 in prostate cancer (88), and

Table 2. Major reports regarding cancer-related miRNA profiling in human HCC

MiRNAs under-expressed in HCC	miRNAs over-expressed in HCC	HCC samples	HCC etiology	Method(s)	References
miR-18, 125a, 195, 199a, 199*, 200a	miR-224	25	Mixed HBV/HCV	MA, NB	Murakami *et al.* (2006)[63]
miR-92-1, 122a, 125a, 125b, 292-3p, 199a	miR-21, 34a, 210, 213, 222, 294, 373, 376a	20	Unknown	MA, NB, RT-qPCR	Meng *et al.* (2007)[68]
miR-122a, 124a, 130a, 141, 142, 143, 146, 181a, 181c, 195, 200b, 223	miR-221	60	Mixed HBV, HCV, ethanol	MA, NB, RT-qPCR	Gramantieri *et al.* (2007)[82]
miR-235	let-7a, 7b, 7c, 7d, 7e, 7f, 7g, 7i, miR-21, 22, 98, 126, 126-3p, 195, 352	10	Mixed non HBV, HCV	MA, NB	Huang *et al.* (2008)[164]
miR-139, 145, 214	miR-9, 9*, 21, 25, 96, 137, 151, 155, 182, 182*, 183, 186, 216, 221, 222, 224, 301, 324-5p, 374	19	Unknown	RT-qPCR	Wang *et al.* (2008)[154]
miR-101, 139, 150, 199a, 199a*, 199b, 200b, 214, 223	miR-18, 21, 33, 130b, 135a, 221, 301	54	Mixed HBV, and/or HCV	RT-qPCR	Jiang *et al.* (2008)[165]
miR-9*, 29c, 95, 104, 106a, 134, 137, 145, 147, 159a, 185, 198, 199b, 204, 218, 302b, 330, 368	let-7g, miR-9a, 10a, 15a, 16, 21, 100, 122, 125b, 299, 326, 370	52	HCV	RT-qPCR	Varnholt *et al.* (2008)[71]
miR-107, 122a, 126*, 200c, 203, 375, 422b	miR-10b, 21, 96, 222, 224	55	Mixed HBV, HCV, ethanol	RT-qPCR	Ladeiro *et al.* (2008)[74]
let-7a, miR-22, 99a, 122a, 126, 130a	miR-15a, 17, 18a, 19b, 20a, 21, 27a, 92, 93, 106b, 148a, 324	19	HBV	NB, RT-qPCR	Connolly *et al.* (2008)[166]
let-7g, miR-1, 9, 15a, 19a, 30a, 30c, 30e, 34a, 122a, 124a, 125b, 126, 148a, 148b, 194	miR-185, 207, 219, 338	241	Mainly HBV	MA, RT-qPCR	Budhu *et al.* (2008)[159] (metastasis-related signature)
miR-29c, 101, 148a, 424	miR-25, 34a, 181a, 221, 222	78	Unknown	MA, RT-qPCR	Li *et al.* (2008)[160]
	miR-181 family miR-17, 20a, 25, 92, 93, 106b	148	HBV	MA, RT-qPCR	Ji *et al.* (2009)[72] (HpSC-HCC Signature)
miR-17-3p, 30a-3p, 30e, 92, 99a, 122a, 125b, 130a, 139, 187, 199a, 199a*, 200a, 200b, 223, 326	miR-21, 98, 183, 221, 222, 301	26	HBV, or HCV	RT-qPCR	Ura *et al.* (2009)[36]
miR-338, let-7a , miR-15a, 27a, 122a, 124a, 125a, 125b, 126, 129, 143, 145, 152, 185, 194, 195, 199a, 200a	miR-16, 17-5p, 24, 25, 107, 128b, 130a, 205, 207, 221, 222, 224	20	HBV, or HCV	MA, RT-qPCR	Huang *et al.* (2009)[167]
101, miR-29c, 99a, 100, 125b, 195, 199a-5p, 199b-3p, 215, 223, 365, 378, 422a, 424, 520c-3p	miR-18a, 18b, 25, 93, 127-3p, 210, 216a, 222, 224, 362-5p, 382, 491-5p, 519b-5p, 527	5	Mainly HBV	MA, NB	Su *et al.* (2009)[146]
	143 (HBV-related HCC)	25	Mixed HBV, non HBV	RT-qPCR	Zhang *et al.* (2009)[37]
miR-26		455	Mainly HBV	MA, RT-qPCR	Ji *et al.* (2009)[168]
miR-124, 203		23	Mixed HBV/HCV	RT-qPCR	Furuta *et al.* (2009)[169]
miR-22, 195, 199a, 199a*, 130a, 422b, 497	miR-15b (recurrence) Let-7a, 7d, miR-25, 30d, 93, 103, 105, 107, 210, 320, 339, 345, 423	25	Mixed HBV, HCV, non HBV/HCV	MA, RT-qPCR	Chung *et al.* (2010)[157]
let-7c	miR-221 miR-21, 34a, 93, 96, 106b, 210, 222, 224, 425, 519a	104	Mixed HBV/HCV	MA, RT-qPCR	Pineau *et al.* (2010)[123]
miR-27a, 100, 125a, 125b, 141, 150, 139, 145, 195, 199a, 199*, 199b, 200b, 214, 302d, 342	miR-9*, 21, 25, 34c, 96, 105, 182, 182*, 183, 184, 185, 190, 191, 193, 216, 219, 222, 224, 301, 320, 330, 331, 374	20	HBV	MA	Wong *et al.* (2010)[133]
	miR-21	30	Mainly HBV	NB	Liu *et al.* (2010)[170]
miR-199-3p, 199-5p		39	Unknown	RT-qPCR	Fornari *et al.* (2010)[116]
miR-152		20	HBV	RT-qPCR	Huang *et al.* (2010)[171]
	miR-183	25	HBV	RT-qPCR	Li *et al.* (2010)[156]
miR-193b		25	HBV	RT-qPCR	Xu *et al.* (2010)[110]
miR-22		160	Mainly HBV	Rt-qPCR	Zhang *et al.* (2010)[112]
miR-22, 99a, 130a, 195, 199a, 199a*, 378, 497	let-7a, 7d, miR-15b, 16, 17-3p, 18a, 21, 30d, 93, 103, 105, 107, 181a, 181b, 185, 191, 193a, 210, 221, 222, 331, 339, 345, 423-3p, 423-5p, 483, 638	30	HBV (70%), HCV (11%), non (19%)	MA, RT-qPCR	Elyakim *et al.* (2010)[127]
miR-29c, 99a, 100, 122, 125b-5p, 143, 199a/b-3p	miR-21	40	HBV	RT-qPCR	Hou *et al.* (2011)[172]
	miR-21, 221, 222	115	Main HBV	RT-qPCR	Yoon *et al.* (2011)[134]

