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Review

MicroRNAs in liver cancer: a model for investigating pathogenesis and novel therapeutic approaches

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MicroRNAs (miRNAs) constitute a large class of short RNAs (e.g., 20–24 nucleotides in length), whose main function is to posttranscriptionally regulate the expression of protein-coding genes. Their importance in tumorigenesis has been demonstrated over the past decade, and correspondingly, they have emerged as potential therapeutic molecules and targets. Liver cancer is one of the most common neoplastic diseases worldwide, and it currently has a poor prognosis owing to largely ineffective therapeutic options. Liver cancer is also an excellent model for testing miRNA-based therapy approaches as it can be easily targeted with the systemic delivery of oligonucleotides. In recent years, the role of miRNAs in hepatocellular carcinoma (HCC) has been established with molecular studies and the development of animal models. These studies have also provided the basis for evaluating the therapeutic potential of miRNAs, or anti-miRNAs. In general, the safety of miRNAs has been proven and antitumor activity has been observed. Moreover, because of the absence or presence of mild side effects, the prophylactic use of miRNA-based approaches may be foreseen.
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Facts

- Hepatocellular carcinoma (HCC) is a primary malignant disease of the liver with poor prognosis.
- With the exception of the multi-kinase inhibitor, sorafenib, there are no effective systemic treatments available for HCC.
- Several miRNAs are differentially expressed in HCC and may be pathogenically relevant.
- Genetically modified animal models have been developed to demonstrate the direct role of miRNAs in the initiation and/or progression of cancer.
- The *in vivo* therapeutic efficacy of certain miRNA mimics or anti-miRNA oligonucleotides has been evaluated.

Open Questions

- Do the animal models that are available faithfully represent the etiology and natural history of human HCC?
- Are methods for the *in vivo* systemic delivery of miRNAs/anti-miRNAs sufficiently developed, and can they be optimized?
- Are miRNA/anti-miRNA molecules effective against liver cancer?
- What are the clinical settings in which miRNA/anti-miRNA molecules may be successfully applied?

MicroRNAs (miRNAs) constitute a large class of short RNAs (e.g., 20–24 nucleotides in length), which have key roles in cell development and differentiation by mediating the post-transcriptional regulation of protein-coding genes.[1] Over the past decade, the critical role of miRNAs in tumorigenesis has been widely investigated and their important regulatory action in several biological processes that are frequently altered in cancer has been described. At present, miRNAs have a well-recognized role in human carcinogenesis, including hepatocarcinogenesis, and accumulating experimental evidence indicates that they may act as oncogenes or tumor suppressor genes.[2,3] It is also well known that the expression of several miRNAs is deregulated in human HCC compared with normal tissue.[4,5] In this context, miRNAs have also emerged as novel molecules or targets for tumor therapy, and liver cancer represents an excellent model for their testing.

HCC is the most common primary liver malignant disease, and the third cause of cancer-related deaths, worldwide.[6] It has a poor prognosis and the therapeutic options currently available are largely ineffective. Molecular studies have revealed several pathogenic mechanisms at the basis of liver cancer. For example, HCC pathogenesis involves multiple

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Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; miRNA, microRNA; DEN, *N*-nitrosodiethylamine; HGF, hepatocyte growth factor; CDK, cyclin-dependent kinase; IAP, inhibitor of apoptosis protein; ROCK2, rho-dependent kinase; AAV, adeno-associated virus; CRAds, conditionally replicating adenovirus

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genetic and epigenetic alterations that lead to the deregulation of several signaling pathways, including the p53, PI3K/Akt/mTOR, Ras/Raf/MEK/ERK, IGFR, and Wnt/β-catenin pathways (for reviews, see Gramantieri *et al.*[7] and Aravalli *et al.*[8]). Recent studies using next-generation sequencing technology have further refined our knowledge of the signaling pathways involved in liver carcinogenesis.[9–11] Regulation of these cellular signaling pathways by miRNAs indicates that this class of short RNAs may present a new set of potentially therapeutic tools for HCC.[12,13]

miRNAs and HCC

Several studies have revealed that the expression of certain miRNAs is deregulated in human HCC compared with matched nonneoplastic tissue (for a comprehensive review, see Negrini *et al.*[14]). Moreover, molecular and cellular studies have established that aberrantly expressed miRNAs can affect crucial cancer-associated pathways.[2,3]

