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MicroRNA involvement in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the third cause of cancer-related death worldwide. Curative options for HCC are limited and exclusively available for patients carrying an early stage HCC. In advanced stages, traditional chemotherapy proved to be only marginally effective or even toxic. Thus, the identification of new treatment options is needed. New targets for non-conventional treatment will necessarily take advantage of progresses on the molecular pathogenesis of HCC. MicroRNAs (miRNAs) are a group of tiny RNAs with a fundamental role in the regulation of gene expression. Aberrant expression of several miRNAs was found to be involved in human hepatocarcinogenesis. miRNA expression signatures were correlated with bio-pathological and clinical features of HCC. In some cases, aberrantly expressed miRNAs could be linked to cancer-associated pathways, indicating a direct role in liver tumourigenesis. For example, up-regulation of mir-221 and mir-21 could promote cell cycle progression, reduce cell death and favour angiogenesis and invasion. These findings suggest that miRNAs could become novel molecular targets for HCC treatment. The demonstration of *in vivo* efficacy and safety of anti-miRNA compounds has opened the way to their use in clinical trials.

Keywords: microRNA • hepatocellular carcinoma • diagnosis • therapy

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, where it represents the third cause of cancer-related death [1]. Because of differences in risk factors, regional incidence is widely variable. It ranges from 5.5 to 14.9 per 100,000 individuals, but it may reach 100 per 100,000 in some regions of Africa and South East Asia. Hepatitis B virus (HBV) infection is the main risk factor in Asia and Africa, whereas Hepatitis C virus (HCV) infection is the main risk factor in western countries and in Japan. Other conditions increasing the risk of chronic liver disease and HCC development are alcohol abuse, aflatoxin B1 or vinyl chloride exposure, primary biliary cirrhosis, diabetes, non-alcoholic fatty liver disease and genetic disorders like haemochromatosis and α1-antitrypsin deficiency. Many of these factors are known causes of liver cirrhosis, which represents a pre-neoplastic condition for HCC. Indeed, in 80–90% of cases, HCC arises on a background of cirrhosis. The remaining HCCs, which arise in an otherwise healthy liver, are thought to develop, at least in part, from the malignant transformation of liver adenomas [2].

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Table 1 Hepatocellular carcinoma classification according to the BCLC staging system

Stage	Features
Very early	Well-differentiated tumours less than 2 cm in size, in cirrhotic patients with well-preserved liver function
Early	Tumours within the Milan criteria (a single HCC less than 5 cm or three HCC nodules less than 3 cm each, [158]) in patients with preserved liver function.
Intermediate	Large, multifocal tumours in patients with preserved liver function, without cancer-related symptoms and macrovascular invasion or extrahepatic spread
Advanced	Large, multifocal tumours in patients with mild cancer-related liver dysfunction and/or vascular invasion or extrahepatic spread
End stage	Tumours with extensive liver involvement, depressed physical status and/or liver function

A worldwide accepted staging system for HCC on cirrhosis, able to provide the proper guide for treatment and overall management of cirrhotic and HCC patients, is lacking. Among the several proposed HCC classification systems, the Barcelona Clinic Liver Cancer (BCLC) (Table 1) is one of the most used, as it offers the advantage of linking tumour staging to the best therapeutic strategy [3].

Treatment strategy is tailored on the basis of the extent of tumour burden, liver function, physical status and potential treatment efficacy. In very early and early stage HCC, potential curative treatments are available. They include surgical resection, percutaneous ablation and liver transplantation. However, only about 30–40% of cirrhotic patients enrolled in surveillance programs are eligible for these types of intervention [4] and, even after a curative treatment, the recurrence rate approaches 70% at 5 years. In advanced HCC, treatment options are even more limited: curative treatments are not available and traditional chemotherapy proved to be only marginally effective or even toxic in either adjuvant or neo-adjuvant settings.

Thus, the identification of new possible targets for the development of non-conventional treatments is urgent and will necessarily take advantage of progresses in the comprehension of the molecular pathogenesis of HCC [5].

Molecular pathways and biological functions altered in hepatocarcinogenesis

HCC results from the deregulation of multiple intracellular and extracellular signalling pathways. Initial steps involve the disruption of a set of interdependent pathways controlling the homeostasis between cell growth and apoptosis. At later stages, cells may acquire angiogenic, invasive and metastatic properties, in a process that involves the interactions of neoplastic cells with the surrounding microenvironment. To develop the above cancer traits, several elements of the Retinoblastoma (RB), p53, rat sarcoma virus oncogene (RAS), wingless-type (WNT) and transforming growth factor (TGF)-β pathways have been frequently found to be genetically or epigenetically altered in HCC.

The cell membrane receptor tyrosine kinase and the downstream RAS-mitogen-activated protein kinase and phosphatidyl inositol 3-kinase (PI3K)-AKT kinase signalling pathways are activated by several growth factors whose role in chronic liver diseases and HCC development is established [6]. By binding to their cell surface receptors, fibroblast growth factor (FGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), TGF-α, epidermal growth factor (EGF) and vascular-endothelial growth factor (VEGF) activate these pathways and ultimately drive cell proliferation and promote cell survival, but also trigger angiogenesis, invasion and metastasis. Overexpression of met proto-oncogene (MET), the HGF receptor, was found in 40–70% of HCCs [7–10]. Overexpression of VEGF was also detected [11, 12] and linked to advanced cancer phenotype [13–17]. Together with VEGF, overexpression of FGF was associated with angiogenic and invasive phenotypes [18–20]. Overexpression of PDGF and its receptor have also been associated with HCC pathogenesis [21, 22]. Among downstream effectors, overexpression of RAS has been demonstrated in HCC [23], while the presence of activating point mutations was infrequently detected [24, 25].

