Accuracy of novel diagnostic biomarkers for hepatocellular carcinoma: An update for clinicians (Review)

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Abstract. Hepatocellular carcinoma (HCC) is the most common liver malignancy and a leading cause of cancer-related mortality worldwide. Accurate detection and differential diagnosis of early HCC can significantly improve patient survival. Currently, detection of HCC in clinical practice is performed by diagnostic imaging techniques and determination of serum biomarkers, most notably α-fetoprotein (AFP), fucosylated AFP and des-γ-carboxyprothrombin. However, these methods display limitations in sensitivity and specificity, especially with respect to early stages of HCC. Recently, high-throughput technologies have elucidated many new pathways involved in hepatocarcinogenesis and have led to the discovery of a plethora of novel, non-invasive serum biomarkers. In particular, the combination of AFP with these new candidate molecules has yielded promising results. In this review, we aimed at recapitulating the most recent (2013-2015) developments in HCC biomarker research. We compared promising novel diagnostic serum protein biomarkers, such as annexin A2, the soluble form of the receptor tyrosine kinase Axl and thioredoxin, as well as their combinations with AFP. High diagnostic performance (area under the curve >0.75) as shown by threshold-independent receiver operating characteristic curve analysis was a prerequisite for inclusion in this review. In addition, we discuss the role and potential of microRNAs in HCC diagnosis and associated methodological challenges.

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Abbreviations: AASLD, American Association for the Study of Liver Disease; AFP, α-fetoprotein; AFP-L3, fucosylated AFP; ALCAM, activated leukocyte cell adhesion molecule; AUC, area under the curve; BCLC, Barcelona Clinic Liver Cancer; CLD, chronic liver disease; ConA-pCD, concanavalin A binding pro-cathepsin D; DCP, des-γ-carboxyprothrombin; EASL, European Association for the Study of the Liver; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial to mesenchymal transition; FDA, Food and Drug Administration; GDF, growth and differentiation factor; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LC, liver cirrhosis; miRNA, microRNA; LTBP, latent TGF-β binding protein; MCM6, minichromosome maintenance complex component 6; MCP-1, monocyte chemoattractant protein-1; miR, microRNA; MMP, matrix metalloproteinase; NAFLD, non-alcoholic fatty liver disease; PON1, paraoxonase 1; PRDX3, peroxiredoxin 3; qMSP, quantitative methylation-specific PCR; qRT-PCR, quantitative reverse-transcription-polymerase chain reaction; RFA, radiofrequency ablation; ROC, receiver operating characteristic; sAxl, soluble Axl; TCF-4, T-cell factor-4; TGF, transforming growth factor; Trx1, thioredoxin; YB-1, Y-box binding protein 1

Key words: diagnostic biomarker, hepatocellular carcinoma, α-fetoprotein, des-γ-carboxyprothrombin, annexin A2, soluble Axl, thioredoxin, minichromosome maintenance

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1. Current diagnosis of hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the most commonly diagnosed liver malignancy and the third leading cause of cancer-related mortality worldwide with rising incidence in Western countries (1,2). In the past decades, HCC staging has been performed according to the Barcelona Clinic Liver Cancer (BCLC) criteria, which mainly rely on imaging techniques and define early and advanced-stage HCC with respect to tumor size and number of nodules (3). According to current guidelines of the European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Disease (AASLD), curative therapies are restricted to early-stage HCC patients only (4). These include liver resection or transplantation, as well as local radiofrequency ablation (RFA) and show a high 5-year survival rate of up to 70% (5). However, the majority of HCC

patients are diagnosed at later stages, restricting therapeutic options to palliative treatment only and leading to a median survival of <1 year (6,7). In this respect, transabdominal ultrasonography is currently the most commonly used tool for HCC detection and surveillance, primarily due to its cost-effectiveness. However, the sensitivity and specificity of ultrasound are highly dependent on operator experience as well as the patient's constitution, with obese patients representing a particular challenge. Furthermore, differential diagnosis of HCC vs. chronic cirrhosis of the liver is difficult and may not always be possible. All these factors contribute to the limited sensitivity of early HCC detection by ultrasonography, ranging from 32 to 65% (8,9). Thus, improved and more accurate detection of HCC at early stages, especially among high-risk groups, such as cirrhosis or hepatitis patients is highly desired.

The detection of biomarkers associated with HCC in body fluids or tissues is the most promising approach to improve diagnostic accuracy and to overcome the disadvantages of current diagnostic strategies. Especially non-invasive techniques relying on blood or serum samples would be beneficial for both patients and clinicians. In addition to ultrasonography, the determination of α-fetoprotein (AFP) levels in serum is the gold standard in HCC detection and has been widely used to complement HCC surveillance (5). However, due to its low diagnostic accuracy, with sensitivities ranging from a mere 18-60% and a specificity of ~85-90%, AFP has recently been excluded from current AASLD HCC surveillance guidelines (4,8,10-13). In line, it has been shown that 80% of small HCC nodules do not display increased AFP levels and that the sensitivity of AFP for tumors smaller than 3 cm is restricted to 25% (14,15). Consequently, several other biomarkers have been suggested to complement AFP and increase the accuracy of HCC detection, most notably des-γ-carboxyprothrombin (DCP), lectin-bound AFP (AFP-L3%), osteopontin (OPN), glypican 3 (GPC3) and Golgi protein-73 (GP73; Table I). However, reports concerning the performance of these markers are conflicting and a comprehensive meta-analysis has shown both DCP and AFP-L3% to be inferior to AFP (16). Furthermore, combination of AFP with these markers only moderately increased diagnostic performance compared to AFP alone (16). For GPC3, available studies were also recently reviewed in a meta-analysis showing lower diagnostic performance as compared to AFP (17). OPN was evaluated in comparison with AFP in early-stage HCC patients and cirrhotic as well as chronic hepatitis B (HBV)-positive controls. In this study, OPN showed almost identical diagnostic performance as AFP (18). With regard to GP73, studies including AFP have been performed which employed various assay methods and applied variable cut-off values (16). Hence, further research is needed to validate these markers and additional candidates that are continuously emerging.

