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Biomarkers for the early diagnosis of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second leading cause of cancer-related deaths worldwide. Although the prognosis of patients with HCC is generally poor, the 5-year survival rate is > 70% if patients are diagnosed at an early stage. However, early diagnosis of HCC is complicated by the coexistence of inflammation and cirrhosis. Thus, novel biomarkers for the early diagnosis of HCC are required. Currently, the diagnosis of HCC without pathological correlation is achieved by analyzing serum α -fetoprotein levels combined with imaging techniques. Advances in genomics and proteomics platforms and biomarker assay techniques over the last decade have resulted in the identification of numerous novel biomarkers and have improved the diagnosis of HCC. The most promising biomarkers, such as glypican-3, osteopontin, Golgi protein-73 and nucleic acids including microRNAs, are most likely to become clinically validated in the near future. These biomarkers are not only useful for early diagnosis of HCC, but also provide insight into the mechanisms driving oncogenesis. In addition, such molecular insight creates the basis for the development of potentially more effective treatment strategies. In this article, we provide an overview of the biomarkers that are currently used for the early diagnosis of HCC.

Key words: α -fetoprotein; α -fetoprotein-L3; Biomarker; Des- γ -carboxyprothrombin; Glypican-3; Golgi protein-73; Hepatocellular carcinoma; MicroRNAs; Osteopontin; Squamous cell carcinoma antigen

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Core tip: Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second leading cause of

cancer-related death worldwide. The poor prognosis of HCC is due to the fact that diagnosis is often made at a late stage in disease development. Thus, the identification of biomarkers for diagnosis at an early stage may result in significant benefits. An up-to-date review of biomarkers that are currently used for the early diagnosis of HCC is provided in this article.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second leading cause of cancer-related deaths. The number of deaths per year for HCC worldwide is similar to the incidence, with nearly 748300 new cases and 695900 deaths per year. HCC most often develops in patients with a history of cirrhosis due to chronic alcohol abuse, non-alcoholic fatty liver disease, or hepatitis C virus (HCV) infection. Continuous cycles of inflammation and healing in hepatocytes are thought to be the underlying cause of the development of HCC. However, the coexistence of inflammation and cirrhosis complicates the early diagnosis of HCC. Therefore, biomarkers that distinguish HCC from inflammation and cirrhosis are desperately needed in order to enhance prognosis of these patients. Furthermore, such biomarkers may influence the development of novel chemopreventive strategies for use during HCC surveillance of patients with cirrhosis.

Contributing to the poor prognosis of HCC is the lack of specific symptoms in the early stages of the disease. More than 60% of patients are diagnosed with late-stage disease after metastasis has occurred^[1], resulting in an overall 5-year survival rate of < 16%^[2]. In contrast, patients diagnosed with early stage disease have a relatively good prognosis, with a 5-year survival rate of > 70%. In patients diagnosed with early stage HCC, such as Barcelona Clinic Liver Cancer (BCLC) stage 0 and A, the 5-year survival rate with surgical intervention was > 93%^[4]. Thus, detection of HCC at an early stage significantly impacts curative treatment regimens.

In Japan, early stage HCC nodules have been detected in more than 60% of patients, due to the routine practice of screening for HCC among high-risk patients^[3]. The diagnosis of HCC without a pathological diagnosis can be achieved by assessing serum α -fetoprotein (AFP) levels and diagnostic imaging, such as computed tomography (CT) and magnetic resonance imaging (MRI)^[5]. Unfortunately, even this approach is inadequate, and very few HCC biomarkers demonstrate sufficient diagnostic performance for

early stage HCC in clinical practice.

The ideal HCC biomarker is one that enables clinicians to diagnose asymptomatic patients and can be widely used in a screening process. In general, a biomarker valuable for clinical use achieves a level of sensitivity and specificity of $\geq 90\%$, and is non-invasive and cost-effective to allow widespread use. The most desirable biomarker is therefore tumor-specific and easily detectable in bodily fluids, such as serum, plasma, and bile.