The Wnt/β-catenin is another signalling pathway that is frequently activated in hepatocarcinogenesis [26]. Its activation leads to β-catenin stabilization, enabling its translocation to the nucleus, where it interacts with T-cell-specific transcription factor (TCF/LEF) transcription factors, which, in turn, activate the transcription of target genes including c-myc, c-met, cyclin D1, VEGF, metalloproteases and others. The Wnt-β-catenin pathway was found to be abnormally activated through several mechanisms in HCC: gain of function mutations at the β-catenin N-terminus in 12–26% of HCCs [27], deletions or mutations or epigenetic alterations of E-cadherin gene, loss of function mutations at the AXIN1 or AXIN2 genes in 8–13% of HCCs [27, 28]. In addition to these mutation events, the increased stability of β-catenin may be related to phosphorylation and consequent inactivation of GSK-3β by growth factors, among which insulin, insulin-like growth factor (IGF)-1, FGF-2, EGF, PDGF, HGF, TGF-β and tumour necrosis factor (TNF)-α. A stabilization of β-catenin may also result from Erk-primed inactivation of GSK-3β or the action of HBV-X protein [29]. Recently, mutations of the β-catenin gene have been described in liver adenomas with a higher risk of malignant transformation [30].

Cell proliferation and survival are main outcomes of signal transduction pathways, but several other downstream effectors are also aberrant in cancer. For example, Rb1, cyclin-dependent kinases (CDKs), cyclins and CDK inhibitors, which directly act coordinately to regulate cell cycle, have been extensively examined in HCC. In HCC, inactivation of the RB1 gene may occur through either loss of chromosome 13, found in about 30% of HCCs [31, 32], or epigenetic mechanisms [33], while point mutations are rare. However, RB1 protein inactivation may derive from the aberrant expression of Gankirin, a protein able to bind RB1 and increase RB1 phosphorylation and its degradation by the proteasome. Gankirin was found up-regulated in all HCCs [34]. Furthermore, among cell cycle controlling elements, cyclins were reported to be up-regulated, while cell cycle negative regulators were often decreased in HCC tissue, compared with surrounding parenchyma [26]. CyclinD1/CDK4 is overexpressed in about 60% of HCCs [35]. Among CDK inhibitors, p16/INK4A, which specifically targets CDK4 and CDK6 thus inactivating CDK4/cyclin D1 complexes [36], is functionally inactivated in a large fraction of HCCs due to deletions at the short arm of chromosome 9 in about 20% of HCCs [27, 31, 32] and methylation of p16/INK4A promoter in 30–70% of cases [37, 38]. Two other proteins acting as tumour suppressor genes in HCC and directly regulating the cell cycle progression are the cyclin-dependent kinase inhibitors of the CDK interacting protein (KIP) family CDKN1B/p27 and CDKN1C/p57. CDKN1B/p27 and CDKN1C/p57 were reported to be down-regulated in HCC tissue in comparison with surrounding cirrhosis [39]. We and other groups have previously reported that about 20 to 50% of HCCs exhibit a loss of maternal allele methylation at the KvDMR1 imprinted locus at 11p15.5, where CDKN1C/p57 gene is located, and this mechanism has been linked to reduction of CDKN1C/p57 expression [40–43]. As it will be described below, one of the major mechanisms involved in the down-regulation of p27 and p57 is now believed to be the overexpression of the microRNAs (miRNAs) miR-221/222, which occurs in about 70% of HCCs [44–46]. On the contrary, some HCCs exhibit high cell proliferation in spite of high p27 expression. In these cases, p27 is thought to be inactivated by sequestration into cyclinD1–CDK4-containing complexes [47].

Abnormal cell cycle control is not only a critical step in HCC maintenance, but it also appears to be a critical early event. Indeed, an increased proliferation rate and proliferating cell fraction characterize liver cirrhosis too, and correlate with a higher propensity to develop HCC.

The contribution of anti-apoptotic factors to hepatocarcinogenesis has been extensively studied [48–50]. In normal hepatocytes, apoptotic signalling may be transduced by either the extrinsic or the intrinsic pathway. Interestingly, TRAIL displays anticancer activity without hepatotoxicity, thus it has been proposed as a possible therapeutic strategy for HCC, which may be further improved by inhibition of proteasome, which was shown to increase susceptibility of HCC cells, but not primary hepatocytes, to TRAIL-induced apoptosis [51]. The intrinsic pathway relies on the mitochondrial-mediated regulation and it is regulated by the Bcl-2 gene family. Although Bcl-2 expression was not detected in HCC immunohistochemical studies [52], Bcl-xL was expressed in all HCC-derived cell lines analysed and in most HCC tissues [53]; furthermore, its overexpression could independently predict a decreased overall- and disease-free survival [54, 55]. Among the pro-apoptotic members of the Bcl-2 family, a down-regulation of the pro-apoptotic genes bax and bcl-Xs was observed in 80% and 64%, respectively, of HCCs [56, 57]. Similarly, the pro-apoptotic Bid was also found down-regulated in all HCCs from HBV-infected patients, and its down-regulation was related to HBV-X protein action [56].

