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MicroRNAs in HBV-related hepatocellular carcinoma: functions and potential clinical applications

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Hepatitis B virus (HBV) infection is still a relevant problem worldwide and many cases of hepatocellular carcinoma (HCC) are related to HBV. The prognosis of HBV-related HCC is poor, particularly for advanced stage diagnosis. Although follow-up strategies were adopted for patients at risk, there is need for an optimal early biomarker for the screening purpose. MicroRNAs (miRNAs) are small non-coding RNAs, tightly connected to cell type and differentiation status and act as genetic regulator which can be involved in oncogenic processes. The alteration in miRNA expression pattern may represent a new opportunity for HBV-related HCC diagnosis and therapies. Some studies focused on miRNA polymorphism responsible for HCC susceptibility; others found several miRNAs deregulated by HBV X protein as well as miRNAs altered in HBV-related HCC tissue and cells. A high variability among results emerged, probably due to different techniques employed, biological substrates, experimental procedures, criteria of miRNAs selection and ethnic provenience of the included patients. Interestingly, circulating miRNAs have been studied as potential HCC-biomarkers but the reported accuracy is still not convincing, particularly in distinguishing patients with HCC from patients with cirrhosis. Hence, the use of miRNAs remains in an experimental phase and more studies are required to define their role in the clinical practice.

KEY WORDS: Biomarkers - Hepatitis B virus - Carcinoma, hepatocellular.

Hepatitis B virus (HBV) belongs to the Hepadnaviridae family of viruses and is the only member that infects humans. HBV-DNA contains 4 open reading frames: preS/S, coding for surface proteins, C for the core proteins, P for the polymerase and X, coding for a protein with regulatory functions, able to alter host cell gene expression.[1]

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The most common route of infection is the contact with chronic carriers' blood. HBV infection is a worldwide health problem: even if a safe and effective vaccine exists, the infection causes from 600,000 to 1 million death/year in the world.[2] The natural history of chronic hepatitis B (CHB) is characterized by a chronic inflammation that evolves to fibrosis, and over time, to cirrhosis, which is the principal risk factor for hepatocellular carcinoma (HCC) development.[3]

HCC is the most frequent primary liver cancer, the sixth malignancy in the world for incidence (749,000 new cases/year), and the third for cancer-related deaths (692,000/year).[4, 5] The incidence of HBV-positive cirrhosis-related HCC varies between 2% and 5%.[6]

The prognosis of HCC is poor, with only 6 months of survival from the time of diagnosis: the incidence and mortality rates are similar because most HCC are diagnosed in an advanced stage.[7] Only few cases are eligible to therapeutic strategies at the time of diagnosis and there is a high rate of relapse after therapy (70-80%).[8] Several chemotherapeutic agents are under evaluation in clinical trials and some basic research studies are investigating the possibility of sensitizing cancer cells to chemotherapy.[9, 10] Follow-up strategies were studied to perform HCC diagnosis

at an earlier stage. The European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Disease (AASLD) guidelines recommend an abdominal ultrasound every 6 months in cirrhotic patients, carried out by experienced personnel.[4] Unfortunately, ultrasound is not accurate enough in the early HCC, with a sensitivity of 63%.[4] Optimal biomarkers for clinical practice are still to be discovered. Alpha-fetoprotein (AFP) is the most used but its increase depends on tumor size and 80% of small HCC does not alter circulating AFP concentration.[11]

MicroRNAs (miRNAs) are small (18-25 nucleotides) non-coding RNAs able to regulate gene expression through repression or degradation of mRNAs.[12] MiRNAs are the biggest class of genetic regulators and they act at a post-transcriptional level with a mechanism based on sequence complementarities between miRNA and target mRNA.[13] Each miRNA can have more than 200 different molecular targets with very different roles, and different miRNAs can share the same target.[11, 14, 15] MiRNAs are involved in the regulation of expression of 60% of protein coding genes.[16]

In this review we will discuss about the possibility of using miRNAs as early marker of HCC.