To establish a formal framework to guide biomarker evaluation and development, a five-phase program was adopted by the Early Detection Research Network (EDRN) of the National Cancer Institute (Table 1)^[6]. Most markers have been evaluated in phase II studies to evaluate their ability to detect early stage HCC, and most, with the exception of AFP, are undergoing further assessment in phase III studies. Further studies with larger sample sizes in multiple clinical centers are needed to confirm that marker-based surveillance reduces morbidity and mortality from HCC. The present review summarizes the various biomarkers that are currently used for early diagnosis of HCC.

LIST OF HCC BIOMARKERS

AFP

AFP has been considered to be the most useful biomarker for HCC evaluation, ever since it was discovered in the serum of HCC patients in 1964^[7]. In addition, it is the only biomarker that has been evaluated in a randomized controlled trial^[8]. AFP is a glycoprotein with a molecular weight of about 70 kDa that transports a variety of molecules, including bilirubin, fatty acids, retinoid, steroids, heavy metals, dyes, flavonoids, phytoestrogens, dioxin, and possibly various drugs^[9]. It is normally produced during fetal and neonatal development by the liver, yolk sac, and in small concentrations, the gastrointestinal tract^[10]. Serum AFP reaches a maximal concentration of 3 g/L at weeks 12 to 16 of fetal life. Protein levels subsequently decrease rapidly, and thereafter only trace amounts are normally detected in serum^[11]. Abnormally high serum AFP concentrations have been correlated with the development of several malignant diseases, most notably HCC^[12,13]. Previously, we reported that through multivariate analysis, a minimum postoperative AFP level was determined to be a significant independent risk factor for recurrence after curative hepatectomy ($P < 0.001$)^[14].

A systematic review evaluating AFP (at a threshold level of 20 ng/mL) in cirrhotic patients showed sensitivities and specificities of 41% to 65% and 80% to 94%, respectively, for HCC at any stage^[15]. However, at this threshold, early stage HCC was detected in only one-third of patients with the disease^[16]. The problem with AFP as a reliable HCC biomarker is that HCC is positive for the protein in only 60%-80% of cases, and false-positives make it difficult to distinguish

Table 1 Phases of biomarker validation for early cancer detection

Phase of biomarker validation	Type of study	Aim	Biomarker
Phase I Phase II	Preclinical exploratory Case-control	Identify promising markers Clinical assay to detect HCC	AFP-L3, DCP, GPC3, OPN, GP73, SCCA, annexin A2 suPAR, MDK, AXL, TRX, nucleic acids, miRNA
Phase III	Retrospective longitudinal	Characterize ability of biomarker to detect HCC before it becomes clinical	
Phase IV	Prospective screening	Identify extent and characteristics of sensitivity and specificity	
Phase V	Randomized control	Determine if biomarker screening can reduce mortality in target population	AFP

Table 2 Early diagnostic values of hepatocellular carcinoma serum biomarkers

Biomarker	Sensitivity, % (95%CI)	Specificity, % (95%CI)	Ref.
AFP	53 (46-59)	90 (87-93)	[17]
AFP-L3	28 (22-34)	97 (93-100)	[17]
DCP	61 (55-68)	70 (65-74)	[17]
GPC3	55.1 (47.9-66.2)	97.0 (95.2-98.2)	[42]
OPN	75 (58-93)	62 (51-73)	[50]
GP73	62	88	[56]
miRNA panel ¹	82.5	83.5	[101]

¹Including miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801.

early stage HCC from other disorders, such as acute hepatitis and cirrhosis, as well as embryonic tumors and certain gastrointestinal tumors. Thus, a lower threshold may be an effective solution for early stage detection. A multicenter case-control study of patients ($n = 836$; HCC, $n = 419$ and cirrhosis, $n = 417$) revealed that AFP exhibited sensitivity and specificity as high as 66% and 82%, respectively, for early stage HCC (BCLC stages 0 and A) at a lower threshold of 10.9 ng/mL (Table 2)^[17]. However, the sensitivity of AFP is only 53% at the commonly used cut-off of 20 ng/mL. Further research may help to optimize the threshold for AFP. However, in order to significantly improve the diagnostic accuracy for HCC, additional biomarkers are needed to complement AFP, especially due to the fact that many patients with benign liver diseases, such as chronic hepatitis, liver cirrhosis and gastrointestinal cancer, also have elevated serum AFP.