Cell proliferation and survival. The discovery of modulated gene targets has helped recognize the contribution of miRNAs to cancer-associated pathways. Unrestricted cellular proliferation and prolonged survival have critical roles in the process of hepatocarcinogenesis. In the cell cycle context, abnormal expression of miRNAs has been shown to alter the functionality of several cell cycle regulators, including RB1, cyclin-dependent kinases (CDKs), cyclins, and CDK inhibitors. RB1 is a target of miR-335.[15] miR-335 is downregulated during mesenchymal stem cell differentiation[16] and upregulated by the Wnt signaling pathway,[16] which is often activated in HCC. RB1 is also a target of miR-221, a miRNA that is frequently overexpressed in HCC.[17,18] These observations suggest that expression of RB1, a key G1–S cell cycle transition checkpoint protein, may be aberrantly regulated by altered miRNA expression. Among the other proteins that control cell cycle progression (Figure 1), cyclins are reported to be upregulated in HCC, whereas negative cell cycle regulators are often downregulated compared with surrounding parenchyma.[19] Overexpression of cyclinD1/CDK4 has been detected in $\sim$60% of HCC cases.[20] Among CDK inhibitors, p16/INK4A is functionally inactivated owing to deletions in the short arm of chromosome 9 in about 20% of HCCs[21] or by promoter methylation in 30–70% of HCC cases.[22] All of these genes are also reported to be affected by altered expression of miRNAs (Figure 1). For example, miR-124 and miR-203, which inhibit the growth of HCC cells via downregulation of CDK6,[23] are methylated and silenced in HCC cell lines and primary tumors.[23] Direct targeting of cyclin D1, CDK6, and E2F3 by miR-195 can also block the G1–S transition, whereas inhibition of miR-195 promotes cell-cycle progression.[24] MiR-195 can also suppress the ability of HCC cells to form colonies *in vitro*, and the development of tumors in nude mice. In most HCC tissues and cell lines, miR-195 expression is reduced,[24] and this may contribute to the upregulation of cyclinD1/CDK that is observed in HCC. MiR-26 is another miRNA whose expression is reduced in HCC,[25] thereby preventing its induction of cell cycle arrest in liver cancer cells via the direct targeting of cyclins D2 and E2.[26] Members of the KIP family of CDK inhibitors, CDKN1A/p21, CDKN1B/p27, and CDKN1C/p57, also act as tumor suppressors in HCC by negatively affecting cell-cycle progression. CDKN1B/p27 and CDKN1C/p57 proteins are downregulated in HCC compared with surrounding cirrhosis.[27] One mechanism that leads to a reduction in CDKN1C/p57 expression is the loss of maternal allele methylation at the KvDMR1-imprinted locus at 11p15.5. This has been found in 20–50% of HCCs.[28] Another mechanism involved in the downregulation of p27 and p57 is the overexpression of miR-221/222, which occurs in about 70–80% of HCCs.[18] Owing to their inhibition of RB1 expression as well, levels of miR-221/222 appear to be major factors in cell-cycle control. CDKN1A/p21 is downregulated by oncogenic members of the miR-17-92 family.[29–33] Among the three clusters of the miR-17-92 family of the human genome, those on chromosomes 13 and 7 are upregulated in HCC.[34]

Disruption of apoptosis in HCC has been extensively reviewed.[35,36] In this context, miRNAs contribute to the altered expression of members of the BCL2 family (Figure 2). For example, upregulation of antiapoptotic members may result from reduced expression of miRNAs, with miR-122 controlling BCL-W and BCL-XL[37] while the latter is also controlled by let-7 members.[38] MCL1 is a target of miR-101,[39] miR-193b,[40] and miR-29,[41,42] and all of these miRNAs are downregulated in HCC. Conversely, proapoptotic members may be inhibited by overexpressed miRNAs. For example, the BH3-only proteins, BMF and BID, are targeted by miR-221 and miR-25, respectively.[29,30,43] These mechanisms have the potential to allow miR-221 to protect cells from 'anoikis', a form of apoptosis induced by the detachment of anchorage-dependent cells from the surrounding extracellular matrix. MiR-25 may also impair the TGF-β tumor suppressor pathway. Both pathways represent critical steps in the process of metastasis.[44,45] miR-483 is a miRNA located within intron 2 of the *IGF2* gene, and it is highly expressed in fetal liver, yet is barely detectable in adult liver.[46] Moreover, it is overexpressed in 30–40% of HCCs either independently or in combination with IGF2. MiR-483-3p has also been shown to promote cell survival by repressing translation of the p53-inducible BH3-only protein, PUMA/BBC3.[47]

Apoptosis is also controlled by molecules not belonging to the BCL2 family. For example, the inhibitor of apoptosis protein (IAP), survivin, is overexpressed in HCC and is controlled by miR-203,[48] a miRNA silenced by aberrant DNA methylation in HCC tissue.[23] Another miRNA, miR-21, appears to promote cell survival under stressful conditions. Indeed, miR-21 is upregulated in HCC, as well as in many other human neoplasms, and is associated with poor prognosis[49,50] and resistance to chemotherapy.[51–53] These effects appears to be linked to the survival advantage imparted by miR-21 via the direct targeting of proapoptotic genes, such as the tumor suppressor lipid-phosphatase, PTEN, which controls the phosphatidyl inositol 3-phosphate kinase (PI3K) signaling pathway[54–56] and programmed cell death 4 (Pdcd4) protein.[53,57–62] The latter also has a role in TGF-β-induced apoptosis.

At the nexus of cell cycle and apoptosis signaling is a molecular pathway that is controlled by the tumor-suppressor protein, p53. Genetic alterations in the *TP53* gene have been

