Gastrointestinal Cancer: Targets and Therapy

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REVIEW

Emerging role of microRNAs in the treatment of hepatocellular carcinoma

This article was published in the following Dove Press journal:
Gastrointestinal Cancer: Targets and Therapy
14 May 2015
Number of times this article has been viewed

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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.[1] In 80%–90% of cases, HCC arises on a background of cirrhosis,[2] a chronic and diffuse hepatic disease that results from continuous liver injury and regeneration.[3] 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.[4–9]

The molecular mechanisms of liver tumorigenesis have been studied, and genetic and epigenetic alterations are reported to dysregulate several important signaling pathways,[10–13] 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.[14–18] 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.[10] Recurrent mutations in the MLL family of histone methyltransferases are also reported to result in alteration of chromatin remodelers.[13]

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Several therapeutic approaches are used nowadays for the management of patients with HCC. According to the Barcelona Clinic Liver Cancer staging system,[19] potential curative treatment of eligible patients with early-stage HCC consists of resection, percutaneous ablation, and liver transplantation.[10] Transarterial chemoembolization was shown to provide survival benefits in patients with intermediate-stage HCC,[20,21] but is not curative.[22] 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.[23,24] 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.[25] 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.[26–28] 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).[29] Evaluation of other molecular targeted agents as a second-line treatment showed unsatisfactory results; both the mTOR inhibitor everolimus[30] (EVOLVE, ClinicalTrials identifier NCT01035229) and the multikinase inhibitor brivanib[31] 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.[32,33]

Several limits have been identified in the settings of recent clinical trials and corrective actions proposed to reduce the risk of trial failure.[34] 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.[35] 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,[36] thus lowering the levels of active miR-122. Hbx protein decreases levels of several tumor suppressor miRNAs, such as miR-34 and let-7,[37] while HBx RNA directly downregulates miR-15a and miR-16-1.[38] Moreover, upregulated gene, upregulation of which is induced by HBx, could induce miR-148a overexpression and promote cell cycle progression and cell migration.[39,40] The mechanisms of miRNA deregulation by hepatitis C virus have been less extensively studied.[41] 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.[42]

Animal models based on miRNAs have confirmed their role in tumorigenesis, both in hematopoietic diseases[43–46] and in solid tumors, such as HCC,[47–49] thereby allowing preclinical studies based on the inhibition or restoration of miRNA (see Callegari et al[50] 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 1 microRNAs with consistently deregulated expression in human hepatocellular carcinoma