MA, Microarray; NB, Northern blot; RT-qPCR, Real time quantitative PCR.

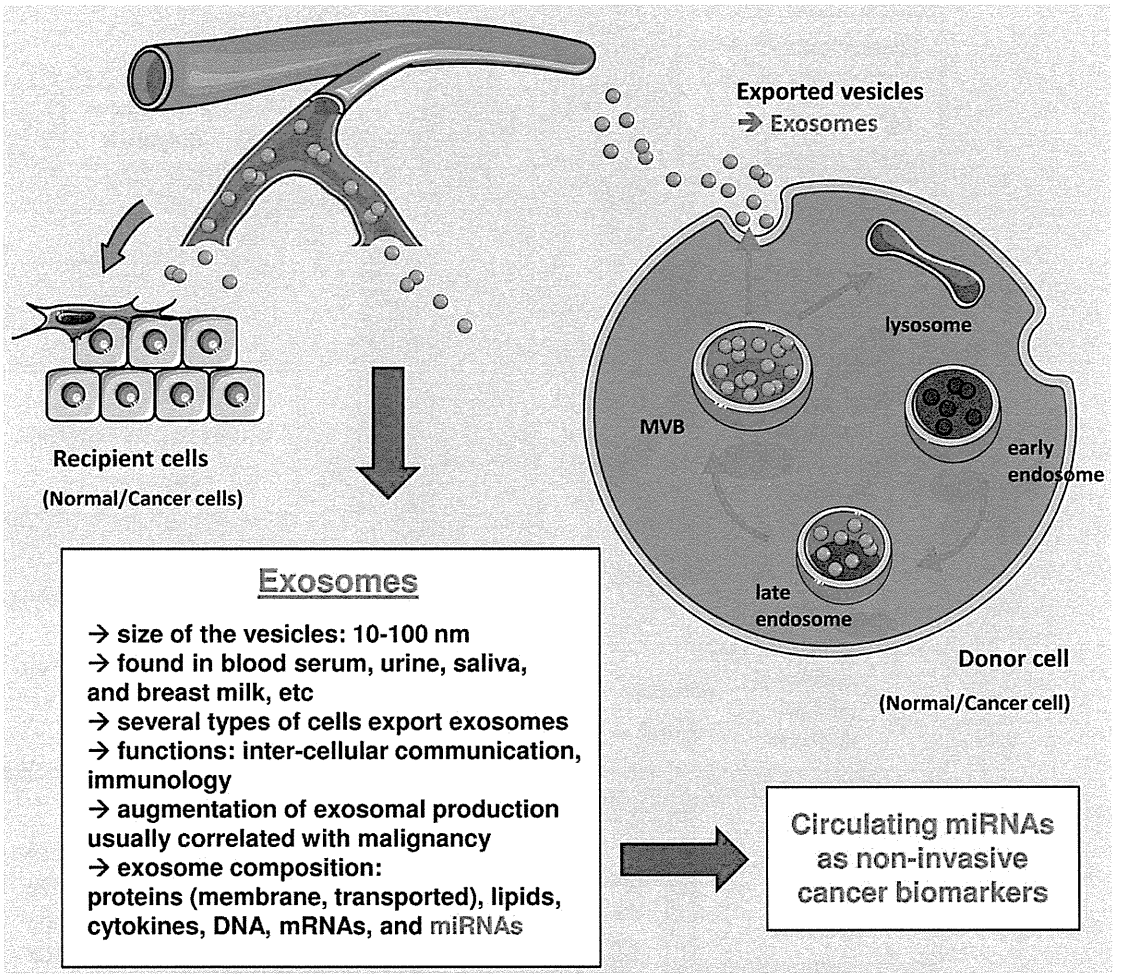

Figure 2. The origin of exosomes. Exosomes are accumulated within the multivesicular bodies (MVBs) as a result of endosome compartmentalization. Then, the vesicles present in the MVBs may undergo degradation (lysosome) or exocytosis. The exocytic MVBs fuse with the membrane after cell stimulation and release exosomes. The mechanisms involved in exosome cargo-sorting processes remain largely unknown.

miR-92 in colorectal cancer (89) as well as miR-25 or miR-223 in various kinds of tumors (90), were efficiently detected in the serum of patients and characterized as potential biomarkers for these diseases.

Concerning the liver, it was first reported in the mouse model that plasma miRNA expression can be associated with hepatocellular injuries induced by drug overdose (91). The effect of acetaminophen was tested and related with at least a 2-fold change of 44 serum miRNAs after treatment. Among them, 57% of the miRNAs showed higher levels in treated plasma samples. Interestingly, the most significant augmentation was observed with miR-122. By using chemical-induced carcinogenesis in a rat model, Sukata and colleagues also highlighted aberrant fluctuations of miRNAs in the serum of the animals, not only in neoplastic lesions, such as HCC, but also in preneoplastic lesions (92). These data suggested that liver cancer-specific miRNAs can circulate through the peripheral blood and potentially represent biomarkers for the early diagnosis of liver cancer. In humans, Zhang *et al.* have characterized the expression of miR-122 in blood serum and demonstrated the relevance of this miRNA for the non-invasive assessment of the HBV (93). Another study also showed that serum miRNA profiles can serve as biomarkers for HBV infection and HBV-positive HCC diagnosis, independently of the cirrhosis etiology. In comparison to normal livers, a specific set of miRNAs was found to be significantly up-regulated in HBV-positive HCC samples (94). Among them, miR-122 was highly up-regulated in the serum of HBV patients but not in those of HCV patients. By employing a combination of these characterized miRNAs as biomarkers, the authors could finally discriminate HCC cases from the controls or the infected non-HCC patients. Other profiling studies have been carried out in order to identify specific HCC-related miRNA profiles in the blood serum of HCC patients that could serve in clinical diagnosis (95,96). In a relevant manner, the levels of 3 miRNAs (miR-21, miR-122, and miR-223) were found to be significantly elevated in the