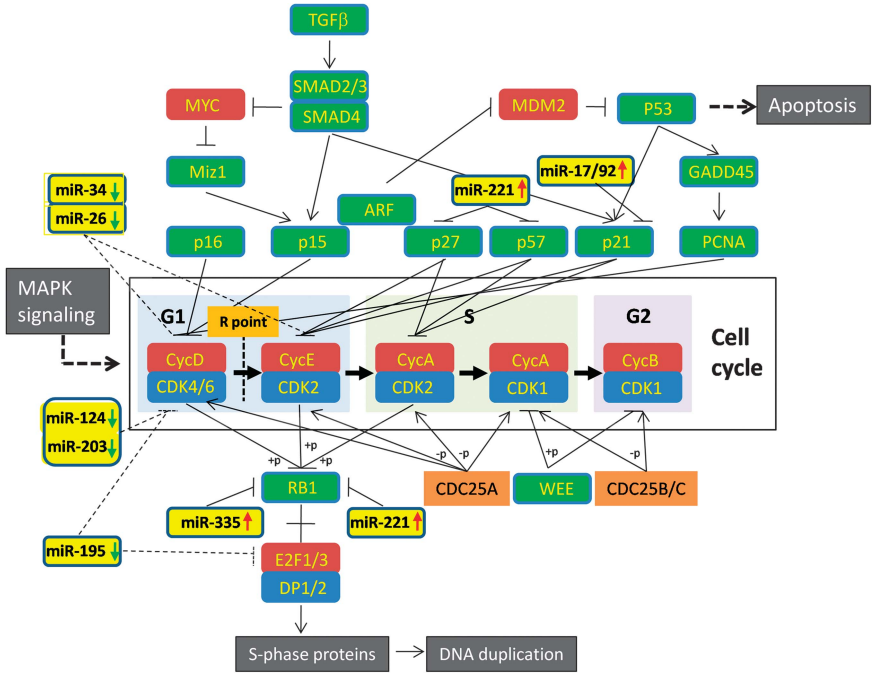

Figure 1 Aberrant miRNA expression in liver cancer promotes cell-cycle progression. A simplified pathway of cell cycle regulation is presented that shows the effects of various aberrantly expressed miRNAs in liver cancer. Upregulated miRNAs are indicated with a red up arrow, and downregulated miRNAs are indicated with a green down arrow. Upregulated miRNAs exert a stronger inhibitory effect on their targets, whereas downregulated miRNAs mediate weaker effects on their targets. Taken together, multiple aberrant miRNAs act on this pathway to support cell cycle progression

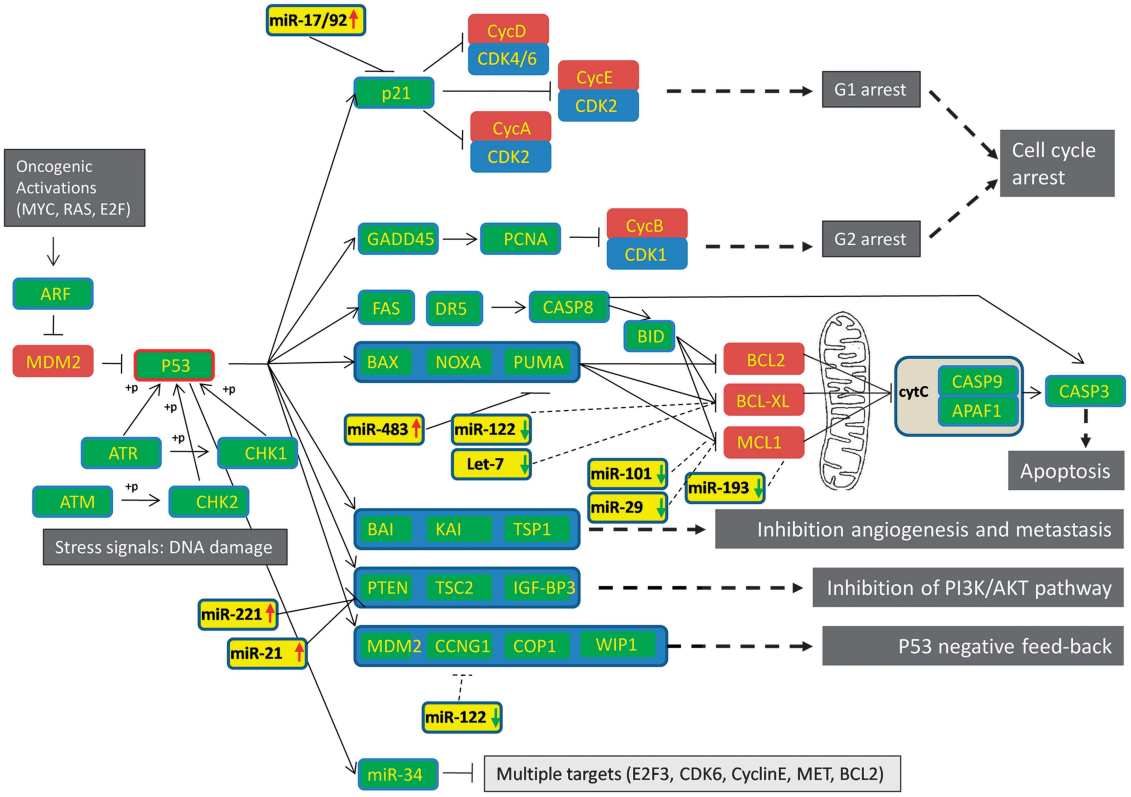

Figure 2 Aberrant miRNA expression in liver cancer affects the functionality of apoptosis pathways. A simplified apoptosis pathway is presented that shows the effects of various miRNAs that are aberrantly expressed in liver cancer. The aberrant expression of multiple miRNAs supports cell survival

extensively described in HCC,[21,63,64] and execution of the p53 pathway in HCC is affected by miRNA expression at various levels (Figure 2). For example, the loss of miR-122 expression in HCC cells leads to repression of p53 via upregulation of cyclin G1.[65,66] MiR-145 also induces p53 activity by an unknown mechanism,[67] and is commonly downregulated in HCC.[65] In many cases, miRNAs that are downstream effectors of p53 are downregulated owing to p53 loss of function. Although not fully proven in HCC, this situation is exemplified by miR-34a that is downregulated in some HCCs, most likely owing to the presence of a nonfunctional p53. However, in other HCCs, miR-34a is upregulated.[54,68] Lack of miR-34a induction can promote several biological effects through increased expression of CDK4, cyclin E2, BCL2, and the tyrosine kinase receptor, MET.[69–74] Other examples of p53-inducible miRNAs include miR-145, whose lack of expression leads to the activation of MYC,[75] or the cluster, miR-192/215.[76,77] Similar to miR-34, either miR-145 or miR-192/215 can promote cell-cycle arrest and/or apoptosis, colony suppression, and can increase CDKN1A/p21 levels.[67,76,77]