miRNA	Genome location	Expression in HCC	Targets
let-7g	3: 52302294–52302377 (–)	Down	BCL2L1, COLIA2
miR-1	20: 61151513–61151583 (+)	Down	MET, FOXP1, HDAC4
miR-23b	9: 97847490–97847586 (+)	Down	uPA, MET
miR-26a	3: 38010895–38010971 (+)	Down	CCND2, CCNE
miR-29	7: 130561506–130561569 (–)	Down	BCL2, MCL1
miR-34a	1: 9211727–9211836 (–)	Down	MET
miR-101	1: 65524117–65524191 (–)	Down	MCL1, FOS
miR-122	18: 56118306–56118390 (+)	Down	CCNG1, SRF, IGF1R, BCL2L2, ADAM10, ADAM17
miR-124	8: 9760898–9760982 (–)	Down	CDK6, VIM, SMYD3, IQGAP1
miR-125a	19: 52196507–52196592 (+)	Down	BMF, ERBB2, ERBB3
miR-125b	11: 121970465–121970552 (–)	Down	
miR-130a	11: 57408671–57408759 (+)	Down	ATXN1, PPARγ
miR-139	11: 72326107–72326174 (–)	Down	RHOK2
miR-145	5: 148810209–148810296 (+)	Down	FSCN1, IRS1, STAT1, YES, MYC, ESR1, KLF4, OCT4, SOX2, MUC1
miR-150	19: 50004042–50004125 (–)	Down	EGR2
miR-193b	16: 14397824–14397906 (+)	Down	MCL1
miR-195	17: 6920934–6921020 (–)	Down	CCND1, CDK6, E2F3
miR-199a-1	19: 10928102–10928172 (–)	Down	KRT7, SET, IKBKB, MAPK1, MET, HES1, Smad1, HIF1A
miR-199a-2	1: 172113675–172113784 (–)	Down	
miR-199b	9: 131007000–131007109 (–)	Down	
miR-200a	1: 1103243–1103332 (+)	Down	ZEB1, ZEB2, beta-catenin
miR-200b	1: 1102484–1102578 (+)	Down	
miR-223	X: 65238712–65238821 (+)	Down	Stathmin1
miR-375	2: 219866367–219866430 (–)	Down	YAP
miR-602	9: 140732871–140732968 (+)	Down	RASSF1A
miR-17-5p	13: 92002859–92002942 (+)	Up	NCOA3, E2F1, BCL2L11, CDKN1A, RBL2, MAPK14, STAT3, CCL1, DNAJC27, FBXO31, GPR137B, NPAT, OBFC2A, RAB12,YES1, ZNFX1, FN1, FNDC3A
miR-18a	13: 92003005–92003075 (+)	Up	NR3C1, CTGF, ESR1
miR-92a	13: 92003568–92003645 (+)	Up	HIF1A, STAT3, CDKN1A, MAPK14, ZBTB7A, E2F1, E2F2, E2F3
miR-106-25	7: 99691616–99691697 (–)	Up	CDKN1A, BIM
miR-21	17: 57918627–57918698 (+)	Up	FasL, SERPINB5, PDCD4, TIMP3, SPRY2, LRRFIP1, RECK, PTEN, BTG2, Peli1, HNRPK, TP63, MARCKS, TPM1
miR-30d	8: 135817119–135817188 (–)	Up	Galphai2
miR-151	8: 141742663–141742752 (–)	Up	RhoGDIA
miR-181b-1	1: 198828002–198828111 (–)	Up	CDX2, GATA6, NLK, TIMP3
miR-135a	3: 52328235–52328324 (–)	Up	APC
miR-221/ miR-222	X: 45605585–45605694 (–)	Up	BMF, CDKN1B, CDKN1C, ESR1, ICAM1, KIT, PTEN, TIMP3, MET, DDIT4, FOXO3
miR-224	X: 151127050–151127130 (–)	Up	CD40, CDC42, CXCR4, KLK10, Smad4, API5
miR-373	19: 54291959–54292027 (+)	Up	MBD2, CD44, LATS2
miR-483-3p	11: 2155364–2155439 (–)	Up	BBC3

Abbreviation: HCC, hepatocellular carcinoma.

studies, showing that multigroup analysis on miRNA panels could give better diagnostic accuracy than single miRNAs.[51] 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 "antagomir" oligonucleotides in mice.[55] 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.[56] Following these studies, a locked nucleic acid-modified anti-miR-122 (miravirsen, SPC3649, Santaris Pharma, Copenhagen, Denmark) entered into clinical trials for the

