Emerging role of microRNAs in the treatment of hepatocellular carcinoma

Elisa Callegari1
Marco Domenicali2
Laura Gramantieri3
Massimo Negrini1
Silvia Sabbioni4

1Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, 2Department of Medical and Surgical Sciences, Alma Mater Studiorum University of Bologna, 3Center for Applied Biomedical Research, S Orsola-Malpighi University Hospital, Bologna, 4Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy

Correspondence: Silvia Sabbioni
Department of Life Sciences and Biotechnology, University of Ferrara, Via Luigi Borsari 46, 44121 Ferrara, Italy
Email sbs@unife.it

Massimo Negrini
Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Via Luigi Borsari 46, 44121 Ferrara, Italy
Email ngm@unife.it

Abstract: Hepatocellular carcinoma is the third leading cause of cancer deaths worldwide. Currently available curative options, such as surgery and transplantation, are not available to patients with advanced stages of disease. Among the potential new treatments being investigated are microRNA (miRNA)-based therapies. A number of preclinical studies have reported antitumor activities of miRNA mimics or anti-miRNA molecules. Optimal in vivo delivery of miRNA molecules is crucial to their action. To this end, significant progress has been made in the development of nanoparticles for in vivo delivery of miRNA molecules. Delivery of these molecules, alone or in combination with other drugs, promises to open new possibilities for therapeutic approaches to hepatocellular carcinoma.

Keywords: hepatocellular carcinoma, microRNA, nanocarriers, therapy

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and the third leading cause of cancer-related deaths worldwide. In 80%–90% of cases, HCC arises on a background of cirrhosis, a chronic and diffuse hepatic disease that results from continuous liver injury and regeneration. The prevalent risk factors for HCC include hepatitis B and C infection and alcohol consumption, which are also the main causes of liver cirrhosis. Other risk factors include exposure to aflatoxin B1 and vinyl chloride, tobacco smoking, diabetes, and genetic disorders such as hemochromatosis and alpha-1 antitrypsin deficiency.

The molecular mechanisms of liver tumorigenesis have been studied, and genetic and epigenetic alterations are reported to dysregulate several important signaling pathways, such as the RAS-mitogen-activated protein kinase and the PI3K/AKT/PTEN signaling pathways, activated by epidermal growth factor, hepatocyte growth factor, platelet-derived growth factor, vascular endothelial growth factor, and fibroblast growth factor. Dysregulation leads to proliferation, cell survival, sustained angiogenesis, invasion, and metastasis. Activation of the Wnt/β-catenin signaling pathway has also been observed, leading to deregulation of cell proliferation, differentiation, and stemness. Several cell cycle regulators, including RB1, cyclin-dependent kinases, cyclins, and cyclin-dependent kinase inhibitors, are aberrantly expressed in HCC, contributing to cell proliferation, while altered expression of BCL2 family members and other genes such as IAP and PTEN contribute to disruption of apoptosis. Recurrent mutations in the MLL family of histone methyltransferases are also reported to result in alteration of chromatin remodelers.
Several therapeutic approaches are used nowadays for the management of patients with HCC. According to the Barcelona Clinic Liver Cancer staging system, potential curative treatment of eligible patients with early-stage HCC consists of resection, percutaneous ablation, and liver transplantation. Transarterial chemoembolization was shown to provide survival benefits in patients with intermediate-stage HCC, but not curative. The prognosis of advanced HCC remains poor, since traditional chemotherapy agents have proven to be marginally effective or even toxic because of limited liver function. A therapeutic option for advanced HCC was offered by the multikinase inhibitor sorafenib, the effectiveness of which in increasing time to progression and overall survival was demonstrated by the SHARP trial in 2008 and confirmed in the Asia-Pacific region trial in 2009. Sorafenib is active against several kinases, such as RAF-1, BRAF, vascular endothelial growth factor receptor 2, platelet-derived growth factor receptor, and c-Kit receptors. This drug displays antiangiogenic and antiproliferative activity for numerous targets.

Nevertheless, resistance to sorafenib after the initial response arises in all patients, and several alternative agents, either alone or in combination with sorafenib, have been investigated in randomized controlled Phase III clinical trials. A combination of transarterial chemoembolization and sorafenib was shown to further improve survival rates in patients with advanced HCC. In contrast, the combination of sorafenib and the epidermal growth factor receptor inhibitor erlotinib did not demonstrate advantages over sorafenib alone (SEARCH trial, ClinicalTrials identifier NCT00901901). The combination of sorafenib and the histone deacetylase inhibitor resminostat is currently being evaluated in patients who are refractory to sorafenib monotherapy (ClinicalTrials identifier NCT00943449). Evaluation of other molecular targeted agents as a second-line treatment showed unsatisfactory results; both the mTOR inhibitor everolimus (EVALE, ClinicalTrials identifier NCT01035229) and the multikinase inhibitor brivanib failed to show survival benefits in patients who did not respond to sorafenib. Similarly, in first-line therapy, alternative multitargeted tyrosine kinase inhibitors (sunitinib, brivanib) conferred no survival benefits compared with sorafenib in the treatment of advanced HCC.

