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REVIEW

Circulating predictive and diagnostic biomarkers for hepatitis B virus-associated hepatocellular carcinoma

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Abstract

Chronic hepatitis B virus (HBV) infected patients have an almost 100-fold increased risk to develop hepatocellular carcinoma (HCC). HCC is the fifth most common and third most deadly cancer worldwide. Up to 50% of newly diagnosed HCC cases are attributed to HBV infection. Early detection improves survival and can be achieved through regular screening. Six-monthly abdominal ultrasound, either alone or in combination with alpha-fetoprotein serum levels, has been widely endorsed for this purpose. Both techniques however yield limited diagnostic accuracy, which is not improved when they are combined. Alternative circulating or histological markers to predict or diagnose HCC are therefore urgently needed. Recent advances in systems biology technologies have enabled the identification of several new putative circulating biomarkers. Although results from studies assessing combinations of these biomarkers are promising, evidence for their clinical utility remains low. In addition, most of the studies conducted so far show limitations in design. Attention must be paid for instance to different ethnicities and different etiologies when studying biomarkers for hepatocellular carcinoma. This review provides an overview on the current understandings and recent progress in the field of diagnostic and predictive circulating biomarkers for hepatocellular carcinoma in chronically infected HBV patients and discusses the future prospects.

Key words: Hepatocellular carcinoma; Hepatitis B virus infection; Biomarkers; Predictive; Diagnostic; Alpha-fetoprotein; Validation; Limitations; MicroRNA

Core tip: Regular screening for hepatocellular carcinoma (HCC) in patients at risk improves their survival rates. Currently available screening methods include abdominal ultrasound and alpha-fetoprotein serum levels, but both methods lack diagnostic accuracy. Recent technological advances have enabled the identification of new predictive and diagnostic hepatitis B virus (HBV)-associated HCC biomarkers. Nevertheless, most of the studies conducted so far show design limitations. This review provides an overview on the current understanding and future prospects of circulating predictive and diagnostic biomarkers for HBV-associated HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and ranks third as cancer-related death cause due to a 5-year survival of only 15%[1]. Moreover, at a time of decreasing overall cancer-related deaths due to an immense progress in cancer diagnostics and treatment options, mortality from hepatocellular carcinoma is increasing[1,2].

Chronic hepatitis B virus (HBV) infection is a major risk factor for HCC development. Prospective cohort studies have revealed an up to 100-fold increased risk for HCC in chronically infected HBV patients[3]. Up to 50% of newly diagnosed HCC cases are attributed to HBV infection, due to both direct and indirect oncogenic effects of the virus[2,4-7]. Integration of HBV DNA in the human genome may result in genomic instability, while inflammation-related oxidative stress, caused by immunological responses, may contribute indirectly to HCC development[6-9].

Four clinical phases can be distinguished during the natural course of a HBV infection: an immune-tolerance phase, an immune active phase, an inactive carrier phase and a hepatitis B e antigen (HBeAg) negative phase[2,10]. Patients in the immune active phase and the HBeAg negative phase are at increased risk for progression towards fibrosis and ultimately the development of cirrhosis, which is a major risk factor for HCC[11,12].

Several years to decades are needed for HCC to develop in a HBV infected liver[13]. Early diagnosis of HCC in HBV patients is challenging but is proven to result in an improved long-term survival due to an increased chance to detect tumors at a resectable stage[14-19].

Importantly, also a significant number of HBV patients develop HCC in a non-cirrhotic liver[20]. Current guidelines therefore advise 6-monthly abdominal ultrasound (US) surveillance for HCC in advanced fibrosis or cirrhotic HBV patients and in non-cirrhotic patients depending on ethnic background and age[21-25]. The technique, however, faces a disappointing 63% sensitivity to detect HCC and is hampered by inter- and intra-observer variability[20]. Finding biomarkers to better predict or diagnose HCC therefore remains an important clinical and research priority.