At the cross-road between cell cycle and apoptosis is the p53 molecular pathway. Genetic alterations of the TP53 gene have been extensively described in HCC, and has been shown to vary according to different geographic areas, possibly reflecting different etiologic mechanisms. The G to T transversion of codon 249 of TP53 gene is the typical alteration found in about 50% of HCCs consequent to aflatoxin B1 exposure [58]. Conversely, mutations of TP53 unrelated to aflatoxin B1 exhibit a lower frequency (10–30%) [27] and are more frequent in advanced cases. In addition to TP53 mutations, several other factors involved in hepatocarcinogenesis have been reported to impair p53 function. In particular, interactions between p53 and HBV-X protein lead to a reduced p53-sequence-specific DNA binding activity, thus reducing p53 transcriptional activity and blocking p53-mediated apoptosis. Concerning HCV, a modulation of p53 promoter transcriptional activity was demonstrated in HCC-derived cell lines and was ascribed to HCV-core protein [59].

Advanced tumour features include the ability of cancer cells to promote uncontrolled angiogenesis and invade tissues and blood vessels. In this regard, HCC is one of the most vascular solid tumours with high propensity for vascular invasion. The above-mentioned aberrant signalling pathways are functional to this feature. Angiogenesis plays a crucial role in HCC development, growth and metastasis and is used as a diagnostic criterion. The switch to an angiogenic phenotype is triggered by the activation of genes like RAS, inactivation of p53, or cellular stress factors like hypoxia, reactive oxygen species and nutrient deprivation.

Signalling pathways crucial for the angiogenic process include growth-factor-mediated pathways such as VEGF and FGF receptor signalling as well as the nitric oxide signalling. VEGF expression is stimulated by hypoxia through the hypoxia inducible factor-1 (HIF-1) and the IGF-2 and it is produced by both endothelial and tumour cells. VEGF exerts a mitogenic effect on endothelial cells and increases vascular permeability. The role of VEGF in the development of neovascularization of HCC has been extensively reported in the transition between low-grade dysplastic nodules to high-grade dysplastic nodules to early HCC. Furthermore VEGF expression correlates with HCC progression, metastatization, tendency towards portal invasion and higher recurrence rate.

In addition to angiogenesis, another distinguished feature of HCC is its propensity towards vascular and tissue invasion. One of the signalling pathways conferring invasive potential to HCC cells, is mediated by the HGF/MET axis. HGF is the most potent growth factor for hepatocytes and, by binding to its transmembrane

