Review Article

Existing and emerging biomarkers in hepatocellular carcinoma: relevance in staging, determination of minimal residual disease, and monitoring treatment response: a narrative review

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Background and Objective: With the development of novel active systemic therapies, the landscape of hepatocellular carcinoma (HCC) management is rapidly changing. However, HCC lacks sensitive and specific biomarkers to predict prognosis, monitor for minimal residual disease after locoregional therapy, and predict treatment response. In this review, we aim to summarize the best supporting evidence for refining existing, and development of novel biomarkers for staging, prognosis, determination of minimal residual disease and monitoring treatment response in HCC, focusing on those with evidence in clinical trials.

Methods: PubMed and Embase databases were searched using the keywords; hepatocellular carcinoma, biomarker, minimal residual disease, surveillance, prognosis, staging, alpha-fetoprotein (AFP), liquid biopsy, treatment response, adjuvant, immunotherapy. Relevant clinical studies were included.

Key Content and Findings: AFP remains the major workhorse as the most widely used biomarker in HCC, however, its lack of wide applicability due to the high proportion of patients with HCC who are AFP negative, limits its value throughout all stages of HCC management. Significant work has been done to combine AFP with other clinical and serologic factors to increase its accuracy and utility as a biomarkers. However, it is likely that other more novel biomarkers such as those obtained through liquid biopsy will provide the prognostic power necessary for applications such as detecting recurrence and predicting treatment response. Liquid biopsy provides not only a wealth of potential biomarkers including circulating tumor cells and cell-free RNA/DNA, but also the ability to examine the mutational characteristics of the tumor with next generation sequencing. While early evidence supports the potential impact of many new biomarkers, validation in large clinical trials is lacking.

Conclusions: This review highlights the paucity of sensitive and specific, widely applicable biomarkers, throughout all phases of management of HCC and summarizes evidence on biomarkers currently in use, as well as those in development and validation. Inclusion of biomarker analysis through clinical trials in HCC is critical to development of optimal therapeutic regimens, and improve patient outcomes.

Keywords: Hepatocellular carcinoma (HCC); biomarker; liquid biopsy; alpha-fetoprotein (AFP)

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Introduction

Hepatocellular carcinoma (HCC) is the seventh most common cancer overall, and the second most common cause of cancer specific mortality in the world (1). For many years there was a steady increase in incidence of HCC in the US (6–7 cases per 100,000). Most recently, there has been a plateau in most demographics believed to be due in large part to the success of antiviral therapies for hepatitis C (2). Surveillance protocols for patients at high risk of HCC (hepatitis B, C, D and cirrhosis), only diagnose one third of HCC cases and greater than half of cases present with Barcelona Clinic Liver Cancer (BCLC) B or higher disease, highlighting the need for better surveillance and more effective systemic therapies (3).

For years, sorafenib, a protein kinase inhibitor was the mainstay of systemic therapy for advanced HCC and offered only a 3-month overall survival (OS) benefit (4). The most significant development comes from the landmark IMbrave 150 trial examining atezolizumab [programmed death ligand 1 (PD-L1) inhibitor] and bevacizumab (vascular endothelial growth factor inhibitor) in unresectable HCC demonstrating improved overall and progression free survival compared to sorafenib. However, with these recent advancements in systemic therapy for unresectable and metastatic HCC, translation into the neoadjuvant and adjuvant space remains limited by the paucity of biomarkers to stage, monitor response to therapy and determine minimal residual disease (*Figure 1*). Alpha-fetoprotein (AFP) remains by far the most prevalent biomarker throughout five major phases of HCC management: (I) early detection through screening of high risk patients; (II) as part of a risk stratification program to decide on optimal therapy (e.g., locoregional therapy, resection, transplant etc.); (III) detection of minimal residual disease after resection who may benefit from adjuvant therapy; (IV) part of post-resection surveillance strategy to monitor for recurrence; and (V) to aid in selection of therapeutic agents to which tumors' may be more sensitive. However, its use and widespread applicability is significantly limited as large multicenter studies of HCC have demonstrated that as high as 40–60% of all HCC patients are AFP negative, thus decreasing its utility as a standalone biomarker (5,6).

Next generation sequencing (NGS) made significant advances in characterizing the genetics of HCC. Though the mutational profile of HCC has demonstrated a paucity of druggable targets as well as significant inter- and intra-tumor heterogeneity (7). Thus, the advancement of adjuvant therapy for HCC has been limited. Additionally, genetic biomarker utilization has been limited by the lack of available tumor tissue in HCC largely due to the fact that its diagnosis can be made with only imaging and AFP level. The promise of liquid biopsy [the detection and molecular analysis of cancer related products in the blood stream, including but not limited to tumor nucleic acids, extracellular vesicles and circulating tumor cells (CTCs)] hopes to address this challenge by obviating the need for tissue or invasive procedures beyond blood draws (8).

In 2022, the newly published BCLC staging model incorporates biomarkers. Additionally, the update incorporates the application of immunotherapy for patients with advanced disease, further emphasizing the need for simple and accurate methods to predict and monitor treatment response (9).

In this review we aim to examine the existing literature describing biomarkers in HCC for staging and prognosis, determination of minimal residual disease as well as uses for monitoring response to systemic therapy. Our aim is to highlight the most well described clinical applications and most promising biomarkers, focusing especially on surveillance post resection or transplantation for HCC. We present this article in accordance with the Narrative Review reporting checklist (available at https://hbsn.amegroups.com/article/view/10.21037/hbsn-22-526/rc).