AFP-L3

AFP exists as three glycoforms, each with different binding capability to lectin *Lens culinaris* agglutinin (LCA): AFP-L1 (non-binding fraction), AFP-L2 (weak binding fraction), and AFP-L3 (binding fraction). AFP-L1 is increased in chronic hepatitis and liver cirrhosis, whereas AFP-L3 is specifically increased in HCC. Because AFP-L3 is derived only from cancer cells, it has been considered a more specific biomarker for HCC^[18,19]. The concentration of AFP-L3 correlates well

with AFP levels, and thus, AFP-L3 has been suggested as a biomarker for early HCC detection, due to its higher specificity than AFP^[20]. For the detection of HCC, AFP-L3 is currently used at a threshold value of 10%. A large multicenter prospective study reported a specificity approaching 92%, but a sensitivity of only about 37% at this threshold for HCC at any stage^[21]. Another multicenter case-control study found that AFP-L3 displayed a specificity of 97% and a sensitivity of 28% for early stage HCC diagnosis (BCLC stages 0 and A)^[17]. Thus, the low sensitivity of AFP-L3 limits its potential as an HCC biomarker, even though specificity is extremely high. Moreover, because AFP-L3 is typically not detected when AFP levels are < 20 ng/mL, AFP-L3 is not relevant for the diagnosis of HCC in individuals with a total AFP concentration of < 20 ng/mL. Thus, the sensitivity for AFP-L3 appears to be adversely affected by the total AFP concentration.

Recent technical advances in higher sensitivity analytical methods with novel and advanced microfluidics-based separation science have improved the sensitivity of the AFP-L3 immunoassay^[22]. The automated immunoassay for AFP-L3 is referred to as "highly sensitive AFP-L3" (hs-AFP-L3). A case-control study of hs-AFP-L3 included patients with benign liver disease ($n = 74$), such as chronic hepatitis and cirrhosis, as well as patients with HCC ($n = 94$). The study compared the performance of conventional AFP-L3 with hs-AFP-L3 and reported that hs-AFP-L3 yielded levels that were significantly higher than conventional AFP-L3, even in patients with single or small (< 20 mm in diameter) HCC nodules/tumors. The sensitivity and specificity of hs-AFP-L3 vs conventional AFP-L3 were 57.0% and 63.5%, and 40.4% and 81.1%, respectively^[23]. These results indicate that hs-AFP-L3% could be a valuable biomarker for detecting early stage HCC and may be used for clinical practice in the near future.

Des- γ -carboxyprothrombin

Prothrombin induced by vitamin K absence II (PIVKA II), known as Des- γ -carboxyprothrombin (DCP), is an abnormal prothrombin molecule that is increased in HCC. During the process of malignant transformation in hepatocytes, the vitamin K-dependent carboxylase

system becomes impaired^[24-27]. It is in fact a defect in posttranslational carboxylation that leads to the production of DCP^[28]. In this process, DCP loses its normal prothrombin function but may take on an important role promoting malignant proliferation in HCC. Many studies have shown that the level of serum DCP in patients with benign and malignant liver diseases deviates significantly from normal, and that its diagnostic sensitivity may be greater than AFP. However, this result remains controversial^[29]. In a test to screen for HCC, when compared to cases of cirrhosis and chronic hepatitis, DCP yielded a sensitivity of 72.7% and a specificity of 90.0%, a result that was comparable to AFP^[25]. Because AFP and DCP are not strictly correlated, i.e. DCP is more specific to HCC and has less tendency to be elevated in other chronic liver diseases, the combination of these markers significantly improves HCC detection, yielding a sensitivity and a specificity of 74.2% and 87.2%, respectively^[30].

Although DCP has demonstrated some potential as a serum biomarker for the early diagnosis of HCC, the possibility requires further investigation particularly in combination with AFP. In a large multicenter case-control study, DCP alone exhibited a sensitivity of 56% in early stage patients^[31]. However, the combination of DCP with AFP increased sensitivity from 65% to 87% at 3 mo before HCC diagnosis; however, the specificity decreased from 84% to 69%.