In this review, we discuss novel biomarkers derived from protein, DNA methylation as well as microRNAs (miRs) and their potential in the differential diagnosis between chronic liver diseases (CLDs), such as chronic hepatitis B or C (HBV, HCV) infection or chronic hepatitis C (HCV) infection and liver cirrhosis (LC) vs. early-stage HCC. In particular, we focus on receiver operating characteristic (ROC) curves, generated by plotting sensitivity against the false-positive rate which represent a threshold-independent approach allowing the proper evaluation and comparison of different biomarkers (19). ROC curves and high corresponding areas under the curve (AUC) values (>0.75), indicating significant diagnostic accuracy, were considered as a requirement for inclusion in this review in combination with significant patient and control numbers (n>50).

Table I. Diagnostic value of current biomarkers for HCC compared to AFP.

Marker	Ref.	Comparison		AUC
DCP	(16)	Mixed (meta-analysis)	DCP	0.79
			AFP	0.83
			Combined	0.87
AFP-L3%	(16)	Mixed (meta-analysis)	AFP-L3	0.71
			AFP	0.83
			Combined	0.83
OPN	(18)	Early-stage HCC vs. LC and CHB	OPN	0.78
			AFP	0.78
			Combined	0.84
GPC3	(17)	Mixed (meta-analysis)	GPC3	0.76
			AFP	0.81
			Combined	0.85
GP73	(16)	Mixed (meta-analysis)	GP73	0.91
			AFP	0.83
			Combined	0.93

AFP, α-fetoprotein; AFP-L3%, lectin-bound AFP; AUC, area under the curve; CHB, chronic hepatitis B; DCP, des-γ-carboxyprothrombin; GP73, Golgi protein-73; GPC3, glypican 3; HCC, hepatocellular carcinoma; LC, liver cirrhosis; OPN, osteopontin.

2. Novel serum proteins as biomarkers for HCC

Annexin A2. Annexin A2 belongs to the calcium-dependent, phospholipid-binding protein family and is located on the surface of endothelial and most epithelial cells (20). It was found to be dysregulated in many cancers, such as colon, lung, gastric, esophageal and breast carcinomas (21-26). In this respect, annexin A2 has been associated with tumor cell proliferation, apoptosis, transcriptional regulation, invasion, metastasis and angiogenesis (27). During hepatocarcinogenesis, annexin A2 expression is upregulated in cirrhotic liver tissue and malignant hepatocytes (28). Serum concentrations of annexin A2 have been suggested as a biomarker for HCC in 2009, but reliable studies have been missing to date (29). One group recently re-evaluated the usefulness of annexin A2 in serum samples from 175 HCCs of all stages, 23 hepatitis, 51 cirrhosis, 19 benign liver tumor patients and 49 healthy controls by enzyme-linked immunosorbent assay (ELISA; Table II) (30). Annexin A2 showed an AUC of 0.79 when comparing HCCs to healthy controls. Discrimination between HCC and grouped cirrhosis as well as benign liver tumor patients achieved an AUC of 0.80. Unfortunately, sensitivity and specificity values were not stated for these comparisons. However, when limited

to early HCC patients (n=95) and cirrhotic controls (n=51), annexin A2 achieved an AUC of 0.80 with a sensitivity of 86.4% and a specificity of 73.5%. Of note, early HCCs were defined as grouped BCLC stage 0 and A, which limits clinical usefulness considering the important differences in the therapeutic options between BCLC 0 and A patients.

sAxl. Axl is a receptor tyrosine kinase and belongs to the TAM family, which is comprised of Axl, Tyro3 and Mer. Axl is expressed in many cell types, including epithelial, mesenchymal and hematopoietic cells and its biological effects depend on cell and tissue context. Overexpression of Axl has been detected in several tumor types and correlates with poor disease outcome (31,32). Binding of Axl by its ligand Gas6 activates a multitude of signaling pathways leading to enhanced proliferation, survival, invasion and metastasis. In HCC, Axl was shown to be upregulated and induced by Hippo/YAP signaling, enhancing invasion and lymphatic metastasis (33,34). We recently discovered that Axl is also involved in TGF-β-mediated HCC progression by modulating TGF-β signaling via activation of c-Jun N-terminal kinase and subsequent phosphorylation of Smad3 (35). Notably, Axl can be proteolytically processed, yielding an 80-kDa soluble protein (sAxl), which is secreted into the extracellular space and can be detected in blood (36). We therefore investigated the potential of sAxl as a non-invasive biomarker of HCC in a multi-center study involving patients from China and Europe (Table II) (37). We collected serum samples from 311 HCC patients of all stages, 30 cirrhotic and 125 healthy controls. Determination of sAxl levels by ELISA and subsequent ROC curve analysis yielded an AUC of 0.834 with a sensitivity of 78.1% and a specificity of 70.8% for all HCC vs. healthy controls. In differential diagnosis of HCC vs. liver cirrhosis, sAxl showed an AUC of 0.815 as well as sensitivities and specificities of 78 and 66.7%, respectively. Importantly, analysis of patients with very early HCC, defined as BCLC 0 (n=26) vs. cirrhotic controls gave an AUC of 0.838 with 80.8% sensitivity and 66.7% specificity. Furthermore, sAxl was able to distinguish AFP-negative HCC (<20 ng/ml, n=137) from liver cirrhosis with an AUC of 0.780, 73% sensitivity and 66.7% specificity. Of note, sAxl values were not increased in breast (n=10), ovarian (n=10), or colorectal (n=62) cancer patients with secondary hepatic malignancies.