Methods

Both PubMed and Embase databases were searched for English language manuscripts using the keywords; hepatocellular carcinoma, biomarker, minimal residual disease, surveillance, prognosis, staging, AFP, liquid biopsy, treatment response, adjuvant, immunotherapy. Relevant studies were reviewed by all authors for appropriateness for inclusion (*Table 1*).

Staging

An optimal staging system should easily provide accurate prognostic information from the patient's history, imaging, and tissue, and should utilize testing that is widely available to guide therapeutic management decisions for patients with HCC. Additionally, in HCC, patient prognosis is in large part driven by underlying liver disease, creating a distinct challenge for the development of accurate prognostic staging systems in contrast to other cancer sites. This

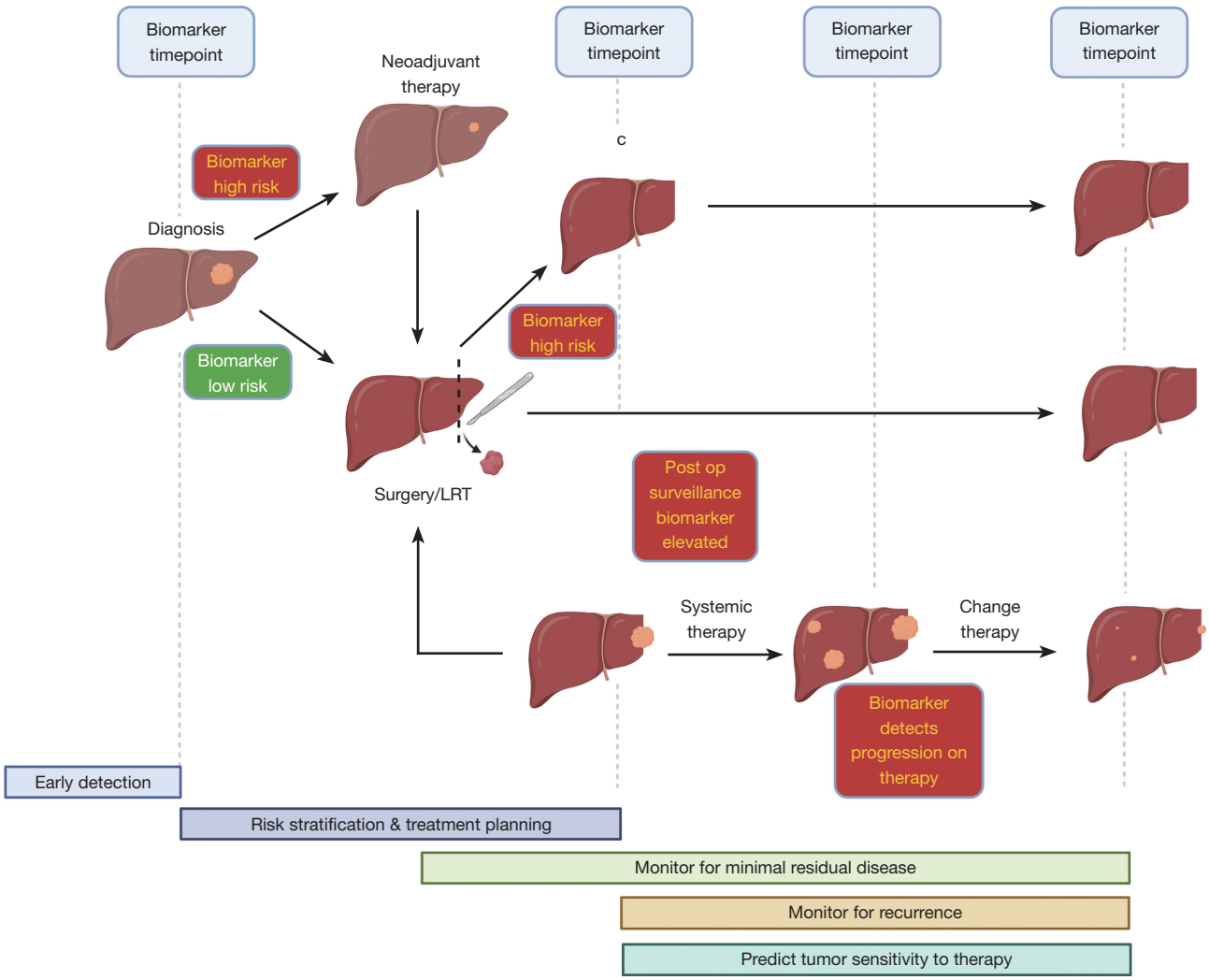

Figure 1 Potential applications of biomarkers throughout all phases of the management of hepatocellular carcinoma: (I) early detection; (II) risk stratification & treatment planning; (III) monitoring for minimal residual disease; (IV) monitoring for recurrence; (V) predicting tumor sensitivity to therapies. LRT, locoregional therapy.

Table 1 The search strategy summary

Items	Specification
Date of search	9/14–9/15/2022
Databases and other sources searched	PubMed, Embase
Search terms used	Hepatocellular carcinoma, biomarker, minimal residual disease, surveillance, prognosis, staging, AFP, liquid biopsy, treatment response, adjuvant, immunotherapy
Timeframe	No limitation
Inclusion and exclusion criteria	Inclusion: all English language publications
Selection process	Search by DAD and PW. Studies reviewed by all authors for appropriate inclusion
Any additional considerations, if applicable	None

AFP, alpha-fetoprotein.