Cancer and miRNA

Cancer pathogenesis consists in a progressive loss of cell function control and life cycle regulation. From recent studies, miRNAs emerge as important regulators of the principal cell functions such as growth, proliferation, differentiation and apoptosis, and they are involved in the oncogenic process acting as oncogenes, tumor suppressor or both.[13, 17]

Calin *et al.* in 2002 were the first to reveal a connection between cancer and miRNAs while studying the relationship between deletions at chromosome 13q14 and chronic lymphocytic leukemia (CLL) pathogenesis. The authors found that miR-15 and miR-16 are both located within a region of chromosome 13q14 that is deleted in more than 65% of all CLL cases. Moreover, miR-15 and miR-16 expression levels correlated with allelic loss suggesting that these miRNAs could play a role in CLL initiation or progression.[18] In addition, three years later, another study showed that these miRNAs were able to inhibit the expression of Bcl2,[19] an antiapoptotic gene.[20]

New technologies for miRNA quantification, such as miRNA microarray, bead-based flux cytometry, real-time PCR with Taqman miRNA assay or the miRAGE facilitated the comparison of miRNA expression patterns from normal and tumor tissues.[21-24] In 2005, a systematic expression analysis of 217 miRNAs from a sample of multiple human cancers showed that distinct patterns of miRNA expression were associated to different oncogenetic mechanisms, supporting the idea that miRNA profiling may reflect different tumor developmental origin.[21] Moreover, hierarchical clustering of miRNA profiling data revealed that cancer samples from colon, liver, pancreas and stomach all clustered together suggesting that miRNA profiles accord to similar embryonic origin of cancer.[21]

Analyzing 228 miRNA profiles in six different types of solid tumors (breast, colon, lung, pancreas, prostate and stomach), Volinia *et al.* found that 21 miRNAs were commonly dysregulated in at least three different types of neoplasia, whereas only miR-21 was significantly over-expressed in all tumors.[25] The cancer miRNA expression signature across the different types of cancer analyzed suggest a common mechanism of tumor development. In addition, these authors investigated the functional significance of miRNA dysregulation in cancer by testing the correlation between several cancer genes and miRNAs expression and they found a significant inverse correlation between protein translation and miRNAs expression.[25] This feature strengthened the hypothesis about the post-transcriptional regulation of cancer-related genes by aberrant miRNAs expression.

Furthermore, epigenetic alterations in miRNA pattern may represent a new opportunity for HCC diagnosis and therapy.[16] MiRNAs may be used as prognostic and diagnostic markers, to discriminate neoplastic tissue from the normal tissues, and they have also been proposed as target for new experimental therapies.[26]

MiRNA in the HCC susceptibility

Recent studies have suggested that miRNA polymorphisms can have a functional role and can be associated to HCC development and progression. MiRNA polymorphism consists of a single nucleotide polymorphism (SNP) in the miRNA gene itself,

and even if it is a rare event, it can influence gene transcription and targets.

Several miRNAs SNPs have been investigated in association to HBV-related liver disease progression and to individual HCC susceptibility. SNPs in miR-149 (rs2292832 C>T) and miR-499 (rs3746444 A>G) were associated with lower HCC risk in HBV infected patients.[27] Moreover, polymorphism rs3859501 C>A in the pri-miRNA region of miR-371, 372 and 373 acted as a protective factor for HCC occurrence both in chronic carriers and in cirrhosis patients.[28]

On the contrary, miR-196a-2 rs11614913 T>C and miR-34b/c rs4938723 T>C were associated to an increased risk for primary HCC in HBV infected patients.[29-31]

Interestingly, miR-101-1 rs7536540 G>C and miR-106b-25 cluster rs999885 A>G were significantly associated with HCC development but the former SNP was also associated to cirrhosis development whereas the latter showed a protective effect on CHB infection.[32, 33]

