REVIEW

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Hepatic Oncology

Biomarker development for hepatocellular carcinoma early detection: current and future perspectives

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Practice points

- Biomarkers for hepatocellular carcinoma (HCC) have the potential to improve early diagnosis and prognosis in patients.
- Several requisite phases of biomarker discovery are necessary before a biomarker is appropriate for use in clinical practice.
- The only biomarker that has gone through all five phases of biomarker development in HCC is α -fetoprotein.
- There are several other candidate biomarkers, including combinations of different biomarkers that are being tested for HCC early detection.
- Historically validation has been limited by lack of large-scale longitudinal cohorts with available samples for testing. Fortunately, two large prospective cohorts are being collected in the USA for such purposes.
- In coming years, with appropriate validation, a biomarker-based precision screening approach to HCC detection has the potential to become the primary method for HCC early detection.

Early detection of hepatocellular carcinoma (HCC) leads to improved survival; however, current early detection strategies for HCC surveillance are ineffective. Thus, there has been interest in developing biomarkers to aid in the early detection HCC. In this review, we discuss the five phases of biomarker discovery that are necessary for clinical implementation. We also describe the most promising investigational biomarkers and their phase of discovery. We review several promising technologies for the early detection of HCC, including miRNA, metabolomics and proteomics. Promisingly, there are samples from multiple longitudinal cohorts of patients with cirrhosis in the USA that are being collected in order to validate candidate biomarkers for HCC. A biomarker-based strategy has the potential to become the primary surveillance method for HCC detection.

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Hepatocellular carcinoma (HCC) is the most common primary hepatic malignancy and is increasing in frequency in several countries around the globe [1]. Unfortunately, HCC is also highly morbid, currently ranking as the third most common cause of cancer-related death worldwide [2]. HCC typically occurs in the setting of underlying chronic liver disease with most cases developing in the setting of cirrhosis or chronic HBV infection [3–5]. While the incidence has been historically highest in Asian countries due to endemic HBV infection, the incidence of HCC has been increasing in many Western countries, both due to the aging cohort of HCV-infected patients and the emergence of nonalcoholic fatty liver disease as a significant cause of chronic liver disease [6].

Once diagnosed HCC prognosis depends the intent of treatment, with curative therapies such as liver resection, local ablative therapies or liver transplantation, typically reserved for early-stage patients [7]. The 5-year survival rate for advanced-stage HCC is less than 10% while early-stage

KEYWORDS

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patients experience significantly better survival, ranging anywhere from 50 to 80% [8–10]. Detection of HCC occurs either due to: active surveillance of at-risk patients (i.e., cirrhosis); incidentally or due to symptoms (e.g., abdominal discomfort, hepatic decompensation) [11]. Active surveillance leads to the highest probability of early-stage detection and several cohort studies have shown that surveillance for HCC is associated with improved early detection rates and overall patient survival [12–15]. HCC is typically diagnosed via contrast-enhanced cross-sectional imaging (e.g., CT scan/MRI) or liver biopsy, however for early detection, the impetus to conduct such testing must exist. The American Association for the Study of Liver Diseases recommends surveillance with abdominal ultrasound with or without α -fetoprotein (AFP) measurement every 6 months in all patients with cirrhosis and in certain patients with chronic HBV infection [16]. Ultrasounds, aside from being inconvenient and time consuming, have numerous limitations included limited sensitivity in patients with central obesity, ascites or cirrhosis and can suffer from interobserver variability in quality, therefore better risk stratification and screening methods for HCC detection are needed [17–20]. The inclusion of AFP in surveillance protocols is controversial due to concerns about specificity leading to false-positives and limited sensitivity in early detection, thus there are varying guidelines as to the optimal approach for surveillance [10,16,21].

Ideally, validated accurate serum-based approaches to improve HCC early detection would be available, but unfortunately there are few that are ready for routine clinical use at this time. There has been little progress made on the development of clinically useful biomarkers for HCC early detection over the last two decades, largely due to lack of available validation cohorts containing longitudinal samples on patients with cirrhosis, some of whom develop HCC. Prior to widespread adoption, biomarkers require several phases of validation [22]. Herein, we will review the stages of biomarker development and the most promising biomarkers for HCC early detection. We will also focus on several emerging technologies that may become useful in HCC early detection in the coming years.