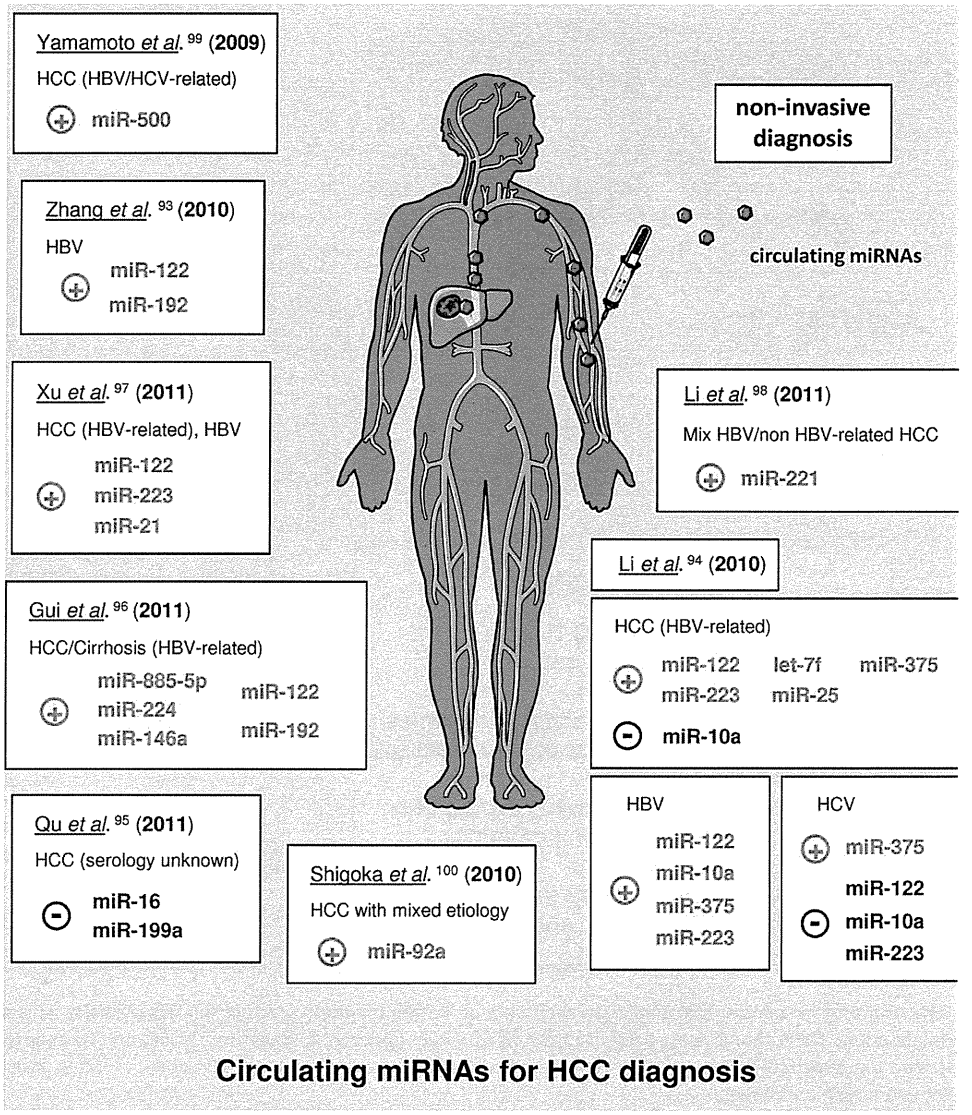

Figure 3. Circulating miRNAs as biomarkers for HCC diagnosis. In a number of studies, the differential expression of circulating miRNAs in chronic hepatitis and HCC in comparison to that in healthy patients has been reported. This figure summarizes the data obtained until now that argues for the clinical potential of serum miRNAs as non-invasive biomarkers in the diagnosis of primary liver cancer. (-) inhibition and (+) promotion traduce serum miRNA modifications as compared to the healthy patients.

serum of patients exhibiting HBV infection or HBV-related HCC (97). Receiver-operator characteristic (ROC) curve analyses suggested that these miRNAs may represent useful markers for routinely discriminating patients with HCC or chronic hepatitis from healthy controls. In addition, miR-21 and miR-122 were significantly higher in the patients with chronic B hepatitis than HBV-related HCC patients. A recent study also emphasized the prognostic significance of serum miR-221, which is frequently over-expressed in HCC. In this report, the high expression of miR-221 was correlated with the size of the tumor and the advancement of the disease (98). Furthermore, the overall survival rate of patients exhibiting a high level of serum miR-221 was significantly lower than that of patients showing low levels of miR-221.

To our knowledge, the major articles focusing on the existence of circulating miRNAs and their suitability for the diagnosis of HCC in humans are referenced in the present review (Figure 3). Our group previously demonstrated that miR-500, which is abundantly expressed in several cell lines and hepatic tumors, can be detected in an increased amount in the sera of HCC patients (99). Remarkably, the elevated rate of serum miR-500 was found to be significantly reduced after surgical treatment. Conversely, Shigoka *et al.* emphasized the low levels of serum miR-92a and their drastic augmentation after tumor resection (100). Taken together, these data suggest that circulating miRNAs are promising biomarkers, potentially useful for the early diagnosis and the monitoring of human