Several limits have been identified in the settings of recent clinical trials and corrective actions proposed to reduce the risk of trial failure. In particular, better selection of enrolled patients, who reflect the molecular and clinical heterogeneity of the disease, has been indicated as a requirement for the correct design of future studies. To this end, despite improvements in the knowledge of the signal pathways involved in HCC, reliable biomarkers for “molecular patient stratification” are lacking and a need exists to identify the main drivers of hepatocarcinogenesis and matched active agents.

Despite awareness of the several signaling pathways involved in HCC, the majority of molecular targeted agents investigated so far in Phase III clinical trials in HCC are antiangiogenic molecules, and therapies that are able to affect multiple targets or different pathways could be more effective. In this context, microRNA (miRNA)-regulatory molecules that are individually able to affect multiple cellular pathways and play a key role in the control of several cancer genes are worth exploring.

**Involvement of miRNA in HCC**

The role of miRNAs in carcinogenesis has been extensively studied during the last decade. The expression of a number of miRNAs was found to be frequently altered in cancer cells, where miRNAs could disturb functions associated with control of proliferation, apoptosis, differentiation, angiogenesis, invasion, and metastasis. The abnormal expression of several miRNAs has also been demonstrated in HCC (Table 1). In some cases, hepatitis viruses were indicated to have a role in dysregulation of cellular miRNA. Hepatitis B virus messenger RNAs, containing an miR-122 complementary site, could act as a sponge that is able to sequester endogenous miR-122, thus lowering the levels of active miR-122. Hbx protein decreases levels of several tumor suppressor miRNAs, such as miR-34 and let-7, while HBx RNA directly downregulates miR-15a and miR-16-1. Moreover, upregulated gene, upregulation of which is induced by HBx, could induce miR-148a overexpression and promote cell cycle progression and cell migration. The mechanisms of miRNA deregulation by hepatitis C virus have been less extensively studied. MiR-141 upregulation, which has been described in hepatitis C virus-infected human hepatocytes in vitro, causes the depletion of DLC-1 protein, a tumor suppressor frequently deleted in HCC.

Animal models based on miRNAs have confirmed their role in tumorigenesis, both in hematopoietic diseases and in solid tumors, such as HCC, thereby allowing preclinical studies based on the inhibition or restoration of miRNA (see Callegari et al for a comprehensive review, and Table 2).

The altered expression of miRNAs in HCC was further investigated to assess their importance as molecular markers for the prognostic stratification of HCC (Table 3). Recently, Yin et al conducted a meta-analysis of several
Table I microRNAs with consistently deregulated expression in human hepatocellular carcinoma

