

WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma**Implications of biomarkers in human hepatocellular carcinoma pathogenesis and therapy**

Li-Li Han, Yi Lv, Hui Guo, Zhi-Ping Ruan, Ke-Jun Nan

Li-Li Han, Hui Guo, Zhi-Ping Ruan, Ke-Jun Nan, Department of Medical Oncology, The First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China

Yi Lv, Departments of Hepatobiliary Surgery, The First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China

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Correspondence to: Ke-Jun Nan, PhD, Department of Medical Oncology, The First Affiliated Hospital of Medical College, Xi'an Jiaotong University, No. 58 Chang'an North Road, Beilin District, Xi'an 710061, Shaanxi Province, China. nankj@163.com

Telephone: +86-29-85324086 Fax: +86-29-85324086

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Abstract

Hepatocellular carcinoma (HCC) is one of the most frequent tumors worldwide and accounts for approximately one-third of all malignancies. In the past decade, advances have been made to improve the prognosis of HCC, including improvement in the clinical diagnosis of early-stage HCC using molecular biomarkers and molecular-targeted therapy to treat advanced HCC. However, the diagnosis, pathogenesis and targeted therapy of HCC are not completely independent, and should be comprehensively studied. For example, a number of tumor markers provide useful clinical information not only for prognosis, but also in pathogenesis and treatment efficacy. Therefore, this review will focus on the role of several specific biomarkers implicated in the pathogenesis of HCC and several promising molecular-targeted

drugs that target the biomarkers of HCC.

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Key words: Biomarkers; Pathogenesis; Hepatocellular carcinoma; Targeted therapy; MicroRNAs

Core tip: Advances made in the prognosis of hepatocellular carcinoma (HCC) in recent years include improvements in clinical diagnosis using biomarkers and the potential of molecular-targeted therapy. The diagnosis, pathogenesis and targeted therapy of HCC should be comprehensively studied. Several biomarkers, including both traditional biomarkers and novel biomarkers, such as microRNAs, are also essential in the pathogenesis of HCC, and represent important new targets for HCC therapy. Ongoing studies and clinical trials suggest that molecular-targeted drugs that target biomarkers and their pathways will be used to treat HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death in men, the sixth leading cause of cancer-related death in women and is the most frequently diagnosed cancer worldwide^[1]. In addition, HCC is one of the deadliest primary cancers, with a 5-year survival rate of 10% or less^[2].

Chronic infections with either hepatitis B virus (HBV) or hepatitis C virus (HCV) greatly increase the relative

risk of HCC. These chronic viral infections are present in more than 70% of HCC cases, and iatrogenic interventions for these viruses significantly reduce the risk of developing HCC^[5]. In addition to HBV and HCV infection, other major pathogenetic factors include alcoholic cirrhosis, non-alcoholic steatohepatitis, metabolic syndrome and fatty liver. These diseases are all associated with impaired liver function in HCC patients, making treatment of HCC very difficult. Furthermore, the early stages of HCC are generally silent, with rapid growth of the tumor^[4].

Despite the possibility of early detection using such methods as serum biomarkers, alpha-fetoprotein (AFP) and ultrasound examination, surgical resection or liver transplantation are not performed in the majority of HCC cases, because surgery can only be carried out at the early and confined stages of HCC. Chemotherapy has a response rate of less than 20%, thus is not a good treatment option. Limited treatment options and late diagnosis may explain the low survival rate of patients with HCC^[5]. Only early diagnosis of HCC can improve the survival rate; therefore, studies on specific biomarkers, particularly novel biomarkers, such as microRNAs, over the last two decades are of profound importance. Both traditional tumor markers including AFP, glypican-3 and transforming growth factor (TGF)- β and novel biomarkers including microRNAs provide useful clinical data, not only on prognosis, but also on pathogenesis and treatment efficacy. Furthermore, specific biomarkers may be potential therapeutic targets.

This review will focus on the role of several specific biomarkers in the pathogenesis and treatment of HCC.

TRADITIONAL MOLECULAR BIOMARKERS AND THEIR IMPORTANCE IN PATHOGENESIS AND THERAPY

Traditional molecular markers used for HCC diagnosis can be classified into three major types: (1) serological markers; (2) cancer stem cell markers; and (3) tumor tissue markers. Of these, serological markers are most commonly used in the clinic, and are closely related to the pathogenesis and targeted therapies of HCC. Therefore, we will discuss several traditional serological molecular biomarkers involved in the pathogenesis and targeted therapies of HCC.

AFP

AFP is abundantly expressed in fetal liver cells, but not in normal adult liver cells, and is the most commonly used HCC serum biomarker. However, its sensitivity and predictive accuracy often depend on the AFP cut-off value, and the results are less than satisfactory. Sensitivity of HCC diagnosis is 60%-80% with a 20 ng/mL AFP cut-off value; however, the sensitivity decreases to 20%-40% for the detection of small tumors^[6]. High serum AFP levels can also be found in several hepatitis infections and

chronic liver conditions. Therefore, AFP is not considered an ideal diagnostic biomarker for HCC^[5].

