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MicroRNAs: new tools for diagnosis, prognosis and therapy in HCC?

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ABSTRACT

MicroRNAs are evolutionary conserved small non-coding 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 on the role of microRNAs 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.
Introduction

Hepatocellular carcinoma (HCC) is the third cause of cancer-related deaths worldwide. Multiple viruses, metabolic alterations leading to chronic inflammation, 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 (RT-PCR) studies indicate a general aberrant activation of signalling 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 also by evaluating the modulation of microRNAs (miRNAs), small non-coding RNAs which 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 microRNAs 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 microRNAs 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.

MicroRNAs and cancer
MicroRNAs are able to control gene expression at a post-transcriptional level, either by blocking mRNA translation or inducing their degradation. Thus, the mechanism of action of microRNAs has revolutionized the concept of gene expression regulation as we now know that mRNA levels in a cell do not strictly correlate with protein expression.

The involvement of microRNAs in cancer pathogenesis is well established as they can behave as oncogenes or tumor suppressor genes depending on the cellular function of their targets (2). Moreover, activation or suppression of specific microRNA families are mechanisms through which oncogenes, such as Myc, or tumor suppressor genes, such as p53, induce or inhibit tumorigenesis (3). Germline mutations have been detected in microRNA 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 (3,4). In addition, epigenetic modifications in microRNA loci, altering their transcription and affecting the metastatic ability of tumor cells have been described (5). The importance of microRNAs in cancer progression is also underlined by the observation that they can influence both the response to chemotherapy (6) and the development of drug resistance (7).

Remarkably, microRNAs might be very useful as cancer biomarkers since they are present in the blood and are very stable. Studies performed in preclinical models and in cancer patients demonstrated that cancer affects microRNA levels in the bloodstream and that specific microRNAs in the serum can be associated with specific tumors (4, 5). 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 microRNA measurement.

Finally, tumor-associated microRNAs 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 microRNA inhibitor (6) gives hope for a possible application of a similar therapeutic approach in clinical oncology.
**MicroRNAs and HCC**

Many reports have shown microRNA deregulation in human HCCs. These works have either compared the cancer miRNome with that of non tumoral tissue or have studied specific microRNAs. The picture stemming from these investigations is not always super-imposable and this can be due to several technical issues. For example, studies have been performed using different techniques such as microarrays, RT-PCR-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 microRNAs but also on their relative amount, Hou et al. (7) analysed the miRNomes of normal human liver and HCC. Interestingly, they found that around 86% of microRNAs were poorly expressed in normal liver, 13% were moderately expressed and less than 1% was abundantly expressed. The three most represented microRNAs were miR-122, miR-192 and miR 199 a/b-3p, accounting for 52%, 16.9% and 4.9% of the miRNome, respectively. Although a tumorigenic role of miR-122 has previously been described (8-12), Hou found that its expression decreased only in half of the HCCs and that it was poorly relevant for patients’ survival; notably, he found a strong decrease of this microRNA 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 microRNAs in HCCs and matched non-neoplastic tissues there were two families: let-7 and miR-100. The let -7 family was usually downregulated in HCC samples, even if an opposite behaviour was observed for let-7a and let-7f in viral-negative HCCs. Concerning the miR-100 family, both miR-100 and miR-99a were downregulated in HCCs of different etiology. The most upregulated microRNA was miR-21, which increased not only in the tumoral tissue, but also in the peritumoral non neoplastic tissue, compared to normal liver. This clearly shows that microRNA expression in the peritumoral non
neoplastic 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 non-hepatocytic cells that express different sets of microRNAs. These findings may explain, at least in part, some of the discrepancies among different studies comparing HCC either to normal healthy liver or to non-neoplastic peritumoral tissue.