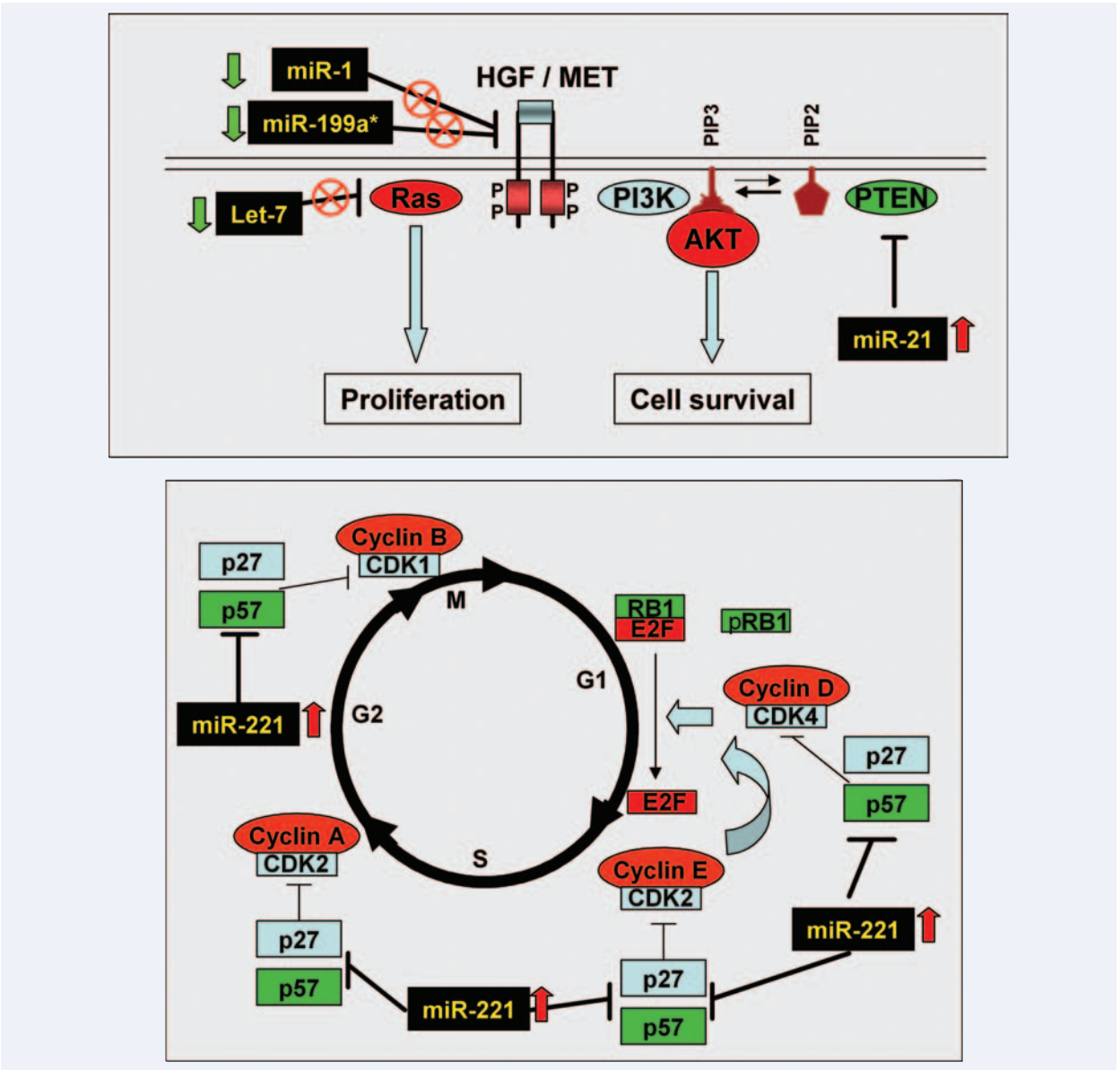

Fig. 1 Schematic representation of pathways affected by miRNAs deregulated in human hepatocellular carcinoma . Receptor tyrosine kinase (here indicated HGF/MET axis solely), RAS and PI3K pathways affected by the down-regulated (downward green arrow) miR-1, miR-199a*, Let-7 and the up-regulated (upward red arrow) miR-21 may lead to cell growth, survival, motility, invasion and metastasis. The cell cycle progression is mainly affected by the miR-221 (and miR-222, not indicated) up-regulation, which can repress the CDK inhibitors CDKN1B/p27 and CDKN1C/p57.

tyrosine kinase receptor, MET, it promotes proliferation and regeneration, migration, survival and angiogenesis and it is involved in the control of invasive growth both during tumourigenesis and in embryonic development [60, 61]. Cytoplasmic downstream effectors of HGF/MET signalling axis include phospholipase Cγ (PLCγ), STATs, PI3K, ERK-1/2 [62]. Among genes targeted by HGF/MET signalling, MMPs and urokinase-type plasminogen activator are thought to play an important role in cell migration [63].

As exemplified above, significant and complex cross-talks among the different pathways exist and are involved in different aspects of HCC development and progression. These cross-talks, largely not understood at the molecular level, could potentially account for the resistance to molecularly targeted drugs, which are able to hit pathways only at one or few sites. In this context, the peculiar function of miRNAs may be important. Indeed, since the expression of many different proteins is affected by the

deregulation of a single miRNA, the targeting of even a single deregulated miRNA for therapeutic purposes may have an effect on several cancer-associated pathways. Thus, recognizing the deregulated miRNAs and their protein-coding gene targets is therefore a relevant task for understanding the molecular basis of liver tumourigenesis and for the development of potentially useful diagnostic and therapeutic tools.

Numerous miRNAs are aberrantly expressed in human HCC

In the recent years, several studies revealed that the expression of miRNAs is deregulated in human HCC in comparison with matched non-neoplastic tissue [64–73]. The list of aberrantly expressed miRNAs in HCC identified in various studies is reported in Table 2. Among these, some miRNAs were identified as aberrantly expressed by more than one study (Table 3), thus indicating that irrespective of the employed technological platform or set of samples, these miRNAs were the most likely involved in liver tumourigenesis. In addition and in support of this hypothesis, among aberrantly expressed miRNAs in HCC, some were also deregulated in other neoplasms. For example, the up-regulation of the miR-221/222 was also reported in colon, pancreas, stomach, bladder carcinomas and glioblastomas, or miR-21 was also reported in ovarian, lung, breast carcinomas and glioblastomas, while the down-regulation of miR-199a, miR-200b and miR-214 was reported in ovarian cancer and miR-199b in ovarian and lung cancer. In support of their role in liver tumourigenesis, gene targets, like the cyclin-dependent inhibitors p27/CDKN1B and p57/CDKN1C, or the PI3K antagonist phosphatase and tensin homolog (PTEN), involved in cell cycle and cell death regulation were experimentally validated targets of miR-221/222 and miR-21.

Among the up-regulated miRNAs, miR-373 was also identified. This oncogenic miRNA was previously found to be up-regulated in testicular cancer and in breast cancer metastasis and protect cells from activated oncogenes [74, 75]. Interestingly, some miRNAs that are up-regulated in HCC, such as miR-21, miR-210, miR-213 and miR-181b, were also found to be up-regulated in hypoxic conditions. Thus, aberrant miRNA expression in HCC may lead to protection from various stress stimuli.

Among the down-regulated miRNA in HCC, several members of the large let-7 family and the miR-143/145 were found. Although for members of the let-7 family there are some controversial reports, these miRNAs emerged as consistently down-regulated in several human cancers [76–82].

Among the down-regulated miRNAs in HCC is the hepato-specific miR-122, which accounts for about 70% of the miRNA population in the adult liver, where it acts as a key regulator of cholesterol and fatty-acid metabolism. Significantly, the down-regulation of miR-122 was detected in more than 70% of HCC [73]. It was shown that the level of miR-122 expression increases in the mouse liver throughout development, to reach the maximum just before birth. Thus, the loss of expression of miR-122 of HCC cells may represent either a differentiation reversion or a block to a less differentiated status of liver cells. There are also indications that the down-regulation of miR-122 may have a more direct role in tumourigenesis through the activation of cyclin G1 [73].