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Genome location</th>
<th>Expression in HCC</th>
<th>Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-7g</td>
<td>5:2302294–52302377</td>
<td>Down</td>
<td>BCL2L1, COLIA2</td>
</tr>
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<td>miR-1</td>
<td>20:6151513–6151583</td>
<td>Down</td>
<td>MET, FOXP1, HDAC4</td>
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<tr>
<td>miR-23b</td>
<td>9:9784790–97847586</td>
<td>Down</td>
<td>uPA, MET</td>
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<tr>
<td>miR-26a</td>
<td>3:38010895–38010971</td>
<td>Down</td>
<td>CCND2, CCNE</td>
</tr>
<tr>
<td>miR-29</td>
<td>7:130561560–130561569</td>
<td>Down</td>
<td>BCL2, MCL1</td>
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<tr>
<td>miR-34a</td>
<td>1:9211727–9211836</td>
<td>Down</td>
<td>MET</td>
</tr>
<tr>
<td>miR-101</td>
<td>11:65524117–65524191</td>
<td>Down</td>
<td>MCL1, FOS</td>
</tr>
<tr>
<td>miR-122</td>
<td>18:56118306–56118390</td>
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<tr>
<td>miR-124</td>
<td>8:9760898–9760982</td>
<td>Down</td>
<td>CDK6, VIM, SMYD3, IQGAP1</td>
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<tr>
<td>miR-125a</td>
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<td>BMF, ERBB2, ERBB3</td>
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<tr>
<td>miR-125b</td>
<td>11:121970465–121970552</td>
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<td></td>
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<tr>
<td>miR-130a</td>
<td>11:57408671–57408759</td>
<td>Down</td>
<td>ATXN1, PPARγ</td>
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<tr>
<td>miR-139</td>
<td>11:72236107–72236174</td>
<td>Down</td>
<td>RHOK2</td>
</tr>
<tr>
<td>miR-145</td>
<td>5:148810299–148810296</td>
<td>Down</td>
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</tr>
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<td>miR-150</td>
<td>19:50004042–50004125</td>
<td>Down</td>
<td>EGR2</td>
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<tr>
<td>miR-193b</td>
<td>16:14397824–14397906</td>
<td>Down</td>
<td>MCL1</td>
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<tr>
<td>miR-195</td>
<td>17:69209324–69210120</td>
<td>Down</td>
<td>CCND1, CDK6, E2F3</td>
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<td>miR-199a-1</td>
<td>19:10928102–10928172</td>
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<tr>
<td>miR-199b</td>
<td>9:131007000–131007109</td>
<td>Down</td>
<td></td>
</tr>
<tr>
<td>miR-200a</td>
<td>1:1103243–1103332</td>
<td>Down</td>
<td>ZEB1, ZEB2, beta-catenin</td>
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<tr>
<td>miR-200b</td>
<td>1:1102484–1102578</td>
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<tr>
<td>miR-223</td>
<td>X:65238712–65238821</td>
<td>Down</td>
<td>Statmin1</td>
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<tr>
<td>miR-375</td>
<td>2:219866367–219866430</td>
<td>Down</td>
<td>YAP</td>
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<tr>
<td>miR-602</td>
<td>9:140732871–140732968</td>
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<td>RASSF1A</td>
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<tr>
<td>miR-17-5p</td>
<td>13:92002859–92002942</td>
<td>Up</td>
<td>NCOA3, E2F1, BCL2L1I, CDKN1A, RBL2, MAPK4, STAT3, CCL1, DNAJC27, FBXO31, GPR137B, NAPAT, OBFC2A, RAB12, YES1, ZNFX1, FNI, FNDC3A</td>
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<td>NRC3, CTGF, ES1</td>
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<tr>
<td>miR-92a</td>
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<td>miR-106-25</td>
<td>7:99691616–99691697</td>
<td>Up</td>
<td>CDKN1A, BIM</td>
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<tr>
<td>miR-21</td>
<td>17:57918627–57918698</td>
<td>Up</td>
<td>FasL, SERPINB5, PDCD4, TIMP3, SPRY2, LRRFIP1, RECK, PTEN, BTG2, Peli1, NHRPK, TP63, MARCKS, TPM1, Galphal2</td>
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<td>miR-30d</td>
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<td>miR-151</td>
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<td>miR-221/222</td>
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<td>miR-224</td>
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<td>miR-373</td>
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<td>miR-483-3p</td>
<td>11:2155364–2155349</td>
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Abbreviation: HCC, hepatocellular carcinoma.

studies, showing that multigroup analysis on miRNA panels could give better diagnostic accuracy than single miRNAs. Moreover, abnormal levels of miRNAs were also found in the serum or plasma of HCC patients in comparison with those in healthy subjects, thus also suggesting a potential role for miRNAs as circulating biomarkers (Table 4). 35,52–54

miRNAs as therapeutic agents

Given the importance of miRNAs in several aspects of tumorigenesis, their therapeutic value has also been investigated. The inhibition of miRNA expression in vivo was for the first time shown to be feasible by the intravenous administration of single-stranded anti-miR-122 “antagonist” oligonucleotides in mice. Subsequently, the safety of a locked nucleic acid-modified anti-miR-122 was assessed in nonhuman primates by showing the absence of toxicity or histopathological changes in the liver after administration of this molecule. Following these studies, a locked nucleic acid-modified anti-miR-122 (miravirsen, SPC3649, Santaris Pharma, Copenhagen, Denmark) entered into clinical trials for the...
Callegari et al Table 2 microRNA-based therapies in preclinical models

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Mouse model</th>
<th>Tumor</th>
<th>Method</th>
<th>Delivery system</th>
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<td>miRNA inhibition</td>
<td>mirVana custom inhibitor</td>
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<td>miRNA inhibition</td>
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<td>TG-221</td>
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<td>miRNA replacement</td>
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<td>dox-repressible modified miR-31 miRNA sponge vector system</td>
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<td>miRNA replacement</td>
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<td>Synthetic miRNAs complexed with siPORT amine; lentivirus</td>
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<td>AAV</td>
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<td>Adenovirus</td>
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<td>miR-143; miR-145</td>
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<td>miR-133a and miR-206</td>
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<td>Oncolytic virus</td>
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<td>miR-124</td>
<td>Orthotopic model of primary human GBM</td>
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<td>miR-124, miR-128, miR-146b and miR-218</td>
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<td>miR-124, miR-128, miR-146b and miR-218</td>
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<td>Glioma</td>
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</tbody>
</table>

Abbreviations: HCC, hepatocellular carcinoma; DEN, diethylnitrosamine; NSCLC, non-small cell lung cancer; AAV, adeno-associated viruses; GBM, glioblastoma multiforme.