MiRNAs in HBx-related oncogenesis

HBV X (HBx) protein, a small transcriptional activator that is essential for viral infectivity, plays a critical role in the development of HCC, and different studies have revealed several HBx altered genes and signaling pathways that contribute to HCC development. HBx can induce epigenetic alterations and can affect miRNA expression profile. The role of HBx protein in the modulation of oncogenic miR-21, miR-222 and tumor suppressor miR-145 in malignant hepatocytes was examined, revealing the down-regulation of these miRNAs in HBx transfected cells.[34] Oncogenic miR-21 was up-regulated by HBx-induced interleukin-6 pathway followed by activation of STAT3 transcriptional factor.[35] Several tumor suppressor miRNAs were down-regulated by HBx, including miR-132, miR-148a, miR-338, miR-122, miR-93 and miR-101.[36-41] On the contrary, other miRNAs that act as oncogene like miR-29a and miR-181a were up-regulated by HBx.[42, 43] Well-known miRNAs with tumor-suppressor activity are those belonging to miR-16 family. It has been shown that HBx directly triggers the down-regulation of miR-15a and miR-16-1 antagonizing p53 or through c-Myc recruitment.[44, 45] Moreover, HBV mRNAs possess a microRNA 15a/16-complementary site that acts as a sponge to sequester endogenous miR-15a/16.[46]

In HCC, HBx is frequently expressed in a truncated form without the carboxyl-terminus (Ct-HBx). A study carried out on human hepatocytes and on a cohort of HBV-associated HCC tissues, reported that Ct-HBx was able to inhibit the transcriptional activity of the promoters of a set of miRNAs with growth-suppressive functions.[47] Some of these miRNAs (miR-26a, -29c, -146a, -190) were also significantly down-regulated in a subset of HCC tissues with Ct-HBx truncation compared to the matched non-tumor tissues.[47] HBx was found to down-regulate Let-7a thus enhancing cell proliferation, viral replication and carcinogenesis.[48] An *in vitro* study demonstrated that miR-224 plays an oncogenic role in hepatoma cell migration and tumor formation through the silencing of its target gene, Smad4.[49] These results were confirmed in liver tumors of HBx gene transgenic mice.[49] Thus, viral genes also may modify host miRNA expression pattern and hence initiate the tumorigenic process.

MiRNAs deregulated in HBV-related HCC tissue

In HCC, miRNAs target genes that codify for proteins involved in the cell cycle, in apoptotic mechanism and in metastasis development.[50, 51] MiRNAs deregulation can alter cell homeostasis through the disruption of several signaling networks leading to alteration in the control of cell processes hence contributing to cancer development (Table I).[52-79] To investigate the role of miRNAs in HCC, analogues and antagonists of miRNAs were experimentally transfected in cancer cells. It has been shown that restoring tumor-suppressor miRNAs cause cell cycle arrest, an increase in apoptosis and a reduced angiogenesis, while the suppression of oncogenic miRNAs blocks cell proliferation, angiogenesis and metastasis.[16]

Shi *et al.* found that miR-22 was down-regulated in HBV-related HCC cell lines as well as in clinical tissues and that its expression was inversely correlated with that of cyclin-dependent kinase inhibitor 1A (CDKN1A).[80] Overexpression of this miRNA *in vitro* is able to suppress CDKN1A expression and to inhibit cell proliferation.[80] Another study detected, in male tumor adjacent tissue, high levels of miR-

TABLE I.—*Deregulated miRNAs in HBV-related HCC tissue.*

miRNA	Alteration	Target	Citation
miR-22	Down-regulated	CDKN1A	80
miR-602	Up-regulated	RASSF1A	83
miR-429	Down-regulated	NOTCH1	84
miR-545/374a	Up-regulated	ESRRG	85
miR-29c	Down-regulated	TNFAIP3	86, 89
miR-152	Down-regulated	DNMT1	87
miR-101	Down-regulated	EZH2	52, 89
miR-103	Up-regulated	PER3, CDK5R1	53, 54, 89
miR-106a	Up-regulated	p130, FAS	55, 56, 89
miR-107	Up-regulated	CDK8, let-7	57, 58, 89
miR-221	Up-regulated	PI3-K/Akt	59, 89
miR-224	Up-regulated	HOXD10, CDC42, CDH1, PAK2, BCL-2, MAPK1	60, 61, 89
Let-7a	Down-regulated	CCR7	62, 90
miR-22	Down-regulated	AKT3, p21	63, 64, 90
miR-99a	Down-regulated	AGO2	65, 90
miR-122a	Down-regulated	PRDXIL, cyclin G1	66, 67, 90
miR-126	Down-regulated	PI3KR2, Crk, PLK2	68, 90
miR-130a	Down-regulated	ERα	69, 90
miR-15a	Up-regulated	Smad7	70, 90
miR-17	Up-regulated	ULK1, ATG7, p62	71, 90
miR-18a	Up-regulated	ERα	82, 90
miR-19b	Up-regulated	P53	72, 90
miR-20a	Up-regulated	Egln3/PHD3	73, 90
miR-27a	Up-regulated	MAP2K4, TRβ1	74, 75, 90
miR-92	Up-regulated	/	90
miR-93	Up-regulated	PTEN, CDKN1A	76, 90
miR-106b	Up-regulated	RhoGTPases, RhoA, RhoC	77, 90
miR-148a	Up-regulated	c-Met, Wnt	78, 79, 90
miR-21	Up-regulated	PDCD4, PTEN	90