MicroRNA and molecular classifications of HCC

Profiling of human tumors based on microRNA expression has identified signatures associated with diagnosis, staging, progression, prognosis and response to treatment. In the attempt to use microRNAs to create a molecular classification of HCC, Murakami analyzed microRNA expression profiles in 25 pairs of HCC and adjacent non-tumorous tissue. He found that three microRNAs exhibited higher expression in the HCC samples, while five were downregulated (13). Classification of samples as HCC or normal, based on this 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. (14) performed a comprehensive genomic analysis by integrating microRNA data with gene expression analysis, copy number changes and assessment of cellular pathway activation by immunohistochemical and mutational analyses. They proposed a microRNA-based classification of 3 subclasses of HCC, displaying either activation of the Wnt pathway or enrichment of interferon-response-related genes or activation of IGF-1R and Akt-pathways.

Sato et al. (15) developed a mathematical model to assess the risk of HCC recurrence after liver resection, based on microRNA expression profiling. They found that the tumor microRNA profile could predict early recurrence, while the microRNA profile of the non tumoral tissue was predictive of late recurrence, suggesting that the tumor microRNA profile represents the malignant potential of primary tumors, associated with the presence of hepatic dissemination. The peritumoral
microRNA profile, instead, reflects the accumulation of genome abnormalities in the remaining non-cancerous liver cells, associated with multicentric \textit{de novo} carcinogenesis.

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

\textbf{MicroRNA and metastasis}

In several types of tumors, as well as in HCC, the analysis of microRNA expression has led to the identification of microRNAs promoting or repressing the metastatic process. Budhu et al. (16) identified a 20-microRNA 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 to other available clinical parameters.

Contrasting results have recently been obtained by Wong et al. (17) who did not find differences in microRNA expression pattern between primary HCCs and venous metastases, but only a marked global reduction of microRNA expression levels in venous metastases, as compared with primary HCCs. Their data suggest that microRNA deregulation is a relatively early event in liver carcinogenesis and that the later global microRNA down-regulation aggravates the preexisting microRNA deregulation to further promote HCC metastasis.

Finally, some studies have analyzed the role of specific microRNAs in the metastatic process of HCC, identifying either pro-metastatic or antimetastatic microRNAs (Supp. Table 2).

\textbf{MicroRNA and response to therapy}

Works performed in many types of cancer have shown that microRNAs can influence the sensitivity of tumors to therapy. This notion holds true also for HCC (Supp. Table 3); restoration of miR-122 in HCC-cells makes them sensitive to adriamycin and vincristine through downregulation of MDR
related genes, the antiapoptotic gene Bcl-w and cyclin B1 (20). The same microRNA, as well as miR-199a-3p, were shown to affect sensitivity of HCC cells to doxorubicin (18, 19). 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 (23). Response to 5-FU was also studied by Tomimaru et al. (20) who found that HCC cells transfected with pre-miR-21 were resistant to IFN-α/5-FU, while cells expressing anti-miR-21 became sensitive to IFN-α/5-FU. Moreover, miR-21 expression in clinical HCC specimens was associated with the clinical response to the IFN-α/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 microRNA down-regulation (25); up-regulation of five ABC genes in HCC patient samples appears to be mediated by 13 microRNAs.

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 downregulate SMAD4 (21), and by miR-26 whose low expression increases patients’ response to interferon therapy (22). Zhou et al. (23) found that sorafenib, a small inhibitor of tyrosine and Raf kinases, recently approved for treatment of advanced HCC, altered the expression of 14 microRNAs; among these miRNAs, is miR-1274a that 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, frequently suppressed in primary HCCs, is able to sensitize HCC cells to sorafenib (29).
**Circulating microRNAs and HCC**

Efforts have been made to develop non-invasive 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 microRNAs can be detected in fluids like free microRNAs or 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 microRNAs in circulation represents a non-invasive way to investigate disease-specific microRNAs and is an alternative and promising approach to current strategies for cancer surveillance (Supp. Table 4).

