

Review

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Molecular diagnosis and therapy of hepatocellular carcinoma: achievements and challenges

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Abstract

Hepatocellular carcinoma (HCC) is often associated with pre-existing chronic liver pathologies of different origin infections of hepatitis B virus (HBV) and hepatitis C virus. Clinically, the diagnosis and therapy for HCC are very important for the prognosis of patients. However, current methods for HCC diagnosis and therapy have no an optimal accuracy due to the tumor heterogeneity and the frequent late diagnosis. This review summarizes the new advances in molecular diagnosis and therapy of HCC, based on the recent novel biomarkers and new therapeutic strategies for HCC, including alpha-fetoprotein-L3, glypican-3, heat shock protein 90, dickkopf WNT signaling pathway inhibitor 1, paraoxonase 1, highly up-regulated in liver cancer. Moreover, epigenetic regulation, signal pathway, cellular and molecular targets for the immunotherapy, tumor microenvironment and genome sequencing analysis may serve as the molecular expression signatures in clinical practice. For promising new treatment strategy of HCC, targeting molecular therapy based on the restoration of tumor suppressor genes lost and inhibition of oncogenic genes is attractive. The new clinical trials for other molecular-targeted agents, including pembrolizumab, nivolumab, tivantinib, lenvatinib, cabozantinib, and ramucirumab, are ongoing in clinic. Interestingly, anti-HBV drugs display an amazing therapy for HBV-related HCC. In future, the global determination of more biomarkers may provide new insights into the diagnosis of HCC. More importantly, the diagnostic markers should be used to trace patient's follow-up disease progression, guiding doctors to judge and prescribe drugs for status of an illness, prognosis and other processes.

Keywords: Molecular diagnosis, therapy, hepatocellular carcinoma, hepatitis B virus, hepatitis C virus

INTRODUCTION

Hepatocellular carcinoma (HCC) is a serious health issue globally. The increased trends of HCC will remain until 2030^[1]. According to the World Health Organization, HCC is the fifth most common cancer



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worldwide and the second most common cause of cancer-related death in 2015. Hepatitis B virus (HBV) or hepatitis C virus (HCV) infection accounting for 80%-90% of all HCC cases are well-known major risk factors for the development of HCC. The other risk factors, such as aflatoxin B1 exposure, alcoholic and non-alcoholic liver cirrhosis, obesity, diabetes, vitamin D deficiency, are involved in HCC occurrence^[2,3]. Although treatment with HBV and HCV infection by some recent antiviral therapies is available, virally mediated hepatocarcinogenesis is still the etiology for the majority of HCC cases worldwide^[4]. In patients with advanced HCC, there is low response to chemotherapy, and sorafenib is the only standard treatment recommended in international guidelines^[5]. Thus, it is very important to identify novel diagnosis biomarkers and therapeutic targets for prognosis of HCC.

Molecular mechanisms of malignant cells will lead to the development of successful HCC therapies. NcRNAs and epigenetic regulation have been considered as a potential non-invasive biomarkers due to their experimental and clinical versatility. Whole-genome sequencing analysis has promoted molecular profiles transduced in expression “signatures” which will help in the comprehension of liver physiopathology. HCC etiology seems to be a factor that should be included in several clinic association studies.

The development of novel and useful biomarkers can be employed as a screening strategy for early diagnosis and prognosis in these high-risk populations, since HCC presents a high mortality rate^[6]. Because late diagnosis, resistance to treatment, tumor recurrence, and metastasis cause to low survival, it is essential for developing novel diagnostics and therapeutics of HCC^[7]. However, current methods for HCC diagnosis and therapy have no an optimal accuracy due to the tumor heterogeneity and the frequent late diagnosis. Therefore, the most urgent needs for early diagnosis and novel therapies of HCC should be developed.

DIAGNOSIS FOR HCC

The present methods for diagnosis of HCC can be divided into the following major aspects: magnetic resonance imaging, abdominal ultrasonography, and contrast-enhanced computed tomography, liver biopsy, and serological test. However, the diagnostic effectiveness of above technologies is not very satisfied, particularly for the diagnosis of small lesions and early diagnosis of HCC. The abdominal ultrasound is an operator-dependent test. Liver biopsy is an invasive method not exempt of mortality risk^[8]. Therefore, the serological test should be developed. In this review, we focused on the advances of gene diagnosis for HCC.