Invasion and metastasis. Advanced tumor features, such as the ability of cancer cells to promote uncontrolled angiogenesis and to invade tissues and blood vessels, are also affected by miRNA deregulation. One of the signaling pathways that confers invasive potential to HCC cells is mediated by the HGF/MET axis. Hepatocyte growth factor (HGF) binds to its transmembrane tyrosine kinase receptor, MET, and promotes hepatocyte proliferation, migration, survival, and angiogenesis. HGF also mediates invasive growth during embryonic development and tumorigenesis.[78,79] Overexpression of MET is found in 40–70% of HCCs[80–83] and is regulated by miR-199-3p (indicated earlier as miR-199a*), miR-34a, miR-23b, and miR-1.[84–88] All these miRNAs are downregulated in HCC, thereby contributing to the upregulation of c-MET. Among the downstream effectors of RTKs, the overexpression of RAS, but rarely activating point mutations, has been demonstrated in HCC.[89,90] All members of the RAS family have been shown to be modulated by various members of the let-7 family, and the latter are downregulated in HCC,[65] as well as in several other human cancers,[91–97] thus suggesting that let-7 potentially contributes to the upregulation of RAS.

As previously mentioned, the PI3K/AKT/PTEN pathway promotes cell survival as well as invasion and metastasis. Correspondingly, the targeting of PTEN by miR-21 and miR-221 can favor an invasive phenotype.[98,99] Indeed, the silencing of PTEN and PDCD4 by miR-21 has resulted in a decrease in apoptosis and an increase in cell invasion. MiR-21, which is upregulated in HCC and in most human malignancies, is linked to invasion and metastasis via its targeting of multiple tumor/metastasis suppressor genes, including tropomyosin 1 (TPM1), maspin, tissue inhibitor of metalloproteinase 3 (*TIMP3*) gene, and RHOB.[54,100–103] In addition, miR-21-mediated inhibition of RECK, a membrane-anchored glycoprotein that negatively regulates matrix metalloproteinase-9, leads to increased cell invasion.[98] In the context of the PI3K/AKT pathway, activation of the mTOR pathway has been found to be common to several human cancers, including HCC.[104] Moreover, when it is overexpressed, it is associated with poor prognosis, invasion, and metastasis.[105] Despite this, a very low rate of genetic alterations that affect the mTOR pathway in HCC has been reported.[104] Recently, mTOR was identified as a direct target of miR-199a-3p, and to be inversely correlated with miR-199a-3p in HCC.[84] The miR-199/214 cluster is of particular interest as it is downregulated in the majority of HCCs that have been studied,[4,54,65,84,106,107] as well as in other human malignancies,[108,109] in cancer-derived cell lines,[110] and in experimental neoplastic and pre-neoplastic conditions.[111]

Other deregulated miRNAs that have a role in the invasive and metastatic properties of HCC cells include miR-122, miR-139, and miR-151. For example, loss of miR-122 enhances cell migration and invasion, and its restoration in metastatic liver cancer cells has been shown to significantly reduce migration, invasion, and anchorage-independent growth *in vitro*, as well as tumorigenesis, angiogenesis, and intrahepatic metastasis *in vivo*.[66,112,113] Invasive and metastatic properties of HCC are also linked to the loss of control of ADAM17 by miR-122. Indeed, specific silencing of ADAM17 resulted in a dramatic reduction in migration and invasion *in vitro*, and reduced tumorigenesis, angiogenesis, and local invasion in the livers of nude mice.[114] These observations are similar to those associated with the restoration of miR-122. Downregulation of miR-122, which has been detected in about 70% of HCCs,[5,54,65] is also associated with the development of intrahepatic metastases[114] and a shorter time to recurrence.[66] Reduced expression of miR-139 has also been detected in HCC,[106,115] and has specifically been linked to the acquisition of metastatic properties.[115] In HCC cells, overexpression of miR-139 has significantly reduced cell migration and invasion *in vitro*, and the incidence and severity of lung metastases *in vivo*. These effects were linked to interactions between miR-139 and the 3′ untranslated region of rho-kinase 2 (ROCK2). In human metastatic HCC, expression of miR-139 is reduced and levels of miR-139 inversely correlate with levels of ROCK2 protein in human HCC samples.[115] MiR-151 is frequently amplified and overexpressed in HCC in combination with its host gene, focal adhesion kinase (*FAK*).[116] It is possible that the host gene, *FAK*, cooperates with miR-151 to enhance cell motility and spreading effects, and this would suggest that miR-151 has a critical role in tumor invasion and metastasis. MiR-151-5p targeting of RhoGDIA, a putative metastasis suppressor, has also been found to increase HCC cell migration and invasion, as well as the activation of Rac1, Cdc42, and Rho GTPases.[116]