Further research has shown that the role of AFP in HCC targeted therapies is a concern. AFP computational secreted network construction and analysis of HCC is very useful to identify novel markers and potential targets for prognosis and therapy^[7,8]. The specific expression level of AFP led to the AFP promoter being used as an HCC targeting promoter to drive the adenovirus *E1A* gene^[9] or suicide genes, such as herpes simplex virus thymidine kinase.

Recent studies showed that AD55-Apoptin, in which the *E1A* gene was driven by the AFP promoter along with a 55 kDa deletion in the E1B gene to form AD55-Apoptin, may be a potential anti-hepatoma agent and has shown marked antitumor efficacy and safety in a cancer targeting gene-viro-therapy system^[10]. Therefore, AFP may contribute to the therapy of HCC.

Glypican-3

Glypican-3 (GP3) is a member of the heat-shock protein family and plays a pivotal role in cell growth, differentiation and migration^[11]. GP3 is expressed by most HCCs. Initially, only GP3 mRNA was reported to be significantly elevated in HCC compared with normal liver and was used as a biomarker^[12]. A study by Capurro *et al.*^[12] in 2003 confirmed these results at the protein level. The authors found that 72% of HCC patients expressed GP3 in serum using immunoblotting and ELISA. Several independent studies confirmed these results^[13-15]. The serological sensitivity and specificity of GP3 as a HCC biomarker were 77% and 96%, respectively, as reported by the International Consensus Group. Consequently, GP3 is used in the clinic to confirm a diagnosis of HCC.

In addition to being a biomarker of HCC, GP3 plays a pivotal role in the pathogenesis and progression of the disease^[11]. GP3 promotes the growth of HCC by regulating the signaling activity of several growth factors, including the Wnt/ β -catenin pathway, which is crucial for the progression of HCC^[16,17]. This is based on the ability of GP3 to increase the binding of Wnt to its signaling receptor^[18,19]. Several reports have confirmed GP3-induced activation of canonical Wnt signaling in HCC cells^[19]. Furthermore, GP3 can stimulate fibroblast growth factor (FGF), which is activated in a significant proportion of HCC^[20].

A humanized anti-GP3 monoclonal antibody has been produced and was safely administered intravenously up to 20 mg/kg per week in a phase I clinical trial of patients with advanced HCC^[21]. In addition, investigators have attempted to inhibit HCC growth by blocking GP3 function using targeted GP3 in immunotherapeutic approaches^[22,23]. Consequently, GP3 not only has a significant role in the diagnosis of HCC, but also an established role in the future therapy of HCC.

Serum Fas/FasL

Fas is a type I membrane protein and belongs to the

tumor necrosis factor (TNF) receptor family, whereas FasL is a member of the TNF family. The Fas/FasL (Fas-ligand) system, which plays a major and unique role in HCC growth and metastasis, is upregulated in various chronic liver diseases and accelerates their progression. Serum Fas (sFas) expression is upregulated in chronic hepatitis B, chronic hepatitis C^[24] and acute liver failure^[25]. Furthermore, sFas is significantly higher in HCC patients compared with patients suffering from chronic hepatitis C or liver cirrhosis. In addition, the level of sFas and FasL increase from chronic hepatitis to cirrhosis^[26]. The linear relationship between expression of Fas in liver tissue and its serum levels suggests that these parameters could be considered predictive markers of tumorigenesis in HCC^[27].

Several independent studies have shown that overexpression of FasL is associated with many tumors, including the advanced stages of HCC^[28]. sFas is believed to act as a decoy receptor that prevents Fas/FasL binding and inhibits Fas-mediated apoptosis^[29]. The sFas protein inhibits the activity of cytotoxic T lymphocytes in a dose-dependent manner, and is capable of inhibiting hepatic apoptosis by binding to FasL or anti-Fas antibodies, and triggering a cascade of intracellular signaling events that end in cell death by apoptosis. Fas/FasL signaling contributes to phenylalanine-induced apoptosis of HCC cells^[30].

Apoptosis and the Fas system are significantly involved in the conversion of liver cirrhosis to HCC. Downregulation of Fas expression and upregulation of FasL expression in hepatocytes, and elevation of serum sFas levels are important in tumor evasion from immune surveillance and in hepatic carcinogenesis. Therefore, attention has been focused on the use of these components of the Fas system as targets for anticancer therapy.

TGF- β

The TGF- β family plays a pivotal role in physiology during embryonic development, as well as in the control of tissue homeostasis in adults, such as regeneration *via* cytokines, which regulates the growth and differentiation of both normal and transformed liver cells^[31].

