MicroRNAs: New Tools for Diagnosis, Prognosis, and Therapy in Hepatocellular Carcinoma?

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MicroRNAs (miRNAs) are evolutionarily conserved small noncoding RNAs involved in the regulation of gene expression and protein translation. Many studies have shown that they play a crucial role in driving organ and tissue differentiation during embryogenesis and in the fine-tuning of fundamental biological processes, such as proliferation and apoptosis. Growing evidence indicates that their deregulation plays an important role in cancer onset and progression as well, where they act as oncogenes or oncosuppressors. In this review, we highlight the most recent findings regarding the role of miRNAs in hepatocellular carcinoma (HCC) by analyzing the possible mechanisms by which they contribute to this neoplasm. Moreover, we discuss the possible role of circulating miRNAs as biomarkers, a field that needs urgent improvement in the clinical surveillance of HCC, and the fascinating possibility of using them as therapeutic targets or drugs themselves. (HEPATOTOLOGY 2013;57:840-847)

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide. Multiple viruses, metabolic alterations leading to chronic inflammation, and epigenetic and genetic changes cooperate in cancer development via a combination of common and distinct etiology-specific pathways. Genome-wide gene expression microarray and quantitative real-time reverse-transcription polymerase chain reaction studies indicate a general aberrant activation of signaling pathways involved in cellular proliferation, survival, differentiation, and angiogenesis, which are heterogeneously present in each HCC. However, what is missing is a signature or a single prominent characteristic pathway that defines this cancer.

Recently, it became clear that the classification and stratification of tumors can be performed by evaluating the modulation of microRNAs (miRNAs), small non-coding RNAs that negatively control gene expression. Notably, microRNA expression profiles are able to classify tumors at different stages and to distinguish among subsets of patients with different molecular pathologies. Although changes in the expression of microRNAs between tumor specimens and the normal corresponding tissues have been investigated in HCC as well, the obtained results are often discordant and do not allow the identification of the microRNAs critical for development and progression of HCC.\(^1\)

Furthermore, among the miRNAs whose expression has changed, several are probably altered not as a cause but as a consequence of the tumorigenic status.

In this review, we summarize the main findings of the role of miRNAs in the context of liver cancer and discuss how this could help our understanding of the mechanisms underlying HCC development and progression and how they may improve diagnosis and treatment.

miRNAs and Cancer

miRNAs are able to control gene expression at a posttranscriptional level, either by blocking mRNA translation or inducing their degradation. The mechanism of action of miRNAs has revolutionized the concept of gene expression regulation, because we now
know that mRNA levels in a cell do not strictly correlate with protein expression.

The involvement of miRNAs in cancer pathogenesis is well established, as they can behave as oncogenes or tumor suppressor genes depending on the cellular function of their targets. Moreover, activation or suppression of specific miRNA families are mechanisms through which oncogenes, such as Myc, or tumor suppressor genes, such as p53, induce or inhibit tumorigenesis. Germline mutations have been detected in miRNA genes and in the binding sequences of target mRNAs. It is tempting to speculate that they might participate in familial predisposition to cancer, especially in those families where a culprit gene has not yet been identified. In addition, epigenetic modifications in miRNA loci, altering their transcription and affecting the metastatic ability of tumor cells have been described. The importance of miRNAs in cancer progression is also underlined by the observation that they can influence both the response to chemotherapy and the development of drug resistance.

Remarkably, miRNAs might be very useful as cancer biomarkers, because they are present in the blood and are very stable. Studies performed in preclinical models and in cancer patients demonstrated that cancer affects miRNA levels in the bloodstream and that specific miRNAs in the serum can be associated with specific tumors. Even if this approach needs further validation, this discovery might open the path to an innovative way of detecting tumors by means of serum or plasma miRNA measurement.

Finally, tumor-associated miRNAs may represent a novel group of viable targets for therapeutic intervention. Further to this, studies attempting to translate work from the bench to the clinic are already well underway. The success obtained in lowering the level of plasma cholesterol in non-human primates by systemic administration of a miRNA inhibitor gives hope for a possible application of a similar therapeutic approach in clinical oncology.

miRNAs and HCC

Many reports have shown miRNA deregulation in human HCCs. These works have either compared the cancer miRNomes with that of nonneoplastic tissue or have studied specific miRNAs. The picture stemming from these investigations is not always superimposable, and this can be due to several technical issues. For example, studies have been performed using different techniques such as microarrays, reverse-transcription polymerase chain reaction–based assays, and next-generation sequencing. Even if reliable, they highlight significant differences that must be considered when analyzing results.