HCC in a non-invasive way. Interestingly, miR-122 and miR-223 appeared redundantly over-expressed in HBV-related HCC as well as HBV-infected patients. However, to validate the clinical relevance of serum miRNAs, further studies with a larger sample size are needed. Especially, standardization methods and the choice of appropriate miRNAs as internal references are crucial. In addition, further studies should also include patients with HCV-related HCC and alcohol-related HCC as well as chemically injured livers in order to refine the characterization.

4. PROGNOSTIC RELEVANCE OF MiRNAs IN LIVER CANCER

In addition to the frequent deregulation observed in the hepatic tumors, an association has also been found between miRNA expression and the clinico-pathological outcome of HCC (101,102,74). An important breakthrough in the clinical management of liver cancer came from the accurate correlation made between the alterations of cancer-related genes and the tumor phenotype. Although HCC lesions can be broadly distinguished by histological or immunological assessment, their prognosis and clinical evolution greatly vary from one individual to another. The use of a miRNA-based classification correlated with the etiology, the pathogenic, and the malignant tendency of the tumor could significantly enhance the molecular diagnosis of HCC and its classification by specifying the tumor-associated phenotypes. In this regard, several teams have reported particular miRNA expression profiles that could be considered as valuable HCC prognostic indicators (103). The achievement of such a field of investigation may provide useful markers in order to predict the HCC behavior of each patient (tumor growth, response to the treatment, and metastatic potential of the tumor). In the following section, a parallel between the cell functions affected in liver cancer and the HCC-related miRNAs aberrantly expressed is presented. In addition, the relevance of miRNA profile evaluation for the prognostic outcome of human HCC is discussed.