Biological and molecular functions affected by miRNA deregulated in HCC

The role of miRNAs either as oncogenes or tumour suppressors in human cancer has been established [83, 84]. Various studies have also begun to elucidate the molecular functional links between miRNA abnormal expression and the hallmarks of malignant transformation: aberrant cell growth, cell death, differentiation, angiogenesis, invasion and metastasis [85]. Here, we summarize the available evidence in HCC.

As previously indicated, MET, the tyrosine kinase HGF receptor, is overexpressed in 40 to 70% of HCCs and is involved in cell motility, invasion and metastasis. Recent reports indicated that MET is post-trascriptionally regulated by miR-199a/a* and miR-1 [86, 87] (Fig. 1). Genes for both miRNAs are methylated in HCCs. Members of the miR-199 family emerged in several studies as frequently down-regulated in HCC (see Table 3). Its *in vivo* significance needs to be confirmed, as the studies were confined to cell lines and the miRNA that was active on MET was the presumed 'non-mature' miR-199a* strand. In spite of the fact that the aberrant down-regulation of miR-1 did not emerge in several miRNA expression studies in HCC, it was recently shown that its expression was silenced in HCC cell lines and primary tumours *via* promoter methylation; in addition, miR-1 ectopic expression could induce apoptosis and inhibit cell cycle in HCC cell lines. Thus, the miR-1 silencing in HCC may remove a restriction element that permits MET overexpression.

The activation of tyrosine kinase receptors (RTKs) initiates a downstream cascade of events that lead cells to proliferate. Crucial elements of this signalling transduction pathway are members of the RAS family of oncogenes. A molecular link between miRNA deregulation and RAS expression has been established. The 3′ untranslated regions (UTRs) of the KRAS, NRAS and HRAS mRNAs contain multiple complementary sites for binding of let-7 members, and forced expression of let-7 in human cancer cells reduces RAS protein levels [78] (Fig. 1). Since let-7 is generally down-regulated in several human cancers, this mechanism could lead to the activation of the RAS pathway. As previously mentioned, it is interesting to note that a frequent overexpression but not point mutations of RAS has been reported in HCC.

Let-7 has also been shown to repress the high-mobility group AT-hook 2 (HMGA2) oncogene [79, 80], which encodes for a high-mobility group protein oncogenic in a variety of tumours, including benign mesenchymal tumours and lung cancers. The effect of let-7 on HMGA2 was dependent on multiple target sites

Table 2 miRNAs aberrantly expressed in hepatocellular carcinoma (HCC) compared to non-tumourous liver tissue detected in at least one report

microRNA	Expression in HCC	miRNA cluster	Chrom
let-7a-1	Down	let-7-a1/let-7f1/let-7d	9q22
let-7a-2	Down	miR125b-1/let7a-2/miR100	11q24
let-7a-3	Down	let-7-a3/let-7b	22q13
let-7b	Down	let-7-a3/let-7b	22q13
let-7c	Down	mir-99a/let-7c/125b-2	21q11
let-7d	Down	let-7-a1/let-7f1/let-7d	9q22
let-7e	Down	mir-99b/let7-e/125a	19q13
let-7f-2	Down	let-7f-2/mir-98	Xp11
let-7g	Down	-	3p21
miR-101	Down	-	1p13
miR-122	Down	-	18q21
miR-125a	Down	mir-99b/let7-e/125a	19q13
miR-125b-1	Down	miR125b-1/let7a-2/miR100	11q24
miR-130	Up	-	11q12
miR-130a	Down	-	11q12
miR-132	Down	mir-212/132	17p13
miR-135a	Up	-	12
miR-136	Down	mir-770/493/337/431/433/127/432/136/370	14q32
miR-139	Down	-	11
miR-143	Down	mir-143/145	5q32–33
miR-145	Down	mir-143/145	5q32–33
miR-150	Down	-	19q13
miR-18	Up	mir-17–92	13q31
miR-181b-1	Up	mir-181-a1/181-b1	1q31–32
miR-195	Down	mir-497/195	17p13
miR-199a-1-5p	Down	-	19p13
miR-199a-2-5p	Down	mir-199a2/214	1q24
miR-199b	Down	-	9q34
miR-200a	Down	mir-200b/200a/429	1
miR-200b	Down	mir-200b/200a/429	1p36
miR-21	Up	-	17q23
miR-210	Up	-	11p15
miR-213	Up	mir-181-a1/181-b1	1q31–32
miR-214	Down	mir-199a2/214	1q23
miR-221	Up	mir221/222	Xp11
miR-222	Up	mir221/222	Xp11
miR-223	Down	-	Xq12–13
miR-224	Up	mir224/452	Xq
miR-301	Up	mir-301/454	17
miR-33	Up	-	22q
miR-34	Up	mir-34b/34c	11q
miR-373	Up	mir-371/372/373	19q
miR-376a	Up	Cluster 34 miRNA	14q32

Results are summarized from the following references: [65–73].