Using next-generation sequencing, which provides data not only on quantitative alterations of the different miRNAs but also on their relative amount, Hou et al. analyzed the miRNomes of normal human liver and HCC. Interestingly, they found that around 86% of miRNAs were poorly expressed in normal liver, 13% were moderately expressed, and less than 1% was abundantly expressed. The three most represented miRNAs were miR-122, miR-192, and miR-199a/b-3p, accounting for 52%, 16.9%, and 4.9% of the miRNome, respectively. Although a tumorigenic role of miR-122 has been described, Hou et al. found that its expression decreased only in half of the HCCs and that it was poorly relevant for survival; notably, he found a strong decrease of this miRNA only in viral-negative HCCs. Similarly, miR-192 did not seem to be significantly deregulated in HCC samples. On the other hand, deregulation of miR-199a/b-3p was observed in 40/40 patients, regardless of the underlying pathology, and its decrement significantly correlated with the poor survival of HCC patients. Among the moderately expressed miRNAs in HCCs and matched nonneoplastic tissues, there were two families: let-7 and miR-100. The let-7 family was usually down-regulated in HCC samples, even if an opposite behavior was observed for let-7a and let-7f in viral-negative HCCs. Concerning the miR-100 family, both miR-100 and miR-99a were down-regulated in HCCs of different etiology. The most up-regulated miRNA was miR-21, which increased not only in the tumoral tissue, but also in the peritumoral nonneoplastic tissue, compared with normal liver. This clearly shows that miRNA expression in the peritumoral nonneoplastic tissue (which is often used as a comparison) is frequently different from that of healthy “normal” liver. This may also be due to the presence in the cirrhotic peritumoral tissues of nonhepatocytic cells that express different sets of miRNAs. These findings may explain, at least in part, some of the discrepancies among different studies comparing HCC either to normal healthy liver or to nonneoplastic peritumoral tissue.

miRNA and Molecular Classifications of HCC

Profiling of human tumors based on miRNA expression has identified signatures associated with diagnosis, staging, progression, prognosis, and response to treatment. In an attempt to use miRNAs to create a molecular classification of HCC, Murakami et al. analyzed miRNA expression profiles in 25 pairs of HCC and adjacent nontumorous tissue. They found that three
miRNAs exhibited higher expression in the HCC samples, whereas five were down-regulated. Classification of samples as HCC or normal, based on these data, provided an overall prediction accuracy of 97.8%. In addition, the expression levels of miR-92, miR-20, and miR-18 were inversely correlated with the degree of HCC differentiation. More recently, Toffanin et al.\textsuperscript{16} performed a comprehensive genomic analysis by integrating miRNA data with gene expression analysis, copy number changes, and assessment of cellular pathway activation by immunohistochemical and mutational analyses. They proposed an miRNA-based classification of three subclasses of HCC, displaying either activation of the Wnt pathway or enrichment of intercellular signaling. Three subclasses of HCC, displaying either activation of the Wnt pathway or enrichment of intercellular signaling were identified, reflecting the accumulation of genome abnormalities in the remaining noncancerous liver cells, associated with multicentric de novo carcinogenesis.

Several studies have examined the prognostic role of individual miRNAs in HCC, their mechanism of action, and the biological effects resulting from their modulation in HCC cells; a list of the most relevant studies is provided in Supporting Table 1.

**miRNA and Metastasis.** In several types of tumors, as well as in HCC, the analysis of miRNA expression has led to the identification of miRNAs promoting or repressing the metastatic process. Budhu et al.\textsuperscript{18} identified a 20-miRNA tumor signature associated with HCC venous metastasis; this signature predicted survival and recurrence of HCC in patients with multinodular or solitary tumors, including those with early-stage disease. Moreover, it was an independent and significant predictor of patient prognosis, when compared with other available clinical parameters.