4.1. miRNAs and tumor growth

The alteration of oncogenes and tumor suppressors involved in cell cycle regulation is an essential step in the development and the progression of cancer. Several studies have reported the role of specific miRNAs in the regulation of the proliferation signaling pathways by a direct interaction with critical cell cycle regulators. Among those miRNAs are tumor suppressor miRNAs targeting cyclin-cyclin-dependent-kinase (Cyclin-CDK) complexes, a class of positive modulators of the cell cycle. As previously exposed, miR-122 has been reported to suppress HCC cell growth by inhibiting cyclin G1 (82). Moreover, miR-122 controls other factors involved in cell cycle progression, such as the serum response factor (SRF), the insulin-like growth factor 1 receptor (Igf1R) (104,105), the tyrosine-protein phosphatase non-receptor type 1 (PTPN1), and the members of the septin family SEPT2 and SEPT9 (106). Recently, miR-122 has also been reported to regulate the balance between proliferation and differentiation in hepatocytes by indirectly activating the expression of hepatic functional genes, such as the cholesterol-7α hydroxylase gene (CYP7A1), through the repression of CUTL1 (107). The major miRNAs identified as regulators of cyclin-CDK complexes are miR-26a, miR-195, miR-124, and miR-185. miR-26a has been shown to induce cell cycle arrest by directly targeting cyclin D2 and E2 in liver cancer cells (108). miR-195 inhibits the G1/S transition by repressing Rb-E2F signaling through the targeting of multiple molecules, including cyclin D1, E2F3, and CDK6 (109). Moreover, CDK6 expression is also down-regulated by miR-124, whereas the cyclin D1 and the oncogene ETS1 were revealed to be directly targeted by miR-193b, inducing cell cycle arrest (110). Recently, two transcriptional targets of Six1, c-myc and cyclin A1, have been reported to be regulated by miR-185 through the translational repression of Six1 frequently up-regulated in HCC (111).

Another class of miRNAs has been identified as major negative regulators of cell cycle progression, such as miR-1, miR-22, miR-375, miR-223, let-7, miR-34a, miR-199a-3p, and miR-29a (Table 3). miR-1, a silenced miRNA in HCC through CpG-island methylation, targets the forkhead box transcription factor (FoxP1), the hepatocyte growth factor receptor (MET, c-MET, or HGFR), and the histone deacetylase 4 (HDAC4) (75). In addition, HDAC4 can also be inhibited by miR-22 (112). miR-375 is an important regulator of the oncogenic yes-associated protein (YAP) (113). miR-223 down-regulates the Stathmin 1 (STMN1), a key microtubule regulatory protein (70). let-7g may act as a tumor suppressor gene that inhibits HCC cell proliferation by down-regulating the oncogene c-Myc and up-regulating the tumor suppressor gene p16 (INK4A) (114). miR-34a was also reported to assure G1 phase regulation in the HCC cell line HepG2 by initiating multiple regulatory processes (115). The PI3K/AKT pathway is another major signaling cascade controlling cell proliferation. PI3K activation can increase the activity of AKT kinase, which promotes cell growth by phosphorylating the mammalian target of rapamycin (mTOR). mTOR has been reported to be inhibited by miR-199a-3p, leading to the enhancement of the G1/S transition (116). More recently, miR-29a was shown to have a positive therapeutic effect in liver cancer cells by inhibiting cell growth and inducing cell apoptosis (117).

Recent evidence suggests that oncogenic miRNAs contribute to tumor cell proliferation in part by regulating checkpoints and cell cycle progression. For instance, miR-106b promotes the G1/S transition by directly targeting p21 (118). On the other hand, miR-125b over-expression has been reported to suppress HCC cell line growth by increasing p21 expression (119). More recently, the G1/S was also reported to be regulated by miR-373 through the inhibition of the protein phosphatase 6 catalytic subunit (PPP6C), a negative cell cycle regulator (120). In line with these findings, the cyclin-dependent kinase inhibitor p27 has been evidenced to be targeted by miR-222 and miR-221 (121-123). These two miRNAs, as well as miR-21, also suppress the expression of the tumor suppressor PTEN (124,68). Furthermore, miR-221 was

Table 3. Aberrantly expressed miRNAs related to tumor growth in HCC (cell proliferation)