Further studies are clearly needed to better assess the effectiveness of this combination of markers in HCC diagnosis. Moreover, DCP has been mainly examined in Asian countries, and experience with DCP in Western countries, particularly Europe, remains limited. Recently, a case-control study to compare the performances of AFP and DCP serum levels for the diagnosis of early stage HCC (BCLC stage A) was conducted in France^[32]. This study included cirrhotic controls ($n = 43$) as well as cases with HCC ($n = 85$), a subset of which ($n = 32$) harbored early stage HCC. DCP (at a threshold value of 42 mAU/mL) performed better than AFP (at a threshold value of 5.5 ng/mL) for early stage HCC diagnosis [area under the curve (AUC) = 0.81, 95%CI: 0.697-0.924 vs AUC = 0.582, 95%CI: 0.443-0.722], with a sensitivity of 77% vs 61%, a specificity of 82% vs 50%, a positive predictive value (PPV) of 76% vs 51%, and a negative predictive value (NPV) of 83% vs 62%, respectively. Thus, the combination of DCP and AFP slightly improved the performance of early stage HCC diagnosis in this French cohort (AUC = 0.826, 95%CI: 0.722-0.929). These results further support the value of DCP as a biomarker in the diagnosis of early stage HCC.

Glypican-3

Glypican-3 (GPC3) belongs to the glypican family of heparan sulfate proteoglycans. It is linked to the cell membrane by a glycosyl-phosphatidylinositol anchor^[33].

GPC3 is involved in cell proliferation, survival, and tumor suppression, but is normally absent in healthy and non-malignant hepatocytes. Interestingly, GPC3 appears to function differently in diverse cancers; while GPC3 is downregulated in breast cancer, ovarian cancer, and lung adenocarcinoma, it is upregulated in HCC^[34,35] where it is thought to stimulate growth by upregulating autocrine/paracrine canonical Wnt signaling^[36]. It has been reported that GPC3 could be detected in as many as 53% of HCC patients^[37], and in our own study it was detected in 40% of HCC patients and 33% of HCC patients seronegative for both AFP and DCP^[38].

As GPC3 is detected in HCC cells but not in benign liver tissues, it has potential as a biomarker for the diagnosis of early stage HCC^[39,40]. Importantly, GPC3 expression appears to be independent of tumor size, as GPC3 exhibited a sensitivity of 56% in patients with early stage tumors that are < 3 cm in size^[41]. In a meta-analysis, the pooled sensitivity and specificity of serum GPC3 for the diagnosis of HCC overall were 55.2% (52.9%-57.4%) and 84.2% (82.2-86.0%), respectively^[42]. More specifically, GPC3 was assessed in the diagnosis of early stage HCC (BCLC 0 and A or TNM stage I), and the observed pooled sensitivity and specificity of serum GPC3 were 55.1% (47.9%-66.2%) and 97.0% (95.2%-98.2%), respectively. For comparison, the pooled sensitivity and specificity of AFP for the same study were 34.7% (26.2%-44.1%) and 87.6% (82.6%-91.6%), respectively. Finally, the combination of GPC3 and AFP was evaluated in this study and found to increase sensitivity to 76% for early stage tumors < 3 cm in size.

The value of GPC3 is not limited to its potential as a serum biomarker. Since GPC3 is uniquely upregulated in HCC, the utility of GPC3 as an immune specific target for cancer immunotherapy has also been tested^[43-45]. Measurable immune responses and antitumor efficacy with good tolerance were shown in a phase I clinical trial of a GPC3 peptide vaccine for patients with advanced HCC^[43].

Osteopontin

Osteopontin (OPN), also known as the transformation-related protein phosphatase, is an integrin-binding glycoprophosphoprotein that is overexpressed in many different types of malignancies, including lung, breast and colon cancer. The protein has been found to play a role in many physiological cellular functions, including migration, invasion, and metastasis^[46]. One of its more critical roles has been suggested to be in the metastatic potential of various cancers^[47]. Normally, OPN is expressed in bile duct epithelium, stellate cells, and Kupffer cells, but not in hepatocytes^[48]. However, elevated expression of serum OPN has been reported in HCC patients compared to normal liver patients or those with liver cirrhosis or chronic hepatitis^[49,50]. In a meta-analysis, the pooled sensitivity and specificity of

OPN were both at 86% for all stages of HCC^[51]. In the study performed by Shang *et al.*^[50], OPN, at a threshold of 91 ng/mL, exhibited an increased sensitivity relative to AFP (74% vs 53%, respectively) when testing for HCC in a cohort including a total of 312 healthy adults and patients with cirrhosis, chronic hepatitis, and HCC. When threshold values of osteopontin at 156 ng/mL and AFP at 20 ng/mL were combined, sensitivity and specificity were even greater (95% and 96%, respectively).