Table 2 Literature reports of % of patient tumors expressing AFP by etiology of HCC

	% of HCC expressing elevated AFP	Reference
Viral		
HBV	88.1% (10 ng/mL cut-off)	(15-17)
	79.6% (11.62 ng/mL cut-off)	
	49.4% (20 ng/mL cut-off)	
HCV	11.6% (10 ng/mL cut-off)	(17-19)
	17.6% (5 ng/mL cut-off)	
	36.4% (20 ng/mL cut-off)	
HBV/HCV co-infection	85.7% (5 ng/mL cut-off)	(19)
HBV/HCV negative	20.5% (5 ng/mL cut-off)	(19)
Non-viral		
Alcoholic liver disease	12.8% (20 ng/mL cut-off)	(17,20)
	65.7% (20 ng/mL cut-off)	
Non-alcoholic steatohepatitis	47.0% (20 ng/mL cut-off)	(20)

AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus.

creates a two-fold need for biomarker application, for staging of existing liver disease, as well as staging of the tumor itself. Multiple existing staging systems including the BCLC, and Cancer of the Liver Italian Program (CLIP) staging system has incorporated biomarkers of liver function including bilirubin, international normalized ratio (INR), and creatinine, as well as the tumor marker AFP. Arguably the most widely used staging system for HCC, the BCLC criteria is also likely the most holistic, including factors on patients over all functional status, liver specific functional status, tumor criteria, and treatment efficacy, providing accurate prognostic information to guide therapy. The guidelines were recently updated in 2022 and have now incorporated the recommendation for use of the albumin-bilirubin (ALBI) score (a formula incorporating only albumin and bilirubin) to aid stratification of liver dysfunction, as well as the use of AFP to identify patients at high risk for recurrence.

AFP

By far the most widely used biomarker that can be elevated in HCC is AFP [reference range, 0–9.2 ng/mL (10)], a protein produced in the fetal liver that declines to a low level by the age of one (11). It is widely available in many in-hospital laboratories, as well as reference labs, and is relatively inexpensive with a price around $33 (12). Most data regarding sensitivity and specificity using AFP comes from screening data, and are 46–59% and 87–93% using a cut-off of 20 ng/mL, respectively (13,14). However, there are significant differences in AFP expression both by etiology of HCC (viral *vs.* non-viral), as well as the histologic subtype (*Table 2*). Macrotrabecular HCC, a more aggressive histologic subtype, has been associated with significantly higher AFP expression levels compared to other subtypes (21). Despite this variation, the majority of staging systems have now incorporated it as a biomarker to augment prognostic power (*Figure 2*).

The most recent staging system to incorporate AFP has been the BCLC system, published recently in 2022. While the recent update does highlight previous studies that associate elevated AFP with poor prognosis, it also acknowledges a lack of sufficiently studied cutoffs, and its incorporation into the model itself remains vague, stating that utilization of the model for prognosis is to be "refined by AFP, ALBI score, Child-Pugh, MELD" (9,22,23). Additionally, the update is in agreement with findings from multiple groups that elevated AFP is associated with higher recurrence risk, and recommends a cut off of 1,000 ng/dL as an exclusion criterion for patients with BCLC-B disease, being considered for extended liver transplant criteria (24-27). The wide variation in the use of AFP in staging regarding both cutoffs, as well as the subsets of patients in which it is applied within a staging system highlight the lack of predictive power of its use as a biomarker, and emphasize the need for more sensitive and specific biomarkers to augment staging prognostication.

ALBI score

Previously the BCLC staging system utilized the Child-Turcotte Pugh (CTP) system for functional hepatic reserve, however, the accessibility of certain factors such as ascites, and encephalopathy can be limited, greatly reducing utility (28). The development of the ALBI score in 2015, utilizing a composite formula of albumin and bilirubin demonstrated its simplicity and utility in further delineating prognostic subgroups among CTP classes (29). An international multicenter validation across BCLC stages in 2016 demonstrated that the ALBI score was a significant predictor of OS after surgical resection (P<0.001),

Staging system	Tumor factors					Liver factors					Patient factor
	Size	Nodes	Metastases	PVT	AFP Cutoff (ng/mL)	Childs-Pugh	Albumin	Bilirubin	Alkaline phosphatase	Ascites	Performance Status
TNM	■	■	■		N/A						
Okuda	■				N/A		■	■		■	
ITALICA	■	■	■		1,000	■					■
NIACE	■	■			200	■					■
BCLC	■	■	■		1,000	■	■	■			■
ALBI					N/A		■	■			
CLIP	■	■		■	4	■					
CUPI	■	■	■		500			■	■	■	
French				■	35			■	■		■
HKLC	■	■	■		N/A	■					■
BALAD					4		■	■			
MESIAH	■		■		Included, no cutoff		■				
JIS	■				400	■					
Bm-JIS	■	■	■		400	■					

■ = included in classification system.