Expression	miRNAs	Target(s)	References
Down-regulated	Let-7g	c-Myc	Lan *et al.* (2011) [114]
in HCC	miR-1	FoxP1, MET, HDAC4	Datta *et al.* (2008) [75]
(anti-	miR-22	HDAC4	Zhang *et al.* (2010) [112]
proliferation)	miR-26a	Cyclin D2, Cyclin E2	Kota *et al.* (2009) [108]
	miR-29a	PPM1D	Meng *et al.* (2011) [117]
	miR-34a	MACF1	Cheng *et al.* (2010) [115]
	miR-122	Cyclin G1	Gramantieri *et al.* (2007) [82]
			Fornari *et al.* (2009) [149]
		SRF	Bai *et al.* (2009) [104]
		IGF1R	Bai *et al.* (2009) [104]
			Zeng *et al.* (2010) [105]
		PTPN1, SEPT2, SEPT9	Boutz *et al.* (2011) [106]
		CUTL1	Xu *et al.* (2010) [107]
	miR-124	CDK6	Furuta *et al.* (2010) [169]
	miR-125b	Not determined	Liang *et al.* (2010) [119]
	miR-185	Six1	Imam *et al.* (2010) [111]
	miR-193b	Cyclin D1, ETS1	Xu *et al.* (2010) [110]
	miR-195	Cyclin D1, E2F3, CDK6	Xu *et al.* (2009) [109]
	miR-199a-3p	mTOR	Fornari *et al.* (2010) [116]
	miR-223	STMN1	Wong *et al.* (2008) [70]
	miR-375	YAP	Liu *et al.* (2010) [113]
Over-expressed	miR-18a	ESR1	Liu *et al.* (2009) [125]
In HCC	miR-21	PTEN	Meng *et al.* (2007) [68]
(pro-	miR-106b	p21	Ivanovska *et al.* (2008) [118]
proliferation)	miR-130b	TP53INP1	Ma *et al.* (2010) [126]
	miR-141	DLC-1	Banaudha *et al.* (2011) [43]
	miR-191	SOX4, IL1A, TMC7	Elyakim *et al.* (2010) [127]
	miR-221	DDIT4	Pineau *et al.* (2010) [123]
		PTEN	Garofalo *et al.* (2009) [124]
		p27	Le Sage *et al.* (2007) [121] Fornari *et al.* (2008) [122] Pineau *et al.* (2010) [123]
		p57	Fornari *et al.* (2008) [122]
	miR-222	PTEN	Garofalo *et al.* (2009) [124]
		p27	Le Sage *et al.* (2007) [121] Pineau *et al.* (2010) [123]
	miR-373	PPP6C	Wu *et al.* (2011) [120]
	miR-517a	Not determined	Toffanin *et al.* (2011) [128]

identified as a negative regulator of the DNA damage-inducible transcript 4 (DDIT4), a modulator of the mTOR pathway (123).

Another class of over-expressed miRNAs has been demonstrated to play pivotal roles in hepatocarcinogenesis via potential growth-enhancer properties. Interestingly, miR-18a prevents the translation of the estrogen receptor-alpha (ER-alpha) by directly targeting ESR1, thereby blocking the protective effects of estrogen and promoting HCC development (125). A recent article has demonstrated that cell growth and the self-renewal of CSCs in the HCC are regulated by miR-130b through inhibition of the tumor protein 53-induced nuclear protein 1 (TP53INP1) (126). Moreover, miR-191 has been proposed as a good candidate target for HCC therapy. Indeed, this miRNA promotes HCC cell proliferation by negatively regulating the expression of the transmembrane channel-like 7 (TMC7), IL1A, a member of the interleukin-1 cytokine family, and SOX4, a DNA damage sensor up-regulated in many cancers that promote cell cycle arrest and apoptosis as well as inhibit tumorigenesis in a p53-dependant manner (127). The tumor suppressor gene DLC-1, a Rho GTPase-activating protein, frequently deleted in HCC and other solid human tumors, was also identified as a direct target of miR-141 (43). Finally, a recent miRNA profiling of HCC samples highlighted miR-517a, which belongs to a family of poorly characterized miRNAs from chromosome 19q13.42, as an oncogenic miRNA that promotes proliferation and tumor progression (128).

4.2. miRNAs and HCC metastasis

Metastasis and recurrence are major concerns for the long-term survival of HCC patients after curative resection. The identification of biomarkers correlated with the underlined mechanisms of these processes may represent a significant advance for the clinical prognosis of HCC patients in order to predict the evolution of the tumor. Over the last years, several miRNAs have been identified as upstream regulators of specific genes involved in the metastasis and the invasion processes, acting as key factors in liver cancer outcome. Among those miRNAs, two subclasses can be distinguished: the pro-metastatic miRNAs that contribute to the migration/invasion of HCC cells and the anti-metastatic miRNAs acting as suppressors of metastasis.

4.2.1. Pro-metastatic miRNAs and their targets in HCC

First, the member of the oncogenic miR-106b family, miR-17-5p, has been reported as over-expressed in HCC, 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) (118,129). miR-151, a frequently amplified miRNA on 8q24.3, co-expressed with the host gene FAK, significantly increases tumor invasion and metastasis of HCC by directly targeting RhoGDIA, a putative metastasis suppressor in HCC, leading to the activation of Rac1, Cdc42, and Rho GTPases. In addition, miR-151 can function synergistically with FAK to enhance

HCC cell motility and spreading (130). Moreover, Galphai 2 (GNAI2) was identified as the direct and functional target of miR-30d, an up-regulated miRNA in HCC that is also amplified on chromosome 8q24 (131). The expression levels of both miR-151 and miR-30d correlate with the intrahepatic metastasis of HCC (130,131). A study performed using metastatic HBV-related HCC 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 (37). More recently, miR-517a has been identified as an over-expressed miRNA in HCC that promotes tumorigenesis and metastatic dissemination *in vivo* (128).