Serum alpha-fetoprotein (AFP) levels are widely used for HCC screening and diagnostics, but the clinical utility to rule out or detect HCC is still a matter of debate. The protein lacks sensitivity and specificity to detect HCC. The recent improvement of systems biology techniques, such as proteomics and genomics, has enabled the identification of several new putative biomarkers[26,27]. This review provides an overview of diagnostic and predictive serum biomarkers for HBV-associated hepatocellular carcinoma and discusses future prospects.

DIAGNOSTIC BIOMARKERS FOR HCC: AN OVERVIEW

An overview of the discussed diagnostic circulating biomarkers with their respective sensitivities and specificities to detect HCC is displayed in Table 1. An overview of their cellular origin is displayed in Figure 1.

Alpha-fetoprotein goes off stage, its glycoforms come on stage

Alpha-fetoprotein is an oncofetal protein produced by the fetal yolk sac and liver[28]. The protein, like albumin, binds exogenous as well as endogenous substances in blood[29]. Physiologically elevated AFP levels are found in pregnant women and newborns, but decrease quickly after birth. Upregulation of AFP later on in life has been associated with various pathological conditions such as acute hepatitis, endodermal sinus tumors and HCC[30-32].

Alpha-fetoprotein was discovered in the late 1950s and has been of interest for the monitoring of HCC development in viral hepatitis patients since the early 1970s[33-35]. For a long time, AFP has been widely used together with abdominal US in routine HCC screening. Nevertheless, the most recent European and American guidelines do not endorse this practice anymore since its diagnostic accuracy is low[21-23]. The protein, most often detected by enzyme-like immunosorbent assays (ELISA), indeed faces a lack of sensitivity and specificity to detect early stage HCC in HBV patients[36]. Only 70% of all HCC's are characterized by markedly elevated AFP levels at the time of diagnosis[37-41]. Large HBV cohort studies showed a maximal sensitivity for AFP of about 75% to detect HCC at optimal cut-off levels[32,41-45].