To determine whether serum or plasma levels of microRNAs have either a diagnostic or a prognostic value in human HCC, Li et al. (24) performed a study on 513 subjects. Serum microRNA expression profiling allowed the identification of 13 microRNAs that were differentially expressed in the sera of HBV+ 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, 6 microRNAs were significantly upregulated in the sera of HBV-HCC patients. Interestingly, 2 of these microRNAs, namely miR-375 and miR-92a, were also present in the previous panel. The use of only 3 of these microRNAs (miR-25, miR-375, and let-7f) as biomarkers could separate HCC cases from controls. In addition, miR-375 alone had a ROC of 0.96 (specificity: 96%; sensitivity: 100%) in HCC prediction. Thus, this study demonstrates that serum microRNA profiles can serve as novel, non-invasive biomarkers for HBV-positive HCC diagnosis.

More recently, Zhou et al. (25) also attempted to identify microRNAs for diagnosing HBV-related HCC. In comparison to the previous study, the analysis was performed on plasma rather than on serum. The study was conducted on 934 individuals and identified a microRNA panel providing a high diagnostic accuracy for HCC. The diagnostic performance of this panel did not depend on the
disease status and 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 hepatitis B and cirrhosis. Remarkably, none of the microRNAs 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 microRNAs. Qi et al. (26) found that miR-122 in serum was higher in HCC patients than in healthy controls and that its levels were reduced in the post-operative 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 microRNA 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. (27) 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 Qi’s work are up-regulated (as those found by Zhou et al. in the plasma), no such increase was observed in the serum by Li et al. Qu et al. (28) 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 3 conventional markers. Liu et al. (29) 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 microRNAs. 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 (30). 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 microRNAs 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 –for example – the best type of sample to be used (plasma, serum or urine) and appropriately-powered sample size, have to be carefully considered.

**MicroRNAs as drugs or therapeutic targets**

Many in vitro and preclinical studies have either reintroduced oncosuppressive microRNAs or inhibited oncogenic microRNAs in cancer cells, showing that these treatments often result in impairment of cell proliferation and invasion or in increased apoptosis. This implies that these microRNAs (in the case of oncosuppressors) or their inhibitors (in the case of oncogenic microRNAs) might be used as therapeutics. One of the advantages of modulating expression of microRNAs, 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 microRNA could strongly enhance the biological efficacy and reduce the risk of resistance to therapy. On the other hand, the effect of microRNAs on gene expression could also result in clinically relevant side effects, due to off-target effects. The other major problem of microRNA-based anticancer therapies is their delivery. In the case of the reintroduction of oncosuppressive microRNAs into cancer cells, a system would be required in which microRNAs
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 microRNAs can be exchanged between cells in paracrine manner, it is important to clarify if this natural biological mechanism could vicariate a relatively inefficient delivery. Figure 1 shows the most widely used strategies to target microRNAs in cancer.

As to HCC, many studies have shown that either exogenous expression of oncosuppressor microRNAs 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 microRNA could block cancer progression in vivo (37). 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 microRNAs may be an important therapeutic strategy.

Recently, two studies have shown the effectiveness of targeting miR-221 in HCC (31, 32). Both used systemic administration of either a cholesterol-modified isoform of miR-221 (33) or anti-miR-221 oligonucleotides (34) 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 HNF4a initiates hepatocellular transformation through a microRNA/inflammatory feedback loop circuit. As this circuit is perturbed in human HCCs, these data raise the possibility that the manipulation of this microRNA feedback-inflammatory loop has therapeutic potential for treating liver cancer.