The β-catenin pathway has a central role in the development of liver cancer and is an essential component of both intercellular junctions and canonical Wnt signaling. Thus, β-catenin is important for the regulation of cell proliferation, differentiation, and stemness[19,117–121] (Figure 3). In HCC, the Wnt/β-catenin pathway has been found to be abnormally activated through gain-of-function mutations at the N-terminus of β-catenin (present in 12–26% of HCCs),[21] by deletions, mutations, or epigenetic alterations of the E-cadherin gene, and by loss-of-function mutations in the *AXIN1* or *AXIN2* genes (present in 8–13% of HCCs).[21,122] The capacity for nuclear β-catenin to promote the epithelial to mesenchymal transition has been shown to induce invasive and metastatic

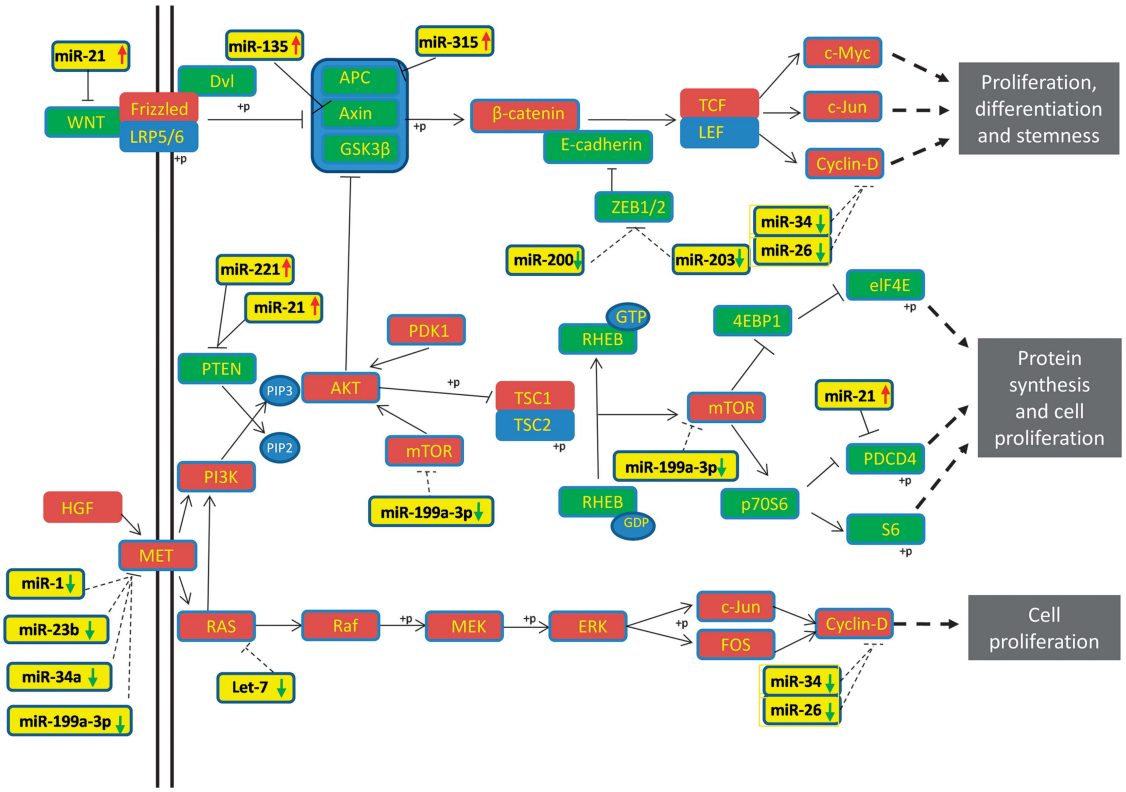

Figure 3 Aberrant miRNA expression in liver cancer supports the activation of various signaling pathways. Simplified diagrams of RAS/MAPK, PI3K/AKT/mTOR, and WNT/β-catenin signaling pathways are shown, along with the effects of various miRNAs that are aberrantly expressed in liver cancer. As a result, cell proliferation, survival, and maintenance of stemness are enhanced

properties of tumor cells.[123] Correspondingly, the silencing of β-catenin reverses the epithelial–mesenchymal transition and represses metastatic potential.[124] In a recent review of miRNA regulation of the Wnt/β-catenin pathway,[125] it is apparent that miR-21, miR-200, miR-315, and miR-135 directly regulate the Wnt/β-catenin core. MiR-135 and miR-315 activate β-catenin by inhibiting the negative regulators, APC and axin, respectively.[126,127] MiR-200a has been shown to regulate β-catenin levels either directly[128] or indirectly by modulating ZEB1/2, and this downregulation of miR-200a in HCC has been reported in various studies,[4,65,106,129,130] thereby supporting its role in mediating an increase in nuclear β-catenin levels and the activation of the pathway in cancer cells. MiR-34a has also been shown to be a negative regulator of the Wnt pathway based on its targeting of WNT1.[131,132] In HCC where miR-34 is downregulated, repression of WNT1 is released. Moreover, in addition to invasion and metastasis, the Wnt/β-catenin pathway is also important for maintaining stemness.[118–120] In this context, it has been proposed that upregulation of miR-181b supports this function at two levels, by repressing the Wnt/β-catenin inhibitor, NLK, and by blocking the hepatic differentiation transcription factors, CDX2 and GATA6,[133] suggesting that liver cancer cells may arise from stem cells that have lost control over their self-renewal potential.