Although the presence of TGF may lead to some false positive results because of its high expression in liver cirrhosis^[32], TGF is still a significant HCC biomarker, especially in patients with HCC caused by chronic hepatitis B; high serum TGF levels generally indicate a poor prognosis^[33]. As a serological biomarker of HCC, TGF has a sensitivity of 68% at a cut-off value of 800 pg/mL, which is much higher than AFP at the same cut-off value, especially in the diagnosis of early stage HCC.

The TGF- β family regulates growth inhibition and induces apoptosis in hepatocytes^[34]. Several independent studies have shown that TGF- β acts as an important tumor suppressor during the early stages of tumor development and as a proto-oncogene during the late phase of carcinogenesis. TGF- β acts as a tumor suppressor by inhibiting cell proliferation, and as a tumor promoter, TGF- β induces an epithelial-mesenchymal transition

(EMT), cell motility and invasion^[35] by regulation of vascular endothelial growth factor (VEGF), extracellular regulated protein kinases (ERK) and hypoxia inducible factor (HIF-1) and over-activation of cyclin and cyclin-dependent kinases^[36].

Carmona-Cuenca *et al*^[37] reported that TGF- β up-regulates the Rac-independent NADPH oxidase, NOX4, which is inhibited by PI3K or mitogen-activated protein kinase (MAPK)/ERK anti-apoptotic signal pathways in both rat and human hepatocytes. This correlates with its proapoptotic activity, leading to mitochondrial-dependent apoptosis^[38]. Furthermore, TGF reduces migration and invasion of HCC cells by upregulating E-cadherin^[39].

In conclusion, any advance in the understanding of the molecular mechanisms that allow HCC cells to escape from TGF- β -induced apoptosis may have potential for future targeted therapy of HCC.

VEGF/VEGF receptor

VEGF was initially identified in 1983 as a protein secreted by tumor cells^[40]. The expression of VEGF and its receptors, which include VEGF receptor (VEGFR)1, VEGFR2, and VEGFR3, is elevated in HCC cell lines and tissues, as well as in the blood circulation of patients with HCC. The combination of a serum AFP value > 19.8 ng/mL and a serum VEGF value > 355.2 pg/mL increased HCC screening sensitivity to 95.5% compared with the individual sensitivities of 68.2% and 86.4%, respectively^[41]. Moreover, VEGF plays an important role in screening patients suitable for liver transplantation, because of its close relationship with microscopic venous invasion and intrahepatic metastasis. Higher VEGF expression in the serum of patients with small HCC (< 5 cm) predicts a poor prognosis after liver transplantation, radiofrequency ablation or transcatheter arterial chemoembolization. Furthermore, other studies have suggested that the expressions of VEGF-C, VEGFR1, and VEGFR3 in peritumoral liver tissue are associated with a unique type of HCC that had a poorer outcome after hepatectomy^[42]. In addition, patients with remotely metastasized tumors showed much higher levels of serum VEGF compared with HCC patients without metastasis.

Numerous studies have shown that angiogenesis is pivotal in the pathogenesis of human HCC. Angiogenesis is mediated by the activation of different pathways in the tumor and endothelium, and the most critical player is VEGFR. As a permeability factor, VEGF promotes extravasation of plasma fibrinogen, leading to the formation of fibrin scaffolding, which facilitates cell migration during invasion. In addition, as an endothelial growth factor, the receptors of VEGF, which induce cell proliferation in an autocrine fashion, activate intracellular signals, such as the RAF/MEK/ERK pathway and the PI3K/AKT/mTOR pathway.

HCC is a vascular tumor, in which increased levels of VEGF and microvessel density have been observed. There are many anti-angiogenic agents in clinical trials of HCC, and most target VEGF and VEGFR^[43]. There is no

Table 1 Several important molecular-targeted therapies that inhibit the vascular endothelial growth factor/vascular endothelial growth factor receptor signaling pathways involved in hepatocellular carcinoma

Names of drug	Targeted signaling pathways	Phase/number of patients	Main side effects and prevalence	Efficiency (mo) (PFS/OS)
Sorafenib	Raf/MAPK/ERK, VEGFR-2, -3, PDGFR	II /137	Diarrhea (8%), hand-foot skin reaction (5%)	5.5/9.2 ^[49]
Bevacizumab	VEGF/VEGFR	II /46	Hypertension (15%), thrombosis (6%), and major bleeding (11%)	6.9/12.4 ^[52]
Sunitinib	VEGFR1, VEGFR2, PDGFR, c-KIT, FLT3, RET kinases	II /45	Fatigue (62%), diarrhea (47%), nausea (44%)	1.5/9.3 ^[54]
Brivanib	VEGFR, FGFR	II /55	Hypertension (33.8%), proteinuria (14.7%), hemorrhage (11.8%)	2.7/10 ^[56]
Linifanib	VEGFR and PDGFR	II /44	Hypertension (25.0%) and fatigue (13.6%)	3.7/9.7 ^[58]
Ramucirumab	VEGFR-2	II /40	Hypertension (14%), gastrointestinal hemorrhage and infusion-related reactions (7% each), and fatigue (5%)	4.0/12 ^[59]