MiRNAs serve as HCC diagnostic markers

As we know, cellular miRNAs released into the extracellular circulation system can be detected by serological test. Owing to circulating miRNAs relevant to HCC, miRNAs may serve as potential biomarkers. Thus, some circulating miRNAs can be considered as representative of certain pathological conditions. Moreover, circulating miRNAs possess accessibility well and high stability in the detection system, particularly for supervision of early stage, pre-symptomatic diseases in at-risk patients^[9]. It has been reported that a serum diagnostic test, based on a 34-miRNAs signature, can recognize the early stage lung cancer with 80% accuracy^[10].

MiRNAs may be used as biomarkers for prognosis or diagnosis in HCC. Down-regulation of miR-let-7g, miR-22, miR-26, miR-29, miR-99a, miR-124, miR-139, miR-145 and miR-199b is involved in the cell's life activities, including proliferation, apoptosis, angiogenesis, disease recurrence, disease-free survival (DFS) and poor prognosis^[11-19]. On the contrary, the increase of miR-10b, miR-17-5p, miR-21, miR-135a, miR-155, miR-182, miR-221 and miR-222 is taken part in the metastasis, angiogenesis and poor prognosis^[20-26]. Additionally, miRNA profiling categorizes HCC into three main parts^[27]. The above discoveries display the important value of miRNA detection in prediction of HCC survival. Some microRNAs may be used for HCC diagnosis. MiR-101 in serum sample was 95.5% for sensitivity and 90.2% for specificity, respectively^[28]. MiR-18a in serum sample was 86.1% for sensitivity and 75% for specificity, respectively^[29]. The expression of miR-25 was

Table 1. Serum diagnostic markers in hepatocellular carcinoma

Biomarkers	Sensitivity (%)	Specificity (%)
AFP ^[6]	60.0	85.0
AFP-L3 ^[6]	84.9	86.4
DCP ^[6]	80.0	81.0
AFP + AFP-L3 + DCP ^[6]	94.3	86.4
PON ^[6]	41.6	85.7
Fuc-PON1 ^[6]	79.1	53.5
Hsp90 α ^[53]	93.32	90.27
Hsp90 α + AFP ^[53]	93.70	94.40
MiR-101 ^[28]	95.5	90.2
MiR-18a ^[29]	86.1	75

AFP: alpha-fetoprotein; AFP-L3: lens culinaris agglutinin (LCA)-reactive AFP; DCP: C-carboxy prothrombin; PON1: paraoxonase 1; Fuc-PON1: PON1-fucosylated level protein; HSP90 α : heat shock protein 90 alpha

significantly up-regulated in HCC tissues, which may be used in HCC prognosis^[30]. And miR-155 reflected tumor recurrence, micro-vascular invasion and recurrence-free survival^[31] [Table 1].

Furthermore, it has been reported that miR-500 is highly expressed in the sera of HCC patients. While, after surgical treatment, the expression level is reduced^[32]. What's more, other miRNAs including miR-25, miR-375 and let-7f, can also be used for distinguishing HCC from normal tissue^[33]. Thus, the levels of extracellular miRNA expression are steady in the body circulation. It suggests that miRNAs may be used as biomarkers for HCC diagnosis.

LncRNAs function as HCC diagnostic markers

Long non-coding RNAs (lncRNAs) are a subgroup of non-coding RNA transcripts greater than 200 nucleotides in length with little or no protein-coding potential. Emerging evidence indicates that lncRNAs may play important regulatory roles in the pathogenesis and progression of human cancers, including HCC. Certain lncRNAs may be used as diagnostic or prognostic markers for HCC, a serious malignancy with increasing morbidity and high mortality rates worldwide. LncRNA HOX transcript antisense intergenic RNA (Hotair) which can bind to lysine-specific demethylase 1 (LSD1) is a 2.2 kilobase ncRNA residing in the HOXC locus. Hotair serves as a scaffold of histone modification complexes including LSD1 and polycomb-repressive complex 2, leading to the development of various tumors^[34,35]. For example, owing to Hotair serving as a scaffold, we found that HBXIP/Hotair/LSD1 complex function as a critical effector of c-Myc in transcriptional activation of downstream target genes^[36]. Silencing Hotair increased response of HepG2 to apoptosis stimulation from TNF- α and chemo-drug Cisplatin and Doxorubicin on a dose-manner^[37]. Highly upregulated in liver cancer (HULC) was detected in 63% (19/30) of the HCC patient's serum, which was much higher than in the healthy control group (10%, 2/20)^[38]. Among the HCC patients, HULC detection frequencies increase with Edmondson grades. The detection rates are 14%, 62%, and 100% for Edmondson grades I-II, II-III, and III-IV, respectively^[38]. HULC was detected more frequently in the plasma of HBV⁺ HCC patients (90%) than in HBV-HCC patients (25%)^[38]. These observations indicate that the presence of HULC is an indication of HCC and its progression. Interestingly, HULC contributes to the abnormal lipid metabolism in HCC cells^[39]. Hepatitis B virus X protein (HBx) is able to raise the expression of HULC in both normal liver L-O2 cells and liver cancer HepG2 cells^[40]. In addition, HULC significantly enhances the hepatocellular proliferation by promoting the HMGA2 expression by sequestration of the microRNA-186 in HCC^[41]. Therefore, the data support the clinical usage of HULC lncRNA as a potential biomarker for HCC diagnosis and prognosis.