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have also demonstrated the therapeutic efficacy of the restoration of tumor suppressor miRNAs that are underexpressed in cancer. miR-26a,67 miR-122,48 and miR-199a68 could effectively and significantly reduce tumor growth by use of adenovirus-associated viruses as delivery vectors in animal models. A Phase I clinical trial was initiated in 2013 to evaluate the safety of a liposome-formulated miR-34 mimic, MRX34 (Mirna Therapeutics, Austin, TX, USA), in patients with primary liver cancer or liver metastases from other cancers (ClinicalTrials identifier NCT01829971).63,69 Recently, Kelner et al established the pharmacokinetic profile of MRX34 in nonhuman primates, providing useful data on the calculation of the first dose in clinical trials of MRX34.70

An additional potential option for miRNA-based therapeutics resides in their use for the control of oncolytic virus replication. On the basis of a first example of a conditional replicative adenovirus regulated by miR-122 to circumvent liver toxicity,71,72 other oncolytic viruses were developed, including miRNA let-7-dependent73 and miR-199-dependent74 conditional replicative adenoviruses. Because of the differential expression of these miRNAs between normal liver cells and liver cancer cells, these viruses were able to replicate in HCC tumor cells but not in normal liver cells, did not produce significant toxicities in normal tissues, and, importantly, have shown the ability to inhibit tumor growth in vivo. As reported in Table 2, a let-7-dependent adenovirus exhibited antitumor activity in an HCC xenograft model, and a miR-199-dependent conditional replicative adenovirus was able to control tumor growth both in an HCC xenograft model and in an HCC transgenic mouse.73,74 For more comprehensive reviews, see Callegari et al.50,54

### Table 3 miRNA alterations with potential prognostic impact in patients with HCC

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Molecular alteration</th>
<th>Clinical significance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 miRNAs</td>
<td>Signature</td>
<td>Venous metastasis, overall survival</td>
<td>131</td>
</tr>
<tr>
<td>19 miRNAs</td>
<td>Signature</td>
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<td>miR-19a, miR-886-5p, miR-126, miR-223, miR-24 and miR-147</td>
<td>Signature</td>
<td>Predictor of overall survival and recurrence-free survival after LT</td>
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<tr>
<td>miR-26a</td>
<td>Downregulation</td>
<td>Poor survival</td>
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<tr>
<td>miR-122</td>
<td>Downregulation</td>
<td>Gain of metastatic properties</td>
<td>135, 136</td>
</tr>
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<td>miR-122</td>
<td>Downregulation</td>
<td>Early recurrence</td>
<td>96</td>
</tr>
<tr>
<td>Let-7 members</td>
<td>Downregulation</td>
<td>Early recurrence</td>
<td>137</td>
</tr>
<tr>
<td>miR-199-3p</td>
<td>Downregulation</td>
<td>Reduced time to recurrence</td>
<td>93</td>
</tr>
<tr>
<td>miR-199b-5p</td>
<td>Downregulation</td>
<td>Poor overall survival and progression-free survival rates</td>
<td>136</td>
</tr>
<tr>
<td>miR-101</td>
<td>Downregulation</td>
<td>Advanced tumor progression, poor prognosis</td>
<td>139</td>
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<tr>
<td>miR-125a</td>
<td>Upregulation</td>
<td>Better survival</td>
<td>140</td>
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<td>miR-92, miR-20, miR-18</td>
<td>Upregulation</td>
<td>Poor differentiation</td>
<td>141</td>
</tr>
<tr>
<td>miR-372</td>
<td>Upregulation</td>
<td>Advanced TNM stage</td>
<td>142</td>
</tr>
<tr>
<td>miR-221</td>
<td>Upregulation</td>
<td>Multinodularity; reduced time to recurrence</td>
<td>143</td>
</tr>
<tr>
<td>miR-221</td>
<td>Upregulation</td>
<td>Gain of metastatic properties</td>
<td>144</td>
</tr>
<tr>
<td>miR-221</td>
<td>Upregulation</td>
<td>High tumor capsular infiltration</td>
<td>145</td>
</tr>
<tr>
<td>miR-17-5p</td>
<td>Upregulation</td>
<td>Multiple tumor nodules; vein invasion; shortened overall survival</td>
<td>146</td>
</tr>
<tr>
<td>miR-155</td>
<td>Upregulation</td>
<td>High recurrence and poor prognosis following OLT</td>
<td>147</td>
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<tr>
<td>miR-203</td>
<td>Upregulation</td>
<td>Good prognosis</td>
<td>148</td>
</tr>
<tr>
<td>miR-18</td>
<td>Upregulation</td>
<td>Poor prognosis</td>
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<tr>
<td>miR-20a</td>
<td>Downregulation</td>
<td>Poor survival and tumor recurrence in HCC patients who underwent LT</td>
<td>150</td>
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<tr>
<td>miR-185</td>
<td>Downregulation</td>
<td>Short overall survival and time to recurrence in early-stage HCC</td>
<td>151</td>
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<tr>
<td>miR-146</td>
<td>Downregulation</td>
<td>Related to clinical TNM stage, metastasis, portal vein tumor embolus, and multiple tumor nodes</td>
<td>152</td>
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<tr>
<td>miR-139</td>
<td>Downregulation</td>
<td>HCC risk association and short-term survival</td>
<td>153</td>
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<tr>
<td>miR-25</td>
<td>Upregulation</td>
<td>Poor prognosis</td>
<td>154</td>
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</table>