VEGFR: Vascular endothelial growth factor receptor; ERK: Extracellular regulated protein kinases; MAPK: Mitogen-activated protein kinase; PDGFR: Platelet-derived growth factor receptor; FGFR: Fibroblast growth factor receptor; PFS: Progression-free survival; OS: Overall survival.

doubt that anti-angiogenesis therapies have shown promise in the treatment of HCC^[44]. Therefore, future studies should identify and characterize these pathways, with the goal of targeting anti-VEGF therapies. Sorafenib, an oral multi-kinase inhibitor, targets VEGF-mediated angiogenesis and is the first drug to prolong the survival of patients with advanced HCC^[45]. This has opened a new era for anti-angiogenic therapies in HCC^[46]. Several important molecular-targeted therapies that inhibit the VEGF/VEGFR signaling pathways involved in HCC are summarized in Table 1.

Sorafenib inhibits Raf/MAPK/ERK signaling, VEGFR-2, -3, and PDGFR, increases apoptosis, decreases angiogenesis and cell proliferation, and inhibits overall tumor cell signaling^[47]. Based on the results of the recent large randomized phase III studies, Sorafenib has been approved by the United States Food and Drug Administration for the treatment of patients with advanced HCC. In the sorafenib HCC assessment randomized protocol (SHARP) trials, the median overall survival (OS) increased from 7.9 mo in the placebo group to 10.7 mo in the sorafenib group. Sorafenib also showed significant benefit in terms of time to progression (TTP), with a median of 5.5 mo in the sorafenib group and 2.8 mo in the placebo group^[48]. It is worth noting that the impact of viral etiology in HCC on the survival of patients is still controversial. Survival benefits of sorafenib are different according to etiologies of HCC, with or without extrahepatic spreading and vascular invasion, as well as other risk factors by subgroup analyses. An Italian study suggested a worse survival for patients with HBV-related HCC, particularly in advanced-stage disease. A meta-analysis of 14 randomized clinical trials of systemic therapy indicated that HBV-related HCC is an independent predictor of better survival^[49].

As the SHARP trial reported, the overall incidence of treatment-related adverse events was 80% in the sorafenib group and 52% in the placebo group. The rate of discontinuation of the study drug due to adverse events

(38% *vs* 37%), grade 3-4 drug-related adverse events and the overall incidence of serious adverse events from any cause (52% *vs* 54%) were similar in the two study groups. The most common serious adverse events of any cause (aside from death) were liver dysfunction (7% and 5%, respectively), diarrhea (5% and 2%) and ascites (5% and 4%).

Sorafenib is currently undergoing investigation in a phase III study (the STORM trial) as an adjuvant therapy for the prevention of recurrence following surgery or local ablation. Moreover, a phase II trial is currently recruiting patients to determine the progression-free survival of patients with advanced or metastatic HCC treated with sorafenib plus tegafur/uracil.

Bevacizumab

Bevacizumab is a recombinant humanized monoclonal antibody that targets the VEGF signaling pathway and blocks tumor vascularization^[50]. A phase II study of 46 patients with unresectable HCC, but no overt extrahepatic metastases or invasion of major blood vessels, treated with bevacizumab, found that six patients (13%) had objective responses (95%CI: 3-23), 65% were progression-free at 6 mo, median PFS was 6.9 mo (95%CI: 6.5-9.1), and median OS was 12.4 mo (95%CI: 9.4-19.9). Grade 3-4 adverse events included hypertension (15%) and thrombosis (6%, including 4% with arterial thrombosis)^[51].

Sunitinib

Sunitinib, a multi-kinase inhibitor, blocks a number of angiogenesis-related signaling pathways, including VEGFR1, VEGFR2, PDGFR, c-KIT, FLT3, and RET kinases^[52]. Sunitinib is a very promising multi-kinase inhibitor, as a study by Koeberle and colleagues showed that in 45 patients treated with a daily dose of 37.5 mg sunitinib, median PFS and OS were 1.5 and 9.3 mo, respectively^[53].