Table 3 miRNAs aberrantly expressed in hepatocellular carcinoma (HCC) reported by more than one study

microRNA class	microRNA	Expression in HCC	miRNA cluster	Chrom	Other cancers	References
miR-18	miR-18	Up	mir-17–92	13q31	No	[70, 159]
miR-21	miR-21	Up	-	17q23.2	Ovarian, glioblastoma, lung, breast	[66, 90, 97, 135–137, 159, 160]
miR-221	miR-221	Up	mir221/222	Xp11.2	Colon, pancreas, stomach, bladder, glioblastoma, thyroid	[69, 73, 90, 91, 94, 97, 135, 159]
miR-222	miR-222	Up	mir221/222	Xp11.2	Stomach, pancreas	[66, 69, 97, 135]
miR-224	miR-224	Up	mir224/452	Xq	Prostate, Thyroid	[66, 68, 70, 161, 162]
miR-122	miR-122	Down	-	18q21	No	[66, 73, 97]
miR-125	miR-125a	Down	mir-99b/let7-e/125a	19q13.4	Breast, Ovarian, Lung	[70, 97, 137, 160, 163]
	miR-125b-1	Down	miR125b-1/let7a-2/miR100	11q24.2	Breast, Ovarian	
miR-130a	miR-130a	Down		11q12	Breast, Lung	[73, 135, 159]
miR-150	miR-150	Down	-	19q13	No	[73, 159]
miR-199	miR-199a-1–5p	Down	-	19p13.2	Ovarian	[70, 73, 97, 136, 159, 160, 163]
	miR-199a-2–5p	Down	mir-199a2/214	1q24.3	Ovarian	
	miR-199b	Down	-	9q34	Ovarian, Lung	
miR-200	miR-200a	Down	mir-200b/200a/429	1p36.3	No	[70, 73, 159, 163]
	miR-200b	Down	mir-200b/200a/429	1p36.3	Ovarian	

in the 3′UTR. It was reported that chromosomal translocations associated with human tumours disrupt repression of HMGA2 by let-7 miRNA, thus demonstrating that disruption of a single miRNA-target interaction and loss of miRNA-directed repression represent mechanisms for the activation of endogenous proto-oncogenes [79]. The disrupted repression of HMGA2 promotes anchorage-independent growth and the growth-suppressive effect of let-7 on lung cancer cells was rescued by overexpression of the HMGA2 ORF without a 3′UTR [79]. The link between the down-regulation of let-7 and the simultaneous up-regulation of HMGA2 was functionally associated with a cancer stem cell signature, at least in the breast cancer cells SKBR3, by Yu *et al.* [82]. These important findings need to be assessed in HCC too. The importance of let-7 down-regulation in cancer is further supported by studies by Takamizawa *et al.* and Akao *et al.* [76, 81], who showed that let-7 can suppress the growth of A549 lung cancer cells and DLD-1 colon cancer cells *in vitro*.

A direct role of miRNAs in controlling cell growth by acting on elements of the cell cycle machinery was provided by studies on miR-221. MiR-221, which was recently shown to be induced by MYCN [88] and repressed by p53 [89], emerged as a significantly up-regulated miRNA in glioblastoma, pancreatic, hepatocellular, kidney, bladder, prostate and thyroid cancer [44–46, 73, 79, 90–94], thus suggesting an oncogenic role in several human neoplasms. Its oncogenic function was substantiated by the discovery of its ability to modulate the expression of the cyclin-dependent kinase inhibitor CDKN1B/p27, a key controller of cell cycle progression [46, 93]. More recently, another cyclin-dependent kinase inhibitor, CDKN1C/p57, was also shown to be target of miR-221 [44, 45], strengthening the role of miR-221 in promoting cell cycle progression (Fig. 1). Moreover, the BH3-only protein BMF was recently proven to be a target of miR-221 (Gramantieri and Negrini, manuscript in preparation). Through this mechanism, miR-221 could protect cells from ‘anoikis’, a form of Apoptosis induced by the detachment of anchorage-dependent cells from the surrounding extracellular matrix. Evading anoikis is a critical step in the process of metastasis [95, 96]. Taken together, the finding that miR-221 could promote cell cycle progression and protect cells from apoptosis outlines the importance of the aberrant expression of one miRNA in cancer, whose action of on various targets could simultaneously affect multiple tumourigenic pathways.

Signal pathways from activated RTKs include also the PI3K AKT. The activation of this pathway leads to the activation of AKT kinases, which phosphorylate several protein targets that in turn

promote cell survival. This pathway is controlled by the tumour suppressor lipid-phosphatase PTEN. It was shown that PTEN is a direct target of miR-21 [97] (Fig. 1), a miRNA that is frequently overexpressed in most types of human cancers. Thus, PTEN could be repressed by overexpression of miR-21, which would lead to cell survival through PI3K-AKT pathway activation.

miR-21 can also down-regulate the tumour suppressor programmed cell death 4 (Pdcd4) [98, 99]. Pdcd4 is believed to have a role in TGF-β induced apoptosis. However, it may also have other functions. It is up-regulated in senescent fibroblasts and it may inhibit proliferation, possibly through the indirect suppression of CDK1/cdc2 kinase. Moreover, it acts as a negative regulator of intravasation, initial step for cancer cell metastasis. Anti-miR-21-transfected RKO cells showed an increase of Pdcd4-protein and reduced invasion, while overexpression of miR-21 in Colo206f significantly reduced Pdcd4-protein amounts and increased invasion. Analyses of primary colorectal cancers revealed that an inverse correlation between miR-21 and Pdcd4-protein exists, suggesting that miR-21/Pdcd4 interaction may be relevant for invasion/intravasation/metastasis of cancer cells.

miR-21 can also down-regulate the tropomyosin-1 [100], which suppresses anchorage-independent growth of MCF-7 breast cancer cells, further supporting the oncogenic mir-21 functions.

miR-373 is up-regulated in HCC. A link between miR-373 and p53 was proven by a study on miR-372 and miR-373, which were found to cooperate with oncogenic RAS to transform primary human cells [75]. The study proved that these miRNAs could confer protection to oncogene-activated p53 pathway. It was shown that primary human cells undergo growth arrest and senescence in response to mitogenic signals from oncogenes such as RAS, by the activation of the p53 pathway, a response that is reversed by the presence of non-functional p53. Voorhoeve *et al.* demonstrated that ectopic expression of miR372/373 was sufficient to allow transformation in the presence of wt p53. Thus the study demonstrated that miR372/373 confers protection to oncogene-activated p53 pathway. Interestingly, this is a characteristic found in testicular germ cell tumours, where, in contrast with other types of tumours, the miR-372/373 cluster is indeed highly expressed in a generally wt-p53 background, suggesting a role in the development of these tumours.