Importantly, this study also investigated the utility of OPN in early diagnosis. For OPN, the AUC for discriminating between early stage HCC (BCLC stage A) and cirrhosis was 0.73. OPN demonstrated a sensitivity and specificity of 75% and 62% for early stage HCC, compared to 46% and 93% for AFP. When combined with AFP, the AUC increased to 0.81. At a threshold of 91 ng/mL for OPN, the combined use of the biomarkers resulted in a sensitivity of 83% and a specificity of 63%. Based on such findings, the value of OPN for diagnosis of early stage HCC is being further investigated in retrospective longitudinal biomarker studies.

Golgi protein-73

Golgi protein-73 (GP73) is a type II Golgi-specific membrane protein that is normally expressed in epithelial cells of various human tissue types, but not hepatocytes. However, GP73 is detected in the serum of patients with liver disease, particularly HCC^[52]. A case-control study demonstrated that serum GP73 in patients with HCC was significantly higher than in healthy adults and hepatitis B virus (HBV) carriers without hepatic disease^[53]. The sensitivity and specificity of serum GP73 for HCC were 74.6% (95%CI: 71.5%-77.6%) and 97.4% (95%CI: 96.8-98.3%), respectively, compared to 58.2% and 85.3%, respectively for AFP. The combination of GP73 and AFP increased the sensitivity and specificity to 89.2% (95%CI: 86.7-91.5%) and 85.2% (95%CI: 83.4%-86.4%), respectively, with an AUC of 0.96. The combined use of GP73 and AFP-L3 for the diagnosis of low serum AFP HCC cases also demonstrated higher sensitivity (94.0%), specificity (93.1%), and better accuracy (93.3%) than either alone^[54].

Investigators have considered GP73 as a potential biomarker also for early diagnosis. Serum GP73 levels showed enhanced sensitivity relative to AFP in the detection of early stage HCC^[55]. In the study of Marrero *et al.*^[56], the sensitivity and specificity of GP73 for early HCC (United Network of Organ Sharing (UNOS) modified TNM stages 1 and 2) were similar (62% and 88%, respectively). Although these studies demonstrated that the sensitivity of GP73 was higher than that of AFP in the diagnosis of early stage HCC, whether the potential clinical value of GP73 as a serum biomarker exceeds that of AFP remains controversial. Regardless, as the elevation of serum GP73 remains

moderate in virus carriers and patients with cirrhosis, GP73 should still be investigated as a potential biomarker for the diagnosis of early HCC in these patients.

Squamous cell carcinoma antigen

Squamous cell carcinoma antigen (SCCA) is a member of the high molecular weight family of serine protease inhibitors that are found in squamous epithelium and isolated from cervical carcinoma. SCCA is highly expressed in epithelial tumors and has a role in protecting tumor cells from apoptosis^[57]. As SCCA is expressed as a consequence of dedifferentiation, it has been considered as a potential marker for HCC. Giannelli *et al.*^[58] evaluated SCCA levels in a cohort of patients ($n = 210$; HCC, $n = 120$ and cirrhosis, $n = 90$) and reported that HCC patients exhibited higher SCCA serum levels than cirrhotic patients. SCCA had a sensitivity of 84.2%, but the specificity was 48.9%. Subsequently, the diagnostic accuracy of SCCA was investigated taking into account only smaller HCC nodules (< 3 cm) and comparing to cirrhosis^[58]. The sensitivity and specificity of SCCA were 56.1% and 74.9%, respectively, with an AUC of 0.7 (95%CI: 66%-74%) at a threshold value of 3.2 ng/mL.

SCCA expression was also tested as an immunohistochemical marker for the diagnosis of HCC. Guido *et al.*^[59] found that the expression of SCCA in HCC and dysplastic nodules was much higher than in regenerative nodules, indicating that the expression of SCCA had already increased early in the development of HCC. Overall, the high sensitivity and low specificity of SCCA were complementary to AFP, rendering SCCA a valuable supplementary marker for the diagnosis of HCC.