22 and showed that miR-22 down-regulates estrogen receptor-alpha (ERα) expression, which in turn, stimulates IL-1α transcription.[81] IL-1α seems to act as a general pro-tumorigenic mediator that is released in chronic liver damage suggesting the activation of signaling pathways such as the NF-κB pathway, which exhibits anti-apoptotic and tumor growth properties.[81] Similarly, Liu *et al.* found that miR-18a targets ESR1 which encodes for ERα leading to repression of mRNA translation.[82]

Yang *et al*. found 14 aberrantly expressed miRNAs in HCC compared with normal liver.[83] Among these, miR-602 expression in HCC was 4.134 fold greater than in normal liver, and similar alterations were found in HBx transfected cells. The authors found that miR-602 targets and inhibits Ras-associated domain family member 1A (RASSF1A), an important tumor-suppressor gene, suggesting a pro-carcinogenetic role of miR-602 in HBV-related hepato-carcinogenesis.[83]

Gao *et al.* found that miR-429 was down-regulated much more in HBV-related HCC than in HCC of other etiologies.[84] By transfecting this miRNA, it was found that the overexpression of miR-429 decreases cell proliferation and induces cell apoptosis by directly targeting NOTCH1, which stimulates proliferation and suppresses apoptosis in HCC cells.[84]

Sixty-six pairs of HBV-related HCC tissues and matched non-cancerous liver tissues were analyzed by Zhao *et al*.[85] MiR-545/374a cluster resulted up-regulated in HBV-related HCC tissue and correlated with prognosis, revealing that these miRNAs were able to promote *in vitro* cell proliferation, cell migration and invasion.[85] Transfection of HBV whole genome or only HBx resulted in an increase in miR-545/374a expression.[85] MiR-29c resulted significantly down-regulated in HBV-related HCC cell lines as well as in clinical tissues, and TNFAIP3, a regulator in inflammation, was identified as a target of miR-29c being inversely correlated with this miRNA.[86]

TABLE II.—*Circulating miRNAs in HBV-related HCC.*

miRNA		AUC	Se	Sp	Citation
miR-375, miR-23b, miR-423, miR-23a, miR-342-3p	HCC *vs*. HC	0.999	96.9%	99.4%	95
miR-10a, miR-125b	HCC *vs*. CHB	0.992	98.5%	98.5%	95
miR-375, miR-25, let-7f	HCC *vs*. HC	0.997	97.9%	99.1%	95
miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, miR-801	HCC *vs*. HC	0.941	83.2%	93.9%	96
	HCC *vs*. CHB	0.842	79.1%	76.4%	
	HCC *vs*. cirrhosis	0.884	75.0%	91.1%	
miR-18a	HCC *vs*. HC	0.881	86.1%	75.0%	97
	HCC *vs*. CHB	0.775	77.2%	70.0%	
miR-122	HCC *vs*. HC	0.869	81.6%	83.3%	98
	HCC *vs*. CHB	0.630	77.6%	57.8%	
miR-21	HCC *vs*. HC	0.87	84.0%	73.5%	99
miR-122	HCC *vs*. HC	0.79	70.7%	69.1%	99
miR-223	HCC *vs*. HC	0.86	80.0%	76.5%	99
miR-15b, miR-130b	HCC *vs*. HC	0.980	98.2%	91.5%	100
miR-206, miR-141-3p, miR-433-3p, miR-1228-5p, miR-199a-5p, miR-122-5p, miR-192-5p, miR-26a-5p	HCC *vs*. HC	0.893	82.8%	83.3%	102
	HCC *vs*. cirrhosis	0.892	81.6%	84.6%	