Biomarkers

A biomarker is defined as any substance, structure or process that can be measured in the body

and influence or predict the incidence of outcome or disease. This is a broad definition that can include any objective patient-based measure such as blood, urine, saliva or any accessible compartment. Biomarkers can be used for screening for early cancer detection, prognosis or to measure treatment response. The ideal biomarker is readily available, reliably measurable, cost-effective, minimally invasive and highly accurate [23]. In cancer, several recent advances have led to the proliferation of candidate biomarkers, from genetic material (e.g., DNA, epigenetic changes, cell-free DNA, RNA, mRNA), serum proteins and circulating metabolites. Several candidate biomarkers are being studied in HCC, however significant challenges exist, largely stemming from HCC molecular heterogeneity [24,25].

Several steps are required to ensure a biomarker is effective for early detection in clinical settings. Five phases of biomarker development exist, as summarized by Pepe *et al.*, from discovery to implementation and measurement of efficacy [26] (Figure 1).

Phase I of development consists of preclinical studies meant for discovery of candidate biomarkers. Examples include exploratory studies of cancer tissue investigating protein or gene expression, analysis blood samples to identify gene expression patterns from in microarray analysis or protein expression profiles on mass spectroscopy. These are primarily performed in order to identify and prioritize candidate biomarkers, ensuring that results are reproducible and effectively distinguish between case and control.

Phase II involves clinical assay development for noninvasive measurements of the biomarker, with the goal of ensuring consistency of the assay as it is measured across laboratories. This phase is also used to characterize if the biomarker is useful in patients accounting for the variance in patient level factors (e.g., etiology of liver, demographic factors) or certain tumor characteristics (e.g., tumor stage). If there are significant patient or tumor level factors which stratify biomarker performance, then this phase of development should begin to define these test characteristics. Testing typically occurs on biobanks of specimens; however, final testing is typically recommended on population-based samples. In the case of HCC, this includes patients at higher risk for HCC development, patients with cirrhosis and chronic hepatitis B. The goal of Phase II is to determine performance characteristics of the

Figure 1. Phases of biomarker development.

biomarker in distinguishing between patients with and without cancer (i.e., case–control design).

Phase III of biomarker development focuses on the ability to detect preclinical disease. Validation studies in this phase consist of specimen collection from cancer case subjects before their clinical diagnosis of cancer, and comparison to those from control subjects (i.e., patients who are at risk but do not develop the cancer). In the case of HCC, longitudinal samples from patients with cirrhosis with characterization of which ones were diagnosed with HCC would be appropriate for validation in this phase of development. The goal of Phase III is to determine the ability of the biomarker to detect preclinical disease, as well as to define the criteria for a positive test. Similar to Phase III, further characterization of the biomarker in certain subpopulations is

undertaken during this phase. If performance is clearly improved in certain subgroups, these groups are typically selected for prospective evaluation in Phase IV.

In Phase IV, the aim is to prospectively determine the stage and nature of tumor detection using the specific biomarker. While Phase III gives information on the performance of detection of preclinical disease, Phase IV determines the tumor characteristics at the time of actual detection in a clinical setting. In this phase, at-risk patients are prospectively screened, leading to actual cancer diagnosis and subsequent treatment. Validation studies in this phase consist of prospective evaluation of the biomarker in an at-risk population in order to determine clinical performance characteristics – specifically the detection rate, particularly of early-stage disease, as well as the false-positive rate and false-negative rates.

Table 1. Candidate biomarkers and phase of biomarker development.

Biomarker	Phase I	Phase II	Phase III	Phase IV	Phase V
AFP					X
Osteopontin			X		
Midkine			X		
GALAD score			X		
AFP-L3		X			
Dickkopf-1		X			
DCP		X			
GPC3		X			
α -1-Fucosidase		X			
Golgi protein-73		X			
Squamous cell carcinoma antigen		X			
miRNA	X	X			
Metabolomics	X				
Proteomics	X	X			
Cell-free DNA	X				

AFP: α -Fetoprotein; DCP: Des-carboxyprothrombin; GPC3: Glypican-3.

The final phase of development, Phase V, evaluates whether screening reduces the burden of the disease in the population. This phase evaluates treatment effectiveness for early-stage disease, compliance with screening, cost-effectiveness of screening and the rates of cancer over-diagnosis. The goal of Phase V is to estimate the reductions in disease-related mortality afforded by the screening test, or biomarker. This is outside the scope of work for most scientists involved in biomarker development, but serves as a critical evaluation of biomarker effectiveness on population health. Biomarkers for HCC, by phase of development, are outlined in **Table 1**.