Other miRNAs belong to the pro-metastatic subclass and have been shown to be highly expressed in HCC, such as miR-21, miR-221, miR-222, and miR-181b (Table 4). miR-21 contributes to HCC growth and spread by repressing the tumor suppressor RHOB (132) as well as by modulating PTEN expression and PTEN-dependent pathways involved in mediating the phenotypic characteristics of cancer cells, such as migration and invasion (68). Moreover, PTEN is targeted by miR-221 and miR-222, which also 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 enhance cellular migration through the activation of the AKT pathway and metalloproteases (124,133). Furthermore, the aberrant expression of miR-221 has been proposed for predicting local recurrence and distant metastasis after curative surgery (134). Finally, TIMP3 has also been reported as a target of miR-181b. Indeed, Wang *et al.* demonstrated the induction of miR-181b induced by TGF-beta and the enhancement of the matrix metalloproteases MMP2 and MMP9 activity by decreasing TIMP3 level, thus promoting the growth, survival, migration, and invasion of HCC cells (52).

4.2.2. Anti-metastatic miRNAs and their targets in HCC

MicroRNAs can actively participate to the epithelial to mesenchymal transition (EMT). As an example, p53 enhances the expression of the miR-200 family, known to repress the EMT-inducer transcription factors ZEB/2 (135). Consequently, p53 mutation leads to miR-200 inhibition and ZEB1/2 up-regulation, promoting EMT. On the other hand, several anti-metastatic-related miRNAs, known to be frequently down-regulated in HCC, have been described to regulate the MET signaling pathway. The MET receptor tyrosine kinase (RTK) is frequently up-regulated in different types of cancers and amplified during the metastatic transition of primary liver tumors. Numerous genes downstream of the MET signaling pathway are involved in the regulation of various cellular functions, including mitosis, proliferation, angiogenesis, tumor cell invasion, and metastasis. Furthermore, the MET-induced gene expression signature is shared by HCC and almost all liver metastases (136). miR-1, a down-regulated miRNA in HCC through CpG-island methylation, has been described as a negative regulator of MET (75). miR-34a was also reported to suppress tumor invasion and migration in HCC patients by directly targeting MET and, thus, to decrease MET-induced phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) (137). miR-199a-3p, another miRNA with MET as a direct target, can inhibit mTOR, leading to G1-phase arrest and reduced invasive capability (116). In addition, miR-23b targets MET and the urokinase-type plasminogen activator (uPA), a critical functional downstream regulated by the HGF/c-Met signaling pathway, thereby decreasing the migration and proliferation abilities of HCC cells (138).

Other anti-metastatic miRNAs have been reported to be down-regulated in HCC such as miR-122, miR-125b, miR-139, miR-193b, let-7g, miR-185, miR-142-3p, miR-181a, miR-34a, and miR-199a-5p (Table 4). miR-122 has been described as a negative regulator of ADAM10 and ADAM17 (a disintegrin and metalloprotease family 10 and 17, respectively), both of which are obviously involved in metastasis (104,81). More recently, miR-122 was reported to target the matrix metalloproteinase MMP7 and paxillin (PXN) (106), the latter of which interacts with a number of proteins involved in the organization of the actin cytoskeleton, which is required for cell motility and implicated in a variety of biological processes, including tumor metastasis. miR-125b also inhibits HCC cell migration and invasion by repressing LIN28B, an RNA-binding protein highly expressed in hepatocellular carcinoma, 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 (119). Recently, miR-139, whose down-regulation in HCC is associated with a poor prognosis and metastatic feature, was reported to interact with Rho-kinase 2 (ROCK2) (139), a frequently over-expressed factor in primary liver cancer that plays a significant role by regulating cytoskeletal events and contributes to the invasion of HCC (140). miR-34a also inhibits cell migration and invasion by repressing the expression of several cytoskeleton proteins (115). let-7g levels in metastatic HCCs were significantly lower than those in the metastasis-free HCCs and inversely correlated with the levels of type I collagen alpha2, which was experimentally validated as a direct target of let-7g (141). The oncogene Six1 is frequently deregulated in aggressive forms of cancers and repressed by miRNA-185, a tumor suppressor miRNA that impedes anchorage-independent growth and cell migration (111). Another miRNA that represses metastatic characteristics in HCC cells is miR-181a, reported to directly target the osteopontin (OPN), a variably expressed and secreted glycophosphoprotein that mediates the growth and metastases of HCC (142). In addition, miR-193b was reported to inhibit the invasion and migration of HCC cells by directly targeting cyclin D1 and ETS1 (110). The discoidin domain receptor-1 (DDR1) tyrosine kinase, involved in the cell invasion-related signaling pathway, represents a potential target of miR-199a-5p, a miRNA significantly down-regulated in 65.2% of HCC tissues (143). Recently, miR-142-3p has been included in the anti-metastatic miRNA subclass because of targeting RAC1, a factor that regulates diverse cellular events in HCC cells, including migration and invasion (144).