Thioredoxin. Thioredoxin (Trx1) is a globular, oxidoreductase enzyme involved in the physiological defense against oxidative stress (38). In this context, Trx1 is capable of reducing disulfide bonds of other proteins in the cytoplasm. However, it can play a different role, depending on its subcellular localization. When localized in the extracellular matrix, Trx1 acts as a chemokine, whereas in the nucleus, it can interact with many transcription factors, thereby regulating gene expression (39,40). The role of Trx1 in cancerogenesis is not yet fully understood, but it was shown to be upregulated in several tumor types. High Trx1 expression can stimulate cancer cell proliferation and angiogenesis through induction of HIF-1α, which in turn increases VEGF-A expression levels (41). Furthermore, upregulation of Trx1 in cancer cells has also been attributed to increased oxidative stress and may even exert a tumor-suppressive function (42). Overexpression of Trx1 has been observed in HCC tissue samples and its levels correlate with tumor cell proliferation and metastasis (43,44). A possible role of Trx1 as a diagnostic serum marker for HCC was recently investigated by ELISA in a training cohort, comprising 180 HCCs of all stages, 120 cirrhosis, 120 CLD patients and 100 healthy controls (Table II) (45). ROC curve analysis of all HCC patients vs. all controls yielded an AUC of 0.946 with 84.3% sensitivity and 91.8% specificity. When the analysis was limited to all HCC vs. pooled LC and CLD patients, the resulting AUC was 0.901 with 78.2% sensitivity and 87.5% specificity. Importantly, the authors also performed an evaluation of very early HCCs (n=38) against LC and CLD patients, resulting in an AUC of 0.844 with a sensitivity of 74.5% and a specificity of 86.7%. The diagnostic performance was also assessed in a validation cohort of similar size, which showed very comparable results. Of note, very early HCCs were defined as well differentiated and smaller than 2 cm. This does not fully comply with BCLC staging criteria, which do not take the histological grade into consideration, but rather includes the number of tumors nodules, vascular invasion and liver function (3).

CD147. CD147, also known as extracellular MMP inducer (EMMPRIN) or Basigin, is a member of the immunoglobulin superfamily and is thought to be involved in several cellular processes, such as intercellular recognition and spermatogenesis (46). CD147 is frequently overexpressed in several cancers, including HCC (47). Importantly, it is capable of inducing several matrix metalloproteinases (MMPs) and induction of MMP-2 and MMP-9 by CD147 can promote invasion and metastasis in HCC (48). Furthermore, membrane-bound CD147 was shown to interact with integrins, thereby modulating migration, invasion, colony formation and MMP secretion of HCC cells (49). Serum levels of soluble CD147 were determined in 62 HCC patients of all stages and 25 healthy controls by ELISA (Table II) (50). CD147 exhibited an AUC of 0.857 with a sensitivity of 83.9% and a specificity of 76.0%. Notably, comparison of very early HCC (BCLC 0; n=12) and healthy controls still gave an AUC of 0.85 with a sensitivity of 83.3%.

CD166. CD166, also known as activated leukocyte cell adhesion molecule (ALCAM), is a transmembrane glycoprotein and belongs to the immunoglobulin superfamily, which was first described as a CD6 ligand on leukocytes (51). It is expressed in many cell types, particularly in immune and epithelial cells, as well as in hematopoietic or mesenchymal stem cells. Aberrant CD166 levels have been observed in colorectal, as well as breast and small cell lung cancer (52). In HCC, CD166 is induced by PI3K signaling and mediates anti-apoptotic effects (53). CD166 concentrations were assessed in sera of 51 HCC patients of unknown stage and 85 healthy controls by ELISA (Table II) (54). No further clinicopathological data were provided. CD166 showed an AUC of 0.986 with 100% sensitivity and 89.41% specificity. Of note, the authors also determined serum CD166 levels in HBV (n=48), HCV (n=40), cirrhosis (n=41), gastric (n=21), breast (n=25) and lung (n=21) cancer patients and found it exclusively elevated levels in HCC patients. Since no ROC curve analysis was performed, these findings require re-evaluation.

Table II. Diagnostic value of novel biomarkers for HCC.