As described above, miRNAs appear to have an essential role in the modulation of complex cross-talk that exists between pathways affecting HCC development and progression. Their understanding may facilitate the development of novel, targeted therapeutic strategies against HCC.

Animal Models of Liver Cancer

The importance of deregulated miRNAs in malignant cell transformation and tumor development has been observed in several studies. In particular, the development of animal models that are genetically modified at miRNA loci have proven the involvement of miRNAs in the initiation and progression of cancers, and provided *in vivo* models to test the efficacy of miRNA-based therapeutic approaches. Most of the models that have been developed are related to hematopoietic diseases, but HCC models have also been developed (Table 1).

miRNA-specific models of HCC. For HCC, various cancer genes, including tumor suppressor genes, oncogenes, and hepatitis B virus or hepatitis C virus (HCV) viral genes, have been employed for the development of mouse models.[134–144] More recently, miRNA-based mouse models predisposed to HCC have been reported. A transgenic mouse model characterized by overexpression of miR-221 in the liver was developed. In these mice, male animals exhibit a strong predisposition for HCC, which includes the emergence of spontaneous nodular liver lesions with age and a strong acceleration of tumor development after treatment with the carcinogen, *N*-nitrosodiethylamine (DEN). Notably, the

Table 1 miRNA-based animal models

miRNA	Mouse model name	Mouse model	Tumor
miR-155	E(mu)-mmu-miR155	Transgenic	B-cell malignancies
miR-155	*mir*-155^{LSLtTA}	Knockin	B-cell malignancies
miR-29a/b	Emu-miR-29	Transgenic	B-cell malignancies
miR15a/16-1	DLEU2/miR15a/16-1	Knockout	Chronic lymphocytic leukemia
miR-21	mir-21$^{LSL\text{-}Tetoff}$	Knockout	B-cell malignancies
miR-125b	Emu/miR-125b	Transgenic	B-cell malignancies
miR-146a	miR-146a KO	Knockout	Myeloproliferative disease
miR-17∼92	Emu-miR-17∼92	Transgenic	B-cell malignancies
miR-122	Mir122-LKO	Knockout	Hepatocellular carcinoma
miR-122	Mir122a$^{-/-}$	Knockout	Hepatocellular carcinoma
miR-140	MiRNA-140$^{-/-}$	Knockout	Hepatocellular carcinoma
miR-221	TG-221	Transgenic	Hepatocellular carcinoma

TG221 mouse was the first transgenic animal model to develop a solid tumor following overexpression of a miRNA.[145] Among the miRNAs that are downregulated in HCC, two studies have reported the development of miR-122 knockout mice. These mice are characterized by hepatic inflammation, fibrosis, and the development of spontaneous liver tumors with age. These studies also demonstrated the tumor suppressor function of miR-122 in the liver, as well as its importance in liver metabolism and hepatocyte differentiation.[146,147]

In addition to these miRNA-specific models, the importance of deregulated miRNA machinery components has also recently been suggested. For DDX20, a DEAD box protein component of miRNA-containing ribonucleoprotein complexes, it suppresses NF-κB activity by regulating miR-140 function.[148] Correspondingly, miR-140 knockout mice[149] are prone to HCC after treatment with DEN compared with control animals, and they have a phenotype that is similar to that for DDX20 deficiency. Taken together, these results suggest that miR-140 may act as a tumor suppressor by regulating the NF-κB pathway.[149] These reports confirm the importance of miRNA deregulation in liver cancer.

Traditional HCC preclinical models. In addition to genetically modified mouse models, traditional preclinical models of liver cancer have been established with chemical treatment. There are two main methods of HCC induction using chemicals: (i) administration of carcinogens alone, such as DEN, over a prolonged period of time or by intraperitoneum injection,[150] or (ii) administration of carcinogens (CCl_4 and DEN) followed by a promotion phase (partial hepatectomy) or administration of alcohol or phenobarbital.[151,152] DEN is frequently used as a carcinogenic agent;[152] however, the target organ in which it induces malignant tumors is species specific. Thus, mice treated with DEN develop liver tumors as well as gastrointestinal,[153] skin, lung,[154] and haematopoietic malignancies.[155] In addition, the time needed to develop HCC after a single DEN injection depends on the administered dose, and the gender, age, and strain of the treated mice.[156]

Other models of chemically induced liver disease involve the administration of CCl_4 in drinking water, by inhalation, or by subcutaneous or intraperitoneal injections.[151,157] CCl_4 treatment has been shown to induce an advanced stage of liver cirrhosis and related complications, such as ascites decompensation, in both rats and mice.[157,158] However, development of HCC has not been reported. In a two-stage chemical model of HCC, CCl_4 administration is accompanied by alcohol supplementation in drinking water.[159] However, only after 104 weeks does this combination induce the development of HCC and metastases.

miRNA-Based Therapies

A comparative analysis of global gene expression patterns for murine and human HCC has shown that many models can reproduce key biological and molecular events observed in the human condition.[160] Accordingly, mouse models have been widely used for the testing of potential therapeutic targets and for preclinical studies (see Li *et al.*[161] for a recent review). The efficacy of miRNA-mediated HCC prevention and therapy has also been evaluated in recent years using various strategies involving miRNA inhibition or miRNA replacement.