As previously mentioned, miR-122 is down-regulated in more than 70% of HCCs. A molecular investigation revealed that cyclin G1 is a direct target of miR-122. Cyclin G1 was initially identified as a transcriptional target of p53 [101–104]. Later, it was found that cyclin G1 can exert a negative regulation on p53 tumour suppressor gene by recruiting the B′ subunit of PP2A phosphatase to dephosphorilate and activate Mdm-2, thus leading to p53 degradation [105, 106]. As a result, cyclin G1 overexpression can enhance cancer cell growth and its silencing suppresses cell proliferation [107–109]. In a mouse model, the absence of cyclin G1 was associated with a lower susceptibility to develop liver tumours, which was associated with an increased p53 tumour suppressor activity [110]. The up-regulation of cyclin G1 consequent to miR-122 down-regulation in human HCC may thus lead to p53 down-regulation and promote tumourigenesis.

miRNA expression profiling in HCC classification and prognostic stratification

miRNA expression could also be used to improve our ability to classify HCC and stratify their prognostic risk. The presently used classifications for HCC are exclusively based on clinical parameters [3, 111–116]. Tumour status (number and size of nodules, presence of vascular invasion and extrahepatic spread), liver function indicators, presence of portal hypertension and performance status are considered for prognostic purposes and for the choice of treatment. Because HCC is heterogeneous both from molecular and clinical perspectives [117, 118], the available classifications for HCC could be improved and redefined by the incorporation of molecular data.

Various molecular factors have been found to correlate with clinical parameters. For example, MET-regulated expression signature defines a subset of human HCCs with poor prognosis and aggressive phenotype [119]. CDKN1B/p27 and CDKN1C/p57 exhibits a relevant prognostic significance in human HCC. CDKN1B/p27 down-regulation is associated with advanced tumour stage, lower survival and higher recurrence rate of small HCC, higher biological aggressiveness, poor differentiation, portal invasion and high proliferative activity [120, 121]. Reduced CDKN1C/p57 labelling index was associated with worse outcomes and lower disease-free survival after surgery, suggesting that CDKN1C/p57 down-regulation might contribute to the progression of HCC through modulation of cell growth [39, 122]. Overexpression of the anti-apoptotic gene bcl-xL [53] independently predicts a decreased overall- and disease-free survival [54, 55]. High throughput technologies have made possible to explore gene expression patterns on large series and to characterise molecular signatures associated with different aetiologies [123–125], stage [126, 127], propensity to recurrence [128–130] and prognosis [131, 132]. Incorporation of these findings in HCC classification could be valuable in improving our ability to stratify HCCs; however, additional validations are still required before their clinical use is granted.

In this context, more recently, patterns of miRNA expression were found to correlate with bio-pathological and clinical parameters, indicating that miRNAs could become useful molecular markers for HCC classification and prognostic stratification. Among deregulated miRNAs, up-regulation of miR-221 was associated with shorter time to recurrence (Gramantieri and Negrini, unpublished). This is not unexpected, giving the fact that miR-221 regulates the expression of p27 and p57 [44], two tumour suppressor proteins whose down-regulation was associated with poor prognostic factors in HCC [39, 120–122]. Recently, up-regulation of miR-210, one of the miRNA that is induced under hypoxia [133],

Table 4 miRNA signatures associated with hepatocellular carcinoma (HCC) metastasis and survival

Metastasis-associated microRNA*	Survival-associated microRNA*	Expression in HCC	miRNA cluster	Chrom
miR-185		Up	-	22q11
miR-207		Up	-	9p21
miR-219–1	Yes	Up	-	6p21
miR-338	Yes	Up	mir-338/mir-657	17q25
let-7g		Down		3p21
miR-1–2	Yes	Down	mir-133a-1/mir-1–2	18q11
miR-122	Yes	Down	-	18q21
miR-124a-2	Yes	Down	-	8q12
miR-125b-2	Yes	Down	mir-99a/let7c/mir-15b-2	21q21
miR-126	Yes	Down	-	9q34
miR-148a	Yes	Down	-	7p15
miR-148b	Yes	Down	-	12q13
miR-15a	Yes	Down	mir-15a/mir-16–1	13q14
miR-194	Yes	Down	mir-194-1/mir-215	1q41
miR-19a	Yes	Down	mir-17/18a/19a/20a/19b-1/92–1	13q31
miR-30a	Yes	Down	mir-30a/30c-2	6q13
miR-30c-1	Yes	Down	mir-30e/mir-30c-1	1p34
miR-30e	Yes	Down	mir-30e/mir-30c-1	1p34
miR-34a		Down	-	1p36
miR-9–2	Yes	Down	-	5q14