Marker	Ref.	Method	No. of patients	No. of controls	Comparison	Sensitivity (%)	Specificity (%)	AUC
Proteins								
Annexin A2	(30)	ELISA	175	70	HCC vs. LC and BLT	ND	ND	0.80
					Early HCC vs. LC	86.4	73.5	0.80
sAxl	(37)	ELISA	Multicenter study					
			Total, 311	155	HCC vs. HC	78.1	70.8	0.83
					HCC vs. LC	78.0	66.7	0.82
					Very early HCC vs. LC	80.8	66.7	0.84
					AFP-negative HCC vs. LC	73.0	66.7	0.78
					Very early HCC vs. HC	83.3	ND	0.85
Thioredoxin	(45)	ELISA	180	340	HCC vs. LC, CLD and HC	84.3	91.8	0.95
					All HCC vs. LC and CLD	78.2	87.5	0.90
					Very early HCC vs. LC and CLD	74.5	86.7	0.84
CD147	(50)	ELISA	62	25	HCC vs. HC	83.9	76.0	0.86
CD166	(54)	ELISA	51	85	HCC vs. HC	100.0	89.4	0.99
EGF	(58)	ELISA	30	20	HCC vs. HCV	63.3	87.5	0.80
GDF15	(63)	ELISA	223	391	HCC vs. LC, HBV, HVC, HC	86.8	72.8	0.84
hCE1	(65)	ELISA	57	27	HCC vs. LC	89.2	77.7	0.92
Ku86 antibody	(70)	ELISA	97	60	HCC vs. HBV and HC	80.5	70.6	0.79
LTBP-2	(76)	ELISA	Multicenter study					
			Thailand, 58	107	HCC vs. CLD and HC	ND	ND	0.94
			Gambia, 50	100	HCC vs. CLD and HC	ND	ND	0.87
			France, 75	150	HCC vs. CLD and HC	ND	ND	0.74
MCM6	(83)	ELISA	61	59	HCC vs. LC	67.2	86.2	0.81
					Small HCC vs. LC	71.4	86.2	0.83
MCP-1	(90)	ELISA	120	110	HCC vs. HBV	73.1	80.9	0.82
Peroxiredoxin 3	(94)	ELISA	98	199	HCC vs. HC	85.9	75.3	0.87
					HCC vs. LC	73.2	69.0	0.72
Talin-1	(99)	ELISA	40	80	HCC vs. HC	100.0	100.0	1.0
					HCC vs. LC	100.0	87.0	0.90
YB-1	(103)	CLIA	105	100	HCC vs. HBV, LC and HC	74.1	63.0	0.76
ConA-pCD	(106)	M-LAC	35	55	HCC vs. LC and HC	85.0	80.0	0.88
					HCC vs. LC	83.0	64.0	0.70
FucPON1	(110)	AAL-ELISA	90	90	HCC vs. LC	80.0	64.4	0.80
DNA methylation								
HOXA9	(115)	qMSP	40	34	HCC vs. HC	73.30	97.10	0.84
INK4A	(118)	Pyro-sequencing	66	43	HCC vs. CLD	65.30	87.20	0.82
microRNAs								
miR-139	(126)	qRT-PCR	31	31	HCC vs. HBV hepatitis	58.61	80.60	0.76
miR-182	(132)	qRT-PCR	103	135	HCC vs. LC, hepatitis, NAFLD	78.64	91.58	0.91
miR-331-3p	(132)	qRT-PCR	103	135	HCC vs. LC, hepatitis, NAFLD	79.61	86.32	0.89
miR-199a-3p	(150)	qRT-PCR	78	156	HCC vs. HC	71.80	86.10	0.88

AAL, *Aleuria aurantia* lectin; AUC, area under the curve; BLT, benign liver tumor; hCE1, human carboxyesterase 1; CLD, chronic liver disease; CLIA, chemiluminescence immunoassay; ConA-pCD, concanavalin A binding procathepsin D; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; GDF15, growth and differentiation factor 15; HBV, hepatitis B virus; HC, healthy controls; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LC, liver cirrhosis; LTBP-2, latent TGF-β binding protein-2; MCM6, minichromosome maintenance complex component 6; MCP-1, monocyte chemoattractant protein-1; M-LAC, multi-lectin affinity chromatography; NAFLD, non-alcoholic fatty liver disease; ND, not determined; PON1, paraoxonase 1; qMSP, quantitative methylation-specific PCR; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; YB-1, Y-box binding protein 1.

EGF. Epidermal growth factor (EGF) signaling is involved in the tumorigenesis of many cancers, including HCC. Alterations in serum EGF levels have been reported, with decreases in non-small cell lung and head and neck carcinomas (55). Increased EGF concentrations were observed in sera from pancreatic and thyroid cancer patients (56,57). Its receptor, EGFR is considered as a major regulator of hepatocarcinogenesis by integrating several proliferation and survival signals. Evaluation of EGF concentrations was performed in sera of 20 HCV-positive controls as well as 30 HCC patients by ELISA (Table II) (58). A sensitivity of 63.3%, a specificity of 87.5% and a corresponding AUC of 0.80 were observed. Notably, EGF concentrations were not significantly increased in early HCC patients and no corresponding ROC curve analysis was performed.

GDF15. Growth differentiation factor 15 (GDF15), also known as macrophage inhibitory cytokine-1 belongs to the transforming growth factor (TGF)-β superfamily (59). Under physiological conditions, it is exclusively expressed in placental tissue but can be induced in activated macrophages by pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-6 (60). In this context, GDF15 may act in a negative autocrine feedback loop, since it suppresses the production of pro-inflammatory cytokines and inhibits the proliferation of blood mononuclear cells (61). Like TGF-β1, GDF15 was shown to play contradictory roles in cancer development, inhibiting early cancerogenesis but promoting tumor progression at later stages (62). Its tumor-promoting effects, such as enhanced migration, angiogenesis or immunosuppression were demonstrated in malignant glioma, myeloma, glioblastoma, melanoma, prostate, gastric and breast cancer (61). In HCC, GDF15 was shown to be upregulated upon HCV infection and was recently investigated concerning its potential as a serum biomarker (Table II) (63). To this end, serum concentrations in 223 HCCs of all stages, 88 liver cirrhosis patients, 51 HBV carriers, 50 HCV carriers and 202 healthy controls were assessed by ELISA. Discrimination of HCC patients from all other conditions (LC, HBV, HCV and healthy controls) achieved an AUC of 0.843 with 86.8% sensitivity and 72.7% specificity. It has to be noted that GDF15 levels were equally elevated in cirrhosis patients and in HCC, as compared to healthy controls. Thus, the diagnostic power for HCC vs. cirrhosis is anticipated to be low, limiting the usefulness of GDF15 for differential diagnosis of high-risk populations.

hCE-1. Human carboxylesterase 1 (hCE-1) is a serine esterase expressed in many tissues. It plays a role in the metabolism of xenobiotic compounds in the liver (64). A recent study explored its diagnostic performance by ELISA in 57 HCC patients of all stages and in 27 cirrhotic controls (Table II) (65). The analysis yielded a sensitivity of 89.2%, a specificity of 77.7% and a corresponding AUC of 0.918. Of note, 37 chronic hepatitis patients were also enrolled in the study but were excluded from this review due to the missing ROC curve analysis. Furthermore, HCC patients were grouped into early and advanced HCC and respective sensitivities and specificities were given. Again, without a threshold-independent approach (i.e., ROC curve analysis), a valid assessment of diagnostic performance was impossible.