MiR-152 is frequently down-regulated in HBV-related HCC tissues compared to adjacent noncancerous hepatic tissues and it is inversely correlated to DNA methyltransferase 1 (DNMT1) mRNA expression.[87] Inhibition of miR-152 increases methylation levels of two tumor suppressor genes, glutathione S-transferase P1 (GSTP1) and E-cadherin 1 (CDH1).[87] MiR-143, miR-34 and miR-19 have been found to be up-regulated in HBV-related HCC and to be associated to a more aggressive cancer phenotype.[88]

The perturbation of miRNA expression during HBV-related disease, from acute infection to HCC development, was analyzed by Zhang and colleagues.[89] The authors found that miRNAs expression pattern during chronic HBV infection was much closer to that of HCC patients than that of patients with acute HBV infection. MiR-103, miR-106a, miR-107, miR-221 and miR-224 were found down-regulated in acute infection and up-regulated in HCC, suggesting a possible role in disease progression. In particular, miR-103 was also found up-regulated in chronic disease, while miR-106a was again down-regulated. MiR-101 and miR-29c showed coherent alteration patterns between acute infection and HCC, indicating their involvement in the establishment and maintenance of the HBV related-disease.[89]

Let-7a, miR-22, miR-99a, miR-122a, miR-126 and miR-130a are down-regulated in HBV-related HCC compared to a normal liver.[90] On the other side, miR-15a, miR-17, miR-18a, miR-19b, miR-20a, miR-27a, miR-92, miR-93, miR-106b and miR-21 exhibit increased expression in 100% of human HBV-related HCC and their suppression causes a 50% reduction in hepatocyte proliferation and anchorage-independent growth.[90] In particular, mir-21 is a very strong oncogene if over-expressed and was found to be altered in HCC as in many other tumors.[25] Moreover, miR-21 promotes migration and invasion of HCC through the miR-21-PDCD4 (programmed cell death 4)-AP-1 feedback loop.[91] Interestingly, the main target of miR-21 is PTEN that enhances cell surviving through PI3K-AKT pathway activation.[92]

Thus, the expression of several miRNAs changes with disease onset and these are interesting candidates for monitoring HCC initiation and progression.

Circulating miRNA in patients with HBV-related HCC

MiRNAs can be either actively or passively released into the blood stream. They can be found included in microvesicles and exosomes, or bound to ribonucleoprotein-complexes, high density lipoproteins, albumin and also to HBV surface antigen (HBsAg).[93] Many miRNAs are deregulated in HBV-related HCC and it is expected that circulating miRNA levels are also affected by HCC development and progression (Table II). Their stability in the circulation and accessibility make miRNAs perfect biomarkers, offering a non-invasive method for screening patients at risk of HCC development.[94]

One of the most relevant study on this topic, carried out on 513 patients, identified 13 miRNAs

able to discriminate HBV-positive patients from HCV-positive subjects and from healthy controls.[95] These miRNAs could also distinguish HCC patients from those with CHB. MiR-375 and miR-92a were specific for HBV infection. Six miRNAs (including miR-375 and miR-92a) were overexpressed in HBV-related HCC patients serum when compared to healthy controls. MiR-375, miR-25 and let-7f, used as biomarkers, were able to discriminate HCC patients from controls and miR-375 alone showed a sensitivity of 100% and a specificity of 96% for the identification of HCC patients.[95]

In 2011, Zhou *et al.* carried out a similar study, using plasma instead of serum for the analyses and enrolled 934 patients grouped into three cohorts (CHB, cirrhosis, HBV-related HCC) and healthy controls.[96] The authors identified a miRNA panel (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a e miR-801) with high diagnostic accuracy and potentially useful for early diagnosis of HBV-related HCC. Indeed, the authors reported area under the curve (AUC) values of 0.888 either for HCC stage 0 and stage A, according to Barcelona Clinic Liver Cancer staging system. Finally, selected miRNAs were able to differentiate HCC from healthy subjects (AUC=0.941), from CHB (AUC=0.842) and cirrhosis (AUC=0.884).[96]