Biomarkers for HCC by phase of development

• Phase V biomarkers

Evaluation of biomarker effectiveness in cancer screening & improving population health

α -Fetoprotein

AFP is the most commonly used biomarker for HCC surveillance and the only biomarker which has been through all five phases of biomarker development. AFP alone, however, is not currently included in societal guidelines for HCC surveillance due to inadequate sensitivity and specificity in the detection of early-stage HCC [27]. As noted, its inclusion in routine surveillance for HCC is controversial between societal guidelines – while AFP has been shown to marginally improve HCC early detection sensitivity, AFP is not universally recommended for routine surveillance in combination with abdominal ultrasound due to concerns about specificity and limitations in its sensitivity [13,16]. AFP level can correlate with serum alanine transaminase (ALT) level, and is typically more sensitive in non-HCV-related liver disease given the baseline elevation of ALT in patients with HCV without HCC, which has historically been the source of poor specificity [28]. Furthermore, up to 40–50% of HCCs do not produce AFP, limiting the sensitivity of AFP alone for HCC detection. Analysis of cohort studies reveals the sensitivity of AFP for detecting early HCC ranges from 39 to 65% in the literature, while the specificity ranges from 76 to 97% [29,30]. Cutoff values for serum AFP vary widely across studies, however, a value of 20 ng/ml is generally accepted as a valid threshold in the early detection of HCC, balancing sensitivity and specificity. The change in AFP value over time has been

shown to improve the prognostic accuracy of AFP, versus single AFP values, in the detection of early-stage HCC (receiver operating curve 0.81 vs 0.76) [31]. Thus, while AFP has been through the five phases of biomarker development, its routine use as a part of the surveillance strategy for HCC early detection remains controversial.

• Phase IV biomarkers

Prospective screening of at-risk individual

At this time, there are no Phase IV biomarkers that have completed testing for HCC early detection. While there are studies underway, the field has been limited due to the lack of adequate Phase III sample availability for proper validation.

• Phase III biomarkers

Longitudinal testing of retrospectively collected specimens

Osteopontin

Osteopontin is an integrin-binding phosphoprotein secreted at low levels by biliary epithelial cells and is overexpressed in many cancers, including lung, breast, colon and HCC [32]. Osteopontin interacts with integrin and CD44 family of receptors to mediate cell signaling that controls inflammatory processes (such as in hepatitis), HCC tumor progression and development of metastasis [33,34]. Plasma osteopontin levels are significantly higher in HCC patients than healthy controls and in patients with chronic liver disease [35]. One study included 120 patients: with HCV without cirrhosis; patients with HCV and cirrhosis; those with HCC; those with NAFLD and finally a healthy age and sex-matched control group. The mean plasma osteopontin level in patients with HCC in the setting of cirrhosis was 401 ng/ml, while the healthy controls had a mean level of 35.1 ng/ml ($p = 0.001$) [36]. Sensitivity and specificity of elevated osteopontin levels have been reported between 75–87% and 62–82%, respectively [37]. In the Phase III validation study of 131 patient with HCC and 76 patient with cirrhosis by Shang *et al.*, osteopontin outperformed AFP for early detection (area-under-the-curve [AUC]: 0.73 [95% CI: 0.62–0.85] vs AUC: 0.68 [95% CI: 0.54–0.82]) [33]. When osteopontin was combined with AFP, the AUC was even higher for early-stage HCC patients (AUC: 0.81 [95% CI: 0.70–0.91]). In a recent meta-analysis, a combination of osteopontin and AFP resulted

in a sensitivity of 82% and specificity of 77% for HCC detection, although the overall proportion of patients included with early-stage disease was small [38].