Ku86 antibody. Ku86 (also known as Ku80), an ATP-dependent DNA helicase II, is part of the DNA-dependent protein kinase, which plays a key role in DNA repair via non-homologous end joining. More specifically, Ku86 dimerizes with Ku70 and binds to the ends of double-strand breaks, thereby acting as a scaffold to facilitate alignment and DNA repair by polymerases, nucleases and ligases (66). Interruption of these functions has been associated with chromosomal aberrations and tumor development in mice (67). In humans, development and progression of several gastrointestinal tumors, including liver cancer, have been linked to reduced Ku86 expression (68,69). One recent study evaluated the occurrence of auto-antibodies against Ku86 by ELISA in 97 patients with HBV-related liver cancer, as well as 30 randomly chosen patients with HBV-related liver cirrhosis and 30 healthy subjects as controls (Table II) (70). The comparison of HCC and grouped healthy as well as cirrhotic controls yielded an AUC of 0.794 with sensitivity and specificity of 80.5 and 70.6%, respectively.

LTBP-2. Latent TGF-β binding protein (LTBP)-2 belongs to the fibrillin family of extracellular matrix proteins. Unlike other LTBPs, it lacks a specific motif required for complex-formation with TGF-β1 and its involvement in modulation of TGF-β signaling remains unclear (71,72). LTBP-2 was shown to be dysregulated in esophageal carcinoma, pancreatic ductal carcinoma and melanoma. In this respect, it affects cell adhesion and overexpression of LTBP-2 impairs colony formation *in vitro*. Paradoxically, high LTBP-2 levels correlate with poor patient outcome (73-75). It was recently suggested as a biomarker for HCC following mass spectrometric profiling (Table II) (76). LTBP-2 levels were subsequently determined in 183 HCC, 274 CLD (HBV- or HCV-positive) and 227 healthy controls from centers in Thailand (52 controls, 49 CLD, 58 HCC), Gambia (50 controls, 50 CLD, 50 HCC) and France (75 controls, 75 CLD, 75 HCC) by ELISA. Results yielded AUCs of 0.94, 0.87 and 0.74 for Thailand, Gambia and France, respectively. No distinction was made between different stages of HCC. ROC analyses of LTBP-2 vs. CLD patients were only shown for HCC patients or CLD controls exhibiting <20 ng/ml AFP and the corresponding patient numbers were not indicated, rendering an evaluation impossible. However, as for healthy controls, a much lower AUC was obtained in samples from France (0.57) as compared to Thailand (0.98) or Gambia (0.85), even though CLD controls were selected from a similar etiological background (HBV- or HCV-positive). Therefore, the diagnostic usefulness of LTBP-2 as a biomarker of HCC remains unclear.

MCM6. Minichromosome maintenance complex component 6 (MCM6) is part of a multimeric protein complex involved in DNA synthesis and replication, specifically during initiation of S phase (77). Non-proliferating, somatic cells are devoid of MCM6 expression and upregulation of MCM family proteins has been associated with several cancers, including HCC (78-82) One group investigated the performance of MCM6 protein levels as a biomarker of HCC by ELISA in sera from 61 HCC patients, including 14 small HCC cases (tumor

size <2 cm), as well as 29 cirrhotic controls and 30 healthy controls (Table II) (83). In this respect, MCM6 showed an AUC of 0.81 when discriminating HCC from cirrhotic controls with a sensitivity of 67.2% and a specificity of 86.2%. Notably, MCM6 exhibited an AUC of 0.825 in small HCCs vs. cirrhosis patients with sensitivity and specificity of 71.4 and 86.2%, respectively. However, one drawback of this study is the missing classification of HCC patients into BCLC stages, which take, besides tumor size, additional clinicopathological parameters into account. Thus, a clear evaluation of MCM6 as a marker for very early HCC is an open issue.

MCP-1. Monocyte chemoattractant protein-1 (MCP-1) is a potent chemotactic factor for monocytes. It is secreted by a variety of cell types and can be induced by oxidative stress, growth factors or cytokines (84). MCP-1 is associated with enhanced tumor infiltration by tumor associated macrophages and enhanced angiogenesis in gastric and breast carcinomas as well as in meningioma (85-87). In HCC, MCP-1 expression correlates with disease progression (88). In particular, hepatic stellate cells and myofibroblasts, which play a crucial role in liver fibrosis and malignant transformation of parenchymal liver cells, secrete large amounts of MCP-1, thus promoting migration and invasion of hepatoma cells (89). One recent study discovered MCP-1 as a promising marker for HCC by determination of serum levels in 120 HCC patients of all stages and 110 HBV-carrying controls by ELISA (Table II) (90). ROC analysis yielded an AUC of 0.823 with a sensitivity and specificity of 73.1 and 80.9%, respectively. Notably, samples were collected from patients who had previously undergone liver resection and were therefore designated as resectable. However, most patients included in this study exhibited advanced BCLC stages, which do not allow curative treatment such as liver resection.

Peroxiredoxin 3. Peroxiredoxin 3 (PRDX3) is a member of the peroxiredoxin protein family, involved in peroxide detoxification (91). It was shown to be overexpressed in mesothelioma, breast cancer, ovarian cancer and liver cancer (92). PRDX3 has also been suggested as a biomarker for HCC progression (93). One group recently investigated PRDX3 as a diagnostic biomarker in the sera of 98 Chinese HCC patients of all stages, 96 cirrhosis patients and 103 healthy controls by ELISA (Table II) (94). ROC analysis of HCC vs. healthy controls gave an AUC of 0.865 with a sensitivity of 85.9% and a specificity of 75.3%. When HCCs were discriminated from liver cirrhosis patients, PRDX3 reached an AUC of 0.717 with 73.2% sensitivity and 69.0% specificity, limiting its potential for differential diagnosis among high-risk populations. Furthermore, different cut-off values for PRDX3 were applied for cirrhotic patients and healthy controls vs. HCC. In addition, AFP performance in cirrhosis vs. HCC patients was not analyzed, rendering a comparison impossible.