Figure 2 Factors included by major hepatocellular carcinoma staging systems, including biomarkers. PVT, portal vein thrombosis; AFP, alpha-feto protein; TNM, Tumor, Nodes, Metastasis; ITALICA, Italian Liver Cancer; NIACE, nodularity, infiltration, AFP, Childs-Pugh score, Eastern Cooperative Oncology Group status; BCLC, Barcelona Clinic Liver Cancer; ALBI, albumin-bilirubin; CLIP, Cancer of the Liver Italian Program; CUPI, Chinese University Prognostic Index; HKLC, Hong Kong Liver Cancer; BALAD, Bilirubin, Albumin, AFP, AFP-L3, DCP; MESIAH, Model to Estimate Survival in Ambulatory HCC patients; JIS, Japanese Integrated Staging; Bm-JIS, Biomarker Integrated Japanese Integrated Staging; HCC, Hepatocellular carcinoma.

transarterial chemoembolization (TACE) (P<0.001) and systemic therapy only with sorafenib (P<0.001), and was independent of BCLC stage (30). Similar to AFP, the updated BCLC criteria includes reference to the ALBI score only in a general sense to refine prognosis, and there are no specific guidelines regarding decision-making based on ALBI grades.

Bilirubin, albumin, AFP-L3, AFP, DCP (BALAD) score

In an effort to provide a more objective staging system, the BALAD score was created and validated in a Japanese cohort in 2006 (31). It is based solely on 5 serum biomarkers: bilirubin, albumin, lens culinaris agglutinin-reactive alpha-fetoprotein (AFP-L3), AFP, and des-gamma-carboxy prothrombin (DCP). Further refinement was done by utilizing continuous forms of each variable rather than splitting each variable by cutoffs as in the original BALAD score, and has been validated in cohorts from both the United Kingdom, and most recently in North America (32,33).

Prognosis

AFP

When AFP is applied in the preoperative setting for prognostication, the data is mixed regarding its accuracy. In a retrospective analysis of 1,182 patients undergoing attempted curative resection in Hong Kong, the median OS was 38.4 months for patients with AFP >400 ng/mL, compared to 132.9 months in patients with an AFP <20 ng/mL (P<0.001). Additionally, in patients undergoing transplant evaluation for HCC, elevated AFP, particularly an AFP of >1,000 ng/mL has been utilized as an exclusion criterion by multiple high-volume centers due to associated with significantly worse outcomes after transplant (34,35). While some studies have found no difference in recurrence free and OS in patients undergoing either curative hepatectomy or thermal ablation for HCC (36,37), very high levels of AFP (>1,000 ng/mL) appear to have a clear association with poor prognostic factors including larger tumors, microvascular invasion, tumor multiplicity, as

well as disease-free and OS (25,38). Reasons for these contrasting results are likely due to a multitude of factors, including different patient cohorts between Asian, European and North American cohorts, not only in race and ethnicity, but in etiology of HCC (39).

AFP-L3

AFP can be separated into three glycoforms based on its affinity with lens culinaris agglutinin (LCA), and are named as such, L1-3. AFP-L3% [reference range, 0–9.9 ng/mL, L3% <10% (40)] is the fraction that is bound by the LCA, and has been suggested as a biomarker more specific to a malignant source of AFP, as opposed to that produced in chronic hepatitis and cirrhosis (41,42). This test costs approximately $128 self-pay and is available through centralized specialty laboratories (12). In the previously mentioned review of patients undergoing curative hepatectomy or ablation, while AFP level was not associated with outcomes, elevated AFP-L3% >10% was associated with decreased survival in both groups (P=0.0171) (31). Additionally, a meta-analysis of 4,465 patients from fifteen studies examining pre-treatment AFP-L3% demonstrated that not only was it associated with both disease-free survival (DFS) [hazard ratio (HR): 1.80, 95% CI: 1.49–2.17) and OS (HR: 1.65, 95% CI: 1.45–1.89), it more importantly maintained this significance in patients with low AFP concentrations (HR: 2.53 95% CI: 1.09–5.89; HR: 1.96, 95% CI: 1.24–3.10; respectively) (43).

DCP

DCP [reference range, 0–7.5 ng/mL (44)] is an abnormal form of the prothrombin protein lacking carboxylated glutamic acid residues, due to an HCC cell specific lack of vitamin K-dependent gamma-glutamyl carboxylase (45). Similar to AFP-L3%, it is also available through reference laboratories for approximately $128 (12). Serum levels have been associated with more aggressive tumor specific factors including tumor differentiation, vascular invasion, intrahepatic metastasis, TNM stage, and size (46). In a systematic review and meta-analysis of six retrospective studies of 943 patients treated with trans-arterial chemotherapy, a lower DCP level was associated with improved OS (HR: 0.653, 95% CI: 0.444–0.960) (47). Interestingly, this effect was not seen in an analysis of 801 patients undergoing either hepatectomy or locoregional ablation with curative intent. In the 345 patients undergoing hepatectomy, there was no association between DCP levels and survival. However, in the 456 patients who underwent locoregional ablation, between AFP, AFP-L3 and DCP, DCP had the strongest association with patient survival (P=0.0004) (31).