**Abbreviations:** HCC, hepatocellular carcinoma; TNM, tumor node metastasis; LT, liver transplant; OLT, orthotopic liver transplant.

### Nanotechnologies for in vivo delivery of miRNAs

In addition to providing encouraging proofs of principle for the application of miRNA-based therapies, the miRNA studies described above revealed weaknesses of the approaches. Although several strategies have been adopted to increase biological stability (chemical modifications of the backbone, glycosylation, nucleic acid locking), limitations surfaced that were...
Table 4 Circulating microRNAs in liver diseases

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Sample</th>
<th>Clinical condition</th>
<th>Clinical value</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-122</td>
<td>Serum</td>
<td>High levels in patients with HCC or chronic hepatitis; lower levels in severe stage of fibrosis</td>
<td>Biomarker for liver injury but not specific for HCC; indicator of fibrosis progression in CHC infection; marker to distinguish CHC patients from healthy controls</td>
<td>155–159</td>
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<tr>
<td>miR-21</td>
<td>Serum/ plasma</td>
<td>High levels in patients with HCC or chronic hepatitis; higher levels in patients with HCC than in patients with chronic hepatitis and healthy volunteers</td>
<td>Biomarker for liver injury but not specifically for HCC; biochemical marker for HCC</td>
<td>156,160–162</td>
</tr>
<tr>
<td>miR-223</td>
<td>Serum</td>
<td>High levels in patients with HCC or chronic hepatitis</td>
<td>Biomarkers for liver injury but not specifically for HCC</td>
<td>160</td>
</tr>
<tr>
<td>miR-885-5p</td>
<td>Serum</td>
<td>High levels in patients with HCC, LC, and CHB</td>
<td>Complementary biomarker for the detection and assessment of liver pathologies</td>
<td>163</td>
</tr>
<tr>
<td>miR-16</td>
<td>Serum</td>
<td>Higher levels in NAFLD patients than in controls</td>
<td>Correlation with liver enzymes levels, fibrosis stage, and inflammation activity; biomarkers of diagnosis and histological disease severity in patients with CHC or NAFLD</td>
<td>156</td>
</tr>
<tr>
<td>miR-34a</td>
<td>Serum</td>
<td>High levels correlated with tumor size, cirrhosis, and tumor stage</td>
<td>Predictive significance for prognosis of HCC patients</td>
<td>162</td>
</tr>
<tr>
<td>miR-221</td>
<td>Serum</td>
<td>Higher levels in tumors during the exploration phase on resected tumor/adjacent non-tumor tissues; lower levels after surgery</td>
<td>Biomarker with clinical value for HCC screening</td>
<td>164</td>
</tr>
<tr>
<td>miR-15b</td>
<td>Serum</td>
<td>High levels in HCV-infected fibrosis patients compared with healthy volunteers or non-HCV-associated liver disease; higher levels in acute and chronic HCV-infected patients as compared with healthy volunteers</td>
<td>Biomarkers for early detection of HCV infection; miR-20a is a predictive biomarker of HCV-mediated fibrosis</td>
<td>165</td>
</tr>
<tr>
<td>miR-130b</td>
<td>Serum</td>
<td>Higher levels in HBV patients with HCC than in healthy controls</td>
<td>Non-invasive biomarker for HBV-related HCC screening</td>
<td>166</td>
</tr>
<tr>
<td>miR-20a</td>
<td>Plasma/serum</td>
<td>High levels in HCV-infected fibrosis patients compared with healthy volunteers or non-HCV-associated liver disease; higher levels in acute and chronic HCV-infected patients as compared with healthy volunteers</td>
<td>Biomarkers for early detection of HCV infection; miR-20a is a predictive biomarker of HCV-mediated fibrosis</td>
<td>165</td>
</tr>
<tr>
<td>miR-92a</td>
<td>Serum</td>
<td>Associated with metastasis status and TNM stages. HCC patients with high expression of serum miR-17-5p show a significantly shortened overall survival</td>
<td>Complementary biomarker for the detection and assessment of liver pathologies</td>