Table 4. Aberrantly expressed miRNAs related to metastasis in HCC

Expression	miRNAs	Target(s)	References
Down-regulated	let-7g	COL1A2	Ji *et al.* (2010) [141]
in HCC	miR-1	MET	Datta *et al.* (2008) [75]
(anti- metastasis)	miR-23b	MET, uPA	Salvi *et al.* (2009) [138]
	miR-34a	MET	Li *et al.* (2009) [137]
		LMNA, GFAP, MACF1, ALDH2, LOC100129335	Cheng *et al.* (2010) [115]
	miR-122	ADAM10	Bai *et al.* (2009) [104]
		ADAM17	Tsai *et al.* (2009) [81]
		MMP7, PXN	Boutz *et al.* (2011) [106]
	miR-125b	LIN28B2	Liang *et al.* (2010) [119]
	miR-139	ROCK2	Wong *et al.* (2011) [139]
	miR-142-3p	RAC1	Wu *et al.* (2011) [144]
	miR-181a	OPN	Bhattacharya *et al.* (2010) [142]
	miR-185	Six1	Imam *et al.* (2010) [111]
	miR-193b	Cyclin D1, ETS1	Xu *et al.* (2010) [110]
	miR-199a-3p	MET, mTOR	Fornari *et al.* (2010) [116]
	miR-199a-5p	DDR1	Shen *et al.* (2010) [143]
Over-expressed	miR-17-5p	Not determined	Yang *et al.* (2010) [129]
In HCC	miR-21	RHOB	Connolly *et al.* (2010) [132]
(pro-metastasis)		PTEN	Meng *et al.* (2007) [68]
	miR-30d	GNAI2	Yao *et al.* (2010) [131]
	miR-143	FNDC3B	Zhang *et al.* (2009) [37]
	miR-151	RhoGDIA	Ding *et al.* (2010) [130]
	miR-181b	TIMP3	Wang *et al.* (2010) [52]
	miR-221	PTEN, TIMP3	Garofalo *et al.* (2009) [124]
	miR-222	PTEN, TIMP3	Garofalo *et al.* (2009) [124]
		PPP2R2A	Wong *et al.* (2010) [133]
	miR-517a	Not determined	Toffanin *et al.* (2011) [128]

4.3. miRNAs, apoptosis, and anti-HCC drug resistance

The apoptotic process is one of the major barriers that must be bypassed by the malignant cell during transformation and tumor progression. Frequently, cancer cells acquire the ability to evade induced apoptosis and, therefore, are able to survive in the tumor environment. The Bcl-2 family is one of the main actors in the control of cell apoptosis by modulating the signaling of the mitochondrial death program. This family is composed of two groups of proteins that exhibit either pro-apoptotic (Bim, Bmf, Bax, Bak, Bid) or anti-apoptotic properties (Bcl-2, Bcl-w, Bcl-xL, Mcl-1). The results of several studies have shown that miRNAs can facilitate the apoptosis bypass by directly targeting the Bcl-2 family genes in HCC cells. Microarray analysis identified the let-7 family as being down-regulated in the HCC line Huh-7 in comparison with primary human hepatocytes (145). More precisely, 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. These data suggest that a low expression of let-7 contributes to the Bcl-xL augmentation observed in the HCC. In addition, let-7c over-expression can enhance the apoptosis of hepatoma cells in cooperation with the anti-cancer drug Sorafenib. In a similar manner, it has been demonstrated that the forced expression of miR-101, normally highly down-regulated in HCC lines and HCC tumors, can exert a pro-apoptotic action by targeting Mcl-1 (146). Another anti-apoptotic Bcl-2 family member has been identified to be directly targeted by the hepatospecific miR-122 (147), a miRNA significantly down-regulated in HCC, as reported previously. Experimentally, the mRNA and protein level of Bcl-w can be repressed by miR-122 over-expression in human HCC lines, subsequently reducing cell viability. Ma *et al.* also showed that adenoviral vector-mediated expression of miR-122 induced the apoptosis and cell cycle arrest of liver cancer cell lines 148). In a therapeutic perspective, Fornari and co-workers assayed the effect of restoring miR-122 expression on triggering chemotherapy-induced apoptosis using doxorubicin and demonstrated that miR-122, as well as cyclin G1 silencing, increases sensitivity to the treatment (149). Moreover, in patients who underwent a surgical resection, lower miR-122 levels were associated with a shorter time to recurrence, whereas higher cyclin G1 expression was related to lower survival rates. Xiong and colleagues also showed that the miR-29 level is significantly reduced in HCC tissues and associated with poor survival rates (150). Conversely, enhanced expression of miR-29 dramatically increases HCC cell sensitivity to various apoptotic signals and suppresses the ability of hepatic cell lines to form tumors *in vivo*. The authors of this work also described that both anti-apoptotic Bcl-2 and Mcl-1 are directly targeted by miR-29, leading to apoptosis promotion. As a consequence, the crucial role of miRNAs in the regulation of apoptotic processes as well as their potential application in prognosis prediction can be considered (Figure 5). Indeed, all the miRNAs presented above are frequently down-regulated in HCC, thus making HCC cells more resistant to chemotherapy-induced apoptosis, at least by the up-regulation of anti-apoptotic gene expression.

On the other hand, miRNAs can also target the pro-apoptotic members of the Bcl-2 family and carry anti-apoptotic effects. For example, miR-25, a member of the miR-106b-25 cluster that is over-expressed in HCC, exerts an anti-apoptotic effect by targeting and inhibiting Bim (151). In addition, 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 (152). In HCC, miR-221 over-expression is associated with a