Talin-1. Talin-1 was first identified as a cytoplasmic binding partner of integrins, essential for cell adhesion and motility (95). In this respect, binding of Talin-1 to the cytoplasmic NPXY motif of β-integrin can lead to integrin activation and affect adhesion, spreading and motility (96). Talin expression has been linked to endometrial as well as prostate cancers (97,98). Its potential as a biomarker for HCC was recently assessed by ELISA in a study including sera of 40 HCC patients of all stages, 40 healthy as well as 40 cirrhotic controls (Table II) (99). Talin-1 exhibited 100% sensitivity and specificity and an AUC of 1.0 in differentiating HCCs from healthy controls. Compared against cirrhotic controls, Talin-1 showed 100% sensitivity, 87% specificity and an AUC of 0.90. The study also evaluated the performance of AFP in the same study population (sensitivity, 80% and specificity, 65%). However, combination of Talin-1 with AFP strongly affected AUC as well as specificity, reducing them to 0.79 and 57%, respectively. In addition, patient characteristics, such as BCLC stage, were not taken into account. Therefore, the suitability of Talin-1 as a tool for detection of early HCC remains uncertain.

YB-1. Y-box binding protein 1 (YB-1) is a pleiotropic transcription/translation factor, belonging to the highly conserved cold-shock domain protein superfamily (100). YB-1 is overexpressed in many cancer types (101). Even though it is predominantly localized in the nucleus, where it is involved in DNA excision repair, it can also be actively secreted in the presence of cytokines and upon oxidative stress (102). One recent study investigated the diagnostic performance of YB-1 in 105 HCC patients of all stages vs. a control cohort comprising 25 HBV, 25 cirrhotic and 50 healthy subjects (Table II) (103). Determination of YB-1 serum concentrations was performed using a newly developed sandwich-type chemiluminescence immunoassay. YB-1 displayed an AUC of 0.764, with a sensitivity of 74.1% and a specificity of 63.0%. Controls were only assessed in combination and no separate analyses of healthy, HBV-bearing or cirrhotic controls vs. HCCs were performed.

ConA-pCD. Alterations in N-linked glycosylation of several proteins have been reported in liver cancer patients (104,105). In this context, the most prominent member of N-linked glycoproteins, AFP-L3% has been intensely studied, ultimately resulting in its approval by the Food and Drug Administration (FDA) for HCC diagnosis in 2005. One recent study aimed at identifying additional N-linked glycoproteins in HCC tissue and serum samples by multilectin affinity chromatography (Table II) (106). This led to the discovery of concanavalin A (ConA) binding procathepsin D (ConA-pCD) as a candidate biomarker. Serum samples from 35 HCC patients as well as 29 cirrhotic and 26 non-cirrhotic controls were further analyzed by western blotting, followed by ConA affinity chromatography. ROC curve analysis of HCC vs. all controls resulted in an AUC of 0.88 with a sensitivity of 85% and a specificity of 80%. Analysis of HCC vs. cirrhotic controls yielded an AUC of 0.70 with sensitivity and specificity of 83 and 64%, respectively.

Fucosylated PON1 (Fuc-PON1). Paraoxonase 1 (PON1) is a calcium-dependent hydrolase, capable of hydrolyzing and thereby detoxifying organophosphorous compounds (107). It is mainly expressed in the liver and secreted into the bloodstream. PON1 polymorphisms have been linked to the development of several malignancies, such as breast, lung and ovarian cancers as well as multiple myeloma (108). In HCC, its expression level was first established as a biomarker for microvascular invasion (109). Notably, PON1 is highly

fucosylated in HCC tissues and its fucosylation status was recently investigated as a diagnostic biomarker by *Aleuria aurantia* lectin-dependent ELISA. Fuc-PON1 levels were determined in sera of 90 liver cirrhosis and 90 HCC patients, all HBV-positive (Table II) (110). ROC analysis yielded an AUC of 0.803 with a sensitivity of 80.0% and a specificity of 64.4%. Of note, the control group was also subdivided and diagnostic performance was separately assessed among AFP-positive and AFP-negative HCC. Here, Fuc-PON1 achieved a lower AUC in AFP-positive (>20 ng/ml) patients (0.788).

3. DNA methylation for HCC diagnosis

HOXA9. Homeobox protein HOXA9 is a member of the homeobox genes, which show precise spatial and temporal regulation during embryonic development and determine the body plan (111). Upon knock out, a reduction of myeloid progenitor cells was observed in mice. HOXA9 is also highly expressed in hematopoietic stem cells and its expression gradually decreases with increasing extent of differentiation (112,113). HOXA9 expression is frequently dysregulated in several cancers. In this context, its expression was found to be increased in colorectal, ovarian and prostate cancer as well as in glioblastoma, whereas decreased expression through promoter methylation was observed in breast cancer (114). One recent study discovered hypermethylation of HOXA9 in HCC samples by array analysis (Table II) (115). Methylation of HOXA9 was subsequently assessed by quantitative methylation-specific PCR (qMSP) in plasma from 40 HCC patients of all stages and 34 healthy controls. Statistical evaluation showed an AUC of 0.835 with 73.3% sensitivity and 97.1% specificity.

p16^{INK4A}. p16^{INK4A} is crucial in cell cycle regulation and considered as a tumor-suppressor. It interacts with cyclin-dependent kinases, thereby inhibiting their ability to phosphorylate and inactivate the retinoblastoma protein, ultimately leading to cell cycle arrest (116). Promoter hypermethylation of p16^{INK4A} and subsequent reduction of p16^{INK4A} levels has been observed in a majority of HCC tissue samples (117). A recent study examined INK4A methylation via pyrosequencing, using circulating cell-free DNA from the blood samples of 66 HCC patients and 43 controls with chronic hepatitis and liver cirrhosis (Table II) (118). The sensitivity and specificity observed for INK4A methylation were 65.3 and 87.2%, respectively, with an AUC of 0.82.