<td>163</td>
</tr>
<tr>
<td>miR-18a</td>
<td>Serum</td>
<td>Higher levels in HBV patients with HCC than in healthy controls</td>
<td>Non-invasive biomarker for HBV-related HCC screening</td>
<td>166</td>
</tr>
<tr>
<td>miR-17-5p</td>
<td>Serum</td>
<td>Associated with metastasis status and TNM stages. HCC patients with high expression of serum miR-17-5p show a significantly shortened overall survival</td>
<td>Biomarker for the prognostic prediction of HCC patients</td>
<td>38</td>
</tr>
<tr>
<td>miR-101</td>
<td>Serum</td>
<td>Inverse correlation between serum miR-101 levels and tissue miR-101 expression levels. High levels in patients with HBV-related HCC versus healthy controls: this increase correlated with hepatitis B surface antigen positivity, HBV DNA levels, and tumor size</td>
<td>Biochemical marker for monitoring the progression of tumor development in HBV-related HCC</td>
<td>167</td>
</tr>
<tr>
<td>miR-101</td>
<td>Serum</td>
<td>Serum miR-101 levels were found to be significantly downregulated in HBV-HCC patients compared with HBV-LC patients, CHB patients, and healthy controls, but were upregulated in HBV-LC patients compared with CHB patients and healthy controls. Consistent with the serum data, the expression of miR-101 was also upregulated and downregulated in the HBV-LC and HBV-HCC tissue samples, respectively</td>
<td>Non-invasive biomarker to differentiate HBV-HCC from HBV-LC</td>
<td>168</td>
</tr>
<tr>
<td>miR-125b-5p and miR-223-3p</td>
<td>Serum</td>
<td>High levels of miR-125b-5p in CHB, HBV-positive cirrhosis, and HBV-positive HCC compared with control group. Low levels of miR-223-3p were detected in same comparisons</td>
<td>Non-invasive biomarkers of HBV-positive HCC in very early disease, even at CHB stage of liver disease</td>
<td>169</td>
</tr>
<tr>
<td>miR-16, let-7f, and miR-21</td>
<td>Serum</td>
<td>Low levels in patients with a tumor more than 5 cm in diameter, correlated with clinical features such as platelets and bilirubin. High levels of serum let-7f in patients with a tumor more than 5 cm in diameter and early recurrence. High levels of miR-21 in female patients with HCC</td>
<td>Potential indicators to estimate tumor size or recurrence of HCC</td>
<td>170</td>
</tr>
<tr>
<td>microRNA-21</td>
<td>Serum</td>
<td>High levels of miR-21 expression correlated with cirrhosis and advanced tumor stage</td>
<td>Potential biomarker for early detection of HCC. No correlation of miR-21 expression with other clinical features including age, sex, and HBV infection</td>
<td>171</td>
</tr>
<tr>
<td>miR-143 and miR-215</td>
<td>Serum</td>
<td>High levels in patients with chronic hepatitis and HCC</td>
<td>Valuable biomarkers for chronic hepatitis (HBV and HCV) and HCC</td>
<td>172</td>
</tr>
</tbody>
</table>

(Continued)
related to stability, stimulation of the immune system, and off-target effects; the need to find a solution thus became evident. The development of more effective delivery approaches was undertaken to improve the potential of miRNA-based therapies after their in vivo systemic administration.

Among the approaches investigated to improve in vivo delivery of drugs, the use of nanoparticles is of particular interest. Different types of nanoparticles exist, range between 20 and 200 nm in diameter, and are formulated with different materials, including lipids, polymers, and inorganic materials. First-generation formulations consisted of nanoparticles associated with anticancer drugs that were administered intravenously to carry the drug to the tumor through “passive tumor targeting”. As a result of the pathophysiologic differences between healthy and tumor-associated blood vessels and to the high production of mediators of permeability in tumors, these nanodrugs could accumulate in tumors through the enhanced permeability and retention effect.