Taken together, the development of lncRNA expression profiling using high-throughput technology for specific HCC biomarkers will no doubt lead to more accurate and precise clinical decision-making having the consequence of better patient care in the future. While the first miRNA (lin-4) was identified two

decades ago, other ncRNAs including lncRNAs, snoRNAs, siRNAs and piRNAs have been surfaced and proved to be essential players in cancer pathogenesis^[42]. Therefore, the combination of lncRNAs with other ncRNAs should not be underestimated in the onset and progression of HCC.

Epigenetic markers

DNA methylation affects the phenotype mainly through expression of the corresponding genes, methylation profiles could reflect the biological characteristics of HCC if “passive methylation” could be eliminated appropriately. A number of results have shown the predictive value of selected methylation events on survival^[43]. After hepatectomy, the methylation map of liver may reflect the recurrence-free survival of HCC patients^[44]. It has been reported that the determination of DNA methylation as a potential tumor marker is able to monitor the circulating tumor DNA in plasma samples^[45]. High levels of trimethylated histone H3 lysine 4 (H3K4me3) were usually accompanied by the decreased overall survival and poor prognosis in HCC^[46]. Another research also indicated that high levels of H3K27me3 forecasted poor prognosis and aggressive tumor characteristics, such as large tumor size, vascular invasion, multiplicity of tumors and poor differentiation^[47]. To better understand the roles in HCC, more studies using accurate detection methods, such as ChIP-sequencing, may be developed to evaluate these specific DNA-protein modifications.

About the traditional biomarkers, because the false negative rate of alpha-fetoprotein (AFP) is about 40% for early-stage HCC patients, 15%-30% of all the patients, even patients with advanced HCC, AFP levels remain normal^[48]. Lens culinaris agglutinin (LCA)-reactive AFP (AFP-L3) as an isoform of AFP may improve the detective rate for small lesion of liver cancer^[49]. It has been reported that C-carboxy prothrombin (DCP) has a higher specificity for HCC than AFP but is less sensitive. Thus, the combination of AFP, AFP-L3, and DCP seems to significantly improve HCC diagnostic accuracy^[50]. The combination of Glypican-3 (GPC3) as a novel tumor marker of HCC with AFP proves the sensitivity but not the specificity in HCC diagnosis^[51]. Moreover, the combination of the NH₂-terminal portion of GPC3 which is also called soluble GPC3 with AFP can improve overall sensitivity from 50% to 72%^[52]. Interestingly, the dynamic changes of plasma Hsp90 α in liver cancer patients can detect the condition of treatment, such as surgery and interventional therapy^[53]. Serum Dickkopf WNT signaling pathway inhibitor 1 (DKK1) was reported to be a useful biomarker for diagnosis of HCC by a large-scale and multicenter study^[54]. Paraoxonase 1 (PON1) has been proposed as a circulating protein biomarker since high serum levels in HCC patients concomitantly infected with HCV infection has been observed^[55]. PON1-fucosylated level protein has been helpful in distinguishing early HCC from liver cirrhosis patients even with low AFP levels^[56].