Finally, Lanford et al. (35) showed that the liver-specific miR-122 is essential for HCV RNA accumulation in liver cells. They chronically treated HCV-infected chimpanzees with a miR-122 specific LNA 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 microRNAs in modulating gene expression has not only changed our concept of gene expression regulation, but has also offered a new opportunity for designing anti-cancer strategies and therapies (Figure 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 microRNAs in cancer development is improving, it is still far from complete. Specifically, several relevant questions need to be solved (Table 1): i) are all microRNAs 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 microRNAs 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. ii) Why do studies on microRNA 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 aetiological 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 microRNA signature in HCC. iii) The emergence of microRNAs 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 towards a resistant phenotype is almost invariably observed in preneoplastic and neoplastic cancer cells; while
therapeutic efforts to treat metabolic disorders have so far addressed ‘druggable’ targets, such as enzymes, the very recent finding that certain microRNAs may represent crucial regulators of metabolism raises the question of whether they may coordinately control metabolism as well. If so, targeting these mRNAs might impact on the metabolic machinery required for the resistant phenotype characteristic of the neoplastic cells. iv) The high stability of microRNAs in circulation makes them perfect biomarkers, especially for detection of early stage, pre-symptomatic diseases. However, the reason for the lack of correspondence between the levels of some microRNAs 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 microRNAs 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 microRNAs 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.

Reference List


Legend to figures

Figure 1. Targeting microRNAs in cancer

Mature microRNAs are obtained from the primary transcript (pri-miRNA) through two sequential cleavages catalysed by two different RNA endonucleases, Drosha and Dicer respectively. The nascent pri-miRNA is first processed into a 70-nucleotide precursor called pre-miRNA; then the pre-miRNA is further cleaved to generate a 20-23 nucleotide mature microRNA. Depending on the degree of complementarity with the target sequence, microRNAs 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 the activation of the RISC complex.

The most widely used strategies to block oncomirs in cancer (right side of the figure) are: (a) antisense oligonucleotides acting as competitive inhibitors of microRNAs; their major drawback is that they are quite unstable. (b) Locked nucleic acid (LNA) constructs showing high affinity for the target, high specificity and high aqueous solubility; (c) miRNA sponges which contain multiple binding sites for the microRNA of interest and act by competing with bona fide targets for microRNA binding. To restore oncosuppressive miRs (left side of the figure), either chemically modified miRNA mimics (d) or miRNA precursors (pre-miRNA) (e) have been developed. To improve their delivery and to have a long lasting expression, they can be incorporated into virus-like particles (mainly adenovirus-associated vectors) (f). *: Chemical modifications.

Figure 2. Potential use of microRNAs in HCC diagnosis, prognosis and treatment. Analysis of microRNAs in patients affected by HCC could be used for (i)
diagnostic purposes: (1) identification of tumor microRNA signatures which could help early diagnosis, by differentiating tumor subtypes; (2) identification of tumor microRNA signatures which could help early diagnosis, by differentiating small neoplastic lesions from the non tumoral tissue; (ii) prognostic purposes: (1) circulating microRNA profiling to identify relapse after treatment (2) identification of tumor microRNA signatures associated with different prognosis; (iii) therapeutic purposes: (1) inhibition of oncomiRs or restoration of oncosuppressive microRNAs (2) identification and modulation of microRNAs able to interfere with response to chemotherapy or to molecular therapy.
Table 1. Open questions

- Lack of studies on preneoplastic and early neoplastic lesions does not allow to discriminate which microRNAs 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 vs. peritumoral tissue)

- Differences among the signatures obtained by the analysis of circulating microRNAs
  - Starting material (plasma vs. serum)
  - Heterogeneity of the pathology
  - Use of different technologies for the analysis

- Use of microRNAs as drugs or drug targets
  - Efficiency of delivery
  - Persistency of response
  - Side effects
microRNAs and Cancer

Diagnosis

1) microRNA signatures to distinguish tumor subtypes
2) microRNA signatures to differentiate pre-neoplastic and neoplastic tissues

Prognosis

1) Circulating microRNAs as biomarkers to monitor follow up
2) Prognostic microRNAs in tumor tissue

Treatment

1) microRNAs as drugs or therapeutic targets
2) microRNAs as modulators of response to treatment
### Supporting Table 1. Prognostic microRNAs in human HCC