An alternative potential biomarker is the variant IgM immune complex that SCCA has been observed to form with IgM (SCCA-IgM IC) when its expression increased in the early phase of hepatocarcinogenesis. SCCA-IgM IC achieved a higher diagnostic performance than determination of the free biomarker, and furthermore was undetectable in the serum of a healthy adult. However, the detection rates of SCCA-IgM IC were 18%, 26%, and 70% in chronic hepatitis, cirrhosis, and HCC respectively^[60]. The sensitivity and specificity of SCCA-IgM determination for HCC were thus 89% and 50%, respectively, with an AUC of 0.66^[61]. Although the AUC was lower than that of the other discussed biomarkers, SCCA-IgM IC was consistently increased in patients with cirrhosis progressing towards HCC development, and sensitivity was higher than AFP^[62]. Thus, SCCA-IgM IC may be a valuable serum marker for early HCC detection in some cases.

Annexin A2

Annexin A2 is a calcium-dependent, phospholipid-binding protein found on the surface of endothelial cells and most epithelial cells^[63,64]. It is upregulated in

many tumor types and has multiple roles in various tumorigenic processes, including angiogenesis, proliferation, apoptosis, cell migration, invasion and adhesion processes, which are essential for cancer metastasis^[65-68]. In HCC, serum concentrations of annexin A2 were found to be frequently elevated compared to healthy controls and individuals with benign liver disease or other malignant tumors^[69-71]. Sun *et al.*^[72] also observed increased concentrations of annexin A2 in 83.2% of early stage HCC (BCLC stages 0 and A) and 78.4% of AFP-negative HCC patients. Annexin A2 (at 17.3 ng/ μ L) demonstrated sensitivity and specificity of 83.2% and 67.5%, respectively in the detection of early stage HCC, and those of AFP (15.64 ng/mL) were 54.7% and 81.3%, respectively. Moreover, the AUC of annexin A2 alone (0.79, 95%CI: 73%-85%) was greater than for AFP alone (0.73, 95%CI: 66%-80%). The combination of annexin A2 and AFP however further improved sensitivity and specificity (87.4% and 68.3%, respectively). Thus, annexin A2 might be an important independent and discriminative serological candidate biomarker for detecting early stage HCC in patients with normal serum AFP.

Soluble urokinase plasminogen activator receptor

Soluble urokinase plasminogen activator receptor (suPAR) is the circulating form of the glycosylphosphatidylinositol-linked membrane protein, urokinase-type plasminogen activator receptor (uPAR). suPAR was recently established as a biomarker for the level of activation of the immune system and cancer metastasis. suPAR serum levels are elevated in patients with ovarian cancer, colon cancer, and HCC^[73-75]. A prospective study was conducted on patients ($n = 267$) with benign liver disease but no signs of HCC on imaging over the course of 7 years in order to determine whether serum suPAR would be a valuable molecular tool for the prediction of the future development of HCC^[73]. This study revealed that within the subgroup of the high-risk European Association for the Study of Liver (EASL), a suPAR concentration of > 9.56 ng/mL yielded sensitivity of 76.0%, specificity of 90.4%, and positive and negative predictive values of 54.3% and 96.2%, respectively, for the eventual development of HCC. Based on these results, suPAR has potential as an early predictor to evaluate the risk of the development of HCC.

Midkine

Midkine (MDK) is a heparin-binding growth factor, initially identified as a retinoic acid responsive gene, which plays a critical role in cell growth, survival, migration, angiogenesis, and carcinogenesis^[76]. In a study performed on patients newly diagnosed with HCC, MDK levels were found to be higher in cases of HCC than cirrhosis (0.625 ng/mL vs 0.15 ng/mL, $P < 0.001$) or healthy controls (0.625 ng/mL vs

0.125 ng/mL, $P < 0.001$)^[77]. The AUC was at 0.941 (95%CI: 0.890-0.992), and for AFP at 0.671 (95%CI: 0.546-0.796) ($P < 0.001$). The sensitivity of MDK (0.387 ng/mL) to discriminate patients with early HCC (BCLC 0 and A) from those with cirrhosis was 90%, which was significantly higher than AFP (20 ng/mL) at 40%.