In addition, a randomized phase III study comparing sunitinib with sorafenib in patients with advanced HCC was conducted. In this large study of 1073 patients, al-

Table 2 Clinical relevance of deregulated microRNAs in hepatocellular carcinoma

Molecular alteration	miRNAs	Clinical significance
Down regulation	miR-139	Poor survival ^[70]
	miR-26	Shorter overall survival ^[82-87]
	miR-124	Gain of metastatic properties early recurrence ^[69]
	miR-145	Advanced tumor progression, poor prognosis ^[71]
	miR-199a-3p	Reduced time to recurrence ^[72]
	miR-199b-5p	Poor overall survival and Progression-free survival rates ^[72]
Up regulation	miR-122	Poor prognosis ^[82-87]
	miR-222	Shorter disease-free survival ^[73]
	miR-135	Poor prognosis worse Edmondson-Steiner grade, vein invasion, shortened overall survival and disease-free survival ^[74]
	miR-221	Multinodularity, reduced time to recurrence, gain of metastatic properties high tumor capsular infiltration ^[79]
	miR-155	Poorer recurrence-free survival and overall survival ^[73]
	miR-182	Intrahepatic metastasis and poor prognosis ^[76]
	miR-10b	Poor prognosis ^[77]
	miR-17-5p	Poor prognosis ^[78]
	miR-21	Poor prognosis ^[79]

though sunitinib failed to demonstrate superiority in OS compared with sorafenib^[54], both PFS and TTP in sunitinib-treated patients were superior to those in sorafenib-treated patients. Unfortunately, the use of sunitinib was discontinued because of adverse effects during phase II and phase III clinical trials.

Brivanib

Brivanib is a dual inhibitor of VEGFR and fibroblast growth factor receptor (FGFR). A phase II study was undertaken to assess the efficacy and safety of brivanib in patients with advanced HCC, and the antitumor activity of brivanib was preliminarily demonstrated. Median PFS and OS were 2.7 mo (95%CI: 1.4-3.0) and 10 mo (95%CI: 6.8-15.2) in the first line-study^[55]. Median OS and TTP as assessed by investigators following second-line treatment with brivanib were 9.79 and 2.7 mo, respectively^[56].

Linifanib (ABT-869)

Linifanib (ABT-869) is a potent inhibitor of VEGFR and PDGFR. Preliminary results from an open label, multicenter phase II study of linifanib in advanced HCC have been reported^[57]. The median TTP/PFS was 112 d and median OS was 295 d (95%CI: 182-333). A phase III study comparing linifanib with sorafenib in advanced HCC is ongoing and the results are pending.

Ramucirumab (IMC-1121B)

Ramucirumab is a recombinant human monoclonal antibody against VEGFR-2. A phase II study demonstrated that the response rate (RR) was 10%, PFS was 4.0 mo, and OS was 12.0 mo in patients who had not received prior systemic therapy^[58]. Furthermore, a phase III study of ramucirumab compared with placebo is ongoing in patients with advanced HCC who have failed or could not tolerate sorafenib.

Cediranib (AZD2171)

Cediranib has potent activity against pan-VEGFR inhibitor, PDGFR and c-Kit. In a phase II study, the median OS was 5.8 mo (95%CI: 3.4-7.3) and TTP was 2.8 mo

(95%CI: 2.3-4.4) in patients with advanced HCC^[59].

Other oral tyrosine-kinase inhibitors Pazopanib (GW786034), TSU-68, Lenvatinib (E7080) and Lenalidomide block a number of angiogenesis-related signaling pathways. Pazopanib targets VEGFR, PDGFR, and c-Kit, TSU-68 targets EGFR-2, PDGFR, and FGFR-2, Lenalidomide inhibits VEGF and FGF, and Lenvatinib (E7080) targets VEGFR1-3, FGFR1-4, RET, KIT, and PDGFR-beta. These agents showed promising results in phase II clinical trials of patients with advanced HCC^[60-63].

NOVEL MOLECULAR BIOMARKERS AND THEIR IMPORTANCE IN PATHOGENESIS AND THERAPY

MicroRNAs

MicroRNAs (miRNAs), a class of noncoding RNAs of 15-25 nucleotides in length found in both plants and animals, have emerged as key posttranscriptional regulators of gene expression by interacting with the 3' UTRs of protein-coding mRNAs^[64]. Clinically, because of their tumor-specific expression and stability both in tissues and in the circulation, miRNAs have been proposed as novel biomarkers in the diagnostic and prognostic stratification of HCC. Numerous studies have reported the key role of miRNAs in tumor cell proliferation, apoptosis, metastasis and drug resistance^[65]. By targeting different genes in tumor development, there is accumulating evidence to indicate the role of miRNAs as tumor suppressors or oncogenes in hepatic malignancies^[66]. Furthermore, the encouraging therapeutic potential of miRNAs has been demonstrated in various studies in recent years.

miRNAs as biomarkers in HCC

The differential expression of miRNAs in HCC cells indicates the potential value of miRNA detection in the prediction of HCC diagnosis and prognosis. Table 2 summarizes studies on the clinical value of miRNA detection in HCC.