4. MicroRNAs to detect HCC

miRs have received increasing attention as a new class of non-invasive biomarkers for many cancers, including HCC (119,120). miRs are small non-coding RNA molecules, which play an important role in post-transcriptional regulation of gene expression by either mRNA degradation or by blocking translation initiation. Importantly, miRs can also be released into the blood stream as free molecules or bound to proteins. In this context, they display exceptional stability against endogenous RNAse activity, which renders them ideally suited for detection and quantification by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (121). Many miRs were shown to be dysregulated in liver cancer and especially miRNA signatures, comprised of three or more miRs have been suggested for highly accurate HCC detection (122). The most novel developments in this field are outlined below.

miR-139. miRNA-139 was shown to be aberrantly expressed in many cancers, including HCC. Notably, it can suppress epithelial-mesenchymal transition (EMT), migration and invasion in HCC via targeting of ZEB1 and ZEB2. Accordingly, it is downregulated in a majority of HCC tissue samples (123). miR-139 was also shown to reduce Rho-kinase 2 expression in HCC, thereby inhibiting migration, invasion and metastasis (124). Furthermore, it regulates proliferation and invasion of HCC cells by targeting T-cell factor (TCF)-4 mRNA, resulting in decreased β-catenin/TCF-4 transcriptional activity (125). This mechanism could also be involved in the suppression of EMT by miR-139. Thus, it is not surprising that miR-139 was recently investigated regarding its performance as a biomarker of HCC by qRT-PCR in sera of 31 HCC patients and 31 chronic HBV patients as controls (Table II) (126). The analysis showed a downregulation of miRNA-139 in HCC patients with an AUC of 0.761, a specificity of 58.1% and a sensitivity of 80.6%. However, clinicopathological and etiological characteristics were not defined.

miR-182. miR-182 belongs to a polycistronic cluster comprised of three miRNAs and located on chromosome 7. miR-182 is predominantly expressed in sensory organs such as the retina, the nose and the inner ear and shows a specific expression pattern during the development of these structures (127). miR-182 was shown to be overexpressed in breast carcinoma, melanoma, glioma, ovarian, prostate and colorectal cancers. In contrast, miR-182 plays a more ambiguous role in lung cancer, where overexpression of miRNA-182 was shown to inhibit cancerogenesis *in vivo* (128). In HCC, upregulation of miR-182 is associated with increased resistance to cisplatin as well as enhanced proliferation and invasive abilities (129-131). miR-182 was recently investigated as a candidate serological biomarker in serum samples of 103 HCC patients of all stages, 95 CLD patients [39 patients with cirrhosis, 47 with chronic hepatitis and 9 with non-alcoholic fatty liver disease (NAFLD)] and 40 healthy controls by qRT-PCR (Table II) (132). ROC curve analysis showed an AUC of 0.911 for the discrimination between HCC and CLDs with a sensitivity of 78.6% and a specificity of 91.6%.

miR-331-3p. miR-331-3p was first discovered during miR expression profiling of chronic and acute lymphocytic leukemia, along with one of its putative targets SOCS1 which regulates STAT activation enhancing cell survival and proliferation (133). miR-331-3p was subsequently shown to be dysregulated in several malignancies, such as prostate, lung, breast and gastric cancer as well as in glioblastoma. Notably, it is also involved in the induction of EMT in prostate cancer (134-138). In HCC, miR-331-3p was suggested as a possible prognostic marker and it was recently shown to promote proliferation and metastasis via targeting of the PH domain and leucine-rich repeat protein phosphatase (139,140). The diagnostic potential of serum miR-331-3p was explored in the same cohort as described for miR-182 (Table II) (132).

Table III. Diagnostic performance of promising markers in early-stage HCC patients vs. high-risk groups.

Marker	Refs.	Comparison		Sensitivity (%)	Specificity (%)	AUC
Annexin A2	(30)	Early HCC vs. LC	Annexin A2	86.4	73.5	0.80
			AFP	ND	ND	0.66
			Combined	ND	ND	0.83
sAxl	(37)	Very early HCC vs. LC	sAxl	80.8	66.7	0.84
			AFP	42.3	93.3	0.66
			Combined	88.5	76.7	0.90
Thioredoxin	(45)	Very early HCC vs. LC and CLD	Thioredoxin	74.5	79.6	0.84
			AFP	70.1	69.8	0.73
			Combined	81.6	87.4	0.88

AFP, α-fetoprotein; AUC, area under the curve; CLD, chronic liver disease; HCC, hepatocellular carcinoma; LC, liver cirrhosis; ND, not determined.