Li *et al.* studied 5 serum miRNAs and reported that the expression level of miR-18a was significantly higher (P<0.01) and that of miR-378 was significantly reduced (p<0.05) in HBV-related HCC patients than in healthy controls.[97] MiR-18 showed a sensitivity of 78% and a specificity of 70% for the diagnosis of HCC among cirrhotic patients or CHB affected patients.[97]

Another elegant study compared circulating miR-122, miR-222, miR-223 and miR-21 expression levels between HBV-positive patients with and without HCC.[98] Interestingly, only miR-122 was over-expressed in HCC patients serum than in healthy controls (P<0.001) and resulted drastically reduced after HCC surgical resection. However, the difference in expression between HBV-positive patients with or without HCC was at the limits of statistical significance (P=0.043).[98] The reason why miR-122 expression results usually reduced in HCC tissue while its circulating level increases in the same patients is still unclear.

In another study, higher expression levels of miR-21 miR-122 and miR-223 were found in HCC patients than in healthy controls.[99] However, elevated serum miRNAs were also detected in patients with CHB. For this reason, these miRNAs may be useful markers for differentiating patients with HCC or chronic hepatitis from healthy subjects, but not patients with HCC from those with chronic hepatitis. Thus, these miRNAs seem to be more correlated to liver damage than to HCC.

MiR-15b and miR-130b were found to be promising markers in HCC screening. By studying the serum of HCC patients, HBV carriers and healthy controls, the combination of miR-15b and miR-130b revealed a sensitivity of 98.2% in detecting HCC.[100] These miRNAs could even identify early stage HCC cases that could not be detected by AFP.[100]

Recently, Giray *et al.* studied the expression pattern of 24 miRNAs in the plasma of 66 patients with HBV-related liver disease (cirrhosis and HCC) and 28 healthy controls.[101] MiR-125b-5p levels resulted over-expressed in cirrhotic patients and in HCC patients when compared to healthy controls. Similarly, miR-223-3p resulted down-regulated in the former groups.[101]

Another miRNA panel (miR-206, miR-141-3p, miR433-3p, miR-1228-5p, miR-199a-5p, miR-122-5p, miR-192-5p, miR-26a-5p) was recently proposed by Tan *et al.* as potential tool for HCC diagnosis.[102] This panel was able to differentiate HCC from healthy subjects and patients with cirrhosis, hence displaying a high level of accuracy. Moreover, the authors compared AUC of miRNA panel with that of AFP and reported significant differences in diagnostic accuracy for discriminating between HCC and cirrhosis (difference between AUCs = 0.184, p=0.0001).[102]

Conclusions

Several studies have pointed out to a link between cancer and miRNAs thus rendering the opportunity to use these novel molecules for cancer diagnosis and therapy attractive. Studies on miRNA developed with new technologies for miRNA quantification and this is reflected by the exponential increase in scientific papers in the last 15 years. Research methods are variable and there is the need for standardization in order to extract the most promising data and the "state of art" in this field.

From the studies regarding miRNA deregulation in HBV-related HCC tissues, a high variability emerged among the results: this is probably caused by the different techniques and criteria employed for

miRNA selection. Moreover, the differences may be due to variations in patient cohorts (differences in sex and ethnicity, or underlying diseases) and to different methods used for miRNA isolation and quantification, thus rendering difficult the identification of a specific miRNA pattern for HBV-related HCC.

Several studies analyzed the accuracy of circulating miRNAs as early biomarkers of HCC. Theoretically, miRNAs may be considered good biomarkers because of their accessibility and high stability into the blood stream. Furthermore, there are still no optimal circulating HCC biomarkers. To date, several miRNAs have been proposed for HCC detection showing high diagnostic accuracy in discriminating patients with HCC, cirrhosis or CHB from healthy controls, but less exciting results has been reported for the discrimination between patients with HCC and patients with cirrhosis, which are those at higher risk of tumor development. In addition, reported results are often conflicting and there are still some technical concerns regarding the use of miRNA as biomarkers. Moreover, serum and plasma are not directly comparable and, more importantly, there is lack of a standard method for miRNAs expression normalization which currently represent the major drawback for the use of such molecules as diagnostic or prognostic markers in clinical practice.

Further studies are needed to improve both our understanding of the intricate miRNAs regulatory network and on their role in cancer development. MiRNAs are indeed promising tools for improving the management of patients with HCC.

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