<table>
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<th>Reference</th>
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<td>miR-182</td>
<td>miR overexpression in HCC correlates with intrahepatic metastasis and poor prognosis</td>
<td>Wang, <em>et al.</em> 2012 (1)</td>
</tr>
<tr>
<td>miR-155, miR-15a,</td>
<td>High expression levels of these miRs are significantly associated with RFS</td>
<td>Huang YH, <em>et al.</em> 2012 (2)</td>
</tr>
<tr>
<td>miR-432, miR-486-3p,</td>
<td></td>
<td></td>
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<tr>
<td>miR-15b, miR-30b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 miR signature</td>
<td>Significant independent predictor of overall survival and recurrence-free survival</td>
<td>Han, <em>et al.</em> 2012 (3)</td>
</tr>
<tr>
<td>miR-10b</td>
<td>High miR-10b is an independent predictor of poor prognosis</td>
<td>Li, <em>et al.</em> 2012 (4)</td>
</tr>
<tr>
<td>C19CM</td>
<td>High C19CM miRNA levels correlate with poor clinico-pathological features, increased risk of recurrence and shorter overall survival</td>
<td>Augello, <em>et al.</em> 2012 (5)</td>
</tr>
<tr>
<td>miR21, miR221</td>
<td>Up-regulation is associated with tumor stage and poor prognosis</td>
<td>Karakatsanis, <em>et al.</em> 2011 (6)</td>
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<tr>
<td>miR-155</td>
<td>High levels in patients with post-OLT recurrence and correlation with micro-vascular invasion. Independent predictor of poor prognosis</td>
<td>Han, <em>et al.</em> 2012 (7)</td>
</tr>
<tr>
<td>67 miRs signature</td>
<td>Discriminates patients with HCC recurrence after transplant from those without recurrence</td>
<td>Barry, <em>et al.</em> 2012 (8)</td>
</tr>
<tr>
<td>miR-135a</td>
<td>High miR-135a expression correlates with low OS and DFS</td>
<td>Liu, <em>et al.</em> 2012 (9)</td>
</tr>
<tr>
<td>miR-99a</td>
<td>Low miR-99a correlates with shorter survival and is an independent prognostic predictor</td>
<td>Li, <em>et al.</em> 2011 (10)</td>
</tr>
<tr>
<td>miR-203</td>
<td>High miR-203 correlates with better RFS and OS. High miR-203 expression is an independent predictor of good prognosis</td>
<td>Chen, <em>et al.</em> 2012 (11)</td>
</tr>
<tr>
<td>DLK1-DIO3 miRNA cluster</td>
<td>Upregulation of this cluster is associated with a stem-cell-like phenotype and poor prognosis</td>
<td>Luk, <em>et al.</em> 2011 (12)</td>
</tr>
<tr>
<td>miR-124</td>
<td>Low expression is significantly associated with a more aggressive phenotype and poor prognosis</td>
<td>Zheng, <em>et al.</em> 2012 (13)</td>
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<tr>
<td>miR-199b-5p</td>
<td>Low expression is associated with poor OS and PFS</td>
<td>Wang, <em>et al.</em> 2011 (14)</td>
</tr>
<tr>
<td>miR signatures in tumor and non-tumor tissues</td>
<td>Tumor signature tends to predict early recurrence; non-tumor tissue signature tends to predict late recurrence.</td>
<td>Sato, <em>et al.</em> 2011 (15)</td>
</tr>
<tr>
<td>miR-139</td>
<td>Downregulation of miR-139 is associated with poor prognosis and features of metastatic tumors</td>
<td>Wong, <em>et al.</em> 2011 (16)</td>
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<td>miR-22</td>
<td>Low miR-22 expression is predictive of poor survival</td>
<td>Zhang, <em>et al.</em> 2010 (17)</td>
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<td>let-7g</td>
<td>Low let-7g expression is predictive of poor survival</td>
<td>Ji, <em>et al.</em> 2010 (18)</td>
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<td>miR-29</td>
<td>miR-29 downregulation is associated with worse DFS</td>
<td>Xiong, <em>et al.</em> 2010 (19)</td>
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<tr>
<td>miR-122</td>
<td>miR-122 is downregulated in tumors characterized by poor prognosis</td>
<td>Coulouarn <em>et al.</em> 2009 (20)</td>
</tr>
</tbody>
</table>

RFS = Recurrence Free Survival; OS = Overall survival; DFS = Disease Free Survival; PFS = Progression Free Survival; OLT = Orthotopic Liver Transplantation.