AXL

AXL is a receptor tyrosine kinase that has been implicated in the proliferation, survival and chemoresistance of many malignancies, including lung, breast, ovarian, colon and pancreatic cancers^[78-82]. AXL is activated by the binding with growth arrest-specific protein 6 to the extracellular domain and undergoes proteolytic processing that results in the release of an 80 kDa soluble form that can be detected in serum^[83]. Increased AXL expression has been identified as a poor prognostic factor for recurrence-free survival, as well as overall survival in colon and pancreatic cancer^[80,82]. The diagnostic value of AXL in early stage diagnosis of HCC (BCLC stage 0) was analyzed in a multicenter study^[84]. The sensitivity of AXL (76.9%) was found to be much higher than that of AFP (38.5%), and AXL outperformed AFP (AUC, 0.848 vs 0.797, respectively) in detecting early stage HCC. Finally, AXL and AFP together reached an extraordinarily high AUC (0.936) in detecting early stage HCC, with sensitivity at 80.8% and specificity at 92.3%.

Thioredoxins

Thioredoxins (TRXs) are thiol oxidoreductases that are ubiquitously expressed and involved in several biological processes such as, regulation of protein states, cellular apoptosis and proliferation, and protection against oxidative stress^[85]. The expression of TRXs is increased in many neoplasms, and has been shown to correlate with prognosis, specifically in lung and colorectal carcinoma^[86,87]. Li *et al.*^[88] reported on the potential availability of a TRX for the detection of early stage HCC (well-differentiated, < 2 cm HCC). In this study, the sensitivity and specificity of TRX (74.9% and 87.5%, respectively) were higher than for AFP (68.6% and 75.2%, respectively). Furthermore, with an AUC of 0.854, TRX outperformed AFP at an AUC of 0.720 in detecting early stage HCC. Again, when combined, TRX and AFP were more accurate in the detection of early stage HCC (AUC, 0.889; sensitivity, 81.3%; specificity, 93.4%).

Nucleic acids

Microarray technology has emerged as a powerful tool to probe nucleic acids for the identification of many clinically relevant molecular biomarkers, bringing a new dimension to disease diagnosis^[89,90]. By screening expression arrays, Shi *et al.*^[91] identified three individual genes associated with HCC development, chemokine (C-X-C motif) receptor 2 (CXCR2), C-C chemokine

receptor type 2 (CCR2) and E1A-binding protein P400 (EP400), and determined their accuracies for detection of the disease: 82.4%, 78.4% and 65%, respectively. Combined measurements of the three gene markers increased the accuracy in the detection of early stage HCC (stages 0 and A) to 86% (sensitivity, 72%; specificity, 95%). Moreover, further improvement in the accuracy (91%; sensitivity, 86%; specificity 95%) occurred when AFP was included in the profile.

MicroRNAs

MicroRNAs (miRNAs) are endogenous, small (17-25 nucleotides), non-coding RNAs that bind to complementary sequences in 3'-untranslated regions of target mRNAs to induce their degradation. They are conserved across species, as miRNAs have been found to regulate diverse processes in worms, flies, and mammals, including humans^[92]. Approximately 500 miRNA genes have been identified and found to be important components of complex functional pathways controlling important cellular processes, such as proliferation, differentiation, and apoptosis. In the development of human cancer, miRNAs have been determined to function both as oncogenes and as tumor suppressor genes^[93]. Because each type of miRNA is stable and can downregulate hundreds of genes at a time, they can control large transcriptional programs that determine fundamental cellular features. Such diversity in functional roles enables miRNAs to be used as diagnostic tools for early cancer detection, risk and prognosis assessment, and as new therapeutic targets^[94].