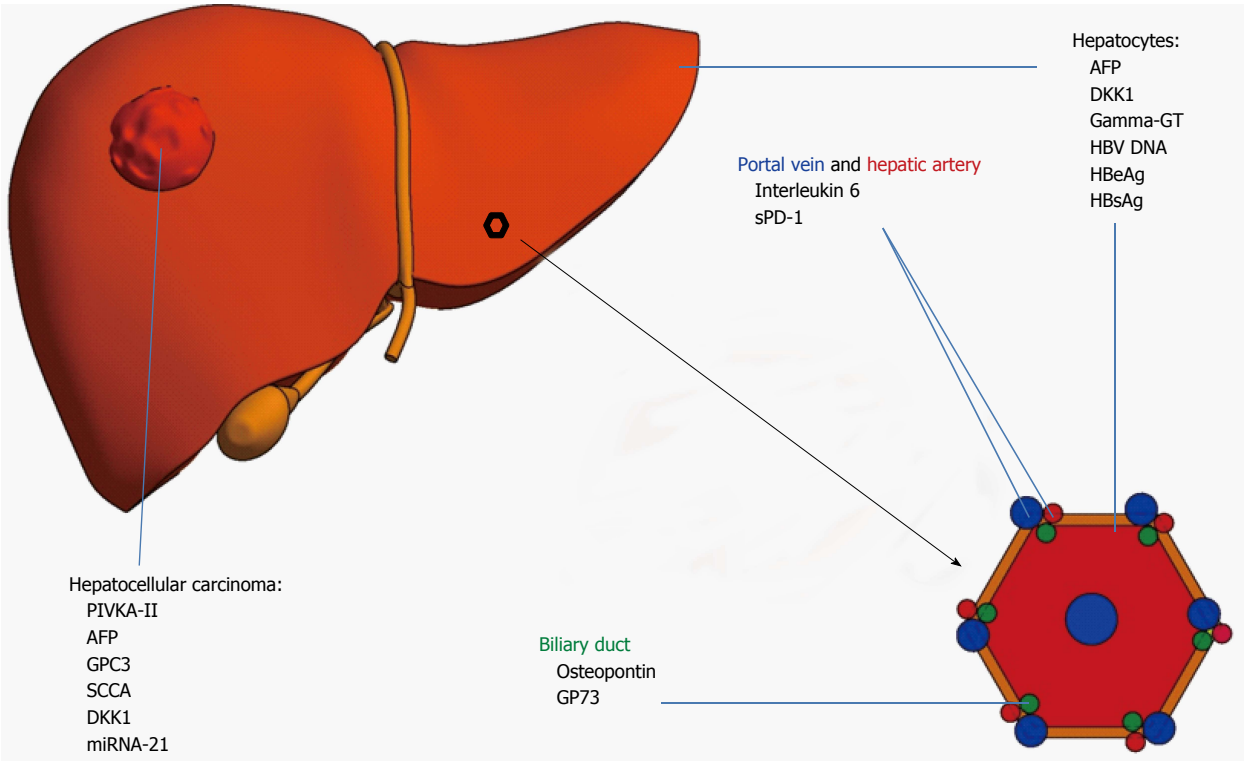

Figure 1 Cellular origin of the discussed predictive and diagnostic biomarkers in a physiological and oncological setting. Predictive biomarkers are displayed in italics. PIVKA-II: Protein induced by vitamin K absence; AFP: Alpha-fetoprotein; GPC3: Glypican-3; SCCA: Squamous cell carcinoma antigen; DKK1: Dickkopf-1 protein; miRNA: MicroRNA; COMP: Cartilage oligomeric matrix protein; GP73: Glycoprotein-73; sPD-1: Soluble programmed death-1; Gamma-GT: Gamma-glutamyltransferase.

Table 1 Diagnostic serum biomarkers for hepatitis B virus-associated hepatocellular carcinoma

Marker	Cut-off[1]	Sensitivity	Specificity	Detection method (most reliable)	Ref.
AFP	7.7-112.0 ng/mL	25%-90%	87%-97%	ELISA	[36,73]
AFP-l3	3%-20%	36%-96%	89%-94%	Liquid-Phase Binding Assay	[46,47,52]
DCP	40-150 mAU/mL	44%-91%	68%-99%	Electrochemiluminescence immunoassay	[36,53]
Osteopontin	9.3-642.5 ng/mL	73%-97%	55%-100%	ELISA	[65]
GP73	78-150 ng/mL	68%-95%	9%-97%	Immunoblotting, Western Blotting or ELISA[2]	[36,71,72]
GPC-3	2-300 ng/mL	36%-100%	40%-100%	ELISA	[36,78]
SCCA	0.12-3.80 ng/mL	42%-80%	50%-88%	ELISA	[36,81]
DKK1	1.01-2.15 ng/mL	69%-91%	62%-91%	ELISA	[86-88]
miRNA-21	NA	84%-90%	71%-92%	qRT-PCR	[83]
miRNA-122	NA	70%-82%	69%-84%	qRT-PCR	[83]

[1]Range of cut-off values used in different studies included in the discussed meta-analyses and systematic reviews; [2]Equally reliable. Relative levels were used with different internal standards to measure miRNA-concentrations. The choice of the most reliable detection method was based on recent studies involving at least 100 patients. Only biomarkers of which at least 4 different studies discussing their diagnostic potential exist, were included. AFP: Alpha-fetoprotein; DCP: Des-gamma carboxy prothrombin; GP73: Golgi-protein 73; GPC-3: Glypican-3; SCCA: Squamous cell carcinoma antigen; DKK1: Dickkopf-1; miRNA: MicroRNA; ELISA: Enzyme-linked immunosorbent assay; qRT-PCR: Quantitative reverse transcriptase polymerase chain reaction; NA: Not applicable.

AFP-l3: The rising star under the AFP glycoforms

AFP is a glycoprotein of which three glycoforms exist: AFP-l1, AFP-l2 and AFP-l3. They are all characterized by an increased binding affinity for *Lens culinaris* agglutinin. AFP-l3, which shows the highest binding affinity is of particular interest as a biomarker for hepatocellular carcinoma. This glycoform is secreted by malignant HCC cells even at early tumor stages and in the absence of elevated AFP levels and can be detected using liquid-phase binding assays[46,47]. In addition, the fraction of AFP-l3 to total AFP in the serum correlates with the degree of malignancy[48].