<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Main finding</th>
<th>Effector</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-7</td>
<td>Inhibits metastasis in vitro and in vivo</td>
<td>PI3K/AKT/mTOR pathway</td>
<td>Fang et al; 2012 (1)</td>
</tr>
<tr>
<td>miR-210</td>
<td>Up-regulated in HCC. Induced by hypoxia. Increases migration and invasion</td>
<td>Vacuole membrane Protein 1 (VMPI)</td>
<td>Ying et al; 2011 (2)</td>
</tr>
<tr>
<td>miR-1395p, miR-101, miR-125b, let-7c, miR-200b miR-21</td>
<td>Downregulated in HCC with metastatic features Negatively regulate HCC metastasis Promotes migration and invasion of HCC cells</td>
<td>miRs epigenetically silenced by EZH2 Up-regulated in HCC. Controls Programmed cell death 4 (PDCD4) Controls osteopontin leves</td>
<td>Au et al; 2012 (3) Zhu et al; 2012 (4)</td>
</tr>
<tr>
<td>miR-96</td>
<td>Promotes migration and invasion of HCC cells</td>
<td></td>
<td>Chen et al; 2012 (5)</td>
</tr>
<tr>
<td>Global miRNA analysis</td>
<td>Global reduction of miRNA expression levels in venous metastases vs. primary HCCs</td>
<td></td>
<td>Wong et al; 2012 (6)</td>
</tr>
<tr>
<td>miR-155</td>
<td>High levels in tumor tissues with post-OLT HCC recurrence and correlation with micro-vascular invasion. Promotes invasion of HCC cells.</td>
<td></td>
<td>Han et al; 2012 (7)</td>
</tr>
<tr>
<td>miR-135a</td>
<td>Favors invasion and metastasis in vitro. In vivo blockade reduces the development of portal vein tumor thrombi</td>
<td>miR-135a is transcribed by FOXM1 and controls metastasis suppressor 1</td>
<td>Liu et al; 2012 (8)</td>
</tr>
<tr>
<td>miR-29b</td>
<td>Downregulation associated with poor recurrence-free survival. Suppresses proangiogenic and invasive ability of HCC cells</td>
<td>Directly controls MMP2</td>
<td>Fang et al; 2011 (9)</td>
</tr>
<tr>
<td>miR-124</td>
<td>Reduced expression associated with poor prognosis. Re-expression in HCC cells inhibits invasion in vitro and metastasis in vivo.</td>
<td>Targets ROCK2 and EZH2</td>
<td>Zheng et al; 2012 (10)</td>
</tr>
<tr>
<td>miR-338-3p</td>
<td>Decreased in aggressive HCC. Re-expression suppresses invasion of HCC cells</td>
<td>Targets Smoothened</td>
<td>Huang et al; 2011 (11)</td>
</tr>
<tr>
<td>miR-198</td>
<td>Downregulated in HCC. Re-expression suppresses HGF-induced invasion of HCC cells</td>
<td>Targets c-MET</td>
<td>Tan et al; 2011 (12)</td>
</tr>
<tr>
<td>miR</td>
<td>Function</td>
<td>miR targets</td>
<td>Ref</td>
</tr>
<tr>
<td>-------</td>
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<td>-------------------------------------------------</td>
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</tr>
<tr>
<td>miR-200, miR-192</td>
<td>Transactivated by p53. Regulate Epithelial mesenchymal transition</td>
<td>miR-200 targets ZEB 1 / 2, miR-192 targets ZEB 2</td>
<td>Kim et al; 2011 (13)</td>
</tr>
<tr>
<td>miR-139</td>
<td>Reduced expression associated with poor prognosis. Re-expression in HCC cells inhibits invasion in vitro and metastasis in vivo.</td>
<td>Targets ROCK2</td>
<td>Wong et al; 2011 (14)</td>
</tr>
<tr>
<td>miR-125b</td>
<td>Suppresses HCC cell growth in vitro and in vivo and inhibits invasion</td>
<td>Targets LIN28B</td>
<td>Liang et al; 2010 (15)</td>
</tr>
<tr>
<td>miR-151-5p</td>
<td>Frequently amplified in HCC. Correlated with intrahepatic metastases. Increases HCC invasion in vitro and in vivo</td>