THERAPY FOR HCC

Prevention and management of HBV infection

Based on hepatocarcinogenesis, prevention of HBV infection is a key step to reduce the incidence of liver cancer. There is a renewed interest regarding the understanding of various steps of the HBV replication cycle, as well as specific virus-host cell interactions, to define new targets and develop new antiviral drugs. Basically, the HBV covalently closed circular DNA (HBV cccDNA) is pivotal for persistent HBV infection and recurrence by the end of treatment. As far as we know, the cccDNA usually organizes into a minichromosome with histone 3 and H4 proteins and other nonhistone proteins, such as HBx, HBV core protein, and host transcription factors^[57]. Even though recent therapies can successfully control the viral replication, but they fail to eliminate cccDNA completely. Demonstrating the molecular mechanisms and screening critical factors involved in cccDNA may make it come true to develop more precisely targeted therapeutic strategies and cure HBV-related HCC patients^[58]. It covers a series of inhibition of viral replication processes such as entry inhibitors, capsid assembly modulators, approaches aiming at the secretion of viral envelope proteins, drugs targeting HBV cccDNA, and siRNAs targeting viral transcripts. Restoration of immune responses is a complementary approach. HBV chronic infection and high viral load have been associated with higher levels of soluble programmed cell death protein 1, and this results

in cytotoxic T-cell inhibition and 6.3-fold increase in risk for HCC development^[59]. Using HBV infection models *in vitro* and *in vivo*, new targets and compounds will be available^[60].

HBx plays a crucial role in the various signal transduction pathways and HBV-induced hepatocarcinogenesis^[61]. HBx accelerates the development of hepatoma^[62]. HBx-elevated male-specific lethal 2 can strengthen HBV replication by regulating cccDNA in liver cancer cells, resulting in the development of HCC^[63]. Moreover, we report that anti-HBx in sera may serve as one of the markers involving HBV-related liver cirrhosis and liver cancer^[64]. Developing drugs targeting HBx is crucial for HBV-related HCC therapy. Two types of drugs, conventional interferon, and nucleoside analogs, have become available for the treatment of chronic hepatitis B infection. We also report that anti-HBV drugs such as entecavir, telbivudine and IFN- α 2b inhibit the tumor growth of HBV-related HCC through depressing HBx^[65]. The finding gives innovative insights into the mechanisms of anti-HBV drugs in HCC therapy.

Molecular targets for the immunotherapy

The research about a cohort of 956 HCC patients, 25% had high expression of programmed death-ligand-1 (PD-L1) and programmed cell death protein-1 (PD-1) in HCC tissues. Moreover, the study found that infiltrating CD8⁺ TILs (tumor-infiltrating lymphocyte) could induce PD-L1 expression *via* IFN- γ ^[66]. Icotinib decreases the growth of hepatoma cells *in vitro* and *in vivo*, relying on EGFR activation and PD-L1 expression^[67]. Thus, the PD-1/PD-L1 pathway is available for prognosis and therapy in HCC. Patients with positive PD-L1 expression had significantly poorer DFS and overall survival (OS) than PD-L1 negative patients. The median DFS and OS were 14.9 and 29.6 months for PD-L1 positive patients compared with not reached and 59.4 months for PD-L1 negative patients, thus confirming the findings of the prognostic value of PD-1/PDL-1 in HCC^[68]. Currently, nivolumab, a monoclonal antibody targeting PD-1, obtained an accelerated FDA approval in view of tumor response and durability for the therapy of HCC patients already treated with sorafenib in the phase 1/2 single-arm CheckMate 040 study^[69]. Nivolumab and pembrolizumab targeted PD-1 are ongoing in clinic^[70].

Clinical trial status of molecular-targeted agents

Tivantinib as the first drug was used to a phase III trial grounded in receptor overexpression analyses after disease progression on sorafenib in HCC^[71]. Lenvatinib as an oral multikinase inhibitor for differentiated thyroid cancer and renal cell cancer treatment initially was approved. In a phase 2 trial of HCC patients in Japan and South Korea, lenvatinib treatment was obtained with a 37% response rate (by mRECIST), a median TTP of 7.4 months, and an available toxicity profile^[72]. In addition, Regorafenib as the first agent showed a good survival benefit over placebo in patients progressing on sorafenib^[73]. Regorafenib acting as an oral multikinase inhibitor, largely interdicted the activity of multiple protein kinases including tumor proliferation, metastasis, angiogenesis, microenvironment, and tumor immunity. Regorafenib exhibited a favorable survival regardless of the last dose of prior sorafenib (HR 0.67 for 800 mg/day; 0.68 for < 800 mg/day)^[74]. Further approvals are coming, with good results from phase 3 trials evaluating cabozantinib and ramucirumab in the second-line setting. Cabozantinib, a small-molecule multikinase inhibitor was better than the placebo in the randomized phase 3 CELESTIAL trial^[75]. Based on the trial analyses 707 advanced HCC patients previously received sorafenib treatment, cabozantinib distinctly increased OS over placebo (10.2 months vs. 8.0 months, respectively, $P = 0.0049$)^[76], as shown in [Table 2](#).