Over 15 studies have addressed the clinical potential of AFP-l3 so far with sensitivity and specificity ranging from 21% to 84% and from 89% to 94% respectively[48-52]. However these studies assessing the clinical potential of AFP-l3 use different cut-off levels, test methods and patient numbers, resulting in a wide range of detected sensitivity. A study from 2009 measuring the fraction of AFP-l3 to total AFP using an automated immunologic analyzer and a cutoff of 10% AFP-l3 in 419 HCC patients and 417 cirrhotic controls, found a sensitivity of 42% to detect HCC[53]. AFP-l3 fractions were measured using Western blotting in another study, involving 388 HCC patients and 212 controls with a cutoff of 15% AFP-l3 to total AFP, resulting in a sensitivity of 21%[54]. In order to unequivocally demonstrate the superiority of AFP-l3 to AFP, large cohort studies using the same cutoff and detection method are needed. Recently AFP-l3 was suggested to be especially useful in the diagnosis of HCC in absence of elevated AFP levels, but further validation is needed[55].

Des-gamma carboxy prothrombin

Des-gamma carboxy prothrombin (DCP) is a non-carboxylated form of prothrombin, also known as protein induced by vitamin K absence (PIVKA-II). Carboxylation takes place in the hepatocytes before the protein is released into the circulation. Release of the non-carboxylated form has been associated with vitamin K deficiency and presence of HCC[56]. Elevated DCP levels, preferably measured using electrochemoluminiscence assays, were found in sera of HCC patients, suggesting proper DCP synthesis in hepatoma cells[36,57,58]. DCP has been investigated as a potential HCC diagnostic biomarker in several studies, showing a comparable to slightly higher diagnostic performance compared to AFP[59-63].

Osteopontin

Osteopontin (OPN) is a glycoprotein that constitutes a major part of the extracellular matrix of bones and teeth. In addition, low levels of the protein are being secreted by biliary epithelial cells. OPN is involved in developmental as well as immunological, tumorigenic and bone homeostatic processes[64]. Overexpression of the protein, detected using ELISA assays, was found in a wide range of tumor types including pancreas cancer, multiple myeloma and HCC[64-66]. Seven retrospective cohort studies have investigated the diagnostic potential of OPN for HCC. So far, OPN does not outperform AFP as a diagnostic marker[65].

Golgi protein-73

Golgi protein-73 (GP73) is a transmembrane protein physiologically located on the Golgi membrane of epithelial cells in different tissues, including the biliary tract[67]. Its function remains largely unknown. Liver damage, caused by viral as well as non-viral agents leads to GP73 upregulation[68,69]. Increasing GP73 serum levels are associated with advanced fibrosis stages in HBV patients[70,71]. A recent meta-analysis showed that GP73's diagnostic accuracy for HCC outperforms that of AFP[72,73]. The protein can be detected using either ELISA assays, immunoblotting or Western blot. Previous studies have shown a comparable efficacy for all three methods[36,71].

Glypican-3

Glypican-3 (GPC3) is a member of the heparan sulfate proteoglycans. It is an oncofetal antigen involved in embryonal morphogenesis[74]. Significant expression in human adults can occur in different tissues including breast and liver and indicates ongoing pathological, mostly carcinogenic processes[75,76]. GPC3 has been proposed as a novel serum marker for HCC[75]. The protein promotes HCC tumor growth through stimulation of the Wnt signaling pathway[77]. A recent meta-analysis showed an acceptable accuracy of the protein to detect HCC with a mean pooled sensitivity and specificity of 56% and 89% respectively[78]. The protein is preferably detected using ELISA assays[36].

Squamous cell carcinoma antigen

Squamous cell carcinoma antigen (SCCA) is a serine protease inhibitor, physiologically located in squamous epithelial cells. It is also expressed by neoplastic epithelial cells, *e.g.,* neoplastic liver cells in which it promotes tumor growth through inhibition of apoptosis[79,80]. Increased serum levels have been detected using ELISA assays in HCC patients[36]. The protein's diagnostic accuracy for HCC has been investigated in over 12 studies and turned out to be moderate with a pooled sensitivity and specificity of 59.0% and 76.0% respectively. Nevertheless some design limitations of these studies such as a small sample size need to be taken into account[81].