Contrasting results have recently been obtained by Wong et al.,\textsuperscript{19} who did not find differences in miRNA expression pattern between primary HCCs and venous metastases, but only a marked global reduction of miRNA expression levels in venous metastases compared with primary HCCs. Their data suggest that miRNA deregulation is a relatively early event in liver carcinogenesis and that the later global miRNA down-regulation aggravates the preexisting miRNA deregulation to further promote HCC metastasis.

Finally, some studies have analyzed the role of specific miRNAs in the metastatic process of HCC, identifying either prometastatic or antimetastatic miRNAs (Supporting Table 2).

**miRNA and Response to Therapy.** Works performed in many types of cancer have shown that miRNAs can influence the sensitivity of tumors to therapy. This notion holds true also for HCC (Supporting Table 3); restoration of miR-122 in HCC cells makes them sensitive to adriamycin and vincristine through down-regulation of Multidrug resistance (MDR)-related genes, the antiapoptotic gene Bcl-w and cyclin B1.\textsuperscript{20} The same miRNA, as well as miR-199a-3p, were shown to affect sensitivity of HCC cells to doxorubicin.\textsuperscript{21,22} DNA methylation of miR-193a-3p, instead, dictates 5-fluorouracil (5-FU) resistance of HCC cells via repression of the serine/arginine-rich splicing factor 2 which, in turn, up-regulates the proapoptotic splicing form of caspase 2.\textsuperscript{23} Response to 5-FU was also studied by Tomimaru et al.,\textsuperscript{24} who found that HCC cells transfected with pre–miR-21 were resistant to interferon-\(\alpha\) (IFN-\(\alpha\))/5-FU, while cells expressing anti–miR-21 became sensitive to IFN-\(\alpha\)/5-FU. Moreover, miR-21 expression in clinical HCC specimens was associated with the clinical response to the IFN-\(\alpha\)/5-FU combination therapy and survival rate.

Many recent studies have demonstrated that resistance to chemotherapy is often due to altered expression of drug transporters. Indeed, up-regulation of adenosine triphosphate–binding cassette (ABC) transporters in HCC occurs prior to chemotherapeutic treatment and is associated with miRNA down-regulation\textsuperscript{25}; up-regulation of five ABC genes in HCC patient samples appears to be mediated by 13 miRNAs.

Molecular therapies have recently entered the clinical scenario and have demonstrated good efficacy in other types of tumors, but resistance to treatment is usually observed in a short period of time. In HCC, where the most widely used biological therapies are interferon and sorafenib, the response to interferon was influenced by miR-146a, which induces resistance to treatment through its ability to down-regulate SMAD4,\textsuperscript{26} and by miR-26 whose low expression increases patients’ response to interferon therapy.\textsuperscript{27} Zhou et al.\textsuperscript{28} found that sorafenib, a small inhibitor of tyrosine and Raf kinases that was approved recently for the treatment of advanced HCC, altered the expression
of 14 miRNAs; among these miRNAs is miR-1274a, which is up-regulated by sorafenib resulting in repression of ADAM9, a protease involved in sorafenib-targeted therapy of HCC. On the other hand, the liver-specific miR-122, which is frequently suppressed in primary HCCs, is able to sensitize HCC cells to sorafenib.29

Circulating miRNAs and HCC. Efforts have been made to develop noninvasive serum biomarkers for the diagnosis of HCC. Despite remarkable advances, the reliability of biomarkers such as alpha-fetoprotein (AFP) or des-g-carboxyprothrombin (DCP) is still debatable. Indeed, their specificity (in particular that of AFP) is low, especially in the context of chronic liver disease. Therefore, novel biomarkers for early HCC diagnosis are greatly needed. The finding that miRNAs can be detected in fluids as free miRNAs or are contained within microvesicles such as exosomes (membrane vesicles secreted by several cells) has opened new opportunities in the search for biomarkers in cancer. Indeed, the possibility of profiling miRNAs in circulation represents a noninvasive way to investigate disease-specific miRNAs and is an alternative and promising approach to current strategies for cancer surveillance (Supporting Table 4).