miRNA inhibition. Krutzfeldt and colleagues were the first to demonstrate that miRNAs could be effectively silenced in mice using specific molecules. In this study, the liver-specific expression of miR-122 was inhibited with an intravenous administration of chemically modified single-stranded RNA, known as 'antagomirs', that were complementary to the target miRNA.[162] The safety of this silencing approach was established in nonhuman primates, where no evidence of liver toxicity was detected.[163] On the basis of these promising results, many studies of miRNA inhibition were subsequently performed. The tumor-promoting activity of miR-221 in HCC was also demonstrated *in vivo*. In one notable study, systemic delivery of a cholesterol-tagged anti-miR-221 to orthotopic HCC tumors was found to reduce tumor cell proliferation and promote survival.[164] In a second study, anti-miR-221 oligonucleotides were found to exhibit significant antitumor activity in the TG221 transgenic mouse model, where a significant reduction in the number and size of tumors in the treated mice was confirmed with histopathological analyses. Significant downregulation of miR-221 levels has also been detected in liver tissues of anti-miR-221-treated mice, thereby confirming the ability of these molecules to inhibit endogenous miR-221[145] (Table 2). The importance of manipulating miRNA levels as a therapeutic tool for treating liver cancer is also supported in the work by Lim *et al.*,[143] where anti-miR inhibition of miR-494 (a miRNA

overexpressed in human HCC) resulted in a significant reduction in tumor size in a primary MYC-driven liver tumor mouse model.

HCV promotes HCC by inducing chronic liver disease and cirrhosis. MiR-122 has been found to support HCV replication.[165–167] Correspondingly, treatment of chronically infected nonhuman primates with an LNA molecule complementary to the miR-122 sequence led to a long-lasting and well-tolerated suppression of HCV viremia. These results support the hypothesis that miR-122 represents a potential target for antiviral intervention and possible liver disease sequels.[168,169] Currently, an anti-miR-122 oligonucleotide, 'miravirsen SPC3649' (Santaris Pharma, A/S, Hørsholm, Denmark), is being evaluated in a phase 2 clinical trial for the treatment of HCV infection (ClinicalTrials.gov Identifier NCT01200420)[170] (Figure 4). However, the concomitant inhibition of the tumor-suppressor activity of miR-122[65,146,147] must also be considered.

miRNA replacement. The miRNA restoration is another strategy that has been used to elucidate the functions of miRNAs, to identify their roles in tumorigenesis, and to provide preclinical tools for the treatment of cancer. One anticancer approach involves the restoration of miRNAs that are generally downregulated in cancer cells. *In vivo* xenograft models have been used to test the tumor suppressor activity of several miRNAs that were delivered by adeno-associated viruses (AAV), nano-sized lipid particles, or by cholesterol-conjugated modified oligonucleotides (Table 2). Hou *et al.*[171] used both cholesterol-conjugated small RNAs and an AAV delivery system to effectively restore miR-199a/b-3p expression in HCC tissues of HCC-bearing nude mice. As a result, tumor growth was inhibited without evidence of toxicity. Lentivirus-mediated expression of miR-199a was also able to inhibit tumor growth in a nude mouse xenograft model,[172] thereby confirming the antitumor effect of this miRNA in HCC and its therapeutic potential.

To provide a better model of human HCC, genetically engineered or chemically induced mouse models have been employed. For example, using MYC-driven mouse models of HCC, the tumor suppressor activity of miR-26a[26] and miR-122[146] have been demonstrated. In a DEN-induced HCC mouse model, the systemic administration of miR-124 led to a reduction in tumor growth and tumor size via induction of apoptosis and deregulation of a miRNA/inflammatory feedback loop circuit involving hepatocyte nuclear factor 4alpha (HNF4α).[173] Taken together, these studies indicate that miRNA mimics represent a promising approach for the treatment of cancer, and could be translated into clinical practice. In fact, MRX34 (Mirna Therapeutics, Inc., Austin, TX, USA), a liposome-formulated mimic of the tumor suppressor miR-34a, is the first miRNA mimic that has entered a multicenter phase I clinical trial aimed at evaluating safety in patients with primary liver cancer or with liver metastasis from other cancers (ClinicalTrials.gov Identifier: NCT01829971)[174] (Figure 4).

Oncolytic viruses. A distinct approach that has become interlaced with miRNA expression in HCC is the field of oncolytic viruses. These vectors have tumor-specific activity that results in the cytolysis of cancer cells, whereas normal tissues exhibit minimal toxicity *in vivo*. Thus, the use of oncolytic viruses represents a promising approach for the treatment of cancer (for a recent review, see Patel *et al.*[175] and Callegari *et al.*[176]). The differential expression of miRNAs in neoplastic *versus* normal tissues has also been used to improve the safety of oncolytic virus-based therapies. For example, to overcome liver toxicity associated with the systemic delivery of oncolytic adenoviruses, regulatory miRNA sequences have been inserted in the genome of conditionally replicating adenoviruses (CRAds). In one case, CRAds regulated by miR-122 were generated to provide liver-specific suppression of viral replication without compromising the replication capacity of the modified virus in cancer