Midkine

Midkine (MDK) is a heparin-binding growth factor that plays a role in cell growth, invasion and angiogenesis during cancer progression and thus it is expressed in many carcinomas, including HCC [39]. Serum levels of MDK are also elevated in patients with HCC, even in those with very early-stage disease; levels decline following curative intent surgery, and rise or remain elevated in patients with incompletely treated or recurrent HCC [40]. A study by Zhu *et al.* included 933 patients (388 with HCC, 545 controls) and found that at a cutoff value of 0.654 ng/ml, the sensitivity of MDK is 87%, while AFP's sensitivity was approximately 52% [41]. The combination of MDK and AFP further improved the detection rate of early-stage HCC from 80% to more than 96.6% in the Zhu *et al.*'s analysis [41]. In an small Phase III study of patients with NASH-related cirrhosis, MDK was not superior to AFP for the early detection of AFP, but was elevated in 59% of patients who were AFP negative in the case-control portion of the study and in 50% of AFP-negative patients in the retrospective-longitudinal portion of the study [40]. The authors did not analyze the combined sensitivity and specificity of AFP and midikine, nevertheless, these are encouraging early data in this increasingly important subgroup of cirrhotic patients [40].

GALAD score

Recognizing that HCC tumor biology is highly heterogeneous, composites of biomarkers and clinical factors associated with risk of HCC have been investigated in order to improve the sensitivity and specificity of HCC early detection. One such panel, named the GALAD score is a Phase III biomarker, and uses objective measures of gender, age, AFP, lens culinaris agglutinin-reactive AFP (AFP-L3) and des-carboxyprothrombin (DCP) [42]. The GALAD score has been validated in patients with HCC with the ability to discern between HCC, cirrhosis and other hepatobiliary malignancies (e.g., cholangiocarcinoma). Several aggregate cohorts were used to provide the Phase III validation of this model – two centers in the UK using data from 833 patients (394 with HCC and

439 with chronic liver disease) and validated in independent cohorts of 6834 patients in Japan, Germany and Hong Kong (2430 with HCC and 4404 with chronic liver disease). 1038 patients across all centers had early-stage HCC, which was defined as tumor size less than 3 cm. Overall sensitivity ranged of GALAD ranged from 80 to 91%, while the specificity ranged from 81 to 90% across the cohorts [43]. There are ongoing studies to provide Phase IV validation of this panel in prospectively collected cohorts.

• Phase II biomarkers

Case-control studies: HCC patients versus cirrhotic controls

Des-carboxyprothrombin

DCP, also known as prothrombin induced by vitamin K absence II, is an abnormal prothrombin produced by hepatocytes as a result of vitamin K deficiency. Levels are elevated in the serum of patients with HCC. The mechanism by which is DCP is elevated in patients with HCC is multifold: vitamin K insufficiency secondary to inadequate intake or dysfunctional intracellular transport mechanisms; selective defects in γ -carboxylase enzyme (which prevents production of normal prothrombin); and cytoskeletal changes that impair vitamin K uptake as the hepatocytes undergo malignant transformation [44]. Sensitivity and specificity of DCP's ability to detect early-stage HCC ranges between from 48 to 62% and 81 to 98%, respectively [45,46]. Vitamin K supplementation can mask HCC diagnosis as it decreases serum levels of DCP. The sensitivity of DCP increases with increasing tumor size, and combining DCP and AFP levels can increase the sensitivity of DCP up to 80% for large tumors (>3 cm) and 70% for small tumors (2–3 cm). DCP is also superior to AFP in discriminating HCC from underlying cirrhosis with a sensitivity of 86% and specificity of 93%. DCP is a better marker for viral etiologies of cirrhosis and HCC with an AUC value 0.76, while the AUC of DCP in non-viral etiology is 0.65 [30]. Despite lack of formal Phase III or IV validation, DCP is used in many countries worldwide for HCC early detection. It is included in the GALAD score, thus it has been validated as part of a panel in that context.

Lens culinaris agglutinin-reactive AFP

AFP-L3 is a glycosylated isoform of AFP that shows the highest binding affinity for lens culinaris agglutinin. AFP-L3 is reported as a

proportion of AFP-L3 to total AFP, with a cut-off of 10% used for HCC detection [47]. This glycoform is secreted by HCC cells even at early tumor stages, and can be used in the absence of elevated AFP levels to detect early-stage HCC [48]. Like DCP, it has undergone Phase III validation as part of the GALAD score and is used for HCC surveillance in many health systems worldwide. While traditional AFP-L3 requires an AFP level above 10 ng/ml for appropriate use, the use of a highly sensitive assay for AFP-L3 (hs-AFP-L3) makes measurements possible in patients with AFP levels as low as 2 ng/ml [49]. AFP-L3 has a better specificity for early HCC detection than AFP (~90%), but its sensitivity is low (37–60%) [46,50]. The introduction of hs-AFP-L3 has improved the sensitivity from 37% to approximately 50% [46,51].