Nanoparticles have also been used to convey molecules such as DNA or RNA oligonucleotides to tumor cells. First studies proving the delivery of oligonucleotides reported the use of stable nucleic acid lipid particles. In a formulation based on cationic lipid nanoparticles (LNPs), the delivery of short interfering RNA (siRNA) antiapoptoprotein B and proprotein convertase subtilisin/kexin type 9 in mice and nonhuman primates was reported to inhibit hypercholesterolemia, with a rapid and dose-dependent gene silencing effect. Various additional formulations of nanoparticles were subsequently proposed to verify the possible use of oligonucleotides as anticancer agents. The main types of nanoparticles used for conveying siRNA and miRNA are cationic liposomes or positively charged polymers, such as polyethylenimine, or water-insoluble polymers, such as poly-lactide-co-glycolide. For example, in a diethylaminoethanol-induced HCC mouse model, systemic administration of miR-124 through liposomes led to a reduction in tumor growth and tumor size via induction of apoptosis and deregulation of an miRNA/inflammatory feedback loop circuit involving hepatocyte nuclear factor 4 alpha (see Table 5 for a list of nanocarrier formulations used in miRNA delivery). Trang et al have used neutral lipid emulsions to mediate the delivery of miR-let-7 and miR-34a in a non-small cell lung cancer model. The delivery of both miRNAs inhibited tumor growth, confirming their role as tumor suppressors and their therapeutic potential in the treatment of lung cancer. Hsu et al used modified cationic LNPs to convey miR-122 in a mouse model of HCC. After intravenous administration, miR-122 was found to accumulate in both normal and tumor liver cells; no toxicity or induced immune response was detected. Significant suppression of tumor growth related to a reduction in expression of molecular targets of miR-122 was also reported. Wang et al also recently discovered that the inclusion of an unsaturated fatty acid, called “helper lipid”, in the formulation of LNPs significantly improved transport of miR-122, resulting in its accumulation in the liver. A formulation of cationic LNPs for the delivery of two different siRNAs, ie, anti-vascular endothelial growth factor and anti-kinesin spindle protein (ALN-VSP02), is currently in a Phase I clinical trial (ClinicalTrials identifiers NCT00882180 and NCT01158079) in patients with hepatic and extrahepatic tumors. The study showed that this formulation of LNP-siRNA is not toxic and is well tolerated.

The delivery of nucleic acid molecules with nanoparticles has been improved by the addition of specific ligands (targeted nanodrugs). This approach made it possible to convey siRNAs/miRNAs to cancer cells that present specific receptors or antigens (“active tumor targeting”). An example of this strategy is given by miR-34, which is encapsulated in a liposome-polycaction-hyaluronic acid nanoparticle formulation modified with a GC4 single-chain variable antibody fragment, a tumor-targeting human monoclonal antibody used against melanoma metastases. Systemic injection of miR-34 liposome-polycaction-hyaluronic acid-modified nanoparticles in mice with metastasis of B16F10 melanoma cells in the lung significantly inhibited the growth of cancer cells.

Table 4 (Continued)

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Sample</th>
<th>Clinical condition</th>
<th>Clinical value</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-R00a, miR-21, miR-122 and miR-224-5p</td>
<td>Serum</td>
<td>High levels of miR-200a, miR-21, miR-122, and miR-224-5p in HCC following TACE was associated with a decreased overall survival</td>
<td>miR-200a may be a promising prognostic biomarker in HCC patients. Patients with a high risk of TACE treatment failure may benefit from measurement of miR-200a in serum</td>
<td>173</td>
</tr>
<tr>
<td>miR-375 and miR-199a-3p</td>
<td>Serum</td>
<td>Low levels in HCC patients in comparison with healthy controls</td>
<td>Potential biomarkers in early-stage HCC</td>
<td>174</td>
</tr>
</tbody>
</table>


Abbreviations: HCC, hepatocellular carcinoma; CHC, chronic hepatitis C; CHB, chronic hepatitis B; LC, liver cirrhosis; NAFLD, non-alcoholic fatty liver disease; HCV, hepatitis C virus; HBV, hepatitis B virus; HBV-HCC, HBV-associated hepatocellular carcinoma; HBV-LC, HBV-associated liver cirrhosis; TACE, transarterial chemoembolization; TNM, tumor node metastasis.
without inducing toxicity. The same miR-34, encapsulated in silica nanoparticles conjugated with antibody anti-GD(2), a cell surface antigen disialoganglioside, was specifically conveyed to neuroblastoma tumor cells in a murine orthotopic xenograft model; there was a consequent significant decrease in tumor growth, increased apoptosis, and reduced vascularization.

In addition to nanotechnology approaches for the delivery and restoration of miRNA levels in target cells, nanoparticles have also been used for transporting anti-miRNAs. For example, interfering nanoparticles have been designed and prepared to convey a chemically stabilized anti-miR-122 molecule into the mouse liver. These nanoparticles present amino acids on their surface, with an excellent binding affinity for negatively charged molecules, such as siRNA or miRNA. This approach led to the specific silencing of miR-122 in the liver without inducing an immune response, and suggested its potential use for the delivery of anti-miRNAs or miRNA mimics for therapy against HCC.