Table 2 microRNA-based therapies in preclinical models

miRNAs	Mouse model	Tumor	Method	Delivery system	Reference
miR-221	Xenograft	Prostate carcinoma	miRNA inhibition	Antagomir	108
miR-221	Xenograft	Melanoma	miRNA inhibition	Antagomir oligonucleotide	109
miR-221	Xenograft	Multiple myeloma	miRNA inhibition	mirVana custom inhibitor	110
miR-221	Orthotopic model	HCC	miRNA inhibition	Cholesterol-modified isoform of anti-miR-221	66
miR-221	TG-221	HCC	miRNA inhibition	anti-miRNA oligonucleotide	47
miR-494	tet-o-MYC; LAP-tTA	HCC	miRNA inhibition	miR-494 anti-miR	111
miR-143	Xenograft	Colorectal cancer	miRNA replacement	Chemically modified benzene(B)-pyridine(P)-analog at the 3′-overhang region	112
miR-101	Xenograft	HCC	miRNA replacement	RNA duplex	113
miR-29b	Xenograft	HCC	miRNA replacement	2′-O-methyl-modified oligoribonucleotides	114
miR-199a/b-3p	Xenograft	HCC	miRNA replacement	AAV	68
miR-199a/b-3p	Xenograft	HCC	miRNA replacement	Lentivirus	115
miR-375	Xenograft	HCC	miRNA replacement	Cholesterol-conjugated 2′-O-methyl-modified miRNA	116
miR-31	Xenograft	Metastasis	miRNA replacement	dox-repressible modified miR-31 miRNA sponge vector system	117
miR-34a	Xenograft	Multiple myeloma	miRNA replacement	Lentivirus	118
Let-7	Xenograft; *LSL-K-ras G12D*	NSCLC	miRNA replacement	Synthetic miRNAs complexed with siPORT amine; lentivirus	119
miR-26a	tet-o-MYC; LAP-tTA	HCC	miRNA replacement	AAV	67
miR-122	tet-o-MYC; LAP-tTAT;	HCC	miRNA replacement	AAV	48
miR-422	Xenograft; DEN-induced HCC mouse model	HCC	miRNA replacement	Oligo mimics; lentivirus	120
Let-7	Xenograft	Colon and lung carcinoma	Oncolytic virus	Vesicular stomatitis virus	121
Let-7	Xenograft	Lung and pancreatic carcinoma	Oncolytic virus	Vaccinia virus	122
Let-7	Xenograft	HCC	Oncolytic virus	Adenovirus	73
miR-122	Xenograft	HCC	Oncolytic virus	Adenovirus	123
miR-122	Xenograft	Lung cancer	Oncolytic virus	Adenovirus	124
miR-143; miR-145	Xenograft	Prostate cancer	Oncolytic virus	Herpes simplex virus	125
miR-122; miR-124; let-7	Xenograft	HCC	Oncolytic virus	Herpes simplex virus	126
miR-7	Xenograft	Gliomas	Oncolytic virus	Measles virus	127
miR-133a and miR-206	Xenograft	Prevents fatal myositis	Oncolytic virus	Coxsackievirus A21	128
miR-199	Xenograft; TG-221	HCC	Oncolytic virus	Adenovirus	74
miR-124	Orthotopic model of primary human GBM	Glioma	Oncolytic virus	Herpes simplex virus	129
miR-124, miR-128, miR-146b and miR-218	Xenografts	Glioma	Oncolytic virus	Adenovirus	130

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

treatment of hepatitis C virus infection,[57] since efficient replication of this virus in the liver depends on miR-122 expression.[58–62] A combination approach has also been proposed for the treatment of hepatitis C virus infection through the use of antiviral drugs (telaprevir and ribavirin) with miRNA-based therapy (miravirsen, ClinicalTrials identifier NCT01872936).[63] Similarly, since hepatitis B virus affects miRNA cellular levels in several ways, new miRNA-based approaches could represent a promising area of investigation for treatment of hepatitis B virus infection and reduction of the risk of HCC.[37]

Although modulation of hepatitis C virus by miravirsen has potential implications for the prevention of HCC, other anti-miRNA molecules have been evaluated in preclinical studies (Table 2). In particular, considering the role of miR-221 in HCC as a tumor promoter,[64,65] anti-miR-221 molecules were used in murine models of HCC, where their therapeutic potential was demonstrated.[47,66] Other studies

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

miRNAs	Molecular alteration	Clinical significance	References
20 miRNAs	Signature	Venous metastasis, overall survival	131
19 miRNAs	Signature	Poor survival	132
miR-19a, miR-886-5p, miR-126, miR-223, miR-24 and miR-147	Signature	Predictor of overall survival and recurrence-free survival after LT	133
miR-26a	Downregulation	Poor survival	134
miR-122	Downregulation	Gain of metastatic properties	135,136
miR-122	Downregulation	Early recurrence	96
Let-7 members	Downregulation	Early recurrence	137
miR-199a-3p	Downregulation	Reduced time to recurrence	93
miR-199b-5p	Downregulation	Poor overall survival and progression-free survival rates	138
miR-101	Downregulation	Advanced tumor progression, poor prognosis	139
miR-125a	Upregulation	Better survival	140
miR-92, miR-20, miR-18	Upregulation	Poor differentiation	141
miR-372	Upregulation	Advanced TNM stage	142
miR-221	Upregulation	Multinodularity; reduced time to recurrence	143
miR-221	Upregulation	Gain of metastatic properties	144
miR-221	Upregulation	High tumor capsular infiltration	145
miR-17-5p	Upregulation	Multiple tumor nodules; vein invasion; shortened overall survival	146
miR-155	Upregulation	High recurrence and poor prognosis following OLT	147
miR-203	Upregulation	Good prognosis	148
miR-18	Upregulation	Poor prognosis	149
miR-20a	Downregulation	Poor survival and tumor recurrence in HCC patients who underwent LT	150
miR-185	Downregulation	Short overall survival and time to recurrence in early-stage HCC	151
miR-146	Downregulation	Related to clinical TNM stage, metastasis, portal vein tumor embolus, and multiple tumor nodes	152
miR-139	Downregulation	HCC risk association and short-term survival	153
miR-25	Upregulation	Poor prognosis	154