Determination of minimal residual disease (MRD) and post-operative surveillance

The term MRD has been long associated with liquid malignancies and represents the small number of cancer cells remaining in the body after treatment. The advent of more sensitive techniques for detecting MRD have made the idea of detecting minimal residual disease in solid cancers more feasible. This is especially important given the recent promising results from the IM Brave 050 trial of atezolizumab plus bevacizumab in the adjuvant setting for patients at high risk of recurrence met their primary endpoint for recurrence-free survival on interim analysis (48). In patients at risk of residual disease after resection, particularly in HCC with microvascular invasion, it has been proposed that a wider resection margin may improve survival by decreasing the chances of MRD, however, data comes from only one randomized controlled trial (RCT), and it remains more likely that outcomes are more reflective of tumor biology rather than margin status (49). A meta-analysis of 7 studies including 1,932 patients undergoing surgical resection found improved 5-year OS in patients with a margin > 1 cm compared with a sub cm margin [odds ratio (OR): 1.76, 95% CI: 1.20–2.59] (50). In addition to margin status alone, it has also been proposed that anatomic resection as compared to non-anatomic resection, may result in improved outcomes by removal of tumor-bearing portal territories. A systematic review and meta-analysis of 9,444 patients suggested improved 5-year disease-free and OS, although no included studies were prospective (51). However, liver resections are limited both by the size and function of the liver remnant, as well as the association of the tumor with critical structures which may preclude the clearing of all microscopic disease, making increasing the margin size in many patients impossible. The presence of microvascular invasion, is only detectable post operatively from surgical specimens, limiting its preoperative detection for prognostication and treatment planning (52). Improved methods of detection of the presence of minimal residual disease after resection would represent a significant improvement in prognostication which now relies mainly on histopathology. Additionally, while these markers may predict the presence of MRD after resection by way of

the surrogate, microvascular invasion, this still does not address ongoing monitoring and detection. Emerging data for adjuvant therapy (48), could serve to select patients at high risk for early recurrence (larger tumors, higher AFP, tumor multiplicity etc.), while other biomarkers and imaging can still be used to monitor for later recurrence (53), Therefore, the development and validation of circulating tumor markers that can work in tandem to enhance existing surveillance imaging strategies including MRI and CT to allow for the early detection of recurrent disease after resection to guide subsequent therapy.

Role of protein markers in the detection of MRD

Given the presence of microvascular invasion on final pathology has been associated with early recurrence and poor prognosis after resection, its detection preoperatively has been proposed as a surrogate for MRD. Prediction of microvascular invasion from preoperative imaging is poor, however, studies of protein biomarkers have shown some ability in predicting microvascular invasion. In patients undergoing liver transplant for HCC, an AFP level greater than 100 was significantly associated with presence of microvascular invasion on pathology (OR: 5.0, 95% CI: 1.4–18.1), and presence of microvascular invasion was associated with risk of early recurrence and death (54). These findings were reproduced in a retrospective review of 1,153 patients undergoing liver resection, both total tumor volume and AFP were found to be independent risk factors associated with the presence of microvascular invasion (55). Given the lack of accuracy from imaging prediction of microvascular invasion, the combination of AFP with imaging was combined to improve preoperative prediction of microvascular invasion and improved the accuracy relative to other existing risk scores (AUC =0.800 in testing cohort n=125) (56). Elevated serum levels of DCP, also known as PIVKA-II (protein induced by vitamin K absence/antagonism-II) were similarly strongly associated with the presence of microvascular invasion on pathology (HR: 3.5, 95% CI: 1.08–11.8). The presence of high DCP tissue expression on immunostaining of surgical specimens was strongly associated with the presence of microvascular invasion as well (P<0.001) (57). In order to strengthen the preoperative prediction power of available tumor characteristics, including AFP, a prediction risk scoring system was devised using an artificial neural network utilizing serum AFP, number of tumor nodules, size of the largest nodule and total tumor volume to predict the presence of microvascular invasion in 250 patients with cirrhosis undergoing resection for HCC. The study found a positive predictive value of 91.9%, but has yet to be validated in an external cohort (58). However, different disease states in the absence of malignancy including chronic liver disease and hepatitis can inherently cause production of the proteins in question making external prospective validation of these risk scores necessary prior to widespread adoption.

Liquid biopsy

The lack of a sensitive and specific circulating biomarker in HCC has been a major limiting factor in monitoring of post operative MRD as well as decision making regarding choice and initiation of systemic therapy (8). The detection of cell-free DNA (cfDNA) and RNA (cfRNA) allows for a whole host of potential biomarkers, from measuring total amount, mutations, integrity, epigenetic changes. Within this circulating nucleic acid, is the subset of circulating tumor DNA (ctDNA) which represents mutations known to be present in the primary tumor. Existing comprehensive commercial tests include but are not limited to Grail Galleri ($949) (59), FoundationOne Liquid Cdx ($5,800) (60) and Guardant360 ($5,000) (61) and while they have the potential to provide a significant amount of information, they do carry a significantly increased cost compared with standard protein biomarkers therefore as new sequencing based tests are developed, a balance must be maintained with depth of sequencing, as this is a main driver of cost and often past a certain depth does not provide added benefit (62). CtDNA has already showed promise in HCC in early detection in the screening phase, a systematic review and meta-analysis demonstrated improved sensitivity (76.0% *vs.* 47.8%) and specificity (92.0% *vs.* 84.0%) when combined with AFP, compared to AFP alone (63,64). One of the earlier applications as a demonstration of the potential power of cfDNA, analyzed serum samples in patients with hepatitis C related HCC and compared it with healthy controls, finding a significantly increased amount of cfDNA in sera from HCC patients, which was superior to AFP and DCP in discriminating between healthy control and HCC patients (65). A systematic review and meta-analysis of 8 studies of ctDNA in Asian patients with HCC found that the presence of pre-treatment ctDNA was independently associated with decreased DFS (HR: 3.01, 95% CI: 1.23–11.30) (66). One specific advantage to the analysis of ctDNA, is the ability to interrogate the molecular pathology