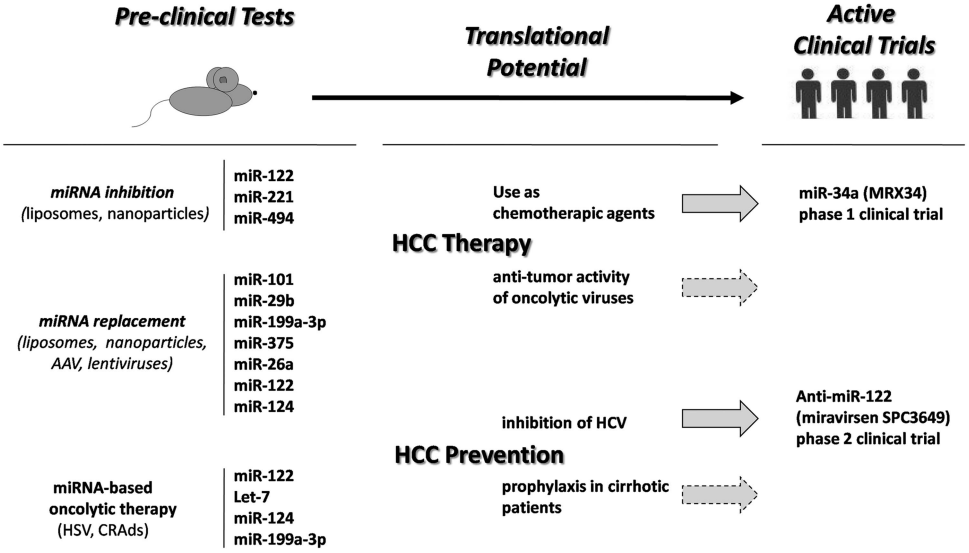

Figure 4 miRNA-based approaches that are moving toward clinical trials. An overview of the many preclinical studies that have produced promising results, as well as the clinical trials that are being planned or are underway

Table 2 miRNA-based therapies in HCC preclinical models

miRNAs	Mouse model	Method	Delivery system
miR-221	Orthotopic model	MiRNA inhibition	Cholesterol-conjugated anti-miR-221
miR-221	TG-221	MiRNA inhibition	Anti-miRNA oligonucleotide
miR-494	tet-o-MYC;LAP-tTA	MiRNA inhibition	Anti-miRNA oligonucleotide
miR-101	Xenograft	MiRNA replacement	RNA-duplex
miR-29b	Xenograft	MiRNA replacement	2′-O-methyl-modified oligoribonucleotides
miR-199a/b-3p	Xenograft	MiRNA replacement	Adeno-associated virus
miR-199a/b-3p	Xenograft	MiRNA replacement	Lentivirus
miR-375	Xenograft	MiRNA replacement	Cholesterol-conjugated 2′-O-methyl-modified miRNA
miR-26a	tet-o-MYC;LAP-tTA	MiRNA replacement	Adeno-associated virus
miR-122	tet-o-MYC;LAP-tTAT;	MiRNA replacement	Adeno-associated virus
miR-122	Xenograft	MiRNA replacement	Cationic lipid nanoparticles
miR-124	DEN-induced HCC mouse model	MiRNA replacement	Liposomes
Let-7	Xenograft	Oncolytic virus	Adenovirus
miR-122	Xenograft	Oncolytic virus	Adenovirus
miR-122; miR-124;	Xenograft	Oncolytic virus	Herpes simplex virus
let-7 miR-199	Xenograft; TG-221	Oncolytic virus	Adenovirus

tissues *in vivo*.[177–179] Other uses of oncolytic viruses have included the generation of a let-7-dependent adenovirus that is able to replicate in tumor cells, and not in normal liver cells, in a HCC xenograft model.[180] Similarly, a miR-199-dependent CRAd was generated to replicate in HCC tumor cells and not in normal liver cells. This virus was able to control tumor growth in a subcutaneous xenograft model in nude mice, and in HCCs arising in immune-competent mice, without causing significant hepatotoxicity[181] (Table 2).

Conclusions

HCC is the most common type of liver cancer diagnosed, and treatment options currently depend on tumor size and staging. For patients with advanced, non-resectable HCC, the only systemic therapy available involves the multi-kinase inhibitor, sorafenib.[182,183] This molecule targets the tyrosine kinase receptors VEGFR and PDGFR, as well as the serine–threonine kinases c-RAF and BRAF. Randomized phase 3 trials with sorafenib have shown an improvement in median overall survival and progression-free survival for HCC patients.[182,184] However, more effective treatments that have less toxic side effects are still needed. The use of miRNAs or anti-miRNAs as antitumor therapeutic molecules has attracted a lot of attention. At present, their short-term safety has been proven in several instances, albeit their efficacy as anticancer agents remains to be fully confirmed. Various studies have demonstrated that miR-26a, miR-199, miR-122, anti-miR-221, and miR-494 reduce tumor growth, and miR-34a has entered a clinical trial. For anti-miR-122, its capacity to inhibit HCV replication has the potential to prevent the development of HCC. Future investigations may also focus on the use of miRNA/anti-miRNA oligonucleotides for the prevention of HCC owing to the limited presence, or absence, of side effects associated with these approaches. To this end, animal models that are predisposed to HCC represent important instruments for testing the effectiveness of miRNA/anti-miRNA molecules.

Animal models of HCC have been established and are widely used for preclinical and translational studies. Moreover, the study of several HCC murine models has provided a comprehensive characterization of the molecular mechanisms of liver carcinogenesis and has also resulted in the development of new therapeutic strategies.[185] Compared with human HCC, genetically modified mouse models generally develop HCC without the development of liver cirrhosis. However, this condition is present in $>80\%$ of human HCC cases. The administration of CCl_4 has been shown to induce advanced stages of liver cirrhosis, although this is not accompanied by the development of HCC.[157] Therefore, CCl_4 treatment of genetically modified mice may lead to the development of models that more closely represent the human condition where HCC develops in cirrhotic livers. Future studies will be needed to investigate these possibilities.

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