<td>Targets Rho GDIA. It synergizes with its host gene FAK</td>
<td>Ding et al; 2010 (16)</td>
</tr>
<tr>
<td>Let-7g</td>
<td>Low in metastatic HCC. Ectopic expression inhibits HCC migration and growth</td>
<td>Targets COL1A2</td>
<td>Ji et al; 2010 (17)</td>
</tr>
<tr>
<td>miR-17-5p</td>
<td>Overexpressed in HCC. Up-regulates migration and proliferation of HCC cells</td>
<td>Activates p38 MAPK pathway and Promotes HSP27 phosphorylation</td>
<td>Yang et al; 2010 (18)</td>
</tr>
<tr>
<td>miR-30d</td>
<td>Reduced expression associates with poor prognosis. Re-expression in HCC cells inhibits invasion in vitro and metastasis in vivo.</td>
<td>Targets Galphai2</td>
<td>Yao et al; 2010 (19)</td>
</tr>
<tr>
<td>miR-122</td>
<td>Reduced expression associated with poor prognosis. Re-expression in HCC cells inhibits invasion in vitro.</td>
<td>miR-122 is under the transcriptional control of HNF1A, HNF3A, HNF3B</td>
<td>Coulouarn et al; 2009 (20)</td>
</tr>
<tr>
<td>miR-23b</td>
<td>miR expression in HCC cells decreases migration and proliferation</td>
<td>Targets uPA and c-MET</td>
<td>Salvi et al; 2009 (21)</td>
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<tr>
<td>miR-143</td>
<td>Increased in metastatic HCC. Promotes invasive and metastatic behavior in vitro and in vivo.</td>
<td>Targets fibronectin type III domain containing 3B c-Met</td>
<td>Zhang et al; 2009 (22)</td>
</tr>
<tr>
<td>miR-34a</td>
<td>Downregulated in HCC. Ectopic expression inhibits Met-dependent cell migration and invasion.</td>
<td></td>
<td>Li et al; 2009 (23)</td>
</tr>
<tr>
<td>20 miRs metastasis signature</td>
<td>Significantly predicts primary HCCs with venous metastases from metastasis-free solitary tumors</td>
<td></td>
<td>Budhu et al; 2008 (24)</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Finding</th>
<th>Mediator</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-122</td>
<td>Increases sensitivity to doxorubicin</td>
<td>NS modulation of mTOR and c-MET</td>
<td>Fornari et al; 2009 (1)</td>
</tr>
<tr>
<td>miR-199a-3p</td>
<td>Restoring miR decreased levels increases sensitivity to doxorubicin-induced apoptosis</td>
<td></td>
<td>Fornari et al; 2010 (2)</td>
</tr>
<tr>
<td>miR-122</td>
<td>Sensitizes HCC cells to Adriamycin and vincristine</td>
<td>downregulates MDR-related genes Bcl-w and cyclin B1 decreases SRSF2</td>
<td>Xu et al; 2011 (3)</td>
</tr>
<tr>
<td>miR-193a-3p</td>
<td>Promotes resistance to 5-FU</td>
<td></td>
<td>Ma et al; 2012 (4)</td>
</tr>
<tr>
<td>miR-122</td>
<td>Sensitizes HCC cells to sorafenib</td>
<td>NS decreases Mcl-1 expression</td>
<td>Bai et al; 2009 (5)</td>
</tr>
<tr>
<td>miR-193b</td>
<td>Decreases the IC(50) to sorafenib</td>
<td>decreases Mcl-1 expression</td>
<td>Braconi et al; 2010 (6)</td>
</tr>
<tr>
<td>miR-1274a</td>
<td>Up-regulated by sorafenib mediates resistance to this drug</td>
<td>downregulates ADAM9</td>
<td>Zhou et al; 2011 (7)</td>
</tr>
<tr>
<td>miR-21</td>
<td>Induces resistance to interferon-α/5FU in HCC cells</td>
<td>PTEN and PDCD4</td>
<td>Tomimaru et al; 2010 (8)</td>
</tr>
<tr>
<td>miR-146a</td>
<td>Suppresses sensitivity to interferon-α in HCC cells</td>
<td>downregulates SMAD4</td>
<td>Tomokuni et al; 2011 (9)</td>
</tr>
<tr>
<td>13 miRs</td>
<td>Mediate multidrug resistance</td>
<td>downregulate ABC (Adenosine Triphosphate Binding Cassette) transporters</td>
<td>Borel et al; 2012 (10)</td>
</tr>
</tbody>
</table>