To determine whether serum or plasma levels of miRNAs have either a diagnostic or a prognostic value in human HCC, Li et al.30 performed a study on 513 subjects. Serum miRNA expression profiling allowed the identification of 13 miRNAs that were differentially expressed in the sera of hepatitis B virus (HBV)-positive patients and accurately discriminated not only HBV-associated HCCs from controls and HCV-associated HCCs, but also HBV-positive HCC cases from HBV cases. Moreover, six miRNAs were significantly up-regulated in the sera of HBV-HCC patients. Interestingly, two of these miRNAs, namely miR-375 and miR-92a, were also present in the previous panel. The use of only three of these miRNAs (miR-25, miR-375, and let-7f) as biomarkers could separate HCC cases from controls. In addition, miR-375 alone had a receiver operating characteristic (ROC) of 0.96 (specificity: 96%; sensitivity: 100%) in HCC prediction. Thus, this study demonstrates that serum miRNA profiles can serve as novel noninvasive biomarkers for HBV-positive HCC diagnosis.

More recently, Zhou et al.31 also attempted to identify miRNAs for diagnosing HBV-related HCC. Compared with the previous study, the analysis was performed on plasma rather than on serum. The study was conducted on 934 individuals and identified an miRNA panel providing a high diagnostic accuracy for HCC. The diagnostic performance of this panel did not depend on the disease status, thus it seems to be of particular clinical value in diagnosing early-stage HBV-related HCCs. Moreover, it could also differentiate HCC from healthy, chronic HBV and cirrhosis. Remarkably, none of the miRNAs included in this panel coincided with those identified by Li et al. The reasons for the different results are not clear but may be related to different materials used (plasma vs. serum).

Other studies focused specifically on candidate miRNAs. Qi et al.32 found that miR-122 in serum was higher in HCC patients than in healthy controls and that its levels were reduced in the postoperative serum samples. Why the expression of miR-122 was generally down-regulated in HCC while its circulating levels increased in the same patients is unclear. One possibility is that the low level of this miRNA in tumor cells is due to increased release. However, this would not explain why high levels of miR-221 increased both in HCC as well as in the serum. Xu et al.33 found that miR-122, miR-21, and miR-223 were high in the serum of patients with HCC but their levels, unlike those found by Zhou et al., could not discriminate between HCC and chronic hepatitis. In this regard, it is puzzling that while serum levels of miR-122 in the work of Qi et al. were up-regulated (as were those observed by Zhou et al. in the plasma), no such increase was observed in the serum by Li et al.30, Qu et al.34 investigated whether serum levels of miR-16, miR-195, and miR-199a, alone or in combination with conventional serum markers, could help differentiate HCC from chronic liver disease. They found that miR-16, as a single marker, had the highest sensitivity for HCC, followed by miR-199a, AFP, DCP, AFP-L3%, and miR-195. As a second-line HCC marker, miR-16 yielded positive HCC predictions in 18 out of 26 HCC patients (most of which had a tumor size smaller than 3 cm) with negative results for all three conventional markers. Liu et al.35 found that miR-15b, miR-21, miR-130b, and miR-183 were highly expressed in 96 tumors and that their levels were markedly reduced after surgery, indicating the tumor-derived source of these circulating miRNAs. In a validation study, combined miR-15b and miR-130 yielded 98.2% sensitivity and 91.5% specificity. The detection sensitivity of the classifier in a subgroup of HCCs with low AFP (<20 ng/mL) was 96.7% and the classifier also identified early-stage HCC cases that could not be detected by AFP.

Finally, expression of serum miR-221 was analyzed to investigate its prognostic value.36 High levels of miR-221 expression were correlated with tumor size,
cirrhosis and tumor stage. In addition, Kaplan-Meier survival analysis showed that the overall survival rate of the high miR-221 expression group (27.6%) was significantly lower than that of the low miR-221 expression group (62.3%).