Dickkopf-1 protein

Dickkopf-1 protein (DKK1) is a glycoprotein secreted by human hepatocytes. Upregulation of DKK1 expression takes place in a wide variety of cancers including prostate cancer, multiple myeloma and hepatocellular carcinoma[82-84]. Overexpression of the protein is detected in tissue as well as serum from hepatocellular carcinoma patients. Although the protein is suggested to be an inhibitor of the Wnt/β-catenin signaling pathway, its exact functions have not been fully elucidated[82,85]. A meta-analysis showed an acceptable diagnostic accuracy of DKK1, comparable to AFP, to detect HCC with a pooled sensitivity and specificity of 65% and 94% respectively[86-88]. Detection of the protein in serum is performed using ELISA assays[87].

miRNA's: Promising biomarkers for HCC detection

MicroRNA's (miRNA) are small non-coding RNA's regulating gene expression by binding to messenger-

RNA (mRNA)[89]. During recent years, circulating miRNAs have gained increasing attention for the early diagnosis and screening of hepatocellular carcinoma[90]. So far, two miRNAs, miRNA-21 and miRNA-122 show particularly high potential in HCC diagnostics[91]. miRNA-122 is a liver specific miRNA, whereas miRNA-21 is produced by different tissues including the colon, liver and heart in which it is involved in respectively tumor growth and cardiac disease development[92-94]. miRNA-21 inhibits tumor suppression by inhibiting tumor suppressor pathway activating phosphatases (*e.g.,* ATK and MAPK), whereas miRNA-122 inhibits tumor growth by acting as a tumor suppressor gene[93,95-97]. A direct correlation was observed between increasing miRNA-21 levels and increased cell proliferation[98]. In addition, high circulating miRNA-21 levels were found to be correlated with more differentiated and progressive hepatocellular carcinoma thus indicating a bad prognosis[94]. Serum miRNA-122 levels correlate inversely with the severity of liver fibrosis[99]. The antitumor properties of miRNA-122 have been successfully applied in a preclinical model to prevent HCC development[96]. The diagnostic accuracy of miRNA-21 slightly outperforms that of miRNA-122 with a pooled sensitivity and specificity of 87% and 80% respectively for miRNA-21 *vs* 68% and 73% for miRNA-122[91].

Other diagnostic biomarkers

Based on systems biology approaches, more markers with diagnostic potential in HCC screening settings have recently been identified, including fucosylated fetuin A, inter-alpha-trypsin inhibitor H4, clusterin, endoglin, soluble Axl, latent TGF-bèta binding-protein 2 as well as peroxireduxin 1, 2 and 3. The evidence for clinical utility of these markers remains low due to a lack of sufficiently large cohort studies[100-108].

In addition, several studies have been published on circulating tumor cells for HCC. However, most of published studies focus on prognosis after HCC diagnosis and prediction of disease progression rather than on the diagnosis of HCC[109-111].

PREDICTION OF THE RISK TO DEVELOP HCC

Three strategies can be applied when assessing the long-term risk for HCC. Firstly, clinical risk scores, *e.g.,* REACH-B and PAGE-B, can be calculated based on viral and host-related (*e.g.,* age and gender) clinical parameters. Most of these models have however been developed in Asian populations and lack validation in non-Asian populations[112,113]. HBeAg positivity and HBV DNA levels above 1 million copies/mL are associated with a 4- and 11-fold increased HCC-risk during 8 and 11 years of follow-up respectively[114-116]. Hepatitis B surface antigen (HBsAg) levels above 1000 IU/mL are accompanied with an up to 6.5-fold increased HCC risk in men and an up to 11-fold increased risk in women within 15 years[117]. HBsAg levels are suggested to be especially useful in case of low HBV DNA levels[118,119].