miRNAs associated with HCC development have been investigated as biomarkers to diagnose the disease. Some of these miRNAs have been shown to accurately predict poor prognosis in HCC^[95]. For example, studies have indicated that miR 200a and miR 200b, two members of the miR 200 family, are deregulated during the development of both HCC and liver fibrosis^[96-98]. The increased levels of serum miR-21 have been used to distinguish cases of HCC from chronic hepatitis and healthy controls. In the case of HCC vs chronic hepatitis, the sensitivity and specificity were 61.1% and 83.3%, respectively, with an AUC of 0.773, and in the case of HCC vs healthy controls the values were 87.3% and 92.0%, respectively, with an AUC of 0.773. Both values were superior to that of AFP as a biomarker in HCC^[99]. Serum miR-15b and miR-130b are additional potential miRNA markers that are significantly upregulated in HCC^[100]. For the detection of HCC, miR-130b exhibited an AUC of 0.913 (sensitivity, 87.7%; specificity, 81.4%). In contrast, while the sensitivity of miR-15b for detecting HCC was extremely high at 98.3%, its specificity was very poor (15.3%). The high sensitivity of serum miR-15b and miR-130b as biomarkers for HCC is potentially favorable, particularly for patients with early stage HCC, who may have low AFP levels. A panel of seven miRNAs (miR-122, miR-192, miR-21, miR-223, miR-

26a, miR-27a and miR-801) has been shown to have high diagnostic accuracy in the early diagnosis of HBV-related HCC (BCLC stage 0 and A; AUC, 0.888)^[101].

A few features, in addition to their expression profiles, make miRNAs particularly attractive as potential biomarkers. First, since many dysregulated miRNAs are highly stable and readily detected in serum and plasma in HCC patients, they may more generally have high AUCs in the detection of HCC as well as any other disease state. Second, miRNAs appear in the urine, which represents a non-invasive and easily obtainable resource for biomarkers. In fact, the detection of five deregulated miRNAs (miR-625, miR-532, miR-618, miR-516-5P, and miR-650) in the urine has already been used to screen high-risk patients for the early detection of HCC^[102]. The presence of miRNAs in body fluids, such as urine, may represent a gold mine of biomarkers for cancer. However, further investigation is necessary to establish specific circulating miRNA(s) as reliable and accurate in the detection of HCC at an early stage.

CONCLUSION

The diagnosis of HCC patients remains difficult, especially early in the development of the disease, and yet early and accurate diagnosis of HCC patients is vital in order to improve prognosis. Promising biomarkers for diagnosis of HCC have been successfully identified in several studies. However, current data suggest that no single biomarker alone is likely to have optimal sensitivity and specificity for the detection of HCC, particularly at early stages of development. In many studies, combinations of several biomarkers have been shown to complement each other and improve the early diagnostic rate. Incorporation of clinical variables, such as age and sex, into models based on combinations of biomarkers could further enhance the predictive performance of the models for HCC detection. More randomized controlled studies investigating such biomarkers will help to validate optimal combinations of them for successful detection of early stage HCC.

Although the emphasis in this review has been on the early detection of HCC, biomarkers have an additional exciting role in the development of personalized treatment. In this regard, any biomarker, even one with low sensitivity, has the potential to serve as an important indicator for a molecularly targeted drug. For example, GPC3-targeted immunotherapy, including a peptide vaccine and antibody, elicited some anti-tumor effect and showed good tolerance^[45]. In our Phase I clinical trial of GPC3-derived peptide vaccines, the disease control rate [partial response (PR) + stable disease (SD)] was 60.6% at two months after initiation of treatment. A median survival of 12.2 mo was observed in patients exhibiting a high frequency of GPC3-specific cytotoxic T lymphocytes (CTLs) with no severe adverse events, compared to 8.5 mo in

individuals with a low GPC3-specific CTL frequency ($P = 0.033$). GPC3 antibodies (GC33) had an SD of more than 26 wk in 4 of 15 (16.7%) patients^[103]. The median overall survival in the group with high expression of GPC3 (49.4 wk) was greater than in the groups with low or no GPC3 expression (13.0 wk).

In conclusion, advances in technologies, such as mass spectrometry and next-generation sequencing, hold great promise for the identification of novel early diagnostic biomarkers for HCC. Circulating miRNAs are particularly intriguing as a whole new class of biomarkers and may outperform traditional serum protein markers. The added advantages are that some changes in miRNAs are detected early and in body fluids so that they can be easily monitored. However, even if any of the markers discussed perform well as biomarkers, therapeutic efficacy remains poor, especially in the absence of imaging. New treatment options and novel imaging modalities are therefore desperately needed. Finally, novel biomarkers may provide important clues to our understanding of oncogenesis, and ultimately lead to better treatment strategies. Simultaneous advancement in these many medical disciplines will hopefully initiate change in the poor prognosis of HCC patients.

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