Altogether, these data show the feasibility of using circulating miRNAs as biomarkers for HCC diagnosis. At present, however, none of these studies have been translated into clinical practice. To fully uncover the clinical perspectives of this field of research, more work has to be done and some critical points, such as the best type of sample to be used (plasma, serum, or urine) and appropriately powered sample size, have to be carefully considered.

miRNAs as Drugs or Therapeutic Targets

Many in vitro and preclinical studies have either reintroduced oncosuppressive miRNAs or inhibited oncogenic miRNAs in cancer cells, showing that these treatments often result in impairment of cell proliferation and invasion or in increased apoptosis. This implies that these miRNAs (in the case of oncosuppressors) or their inhibitors (in the case of oncogenic miRNAs) might be used as therapeutics. One of the advantages of modulating expression of miRNAs, as opposed to genes, resides in their ability to simultaneously target multiple genes and pathways. Moreover, targeting critical genes (and their related pathways) with more than one oncosuppressive miRNA could strongly enhance the biological efficacy and reduce the risk of resistance to therapy. On the other hand, the effect of miRNAs on gene expression could also result in clinically relevant side effects due to off-target effects. The other major problem of miRNA-based anticancer therapies is their delivery. In the case of the reintroduction of oncosuppressive miRNAs into cancer cells, a system would be required in which miRNAs could be delivered to all the tumor cells, otherwise untreated cells would sustain tumor recurrence. At present, such an efficient system of delivery is not available. Interestingly, as miRNAs can be exchanged between cells in paracrine manner, it is important to clarify whether this natural biological mechanism could vicariate a relatively inefficient delivery. Figure 1 shows the most widely used strategies to target miRNAs in cancer.

Regarding HCC, many studies have shown that either exogenous expression of oncosuppressor miRNAs or inhibition of oncomiRs resulted in impaired growth or invasive ability of HCC cell lines in vitro or in xenografts. Furthermore, re-expression of a tumor suppressor miRNA could block cancer progression in vivo. Indeed, systemic administration of miR-26a in a mouse model of HCC using adeno-associated virus resulted in inhibition of cancer cell proliferation, induction of tumor-specific apoptosis and protection from disease progression without toxicity. This finding suggests that delivery of miRNAs may be an important therapeutic strategy.

Recently, two studies have shown the effectiveness of targeting miR-221 in HCC. Both used systemic administration of either a cholesterol-modified isoform of anti-miR-221 or anti–miR-221 oligonucleotides and observed an antitumoral effect, leading to prolonged mouse survival or a reduction in the number and size of tumor nodules. Furthermore, miR-124 administration inhibited and prevented DEN-induced HCC in mice, supporting the notion that systemic delivery of miR-124 may be a clinically viable anticancer therapeutic approach. This study also demonstrated that transient inhibition of hepatocyte nuclear factor 4a initiates hepatocellular transformation through a miRNA/inflammatory feedback loop circuit. As this circuit is perturbed in human HCCs, these data raise the possibility that the manipulation of this miRNA feedback-inflammatory loop has therapeutic potential for treating liver cancer.

Finally, Lanford et al. showed that the liver-specific miR-122 is essential for HCV RNA accumulation in liver cells. They chronically treated HCV-infected chimpanzees with an miR-122 specific locked nucleic acid oligonucleotide and observed suppression of viremia, without overt toxicity, thus implying that miR-122 is essential for accumulation of HCV RNA in vivo. MiR-122 targeting may then represent a strategy to prevent the onset of chronic hepatitis, a major HCC risk factor.

Conclusions

Discovery of the critical role of miRNAs in modulating gene expression has not only changed our concept of gene expression regulation, but has also offered a new opportunity for designing anticancer strategies and therapies (Fig. 2). However, it is essential to determine the safety of these treatments and to gain insight into the side effects these therapies may have. Indeed, although our understanding of the role of miRNAs in cancer development is improving, it is still far from complete. Specifically, several relevant questions need to be solved (Table 1).

First, are all miRNAs found deregulated in HCC critical for tumor development, or is their deregulation
simply the consequence of metabolic and structural rearrangements of fully transformed cancer cells? Studies should continue to focus on dissecting the carcinogenic process to identify miRNAs that are modified in the early phases of the process. The paucity of studies on HCC at the initial stages in humans is probably due to the clinical difficulty of diagnosing and collecting enough material to study early lesions. In this context, animal models of hepatocarcinogenesis, in which discrete lesions at different stages of progression can be identified and analyzed, will be extremely helpful.

Second, why do studies on miRNA profiling by different groups often fail to provide reproducible results and are frequently contradictory? Is this due to the intrinsic heterogeneity of human HCCs, to the different etiological agents or to the type of technology used? New experimental strategies using system biology methodologies aimed at classifying and comparing the conditions underlying different studies by different groups are likely to provide an explanation to the apparently contradictory results and to help identify a precise miRNA signature in HCC.