Secondly, genome-wide association studies have enabled the linkage of genetic variants to specific disease outcomes. Single nucleotide polymorphisms (SNPs) in a wide range of genes, including the Interleukin-21 and the CRP-gene, have been associated with an increased susceptibility for HCC over a variable time course[120-124]. Increasing evidence indicates that SNP's in the STAT4, MDM2 and HFE gene, determined on whole blood, are germline risk factors for HCC[125,126]. On the other hand, also somatically acquired mutations, *e.g.,* in the TP53 gene, have been associated with an increased risk for HCC[127]. All together these findings are strongly suggestive for interindividual differences in the genetic predisposition for HCC development, a predisposition that can be boosted by additional somatic mutations.

Thirdly, circulating biomolecules would be ideal as a non-invasive, predictive biomarker for HCC. An overview on the discussed predictive biomarkers including their respective increase in HCC risk is displayed in Table 2. The clinical utility of Gamma-Glutamyl Transferase (Gamma-GT) Iso-enzyme II was first evaluated as a predictive HCC marker in 1992. Patients showing persistently elevated levels of Gamma-GT Iso-enzyme II at presentation had a 86.7% risk to develop HCC within 10 years' time[128]. Gamma-GT levels above 41 U/L and AFP-levels > 5 ng/mL have later been associated with an 8-fold increased risk for HCC in a large HBV cohort followed up for 6 years[129]. The usefulness of AFP-levels for HCC prediction has, however, been assessed in several other studies with contradictory results[128-130].

Cartilage oligomeric matrix protein (COMP) is an extracellular matrix protein involved in tissue genesis and remodeling[131]. The protein is released into the circulation upon cartilage damage[132]. Overexpression of the protein in serum from HCC patients suggested that serum COMP levels reflected an individual's fibrosis stage and subsequent risk for HCC[133]. A recent study in Greece supports this hypothesis: COMP positivity (> 15 U/L) was associated with a 3-fold increased HCC risk during a median follow-up of 8 years[132,134].

In a study of 27 serum cytokines and growth factors, interleukin-6 (IL-6) levels were found to predict cancer development within a timeframe of 8 to 11 years with moderate accuracy[135]. Levels above 7 pg/mL were associated with a 3-fold increased HCC risk. As IL-6 induces C-reactive protein (CRP), the potential value of CRP in HCC risk prediction was assessed, but turned out to be disappointing[136,137]. Recently, soluble programmed death-1 (sPD-1), a soluble form of the membrane-bound programmed death 1 on T cells with a largely unknown function was put forward as a marker[138,139]. sPD-1 levels above 637.6 pg/mL at baseline reflected a 2-fold increased risk to develop HCC during a median follow-up time of 20 years, when

Table 2 Predictive serum biomarkers for hepatitis B virus-associated hepatocellular carcinoma

	Marker	Cut-off	Increased risk for HCC	Control group[1]	Follow-up time	Ref.
Viral	HBeAg	Positive	4-fold	HBeAg negative HBV patients	8 yr	[114]
	HBV DNA	> 1 million copies/mL	11-fold	HBV patients with HBV DNA < 300 copies/mL	11 yr	[115]
	HBsAg	> 1000 IU/mL	3-fold	HBV patients with HBsAg 5-9 IU/mL	14.7 yr	[117]
Host	Gamma-GT Iso-enzyme II	Positive	86-fold	GGT Iso-enzyme II negative HBV patients	10 yr	[128]
	Gamma-GT	> 41 U/L	8-fold	HBV patients with Gamma-GT ⩾ 41 U/L	5.9 yr	[129]
	AFP	> 5 ng/mL	8-fold	HBV patients with AFP ⩽ 5 ng/mL	5.9 yr	[129]
	COMP	Positive	3-fold	COMP negative HBV and HCV patients	8 yr	[134]
	IL-6	> 7 pg/mL	3-fold	HBV patients with IL-6 < 7 pg/mL	7.25 yr	[135]
	sPD-1	> 637.6 pg/mL	2-fold	HBV patients with sPD-1 < 117.3 pg/mL	20 yr	[138]

[1]Control group: group included in the study, to which the increased HCC risk was calculated. HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; Gamma-GT: Gamma glutamyltransferase; AFP: Alpha-fetoprotein; COMP: Cartilage oligomeric matrix protein; IL-6: Interleukin 6; sPD-1: Soluble programmed death 1.

compared to sPD-1 levels below 117.3 pg/mL[138].