Downregulation of miR-99a^[67], -124^[68], -139^[69],

Table 3 MicroRNAs deregulated in hepatocellular carcinoma

miRNAs	Expression in HCC	Target	Involvement in cellular processes
miR-199	Down	Met, mTOR, PAK4, DDR1, caveolin-2	Proliferation, autophagy, metastasis, invasion; autophagy, drug resistance; cell growth ^[95]
miR-449	Down	c-Met	Proliferation; apoptosis ^[97]
miR-122	Down	Bcl-w, ADAM-1, Wnt-1	Apoptosis; metastasis; angiogenesis ^[100-102]
miR-34a	Down	c-Met; CCL22	Metastasis ^[103]
miR-200a	Down	HDAC4	Proliferation; metastasis ^[104]
miRs-let-7a, -7b, -7c, -7d, -7f-1	Down	Caspase-3, HMG2A, C-myc, Bcl-xl	Proliferation, apoptosis ^[112]
miR-125a, -125b	Down	MMP11, SIRT7, VEGF-A, LIN28B2, Bcl-2, Mcl-1, Bcl-w	Angiogenesis, apoptosis, metastasis, proliferation ^[115,116]
miR-29	Down	SPARC	Proliferation ^[114]
miR-224	Up	RKIP; CDC42; CDH1; PAK2; BCL-2; MAPK1; API-5	Metastasis; proliferation; apoptosis ^[113]
miR-519d	Up	CDKN1A/p21; PTEN; AKT3; TIMP2	Proliferation; invasion; apoptosis ^[109]
miR-216a	Up	TSCL1, PTEN	Tumorigenesis ^[107]
miR-148a	Up	PTEN	Tumorigenesis ^[108]
miR-21	Up	PTEN, RhoB, PDCD4	Drug resistance, metastasis ^[105,106]
miR-221	Up	Bmf, DDIT4, Armt, CDKN1B/p27, CDKN1C/p57	Angiogenesis, apoptosis, proliferation ^[98,99]

HCC: Hepatocellular carcinoma.

-145^[70] and -199b^[71] was significantly associated with poor prognosis, shorter disease-free survival and features of metastatic tumors including venous invasion, microsatellite formation, absence of tumor encapsulation and reduced differentiation. Conversely, high levels of miR-222^[72], -135a^[73], -155^[74], -182^[75], -10b^[76], -17-5p^[77], -221 and -21^[78], correlated with poor prognosis, such as increased risk of tumor recurrence and shorter overall survival.

In addition, for classification purposes, miR-200c, miR-141 and miR-126, alone or in combination, could be used to distinguish primary HCC compared to tumor metastases to the liver with very high accuracy; moreover, the ratio of miR-205 to miR-194 expression could be used to distinguish between gastrointestinal tumors and metastases outside the gastrointestinal system^[79].

Extracellular miRNAs in the circulation are stable because RNase in the blood protects them from enzymatic cleavage, suggesting that the expression profile of miRNAs in serum or plasma may also serve as novel diagnostic markers^[80]. To date, more than 20 circulating miRNAs have been associated with HCC detection, of these miRNAs, the levels of miR-122, -192, -21, -223, -26a, -27a and -801a were significantly higher in patients with HCC and could help detect early-stage HCC with high diagnostic accuracy^[81-86]. However, because increased levels are also detected in chronic hepatitis, their usefulness as clinical tumor markers needs to be further validated. Several independent studies showed that the amount of miR-500 in the serum of HCC patients increased and then returned to normal after surgical treatment. Moreover, the relative amount of miR-92a in the plasma of HCC patients was reduced compared with that in healthy persons^[87]. In addition, the expression profile of miR-25, miR-375, and let-7f in serum could identify HCC cases^[88]. These studies confirmed the potential use of miR-

NAs as sensitive markers for the detection of underlying HCC and for prognostic stratification of the disease.

Involvement of miRNAs in HCC pathogenesis

In addition to being biomarkers of HCC, miRNAs play a pivotal role in the pathogenesis and progression of this disease.

MiRNAs and HCC-associated virus infection: miRNAs regulate HBV and HCV infection, which significantly increase the relative risk of HCC, at the transcription level either by targeting crucial cellular transcription factors required for HBV gene expression or by directly binding to HBV transcripts^[89]. For example miR-1, miR-152 and miR-148a play protective roles in HBV-induced HCC^[66]. Similarly, miR-196 inhibits HCV transcription by upregulating heme oxygenase (decycling) 1 (HMOX1) expression^[90]. In addition, miR-217 promotes hepatocyte ethanol-induced fat accumulation, which is another risk factor for HCC^[91].