* These data were from Budhu *et al.* [72]

was associated with reduced disease-free and overall survival in breast cancer [134]. Since miR-210 is also up-regulated in HCC [97] as well as in a variety of solid cancers [135–138], its prognostic value may possibly be relevant for other cancers too, including HCC. The study by Ladeiro *et al.* [66] could identify miRNA signatures able to classify liver samples according to histology (tumour/non-tumour; benign/malignant; inflammatory adenomas and focal nodular hyperplasia), aetiology (alcohol consumption or HBV infection) and cancer gene mutations (β-catenin and hepatocyte nuclear factor 1α). The study by Budhu *et al.* [72] revealed a 20-miRNA signature associated with venous invasion (Table 4). Significantly, the same signature could also correlate with disease-free and overall survival. The results by Budhu *et al.* may be particularly useful to classify patients with HCC at early stage, which may provide a more rational approach to treatment intervention. Hence, either alone or in combination with other parameters, miRNA expression patterns may potentially become useful markers for HCC classification and prognostic risk stratification.

miRNA in HCC therapy?

To identify novel therapeutic approaches for the treatment of HCC, targeting genes associated with molecular pathways involved in human tumourigenesis has become the most rational approach. In HCC, targeting RAS by antisense oligonucleotides could inhibit hepatocarcinogenesis by restoring the apoptotic pathway. Inhibition of MET could control migration and invasion of HCC cells [139]. The use of imatinib mesylate, a platelet-derived growth factor receptor (PDGFR) and other tyrosine kinases inhibitor, has been suggested [140–142]; however, in spite of initial positive results [143], phase 2 clinical trials did not reveal any significant efficacy [144, 145].

New molecularly targeted agents with a selective action and few side effects on residual liver function are under investigation: taking advantage of the progresses in understanding the molecular pathogenesis of HCC, kinases inhibitors, anti-angiogenic

compounds, mTOR inhibitors and other molecularly targeted agents have been and are currently under evaluation in phase 2 and 3 trials. At present, the only phase 3 clinical trial so far completed compared the oral multikinase inhibitor sorafenib, which is known to block the kinase activities of VEGFR, PDGFR and Raf [146], with placebo in patients with advanced HCC. Treated patients displayed a median overall survival of 10.7 months *versus* 7.9 months in the placebo group, setting the standards for future investigations [147, 148]. This moderate improvement in therapeutic efficacy and overall survival may represent a significant progress, as most of the studies did not rely upon molecular characterization of the different gene targets prior to inclusion in clinical studies, which might have aid the selection of the best target population for the different treatment options.

The discovery that miRNAs play an important role in hepatocarcinogenesis has laid the foundation for their exploitation for molecular therapy. Feasibility of the approach has been proved. Song *et al*. used anti-Fas siRNA to protect mice from induced acute liver injury and, similarly, Zender *et al*. used anti-caspase-8 siRNA to protect mice against Fas ligand-induced liver injury [149, 150]. Both of these studies demonstrated a survival benefit using siRNA approach without significant side effects. These studies also confirmed a high hepatic uptake of siRNA following their systemic administration. Although several studies have established the potential usefulness of miRNA-based therapy in cancer [76, 81, 151, 152], up to now there has not been any report of using agents that mimic miRNAs in animal or clinical models. More recently, anti-miRNA oligonucleotides (AMOs) have been developed. Among miRNAs up-regulated in HCC, transfection of cultured glioblastoma and breast cancer cells with AMOs anti-miR-21 induced inhibition of miR-21 accompanied by suppression of cell growth, associated with increased apoptosis [153, 154]. MiR-21 is overexpressed in colangiocarcinoma and its inhibition by AMOs increases sensitivity to the chemotherapeutic agent gemcitabine [155]. Either cholesterol-bound oligonucleotides (antagomirs), or LNA-modified oligonucleotides (LNA anti-miR) were also found to be effective in stably suppressing miRNA activity in the *in vivo* condition [156, 157]. In addition, liver appeared to be the organ most efficiently and consistently targeted by intravenous injection of AMOs. Recently, a study performed in African green monkeys assessed safety and efficacy of the approach. Efficient silencing of miR-122 was achieved by three doses of 10 mg/kg LNA-anti-miR, leading to a long-lasting and reversible decrease in total plasma cholesterol without any evidence for associated toxicities or histopathological changes in the liver of the animals. Thus, by proving feasibility, safety and efficacy for the use of AMOs in a pre-clinical setting, these studies established the basis for their use as therapeutic molecules in clinical trials. The potentiality for therapeutic implementation of small RNAs or AMOs in clinical practice is enormous. Moreover, as far as treatment of liver and HCC is concerned, the limitations encountered for other organs related to an effective *in vivo* delivery of the drugs is partly overcome by the current clinical application of techniques able to deliver the drugs directly into the hepatic artery branches.

Concluding remarks

The discovery of aberrantly expressed miRNAs in HCC has helped to reveal novel mechanisms in liver tumourigenesis. Understanding molecular tumourigenesis has established the basis for the development of more rational classification and therapeutic approaches. In HCC, aberrantly expressed miRNAs could be associated with bio-pathological and clinical features, making miRNA expression a potentially useful tool for HCC classification and prognostic stratification, in particular in early HCC, where the availability of potentially curative approaches requires a more sophisticated diagnostic approach. Finally, by revealing which miRNA are up- or down-regulated in liver tumourigenesis, new potential therapeutic targets have been revealed. Since technological advancements have shown that the use of miRNAs or AMOs as potentially therapeutic molecules is feasible and safe, their experimentation may become an important area of clinical investigation in the years to come.

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