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

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-199a[68] could effectively and significantly reduce tumor growth by use of adeno-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, Kelnar 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-dependent[73] and miR-199-dependent[74] 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]

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

miRNAs	Sample	Clinical condition	Clinical value	References
miR-122	Serum	High levels in patients with HCC or chronic hepatitis; lower levels in severe stage of fibrosis	Biomarker for liver injury but not specific for HCC; indicator of fibrosis progression in CHC infection; marker to distinguish CHC patients from healthy controls	155–159
miR-21	Serum/ plasma	High levels in patients with HCC or chronic hepatitis; higher levels in patients with HCC than in patients with chronic hepatitis and healthy volunteers	Biomarker for liver injury but not specific for HCC; biochemical marker for HCC	156,160–162
miR-223	Serum	High levels in patients with HCC or chronic hepatitis	Biomarkers for liver injury but not specifically for HCC	160
miR-885-5p	Serum	High levels in patients with HCC, LC, and CHB	Complementary biomarker for the detection and assessment of liver pathologies	163
miR-16 miR-34a	Serum	Higher levels in NAFLD patients than in controls	Correlation with liver enzymes levels, fibrosis stage, and inflammation activity; biomarkers of diagnosis and histological disease severity in patients with CHC or NAFLD	156
miR-221	Serum	High levels correlated with tumor size, cirrhosis, and tumor stage	Predictive significance for prognosis of HCC patients	162
miR-15b miR-130b	Serum	Higher levels in tumors during the exploration phase on resected tumor/adjacent non-tumor tissues; lower levels after surgery	Biomarker with clinical value for HCC screening	164
miR-20a miR-92a	Plasma/ serum	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	Biomarkers for early detection of HCV infection; miR-20a is a predictive biomarker of HCV-mediated fibrosis	165
miR-18a	Serum	Higher levels in HBV patients with HCC than in healthy controls	Non-invasive biomarker for HBV-related HCC screening	166
miR-17-5p	Serum	Associated with metastasis status and TNM stages. HCC patients with high expression of serum miR-17-5p show a significantly shortened overall survival	Biomarker for the prognostic prediction of HCC patients	38
miR-101	Serum	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	Biochemical marker for monitoring the progression of tumor development in HBV-related HCC	167
miR-101	Serum	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	Non-invasive biomarker to differentiate HBV-HCC from HBV-LC	168
miR-125b-5p and miR223-3p	Serum	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	Non-invasive biomarkers of HBV-positive HCC in very early disease, even at CHB stage of liver disease	169
miR-16, let-7f, and miR-21	Serum	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	Potential indicators to estimate tumor size or recurrence of HCC	170
microRNA-21	Serum	High levels of miR-21 expression correlated with cirrhosis and advanced tumor stage	Potential biomarker for early detection of HCC. No correlation of miR-21 expression with other clinical features including age, sex, and HBV infection	171
miR-143 and miR-215	Serum	High levels in patients with chronic hepatitis and HCC	Valuable biomarkers for chronic hepatitis (HBV and HCV) and HCC	172

(*Continued*)

Table 4 (*Continued*)

miRNAs	Sample	Clinical condition	Clinical value	References
miR-200a, miR-21, miR-122 and miR-224-5p	Serum	High levels of miR-200a, miR-21, miR-122, and miR-224-5p in HCC following TACE was associated with a decreased overall survival	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	173
miR-375 and miR-199a-3p	Serum	Low levels in HCC patients in comparison with healthy controls	Potential biomarkers in early-stage HCC	174

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.