MiRNAs and HCC-associated molecular pathways:

Numerous studies have demonstrated that HCC develops *via* deregulation of various molecular pathways, such as PTEN/PI3K/AKT/mTOR, p53, RAS/MAPK, WNT/ β -catenin, MET, CDKN1B/p27/Kip1, CDKN1C/p57/Kip2 and transforming growth factor beta. MiRNAs play key roles in regulating these diverse molecular pathways, similar to transcription factors. It has been reported that miRNAs exert their functions either as oncogenes or tumor suppressor genes in HCC. Deregulated miRNAs in cancer cells contribute to diverse molecular pathways, including sustained proliferative signaling, survival, angiogenesis, invasion, and metastasis^[92] (Table 3).

MiR-199 regulates several targets, including target genes, such as MET, mTOR, HIF-1 α 48-51 and trans-

membrane glycoprotein CD44. Restoring the expression of miRNA-199 in HCC cells led to reduced invasive capability, enhanced susceptibility to hypoxia, and increased sensitivity to doxorubicin-induced apoptosis^[93]. Similarly, downregulation of miR-199a in patients with HCC is associated with a higher recurrence rate and shorter time to recurrence after surgery. Therefore, miR-199 was suggested to act as a tumor suppressor gene in HCC. C-Met can also be suppressed by other miRNAs, including miR-198^[94] and miR-449^[95].

MiR-221 affects several cancer pathways, such as cyclin-dependent kinase inhibitors CDKN1B/p27 and CDKN1C/p57, and the PTEN-PI3K-AKT-mTOR pathway. Other important targets include the BH3-only protein, Bcl-2-modifying factor (Bmf) and DNA damage-inducible transcript TIMP3, a tissue inhibitor of metalloproteases^[96]. Overexpression of miR-221 increased growth, proliferation, migration, and invasion in HCC cells. Similarly, overexpression of miR-221 promoted tumor progression and shortened the survival of animals with cancer. Recently, a study using a transgenic mouse model with overexpression of miR-221 demonstrated that cancer in these animals was partly inhibited by anti-miR-221 oligonucleotides. Taken together, miR-199 is thought to be a tumor oncogene in HCC^[97].

MiR-122, a negative regulator of p53, is downregulated in approximately 70% of HCCs, suggesting that it has tumor suppressor function. Studies using miR-122 knockout mice demonstrated a direct role for miR-122 in liver cancer^[98,99]. It was shown that high expression of miR-122 induced apoptosis, arrested the cancer cell cycle, inhibited tumorigenicity and sensitized HCC cells to sorafenib or doxorubicin^[100]. On the other hand, loss of miR-122 expression in patients with HCC correlated with metastasis and recurrence.

MiR-34a, another downstream target of tumor suppressor p53, functions as a link between p53 signaling and cell cycle regulation by targeting cyclin D1, cyclin-dependent kinase 4 (CDK4) and CDK2 in HCC^[101]. In addition, p53 upregulates the miR-200 and miR-192 families of miRNAs to inhibit ZEB1/2-mediated EMT^[102].

MiR-21, which downregulates the expression of tumor suppressor phosphatase and tensin homolog (PTEN)^[103], is also a potent oncogene in HCC. Overexpression of miR-21 in HCC cells can increase tumor cell proliferation and migration. In addition, miR-21 plays a role in the inhibition of cell proliferation and increased apoptosis *in vivo*^[104].

PTEN is downregulated by many other miRNAs in HCC, such as miR-216a^[105], miR-148a^[106], miR-519d^[107], and miR-29a^[108], leading to activation of the PI3K/AKT/mTOR pathway. Moreover, miR-7 regulates the PTEN/PI3K/Akt pathway by targeting phosphoinositide-3-kinase (PIK3CD), mTOR, and p70S6K^[109].

Let-7 miRNAs negatively regulate B-cell lymphoma-extra large (Bcl-xL) expression and enhance the sensitivity of HCC cells to apoptosis induced by Mcl-1-targeting anticancer drugs^[110]. On the other hand, B-cell lymphoma

2 (Bcl-2), induced myeloid leukemia cell differentiation protein (Mcl-1) and Bcl-2-like protein 2 (Bcl-w) are targets of miR-224^[111], miR-29^[112] and miR-125b, respectively^[113,114].

Potential roles of miRNAs in HCC therapy

Recent studies have suggested that strategies based on the modulation of miRNA activity may provide a novel approach to treating HCC.

MiRNA inhibition: several independent studies have demonstrated that inhibition of miR-122 by administering anti-miRNA oligonucleotides in nonhuman primates resulted in a reduction in miRNA activity in the adult liver without any evidence of toxicity, and led to prolonged survival or a reduction in the number and size of tumor nodules^[115,116]. It was confirmed that AAV-mediated delivery of miR-122 inhibited tumorigenesis in a myc mouse HCC model^[100]. Treatment with an LNA-modified anti-miR-122 was well tolerated and led to stable suppression of HCV infection in cynomolgus monkey models^[117]. In addition, an antisense inhibitor of miR-122 could produce a dose-dependent and prolonged decrease in HCV RNA levels in patients with chronic HCV genotype 1 infection, which is a major cause of hepatocellular carcinoma in many Western countries^[118].