Dickkopf-1

Dickkopf-1 (DKK1) is a glycoprotein that is a secretory antagonist of the Wnt/B-catenin signaling pathway [52]. Its exact function is not fully understood but upregulation of DKK1 expression occurs in a wide variety of cancers, including multiple myeloma, prostate cancer and HCC [53]. DKK1 levels are also increased in the serum of patients with HCC as compared with cirrhotic and noncirrhotic controls. Shen *et al.* reported DKK1 and AFP values in a cohort of 1284 patients (831 in the test cohort and 453 in the validation cohort). DKK1 concentrations were significantly higher in patients with HCC in the test cohort than in all the controls, and values did not differ significantly between the control groups ($p < 0.001$) [54]. The sensitivity for detection of early-stage HCC was 73.8% and the specificity was 87.2% in the validation cohort. DKK1 measurement was more useful for detection of AFP-negative HCC, but combining DKK1 levels with AFP enhanced the detection rate of early-stage HCC as it appears to aid in detection in HCCs, which do not produce AFP [54]. A Phase II study in predominantly HBV-infected patients in South Korea ($n = 208$), the combination of AFP and DKK1 was just slightly better than AFP alone for the detection of early-stage HCC (AUC: 0.693 vs 0.691) [55].

Glypican-3

Glypican 3, or GPC-3, is a heparin sulfate proteoglycan that plays an important role in cell proliferation and tumor suppression. For example, during embryonic development, GPC-3

binds to growth factor receptors and controls cellular proliferation. It is also known to regulate cell growth by modulating activities of several tyrosine kinases and through the Wnt signaling pathway [56,57]. GPC-3 is upregulated in some HCC tissue, and there is increased secretion from HCC-derived cell lines (as opposed to from normal hepatocytes, or those hepatocytes found in benign liver disease) [58]. GPC-3 has been proposed to be a complementary serologic biomarker to AFP as GPC-3 has better diagnostic performance due to its ability to accurately distinguish between patients with small, well-differentiated HCC and those with underlying cirrhosis [59]. While the sensitivity of GPC-3 for detection of early-stage HCC is low when used alone (~50–55%), this rises to a sensitivity of around 80% when combined with AFP. The specificity of GPC-3, even in combination with AFP, is around 75% [59,60].

α -1-Fucosidase

α -1-Fucosidase (AFU) is a lysosomal enzyme that can be detected in the serum of healthy adults, although levels are increased in the serum of patients with HCC. AFU does not correlate well with tumor size or the AFP measurement. In one series, AFU activity was reported to be elevated in 85% of patients at least 6 months before the detection of HCC by transabdominal ultrasound [61,62]. The sensitivity and specificity of AFU in the early detection of HCC is 82 and 71%, respectively. Combining AFU and AFP raises the sensitivity to 95% and the specificity to 99% [63]. However, the specificity of AFU is poor as it is also overexpressed in diabetes, pancreatitis and hypothyroidism, and varies across patient race/ethnicities [61].

Golgi protein-73

Golgi protein-73 (GP-73) is a transmembrane protein that is primarily expressed in human epithelial cells. In the normal human liver, GP-73 is expressed in biliary epithelial cells, with negligible secretion by hepatocytes. However, in the setting of chronic liver disease, there is upregulated expression of GP-73 in hepatocytes. Increased GP-73 levels have also been associated with advanced fibrosis in patients with HBV and HCV infection [64]. In a study by Marrero *et al.*, GP-73 was found to have a sensitivity and specificity of 69 and 86%, respectively, for distinguishing between HCC and those with underlying cirrhosis [65]. The sensitivity and specificity for detecting early-stage HCC was similar at 62

and 88%, respectively. Combining GP-73 and AFP increased sensitivity and specificity to 98 and 85% for differentiating HCC from cirrhosis [65]. GP-73 has limited early published data evaluating its role in the early detection of HCC.