of the tumor, without an invasive biopsy, which are rarely done in HCC. In one study of 34 patients in China who underwent liver resection for HCC, and had postoperative ctDNA measured within 90 days of surgery, as well as other protein biomarkers for comparison (AFP, AFP-L3, DCP) to evaluate the potential for identification of MRD. Of the 17 patients that had an early recurrence (<1 year), ctDNA identified 10 (58.8%), approximately double the amount detected by each individual protein marker (67). Another benefit of ctDNA is the combination of powerful sequencing technology such as NGS to detect tumor mutations, which can be monitored longitudinally during the post operative period, or during systemic therapy to monitor response. Indeed, this was performed on 3 patients undergoing TACE and resection, interestingly, in one patient a somatic mutation (HCK p.V174M), which was initially detected after initial TACE treatment, then became undetectable following surgery for initial recurrence, followed by a rapid increase after second recurrence, demonstrating a proof of concept for post operative monitoring (68). Most recently, in a prospective trial of 41 patients with HCC, those with detectable ctDNA preoperatively, were more likely to have an early recurrence than those without, when adjusting for BCLC stage ($P<0.05$). Additionally, patients with detectable ctDNA at 1- and 4-month time points were more likely to have a shorter time to recurrence (69). Although small, these studies represent an important base from which to build, and the roadmap has already been partially laid out by the implementation of ctDNA in the management of colorectal cancer which has already produced some promising randomized controlled trial data. In a phase II randomized controlled trial of 455 patients undergoing colectomy for stage II colon cancer, patients were randomized to either post operative monitoring with ctDNA [45/291 (15.5%) ctDNA+], versus standard management. Two-year recurrence free survival was not different between ctDNA and standard groups (93.5% and 92.4%, respectively), and importantly, a lower proportion of patients in the ctDNA group received chemotherapy (15% *vs.* 28%) (70). As more data from implementation in other cancers is produced, it will allow for improved design of clinical trials and application in HCC, that will be more likely to succeed.

Another promising type of liquid biopsy is the detection of CTCs, which has been aided by the recent improvements in NGS, single cell sequencing, and microfluidic technologies (71). The role of CTCs in HCC aims to follow the advancements in applications in colorectal cancer. As in colorectal cancer, CTCs in HCC can be detected in a high percentage of patients, even in those with early-stage disease and in contrast to technologies requiring sequencing, CTC counts are significantly less expensive [$564, ARUP laboratories (12)]. Similar to ctDNA, data regarding sensitivity and specificity of CTC in HCC-specific applications are still emerging, however, in HCC diagnosis, early results are promising with one study reporting a sensitivity and specificity of 75.5% and 86.1%, respectively using a cut-off of 4 CTC/5 mL (72). In one study of 112 patients with HCC undergoing curative resection, CTCs were able to be detected preoperatively in 101 (90.2%) of patients, demonstrating that this method would be widely applicable to a variety of HCC patients. Additionally, they found that patients with a CTC count ≥16 and mesenchymal CTC >2% was a predictor of early recurrence, multifocal intrahepatic recurrence and lung metastasis (73). Similarly, in a prospective study of 42 patients undergoing resection of hepatitis B related HCC, post operative CTC counts of both >2 and >5, as well as increase between pre- and post-operative CTC count, were all associated with decreased progression free survival ($P<0.05$ for each) (74).

Prediction and monitoring of treatment response

Biomarkers are often utilized in predicting response to immunotherapeutic treatments for a variety of cancers (75). A variety of biomarkers at the soluble, cellular, and genomic levels have been investigated in order to better understand which subsets of patients will best respond to immunotherapy. Examples of these include serum proteins, tumor-specific receptor expression patterns, circulating cells, host genomic factors, and aspects of the tumor microenvironment (76). For HCC, particular biomarkers and genetic characteristics have demonstrated a role during clinical trials in predicting efficacy of immunotherapy (*Table 3*). Additionally, the presence of microbial signatures in the gut microbiome have also been shown to correlate to responses towards cancer immunotherapy.

AFP

In addition to its diagnostic and prognostic applications, AFP has been described to predict responses to immunotherapy for HCC patients. In a multicenter study of HCC patients receiving programmed cell death protein-1 (PD-1) blockade treatment, AFP was identified

Table 3 Clinical trials that incorporated biomarkers to predict and monitor treatment response to immunotherapy

Biomarker	Trial name	Intervention/Treatment	Phase	Pertinent findings
AFP	GO30140 (NCT02715531)	Atezolizumab + bevacizumab	Ib	AFP decrease ≥75% and increase ≤10% from baseline at 6 weeks used to identify responders and patients with disease control, respectively (77)
	IMbrave 150 (NCT03434379)	Atezolizumab + bevacizumab	III	AFP decrease ≥75% and increase ≤10% from baseline at 6 weeks used to identify responders and patients with disease control, respectively (78)
	Celestial (NCT01908426)	Cabozantinib	III	Greater treatment efficacy for patients with AFP ≥400 ng/mL compared to those under this threshold (79)
	REACH (NCT01140347)	Ramucirumab	III	Increased overall survival with second-line treatment of ramucirumab compared to placebo in patients with AFP ≥400 ng/mL (80)
	REACH-2 (NCT02435433)	Ramucirumab	III	Increased overall survival with second-line treatment of ramucirumab compared to placebo in patients with AFP ≥400 ng/mL (81)
ctDNA/CTC	SORAMIC (NCT01126645)	Sorafenib + radioembolization or radiofrequency ablation	II	Significant correlation seen between higher circulating free DNA levels and survival (82)
PD-L1	CheckMate 040 (NCT01658878)	Nivolumab + ipilimumab	I/II	No significant difference between response rates in PD-L1 positive (≥1%) and negative patients (<1%) (83)
	KEYNOTE-224 (NCT02702414)	Pembrolizumab	II	Positive PD-L1 expression associated with improved response (84)
	CheckMate 459 (NCT02576509)	Nivolumab *vs.* sorafenib	III	In PD-L1 positive patients, nivolumab monotherapy was shown to produce a higher response rate compared to sorafenib (85)
TMB/MSI	GO30140 (NCT02715531)	Atezolizumab + bevacizumab	Ib	TMB was not associated with treatment response or progression-free survival (77)