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.[75] 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 pathophysiological 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.[76]

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) antiapolipoprotein 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.[77,78] 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.[79] For example, in a diethylnitrosamine-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[80] (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.[81] 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.[82] 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.[83] 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.[84]

The delivery of nucleic acid molecules with nanoparticles has been improved by the addition of specific ligands (targeted nanodrugs).[85] 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-polycation-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-polycation-hyaluronic acid-modified nanoparticles in mice with metastasis of B16F10 melanoma cells in the lung significantly inhibited the growth of cancer cells

Table 5 Representative nanocarrier delivery approaches for microRNA-based therapies in vivo

miRNAs	Mouse model	Tumor	Method	Nanoparticle delivery system	Reference
miR-21	Xenograft	Multiple myeloma	miRNA inhibition	NLE-formulated synthetic oligo	175
miR-155	*mir-155*^{LSLtTA}	B-cell lymphomas	miRNA inhibition	Anti-miR-loaded PLGA nanoparticles	176
miR-296	Xenograft	Angiogenesis	miRNA inhibition	cRGD-LPH-NP; AMOs	177
miR-34	Xenograft	Metastasis	miRNA replacement	LPH NP-modified with GC4 single-chain variable antibody fragment (scFv)	86
miR-34	Xenograft	Neuroblastoma	miRNA replacement	Silica nanoparticles conjugated with an antibody GD (2)	87
miR-122	Xenograft	HCC	miRNA replacement	Cationic lipid nanoparticles	82
miR-124	DEN-induced HCC mouse model	HCC	miRNA replacement	Liposomes	80
miR-34a	Kras$^{LSL\text{-}G12D/+}$; Trp53$^{LSL\text{-}R172H/+}$	Lung adenocarcinoma	miRNA replacement	NLE particle-delivered miRNA mimic	178
miR-29b	Xenograft	NSCLC	miRNA replacement	Cationic lipoplexes	179
miR-34a; let-7	Xenograft; *LSL-K-ras G12D*	NSCLC	miRNA replacement	NLE particle-delivered miRNA mimic	81
miR-29b	Engraft	AML	miRNA replacement	Transferrin-conjugated anionic lipopolyplex nanoparticles (TF-Np-miR)	180
miR145; miR-33	Xenograft	Colon carcinoma	miRNA replacement	PEI/miRNA complex	181
miR-143; miR-145	Xenograft	Colorectal cancer	miRNA replacement	Synthetic miRNA encapsulated with cationic liposomes (LipoTrust)	182
miR-7	Xenograft	Angiogenesis; glioblastoma	miRNA replacement	Integrin-targeted biodegradable polymeric nanoparticles	183

Abbreviations: AMOs, anti-miRNA oligonucleotides; HCC, hepatocellular carcinoma; DEN, diethylnitrosamine; NSCLC, non-small cell lung cancer; AML, acute myeloid leukemia; NLE, neutral lipid emulsion; PLGA, poly-lactide-co-glycolide; NP, nanoparticles; PEI, polyethylenimine; LPH, liposome-polycation-hyaluronic acid.

without inducing toxicity.[86] 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.[87]

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.[88,89] 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. An optimized 2′-OMe-4′-modified thioribonucleoside anti-miR-122 has been conveyed to the mouse liver by using liposomes conjugated with a pH-sensitive cationic lipid, YSK05 (YSK05-MEND),[90] which proved to be effective in inhibiting miR-122 at low doses.[91] Another formulation based on LNPs conjugated with a ligand for the asialoglycoprotein receptor and peptide gramicidin A has recently been developed to inhibit miR-155. Its intravenous injection has allowed the efficient diffusion of molecules of anti-miR155 to the liver of wild-type mice, leading to a preferential accumulation of anti-miR-155 in hepatocytes and upregulation of miR-155 target genes.[92]

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.[93,94] 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 sorafenib[95] 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 al[98]). 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 growth[105] 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 cirrhosis[106] 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.

Acknowledgments

This work was supported by funding from the Associazione Italiana per la Ricerca sul Cancro (AIRC IG15615) and from the University of Ferrara (FAR projects 2012–2013) to MN.

Disclosure

The authors report no conflicts of interest in this work.

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