Squamous cell carcinoma antigen

Squamous cell carcinoma antigen (SCCA) is a serine protease inhibitor that is present in squamous epithelium. Increased levels of SCCA have been detected in several epithelial cancers such as those of the head, neck, lung and cervix [66]. SCCA is also expressed by neoplastic epithelial cells, including neoplastic hepatocytes in which it promotes tumor growth through the inhibition of apoptosis. In the diagnosis of HCC, SCCA is sensitive (89%), however suffers from poor specificity (50%) in differentiating from cirrhosis [67,68]. In a meta-analysis, when comparing receiver-operating curve characteristics, SCCA was found to be inferior to AFP for the early detection of HCC ($p = 0.001$), however, its combination with AFP for HCC early detection remains poor characterized [69].

Emerging technologies

miRNA

miRNAs are small non-coding RNAs that play important regulatory roles in various processes such as cell development, differentiation and proliferation [70]. The aberrant expression of these miRNAs can contribute to oncogenesis and cancer progression. miRNAs can circulate in a cell-free form in body fluids, including serum and plasma, which protect them against degradation by RNase. Due to their inherent stability and their proven role in tumor proliferation, circulating miRNAs have great promise as a noninvasive biomarker for the diagnosis of HCC. Two specific miRNA, miRNA-21 and miRNA-199a, have been proposed as potential biomarkers for the early diagnosis of HCC. Serum levels of miRNA-21 have been found to be elevated in HCC patients, and also show early promise in differentiating between cirrhosis status and HCC tumor stage in small Phase II studies [71–73]. There are several additional candidate miRNAs under investigation for the early detection and prognostication of HCC, and many are being studied individually or as components of miRNA panels combined with other biomarkers in Phase I and Phase II studies [74,75]. There are challenges with miRNA analyses related to incomplete annotation, however, several efforts

are underway in order to attempt to ensure uniformity in characterization of miRNA molecules [76,77].

Metabolomics

Metabolomics is a field in systems biology designed to provide a comprehensive analysis of low-molecular-weight endogenous metabolites in a biological sample, with the goal of mapping of disturbances in biochemical changes in disease, and to provide an opportunity to develop predictive biomarkers [78]. Metabolomics can allow for comprehensive measurements of small molecules, which are a by-product of metabolic reactions, in accessible biologic samples, such as serum and urine, in order to discover diagnostic biomarkers that can distinguish between disease and nondiseased states [79,80]. In the field of HCC detection, advanced chromatography and mass spectrometry technologies allow the detection of small-molecule metabolites produced by dysregulated metabolic pathways during hepatocarcinogenesis [81,82]. For early detection of HCC, metabolomics in combination with AFP could be an efficient and convenient tool, although development of these markers remains in the preclinical Phase I phase. Through a metabolomics approach, Wang *et al.* described the identification of 13 potential biomarkers and corresponding pathways that were significantly aberrant in patients with HCC [83]. Of the candidate biomarkers, glycochenodeoxycholic acid has been proposed as a potential indicator for HCC diagnosis [84]. The field of metabolomics for biomarker discovery has been limited by poor validation of potential markers in clinical practice. Several factors may play an important role in the concentrations of markers in an individual patient, such as environmental or behavioral factors (e.g., diet), which has limited the field [85].

Proteomics

Proteomics, or the large-scale study of proteins, is an emerging field in the development of biomarkers. Proteomics has historically been limited by both the sheer number and individual heterogeneity in circulating proteins present in the serum and cumbersome technology required to conduct adequate analysis (i.e., mass spectroscopy) that has limited the speed at which candidate proteins could be evaluated. Recent advances in high-throughput proteomic analysis have in part mitigated these challenges [86]. Protein post-transcriptional