Anti-miR-221 was previously shown to have anti-tumor activity in prostate carcinoma, melanoma and multiple myeloma^[119]. Two recent independent studies using mouse models of HCC showed that anti-miR-221 molecules reduced the proliferation of tumor cells, promoted survival and achieved a significant reduction in the number and size of tumors compared with untreated mice^[116,117].

In addition, separate restoration of miR-143 and miR-124 significantly inhibited tumorigenesis and metastasis *in vivo*^[120].

Miravirsin SPC3649, the first miRNA-targeted drug, has been used in phase I investigations and is currently in a phase II clinical trial for the treatment of HCV infection^[121].

MiRNA replacement: further studies demonstrating the antitumor effects of miRNAs have been reported in other types of tumors and experimental settings^[122]. Thus, in addition to the inhibition of oncomirs, another approach to treating cancer is based on restoration of tumor suppressor miRNAs.

MiR-31 regulates the invasive properties of tumor cells, and elicits metastatic regression in already-established metastases^[123].

AAV8 miR-199, which effectively restored miR-199a/b-3p, was reported to reduce tumor size in HCC^[124].

Cholesterol-conjugated 2'-O-methyl, which effectively restored miR-375, significantly suppressed the growth of HCC in a mouse model^[125].

MiR-34a is the first microRNA replacement therapy scheduled for use in the clinic^[126]. In earlier studies, miR-34a was observed to prevent tumor growth and

progression in mouse models of lung adenocarcinoma and multiple myeloma xenografts induced by K-ras and p53^[127,128]. Recently, miR-34 combined with the cytokine, interleukin-24, showed synergistic antitumor activity in a mouse model of HCC, indicating its possible use in HCC therapy^[129].

Such miR replacement therapies have been demonstrated in the case of miRs-26a^[130] and -124^[124] in mouse models of HCC.

MiRNAs influencing the chemosensitivity of HCC cells: miRNAs regulate the chemosensitivity of cancer cells. Tumors with high expression of oncomiR-21^[131] and -181b^[132] were resistant to IFN-5FU combination therapy and doxorubicin treatment, respectively. Therefore, antagomirs targeting miR-21 or miR-181b may be useful to increase drug efficacy. In contrast, restoration of miR-122 in HCC cells resulted in HCC cells being more sensitive to sorafenib treatment *via* downregulation of the expression of multidrug resistance (MDR) proteins^[133]. In addition, recent studies showed that microRNA-200a/b influenced the therapeutic effects of curcumin in HCC cells, and miR-23a potentiated HCC cell response to etoposide *via* the inhibition of topoisomerase 1 expression.

Anti-miRNA oligonucleotides and miRNA mimics have antitumor activities. By confirming the feasibility as well as the safety and efficacy of these molecules, these studies established the basis for the use of anti-miRNAs as efficient therapeutic targets of HCC in clinical trials. Further studies are expected to assess the clinical value of miRNA-based approaches in HCC.

Potential mRNA biomarkers in HCC

Recently, molecularly-detected peripheral blood mRNA transcripts have been considered as new cancer biomarkers^[134], especially with the application of new generation qPCR and sequencing platforms in mRNA biomarker analysis^[135]. AFP and GPC3 mRNA in peripheral blood had clinical value as predictors of HCC^[136]. Furthermore, some RNA-binding proteins regulated the expression of target spots through mRNA stability^[137]; thus, mRNA biomarkers may represent potential therapeutic targets in HCC.

It is true that among the new molecules, miRNAs have a more clear pathophysiological association with HCC and a better diagnostic accuracy for HCC; however, it is also true that new biomarkers/molecules may not be easily available in all clinical settings (especially primary and secondary clinical settings) and accessibility may not be straightforward everywhere. Therefore, traditional molecular markers such as AFP, widely available and a relatively cheap biomarker for screening, may remain the main clinical marker for the foreseeable future.

CONCLUSION

Exhaustive research on the molecular biology of HCC in recent years has resulted in two major steps to improve

the management of HCC: (1) a wide variety of molecular markers have been shown to be excellent diagnostic tools for HCC; and (2) improvements in the identification of several key molecular pathways involved in the pathogenesis of HCC have led to the development of new targeted therapies for HCC.

However, the diagnosis, pathogenesis and targeted therapy of HCC are not completely independent, and should be comprehensively studied. For example, a number of molecular markers, which have a significant impact on the molecular pathogenesis of HCC, were shown to be promising therapeutic targets for this disease, providing the rationale to develop new effective treatments.

There is new hope for improving the survival of patients with advanced HCC. However, additional clinical trials are required to improve the treatment of HCC patients.

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