AFP, alpha-feto protein; ctDNA, circulating tumor DNA; CTC, circulating tumor cells; PD-L1, programmed death ligand-1; TMB, tumor mutational burden; MSI, microsatellite instability.

to be a marker associated with therapy response. Namely, baseline pre-treatment AFP levels less than 400 ng/mL were shown to be associated with significantly longer median progression-free survival (PFS) (P<0.05) and OS (P<0.0001) in patients treated with anti-PD-1 (86). Despite the correlation of advanced disease and elevated levels of AFP, increases in this biomarker have also been associated with greater efficacy of cabozantinib treatment in HCC patients with an AFP ≥400 ng/mL compared to those with AFP under this threshold, although mechanisms driving this association remain unclear and require further study (79). In addition, both the REACH and REACH-2 trials demonstrated increased OS (P<0.05) with second-line treatment of ramucirumab compared to placebo in patients with elevated AFP levels (≥400 ng/mL) following intolerance or cancer progression with sorafenib (80,81). Meanwhile, the initial REACH trial showed no statistically significant difference in OS for patients with AFP <400 ng/mL receiving ramucirumab or placebo (80).

AFP also plays a role in monitoring response to immunotherapies. In a study assessing treatment response to atezolizumab + bevacizumab for unresectable HCC, AFP was demonstrated to be a potential biomarker of predicting OS and PFS. AFP cutoffs of ≥75% decrease and ≤10% increase from baseline at 6 weeks were used to determine responders to treatment and those who had disease control, respectively. For the ≥75% decrease cutoff, the sensitivity was 0.59 and sensitivity was 0.89, whereas the sensitivity was 0.77 and specificity was 0.44 for the ≤10% increase cutoff of disease control (87). Additionally, Hsu *et al.* showed that

≥20% decline in serum AFP levels within three months of treatment was a predictor for objective response (P=0.042) and PFS (P=0.001) (88). Despite some use in certain populations, AFP, as in other applications, lacks sensitivity and wide applicability, highlighting the need for increased biomarker discovery and validation.

CtDNA and CTC

Liquid biopsy techniques have recently been highlighted as methods to identify ctDNA and CTC in patient blood. Findings from these assays have been suggested as biomarkers for metastasis or recurrence, and efforts have also been made to study ctDNA as a predictor for treatment response (89-92). For advanced non-small cell lung cancer (NSCLC), rapid decreases in plasma ctDNA were associated with a significantly higher response rate in patients treated with first line pembrolizumab based therapy (93). Additionally, in prostate cancer, CTC enumeration has been described as a reliable predictor of prognosis and treatment response (94,95). In HCC, Ikeda *et al.* have suggested ctDNA as a non-invasive test to identify targetable mutations in tumors for treatment (96). A subset study from the SORAMIC trial also explored the value of using cfDNA and ctDNA in advanced HCC and described its use as a potential biomarker for predicting treatment response (97). In a longitudinal analysis of patients with locally advanced and metastatic HCC, changes in CTC count were shown to be correlated with treatment responses to a variety of systemic therapies (70% sorafenib) and was especially useful for disease monitoring in patients without elevated serum AFP levels (98). Techniques to detect ctDNA and CTC demonstrate promise in various cancer types, but further refinement is required to determine its utility in predicting treatment responses in HCC.

PD-L1

Around 10% of tumor cells demonstrate PD-L1 expression in HCC, which is relatively low compared with a variety of other tumor types, including breast [27%, (99)], pancreatic [19%, (100)], gastric [59%, (101)] (102,103). Although PD-L1 is expressed at a relatively low level, this biomarker has nevertheless been extensively explored in the context of immunotherapy. In the phase III CheckMate 459 trial involving patients with PD-L1 positive advanced HCC, nivolumab monotherapy was shown to produce a higher response rate compared to sorafenib as front-line treatment, although significant improvements in OS were not seen (85). Similarly, findings from the phase II KEYNOTE-224 trial demonstrated that positive PD-L1 expression was associated with improved response rates in patients receiving pembrolizumab monotherapy who had been previously treated with sorafenib (84). Conversely, the phase I/II CheckMate 040 trial reported no statistically significant difference between response rates in PD-L1 positive and negative patients (<1%) (104). Notably all three of these trials used a definition of ≥1% PD-L1 expression for outcome analysis, a very low threshold. Overall, there is not an unequivocal body of evidence towards the utility of PD-L1 as a predictive biomarker for immunotherapeutic treatments in HCC and its true value remains to be determined.