Third, the emergence of miRNAs as important regulators of metabolism has raised much interest not only from a scientific point of view but also from a clinical perspective. Indeed, a metabolic shift toward a resistant phenotype is almost invariably observed in preneoplastic and neoplastic cancer cells; although therapeutic efforts to treat metabolic disorders have so far addressed “druggable” targets (e.g., enzymes), the very recent finding that certain miRNAs may represent crucial regulators of metabolism raises the question of

Fig. 1. Targeting miRNAs in cancer. Mature miRNAs are obtained from the primary transcript through two sequential cleavages catalyzed by two different RNA endonucleases, Drosha and Dicer. The nascent primary miRNA (pri-miRNA) is then processed into a 70-nucleotide precursor (pre-miRNA); then the pre-miRNA is further cleaved to generate a 20-23 nucleotide mature miRNA. Depending on the degree of complementarity with the target sequence, miRNAs can hinder protein synthesis from a transcript either by interfering with the assembly of the ribosomes around the mRNA or by committing mRNAs to degradation through activation of the RNA-induced silencing complex (RISC). (a-c) The most widely used strategies to block oncomirs in cancer are: (a) antisense oligonucleotides acting as competitive inhibitors of miRNAs (their major drawback is that they are quite unstable); (b) Locked nucleic acid constructs showing high affinity for the target, high specificity, and high aqueous solubility; and (c) miRNA sponges that contain multiple binding sites for the miRNA of interest and act by competing with bona fide targets for miRNA binding. (d,e) To restore oncosuppressive miRs, either chemically modified miRNA mimics (d) or pre-miRNA (e) have been developed. (f) To improve their delivery and to have a long-lasting expression, they can be incorporated into virus-like particles (mainly adenovirus-associated vectors). *Chemical modifications.
whether they may coordinately control metabolism as well. If so, targeting these mRNAs might affect the metabolic machinery required for the resistant phenotype characteristic of the neoplastic cells.

Finally, the high stability of miRNAs in circulation makes them perfect biomarkers, especially for detection of early stage, presymptomatic diseases. However, the reason for the lack of correspondence between the levels of some miRNAs in HCC and in the patients’ fluids is still incompletely clear. Apart from these still unexplained findings, the fluid most reliable for the detection of miRNAs as possible cancer biomarkers has yet to be established before translation into clinical practice.

In view of the many unanswered questions, a greater understanding of the molecular mechanisms by which miRNAs regulate tumorigenesis is both a priority and a fascinating scientific challenge that may promote the development of innovative concepts in the diagnosis and treatment of cancer.

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Table 1. Open Questions

| Lack of studies on preneoplastic and early neoplastic lesions does not allow to discriminate which miRNAs are real drivers of the carcinogenic process |
| Analysis of early tumor steps in humans |
| Use of animal models |
| Differences among the tumor signatures obtained by different groups |
| Heterogeneity of the pathology |
| Use of different technologies for the analysis |
| Use of different material as comparison (healthy liver versus peritumoral tissue) |
| Differences among the signatures obtained by the analysis of circulating microRNAs |
| Starting material (plasma versus serum) |
| Heterogeneity of the pathology |
| Use of different technologies for the analysis |
| Use of miRNAs as drugs or drug targets |
| Efficiency of delivery |
| Persistency of response |
| Side effects |

Fig. 2. Potential use of miRNAs in HCC diagnosis, prognosis, and treatment. Analysis of miRNAs in patients affected by HCC could be used for (a) diagnostic purposes via (1) identification of tumor miRNA signatures that could help early diagnosis by differentiating tumor subtypes and (2) identification of tumor miRNA signatures that could help early diagnosis by differentiating small neoplastic lesions from the nontumoral tissue; (b) prognostic purposes via (1) profiling of circulating miRNAs to identify relapse after treatment and (2) identification of tumor miRNA signatures associated with different prognosis; or (c) therapeutic purposes via (1) inhibition of oncomiRs or restoration of oncosuppressive miRNAs and (2) identification and modulation of miRNAs able to interfere with response to chemotherapy or to molecular therapy.

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