To date, no studies have compared different formulations in the same preclinical model or with the same molecule, so a comparative assessment of the different formulations in terms of efficacy and safety of delivery is difficult. However, data emerging from recent studies strongly suggest the usefulness of nanotechnology approaches for implementing miRNA-based therapeutics against HCC.

### miRNA and chemoresistance

A potentially important area of investigation and application of miRNA-based therapies is represented by resistance to drugs. Drug resistance is one of the main obstacles to the treatment of tumors, as most patients become insensitive to therapies that have until then been effective. This insensitivity causes treatment failure and tumor relapse.

Altered levels of miRNAs in cancer may significantly affect the sensitivity to chemotherapy drugs. For example, the sensitivity of HCC cells to apoptosis induced by doxorubicin is significantly influenced by two miRNAs, miR-199a-3p and
miR-26b. Evaluation of miRNA levels that predict the response to therapy could be needed to set the correct treatment for a patient. To overcome acquired resistance to sorafenib, for example, the synergistic combination with other molecules may potentially represent an improvement in therapeutic options. In fact, it has been shown that enforced expression of miR-122 sensitizes HCC cells to sorafenib93 or doxorubicin,96 while restoration of miR-34a in HCC cells treated with sorafenib increased apoptosis induced by the drug.97

In this context, nanotechnology approaches may represent a useful and important aid. Nanoparticle formulations combining chemotherapy drugs and siRNAs have been proposed to prevent drug resistance, for example, the use of anti-Myc combined with doxorubicin against fibrosarcoma cells in vitro and in vivo, or anti-Bcl2 combined with doxorubicin against glioma cells in vitro and in vivo (reviewed recently in Gandhi et al94). miRNA-based molecules have also been used in combination with chemotherapeutic drugs in preclinical models through cationic LNPs or hyaluronic acid-chitosan nanoparticles. For example, the synergistic effect of miR-34a in combination with paclitaxel has been shown to inhibit growth of melanoma metastases to the lung,99 and growth of breast cancer when miR-34a was used in combination with doxorubicin.100 A polymer formulation has been developed to co-channel miR-205 and gemcitabine against pancreatic cancer.101–103 Intratumoral administration of these polyplexes in xenografted mice resulted in a significant reduction in the growth rate and weight of tumors compared with that in the control groups and showed a high biocompatibility of the micelles in vivo.104 Qian et al have synthesized new amphiphilic star-branched copolymers (PLA-PDMAEMA) for co-channeling of an inhibitor of miR-21 and doxorubicin into xenografts of glioma cells, leading to a significant synergistic inhibition of tumor growth105 and demonstrating the potential of the therapeutic combination.

These data suggest that use of molecules based on miRNA (miRNA mimics or anti-miRNAs) may intensify the therapeutic effects of chemotherapy drugs by specifically hitting crucial signaling pathways, thereby helping to enhance their efficacy and/or preventing resistance. Although not yet applied to HCC, the principles that miRNAs have established can open the way to studies for their application in HCC as well.

Conclusion
Knowledge of the role of miRNAs in HCC established the foundation for studying the antitumor activity of miRNA mimics or anti-miRNAs in HCC. These studies were made possible by the availability of animal models, induced either by use of carcinogens or by genetic modification. At least one of the existing models, the miRNA deregulation profile of which resembles that found in the human counterpart,47 may be adequate to investigate miRNA-based therapies. Still, some critical issues have also emerged. It is well known that human HCC occurs on a background of cirrhosis in 80% of cases, while this feature is generally not found in available animal models, which develop HCC in the absence of cirrhosis106 or develop cirrhosis but not HCC.106,107 Thus, models that could reproduce the natural history of human HCC could also improve preclinical studies aimed at prevention and treatment of HCC, possibly allowing the testing of new anticancer therapies on a cirrhotic background, which may help to limit failure in future clinical trials.

Another limitation of new therapies based on miRNA is the in vivo instability of the molecules. In addition to chemical modifications, a crucial point for therapy based on miRNAs is represented by the necessity of optimal delivery systems. Recent advances made in the field of nanocarriers have shown the way, ensuring greater protection of oligonucleotides and better targeted delivery to cancer cells, allowing more efficient transport of these molecules in vivo.

Several studies have also indicated that miRNAs can potentially sensitize cancer cells to chemotherapy. In vitro and in vivo evidence indicates increased therapeutic efficacy of the co-conveying miRNA/drug, suggesting a possible use of miRNAs in combination with drugs currently in clinical use. There are still no results for HCC, but available data are encouraging and the possibility of new systems for co-channeling miRNA/drug therapy against HCC with better characteristics in terms of efficacy or tolerability appears to be an important area of investigation.

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Disclosure
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