NS = not specified; 5-FU= 5- Fluorouracil
Reference List


### Supporting Table 4. Circulating microRNAs as prognostic biomarkers for HCC

<table>
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<tr>
<th>Experimental Setting</th>
<th>Material</th>
<th>Main finding</th>
<th>Reference</th>
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<tr>
<td>120 HCC, 135 HBV+, 48 HCV and 210 healthy controls</td>
<td>serum</td>
<td>Combined miR-25, miR-375 and let-7f upregulation in HBV and HCC</td>
<td>Li et al; 2010 (1)</td>
</tr>
<tr>
<td>10 HCC patients and 10 healthy controls</td>
<td>plasma</td>
<td>miR-92a decrease in HCC patients</td>
<td>Shigoka et al; 2010 (2)</td>
</tr>
<tr>
<td>46 HCC patients and 20 healthy controls</td>
<td>serum</td>
<td>Upregulation of miR221 has prognostic value</td>
<td>Li et al; 2011(3)</td>
</tr>
<tr>
<td>105 HCC, 107 CLD patients and 71 healthy controls</td>
<td>serum</td>
<td>Downregulation of miR-16 and miR-199a</td>
<td>Qu et al; 2011(4)</td>
</tr>
<tr>
<td>101 HCC patients, 48 CHB and 89 healthy controls</td>
<td>serum</td>
<td>Upregulation of miR-21, miR-122 and miR-123 in HCC and CHB</td>
<td>Xu et al; 2011(5)</td>
</tr>
<tr>
<td>10 HBV+ HCC patients and 10 healthy controls</td>
<td>serum</td>
<td>miR-122 upregulation in HCC and CHB</td>
<td>Qi et al; 2011(6)</td>
</tr>
<tr>
<td>457 HCC, 169 CHB, 141 cirrhotic patients and 167 healthy controls</td>
<td>plasma</td>
<td>Identification of a plasma miR-panel* with diagnostic value</td>
<td>Zhou et al; 2011(7)</td>
</tr>
<tr>
<td>57 HCC patients and 30 healthy controls</td>
<td>serum</td>
<td>Combined increase of miR-15b and miR-130b as a classifier for HCC detection</td>
<td>Liu et al; 2012(8)</td>
</tr>
<tr>
<td>32 HCC HCV+, 74 HCV+ and 12 healthy controls</td>
<td>urine</td>
<td>miR-618/650 increase for early diagnosis</td>
<td>Abdalla et al; 2012(9)</td>
</tr>
</tbody>
</table>

HCC: hepatocellular carcinoma; HBV: Hepatitis B Virus; HCV: Hepatitis C virus; CHB: Chronic hepatitis B; *miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801
Reference List