modification, especially glycosylation, has been shown to change in different disease states. Several groups have analyzed core-fucosylation that is being used as a potential marker for various cancers, including pancreatic, lung and liver cancer [87–91]. Site-specific core-fucosylation of serum proteins can be used as markers to distinguish disease based on etiology, and to distinguish cirrhosis from HCC. For example, Lubman and co-workers identified 1300 core-fucosylated peptides in which 613 distinct core-fucosylated proteins were identified; of these, 20 core-fucosylated peptides were differentially expressed in alcohol-related HCC compared with alcohol-related cirrhosis, and 26 core-fucosylated peptides were different in HCV-related HCC as compared with HCV-related cirrhosis [88]. Of particular interest is fucosylated haptoglobin, which has been proposed as a marker for detection of early HCC. A unique pattern of bi-fucosylated tetra-antennary glycan was identified in HCC patients; the bi-fucosylation level was distinctly elevated in HCC patients versus in those with cirrhosis alone [92]. Wang *et al.* recently published the performance of a glycosylated kininogen added into to a previously established Doyelstown algorithm (log AFP, age, gender, alkaline phosphatase and alanine aminotransferase) in the detection of HCC with a particular focus on AFP-negative HCC. The authors performed a Phase II case–control study of 115 patients with HCC and 93 controls and they found their algorithm had a higher AUC than for AFP alone (0.977 vs 0.828) with an 89% detection rate in AFP-negative HCC [93,94]. This and other proteomic markers have been validated in smaller Phase II studies validation studies, however, there are several larger-scale Phase II and Phase III validation studies in progress for better characterization of these markers prior to Phase IV testing.

Other serum-based emerging technologies

Cell death and apoptosis results in release of cellular components, including cell-free DNA microparticles into the bloodstream. Cell-free DNA has been a biomarker of interest in several cancers, including HCC [95]. A meta-analysis of the existing data of use of cell-free DNA for HCC detection showed overall moderate performance characteristics, however, this technology is rapidly evolving and use of platforms incorporating machine-learning techniques may

improve its performance in early detection of HCC [96,97]. Additional serum-based markers including extracellular vesicles released by tumor tissue containing multiple tumor-associated proteins (tumor associated microparticles) have been explored in HCC detection. Early Phase I data show some promise for serum-based detection of HCC in patients undergoing surgical resection [98].

Challenges & conclusion

Several candidate biomarkers for HCC early detection currently exist and are in development, however, most have not been through the requisite phases of biomarker development, so that they can be used routinely in a clinical setting. There are methodologic challenges that remain in the field including incomplete characterization of cohorts, biased and incomplete prospective cohort sample collection and small-sample-sized validations sets. Phenotyping cohorts and appropriate use of at-risk controls is vital toward proper validation of biomarkers. Patients in the control arms of validation studies should have cirrhosis (i.e., at-risk population), however, they must be appropriately phenotyped in order to ensure they do not have early-stage HCC, otherwise validation will fail. Similarly, in the case of serial prospective collection, if cohort follow-up is incomplete, the risk of ascertainment bias for sample collection is significant. Finally, the question of sample size in validation is an important one. There is significant patients level and tumor level heterogeneity in patients with cirrhosis and HCC. This is a truly global disease and thus all races and ethnicities are at risk, and there are likely important genetic and epigenetic differences between individuals, not to mention differences in disease etiology and environmental factors. Homogenous validation sets may not be appropriate for routine clinical use of biomarkers. There may be biomarkers that only have adequate performance characteristics in subset of patients based on racial ethnic background, or by disease etiology. To date, aside from AFP, there have been very few comparative analyses of biomarker performance across patient groups. These challenges must be acknowledged in the development of biomarkers – ideally large diverse well-characterized prospectively collected datasets would be available for validation of these biomarkers [99].

Fortunately, serial samples from several longitudinal cohorts are being collected to allow for widespread validation of these biomarkers. Two of

the largest in North America include the National Institutes of Health sponsored Early Detection Research Network hepatocellular carcinoma collection, which is prospectively collecting samples from patient with cirrhosis with a target enrollment of 1500 patients, which will complete enrollment at the end of 2017. A larger cohort of cirrhosis patient sample has begun collection in several centers in Texas through the Cancer Prevention Research Institute of Texas. Both of these cohorts will contain a diversity of disease etiology and patient racial/ethnic background. In sum, once these cohorts complete enrollment and have sufficient patients whom have developed HCC, they will provide valuable samples for candidate biomarker validation. Thus, the future of biomarkers for the early detection of HCC is promising. The availability of large biomarker validation cohorts, in addition to the advent of big data techniques such as machine learning, will help in developing diagnostic algorithms combining biomarkers and clinical information for the early detection of HCC [100].

Future perspective over the next 10 years

In all likelihood, given the genetic heterogeneity seen in HCC, a combination of biomarkers along with patient demographic or genetic risk stratification will allow for precision screening for HCC with biomarkers alone, precluding the need for routine ultrasound surveillance in at-risk patients. With improved diagnostic testing and risk stratification, the promise of improved early detection and patient outcomes of HCC is prime to be realized.

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