DISCUSSION

Despite its disappointing sensitivity and specificity, AFP still remains the most widely used serum HCC biomarker. Some newly discovered circulating biomarkers, *e.g.,* AFP-l3, DCP and microRNA's show promising potential for implementation in clinical practice. However, only GP73 strongly outperforms AFP in terms of diagnostic accuracy. Large variations in sensitivity and specificity are noticed between different studies assessing the same biomarker (Table 1).

Due to the heterogeneity of HCC, one single biomarker with 100% sensitivity and specificity in all HCC cases will be hard to find. A more rational approach to increase the diagnostic accuracy might be the combination of different biomarkers[41,52,55,62,65,72,140-142]. The most recent APASL guidelines indeed recommend the combined use of AFP, AFP-l3 and DCP in HCC screening[22,142]. In favor of this approach, a meta-analysis showed that combined testing of GP73 and AFP increased the pooled sensitivity without decreasing the specificity to detect hepatocellular carcinoma. The pooled sensitivity and specificity were 87% and 85% respectively when biomarkers were combined, compared to 77% and 91% for GP73 and 62% and 84% for AFP when used alone[72].

All currently identified circulating biomarkers and their combinations definitely need more validation studies. Most of the biomarker discovery studies have been performed in cohorts of a few 100 patients. The majority of identified markers has so far not been subject of large, external validation studies[100-103,108]. Five subsequent steps are to be followed in cancer biomarker discovery. The first step is the implementation of preclinical exploratory studies. Step 2 is the development of a clinical assay. Step 3 involves retrospective studies, step 4 prospective studies and step 5 large, randomized controlled trials. Biomarkers identified in step 1 must pass all other steps before they can be termed validated biomarkers[143]. So far, only AFP has reached step 5[15].

In addition, the studies that have been conducted over the last decades are hampered by limitations in their study design. As an example the patient cohorts for HCC biomarker discovery studies are often heterogeneous regarding liver disease etiology and ethnicity. In their paper, da Costa *et al*[144] proved the need to validate biomarkers in different ethnic populations. They investigated the potential of osteopontin and latent Transforming Growth Factor beta binding-protein in HCC diagnosis in separate cohorts in Gambia, Korea, Thailand and France. The sensitivity and specificity of both markers differed (> 10%) among ethnicities. The onset of HCC occurs at a median age of 45 in sub-Saharan African people, whereas a mean age of 52 to 65 has been observed in the rest of the world[145,146]. In addition, HCC incidence varies among HBV and hepatitis C virus (HCV) patients, highlighting the importance of homogeneous patient cohorts. Future biomarker discovery and validation studies should therefore distinguish between different ethnicities and etiologies as this most probably explains the variation in sensitivities and specificities noticed between studies assessing the same biomarker (Table 1).

The inclusion of clinical parameters into biomarker scores could increase their performance. One study demonstrated that incorporation of age into combined models of biomarker testing significantly improved the diagnostic performance for HCC[140].

From a clinical point of view, however, predictive serum biomarkers would be preferred over diagnostic biomarkers to tailor HCC surveillance according to the individual needs. Proteomic approaches are encouraging, but also need a validation in larger cohorts[147,148]. Expression of Heat-shock Protein 27 was *e.g.,* detected in 90% of sera from HCC patients and in 0% of sera from non-HCC patients, which seems promising[148]. Other groups have focused on genomics and have identified a gene signature in

liver tissue of HCV infected patients predictive of HCC development[149,150]. It could be of interest to identify corresponding secretory biomarkers in blood.

CONCLUSION

Monitoring HCC development in chronic hepatitis B patients based on serum biomarkers remains challenging. During recent years, new predictive and diagnostic circulating biomarkers have been proposed. Combinations of these biomarkers show a higher potential for implementation in clinical practice, but large validation studies in homogeneous ethnic and etiological populations are urgently needed to unequivocally demonstrate their clinical utility.

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