Tumor mutational burden (TMB) and microsatellite instability (MSI)

TMB, the total number of mutations in a tumor genome, has been assessed as another possible biomarker for identifying patients with tumors that may be sensitive to biologic and immunotherapy treatments. Tumors with higher number of mutations have been associated with greater levels of neoantigens that may be targeted in immune responses (105). While there has not been a comprehensive assessment of TMB's role in predicting tumor sensitivity to immunotherapeutic treatments for HCC, various studies have been conducted across multiple types of cancers that have concluded that high TMB is associated with improved survival and greater rates of treatment response in the context of immune checkpoint inhibitors (106,107). For HCC, an assessment of 755 patients with advanced HCC showed a median TMB of 4 mutations/Mb, with only 6 tumors (0.8%) having high TMB (≥20 mutations/Mb). A further small case series (n=17) by the same group showed that there was no association between TMB and tumor response to immune checkpoint inhibitors (108). Thus, further studies are needed to refine the threshold for high TMB and to assess the predictive value of TMB in stratifying and selecting patients that may benefit from biologic and immunotherapies.

DNA mismatch repair occurs as a safeguard in cases of DNA replication errors, however, deficiencies in this mechanism produce a phenotype of MSI leading to a greater probability of mutations (109). Similar to TMB, higher amounts of mutations and a lack of mismatch repair would increase the levels of neoantigens produced

to generate immune responses and vulnerability towards immunotherapies. Many studies have linked MSI-high status to increased sensitivity towards immune checkpoint blockade and tumor response rates in various cancer types (110). In HCC, various studies exploring the frequency of MSI-high status, defined as MSI ≥30%, have demonstrated a relatively low prevalence varying from 0% to 18% of patients (111). Thus, like with high TMB, the low frequency of MSI-high status in HCC patients has stalled the performance of sufficiently powered studies to assess the association between MSI-high and predicting sensitivity to immunotherapy. However, given MSI's demonstrated value in various other cancers, further studies should be conducted to assess its value as a biomarker for predicting tumor sensitivity to immunotherapeutic treatments.

Gut microbiome

In addition to its various roles in regulating other disease processes, the gut microbiota plays an integral role in managing innate and adaptive immune responses, especially in the context of cancer. Alterations in the composition of the gut microbiome have been associated with resistance towards chemotherapy and immune checkpoint inhibitor treatments, as antibiotic removal of gut microbiota have led to cyclophosphamide resistance in mice models (112,113). In humans, a systematic review and meta-analysis of 12,794 cancer patients with a variety of tumor types found that use of antibiotic therapy prior to immunotherapy, was associated with decreased response rates, as well as decreased progression-free and OS (114). Additionally, the enrichment of particular bacterial species, such as *Bifidobacterium pseudolongum*, *Lactobacillus johnsonii*, and *Olsenella*, have been shown to significantly improve immune checkpoint inhibitors efficacy in mouse models in multiple tumor types (115). In HCC specifically, a study examining strains of bacteria in patients receiving immune checkpoint inhibitor treatment for unresectable HCC, appreciable differences were identified in patients with objective responses to treatment and progressive disease (116). *Prevotella 9* was identified more frequently with progressive disease, whereas strains of *Lachnoclostridium*, *Lachnospiraceae*, and *Veillonella* were more prevalent in patients with objective responses. Further analysis demonstrated that a microbial signature of *Lachnoclostridium* enrichment and *Prevotella 9* depletion independently predicted greater OS in these patients. Similarly, in HCC patients treated with anti-PD-1, Zheng *et al.* showed that *Akkermansia muciniphila* and *Ruminococcaceae* spp were enriched in treatment responders, whereas *Proteobacteria* was the predominant bacterial species found in non-responders (117). Overall, these studies highlight the utility of gut microbiota as a potential target to enhance immunotherapeutic treatment responses and as a biomarker for disease monitoring in HCC patients.

Other biomarkers

It is important to note that biomarkers are not limited to tissue based analysis. Imaging already plays a central role in the current management of HCC, both before and after diagnosis. Therefore, advances in tissue based biomarkers will assuredly be accompanied by ongoing advances in imaging technology, and importantly, by new applications of artificial intelligence (AI) throughout all phases of HCC management. Use of AI allows for the rapid and comprehensive incorporation of information from massive datasets, that creates the potential for integration of multiple sources of information such as patient characteristics, histopathologic data, molecular profiling, and imaging features, to aid in clinical decision-making (118,119). Additionally, the field of radiomics (the extraction of mineable data from medical imaging) has significant potential for the development of an imaging based biomarker. Importantly, AI applications in this field should allow for significant increases in data mining power. Radiomic approaches have already been tested in the retrospective setting in HCC, including one of the largest to date which used a training cohort of 177 patients from which radiomic features were extracted from preoperative CT scans and combined with pre- and post-operative clinical features to predict recurrence. The model was then validated in an external cohort of 118 patients at two other institutions and had a concordance index of ≥0.77, ($P<0.05$) (120). As we look beyond AFP as the workhorse of prognostication in HCC, it is important to maintain a comprehensive view of existing as well as emerging technologies, to allow for optimal growth and development of biomarkers.

Conclusions

With the convergence of both the development of new active systemic therapies including chemo- and immunotherapy, as well as technologies for interrogating tumor molecular pathology, we may be approaching a new age in the management of HCC, where both surgical, locoregional and systemic therapies can not only be tailored

to tumor biology, but sequentially monitored by novel biomarkers. There is a need for well-developed clinical trials to test and validate existing biomarkers such as ctDNA and CTC that have shown such promise in other malignancies.

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