Sorafenib

Sorafenib as a molecular-targeted agent can attenuate HCC proliferation and angiogenesis by inhibiting RAF serine threonine kinase and VEGF, PDGF, Flt-3, c-Kit receptor tyrosine kinase, getting approved in Europe and North America in 2007 and in Japan on May 20, 2009. To our delight, a subanalysis of the SHARP study, such as sorafenib in combination with resection, ablation, transcatheter arterial chemoembolization or hepatic arterial infusion chemotherapy, will overtly extend the overall survival in early-, intermediate- or

Table 2. Overview of clinical trial agents for hepatocellular carcinoma therapy

Agents	Developmental status	Targets	Outcomes
Nivolumab	Phase III	PD-1 ^[69]	Ongoing ^[70]
Pembrolizumab	Phase III	PD-1 ^[76]	Ongoing ^[70]
Ramucirumab	Phase III	VEGFR2 ^[77]	Survival benefit for the subgroup with AFP \geq 400 ng/mL ^[79]
Lenvatinib	Phase III	VEGFR1-3, FGFR1-4, PDGFR α , RET and KIT ^[70]	Non-inferior OS, improved PFS, TTP, and ORR ^[70]
Cabozantinib	Phase III	TIE-1, TIE-2, FLT3, c-MET, KIT, RET and VEGFR ^[78]	Significant improvement in OS, PFS, and ORR ^[75]
Tivantinib	Phase III	c-MET ^[71]	Improved OS and PFS ^[71]

HCC: hepatocellular carcinoma; OS: overall survival; ORR: objective response rate; PFS: progression-free survival; TKI: tyrosine kinase inhibitor; TTP: time to progression

advanced-stage HCCs^[80]. However, it increases the potential risk of invasion and metastasis of HCC although it significantly delays tumor progression time^[81].

FUTURE CHALLENGES

Although the effective diagnosis and therapies have been developed in HCC at present, it is unsatisfied to improve the patients' survival. The challenges in the field of diagnosis and therapy for HCC are still ongoing. Therefore, developing new diagnostic approach and drugs are urgent. About gene diagnosis of HCC, high-throughput means combined with bioinformatics methods will be used to find out the root cause of HCC in large-scale sample research. Clinically, it is necessary to monitor the biomarkers in the development of HCC involving treatment and prognosis, but not only in the early stage. It is vital to develop kits for determining the replication activity of HBV (or HCV) and HBV cccDNA to evaluate the risk of HCC incidence. For HCC therapy, identifying innovative targets and combination with multiple drugs are still needed in the treatment strategy. Effective combination of antiviral therapies with anti-inflammation drugs involving inflammation factors is available to treat chronic HBV-related HCC. It is necessary to examine the sensitivity, specificity, predictive value positive, predictive value negative, and validity of any candidate biomarker in a large pool of HCC patients with or without HBV infection, furthermore, it is also important to follow up large patients with HBV or other risk factor exposure for the prediction of occurrence and postoperative recurrence of HCC using representative markers. If biomarkers are valid, it is necessary to develop kits for molecular diagnosis, monitoring the efficacy, prognosis and treatments of HCC patients.

CONCLUSION

In summary, this review lists the recent progresses in gene diagnosis and therapy for HCC. The achievements include the recent novel biomarkers and novel therapeutic strategies for HCC, such as AFP, AFP-L3, GPC3, HSP90, DKK1, PON1, *etc.* Moreover, epigenetic regulation, signal pathway, cellular and molecular targets for the immunotherapy, tumor microenvironment and genome sequencing analysis may also serve as the molecular expression signatures in clinical practice. More studies are necessary to find new biomarkers for prognosis and treatment response in patients under standard treatment of sorafenib. The new clinical trials for other molecular-targeted agents, including pembrolizumab, nivolumab, tivantinib, lenvatinib, cabozantinib, and ramucirumab, are ongoing in clinic. Anti-HBV drugs are available in the therapy of HBV-related HCC.

DECLARATIONS

Authors' contributions

Drafted the outline of this review: Zhang XD
 Drafted the manuscript: Zhang XD, Zhao M
 Finalized the manuscript: Zhang XD, Zhao M

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All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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