In this context, miR-331-3p was upregulated and showed comparable diagnostic performance, with an AUC of 0.890, a sensitivity of 79.6% and a specificity of 86.3% when discriminating HCC from CLDs.

miR-199a-3p. Altered expression of miR-199a-3p has been shown in a variety of tumors, such as ovarian, breast, esophageal, colorectal and gastric cancer, as well as in osteosarcoma. In this respect, it was also suggested as a serum biomarker for colorectal carcinoma (141-147). Furthermore, both higher and lower expression levels have been observed in different tumor types as compared to normal tissue and accordingly, tumor-suppressive as well as tumor-promoting abilities of miR-199a-3p have been described. Notably, miR-199a-3p interferes with the expression of Axl, thereby inhibiting the progression and metastasis of osteosarcoma (144). In HCC, miR-199a-3p regulates mTOR and c-Met signaling, thereby attenuating the invasive potential and increasing chemosensitivity. Accordingly, miR-199a-3p is often downregulated in human HCC (148). It targets CD44 and subsequently reduces proliferation in CD44-positive HCC cells (149). Decreased serum levels of miR-199a-3p were investigated for their diagnostic value in the sera of 78 HCC patients of all stages and in 156 healthy controls by qRT-PCR (Table II) (150). ROC curve analysis yielded an AUC of 0.883 with 71.8% sensitivity and 86.1% specificity.

5. Conclusion

The clinicopathological and etiological characteristics specific for HCC represent a massive challenge for validating and establishing promising candidate biomarkers in clinical practice. HCC can develop in response to direct carcinogenic events, such as Aflatoxin B_1 adducts, but in the majority of cases, liver cancerogenesis is a multi-step process driven by chronic inflammation due to oxidative stress caused by HBV or HCV infection, alcohol abuse and/or NAFLD. These insults cause senescence of hepatocytes and concomitant activation of stellate cells, leading to fibrosis and ultimately cirrhosis of the liver, which is considered a pre-malignant state and the major risk factor for HCC (151). Thus, biomarkers permitting accurate differential diagnosis between cirrhotic and cancer patients are most desirable. Additionally, HCCs exhibit a vast genetic and pathological heterogeneity due to the variety of underlying CLDs, which must be especially considered when evaluating the performance of a biomarker. Furthermore, the accuracy of biomarkers must be viewed in light of the etiology as well as the respective controls since the prevalence of different etiologies shows great variation depending on the geography. Notably, HBV is the leading cause of HCC in Asia, whereas alcohol-, NAFLD- and HCV-induced HCCs are more prevalent in Western countries (152).

HCC detection at the earliest stage is of outmost relevance for anticancer therapy. The BCLC staging system classifies early HCC as a solitary tumor of up to three nodules, each below 3 cm in size within preserved liver functions (153). In these cases, curative therapeutic interventions by surgical resection, liver transplantation or percutaneous ablation show high 5-year survival rates. Yet, every 3-month waiting period for a liver transplant is expected to increase pre-transplantation mortality by 10%. Furthermore, liver resection poses a risk of recurrence depending on tumor size and number (5,154). In this respect, minimally invasive percutaneous ablation methods achieve complete tumor necrosis in 100% of solitary HCC <2 cm, whereas the success rate is 70-80% in HCC <3 cm (5). Furthermore, a study examining 218 patients with tumors <2 cm showed sustained responses following RFA in 97% of patients during the 31-month follow-up period (155). Therefore, HCC detection at the earliest stage, defined as BCLC 0 (a solitary tumor <2 cm) could minimize waiting periods and many risks associated with liver resection or transplantation. Thus, biomarkers showing high diagnostic power in both HCC detection and distinction from cirrhosis, while taking tumor stage and patient etiology into account, are the most promising. Annexin A2, MCM6, sAxl and thioredoxin were evaluated in early or very early HCCs vs. cirrhotic or CLD patients and achieved high AUCs. LTBP-2 and sAxl were also investigated in Eastern as well as Western patient populations. However, LTBP-2 exhibited a drop in diagnostic performance in European HCC patients and no independent comparison with cirrhotic controls was provided, whereas sAxl performed equally well in all populations examined.

Moreover, most diagnostic strategies using serum samples rely on the combination of novel biomarkers with established ones, especially AFP, to increase diagnostic performance. In this context, annexin A2 showed a combined AUC of 0.83 for early HCC. In the case of sAxl and thioredoxin, combined AUCs of 0.901 and 0.875 were achieved for very early HCC (<2 cm), respectively, emphasizing their potential clinical relevance (Table III) (37,45). For thioredoxin and AFP, these results could be verified in a validation cohort, with a combined AUC of 0.870. The authors also reported that 69.2% of AFP-negative patients were detectable by thioredoxin. However, no corresponding ROC analysis was performed (45). Although no matched validation cohort was evaluated for sAxl and its combination with AFP, it achieved high performance in all patient populations evaluated in the multicenter study. Furthermore, sAxl displayed a sensitivity of 86.7% and an AUC of 0.858 for very early, AFP-negative HCC, underlining its potential in complementing AFP (37). For MCM6, the combined AUC was not indicated, but the specificity dropped to 50.8% (83).

Although miRs exhibit exceptional diagnostic accuracy, several methodological limitations must be considered for this new class of HCC biomarkers. For example, sample collection and processing have a significant impact on the result of miR quantification, since platelets contain a wide spectrum of miRs, which may be released into the sample during coagulation (156). Thus, significant differences can arise between serum and plasma samples. The fasting status may further affect the miR status, as their carriers, such as proteins or lipoprotein particles, may be more or less abundant (157). Furthermore, different extraction methods can strongly bias the resulting miR levels and subsequent quantification (158). In addition, qRT-PCR analysis as the most common method of miR detection lacks universally accepted normalization controls (159). These restrictions currently limit the usability and reproducibility of miR data for the detection of HCC.

Despite increasing overall complexity, the availability of high throughput technologies has considerably improved our understanding of HCC pathogenesis. As a result, new molecules and strategies in HCC biomarker research are continuously emerging. The analysis of exosomes, small membranous vesicles of 30-100 nm in diameter, is among the most promising strategy in this respect. They are derived from multi-vesicular bodies and can be secreted into the extracellular space by most cell types, including HCC cells (160). Exosomes can transport a variety of molecules such as proteins, mRNAs and miRs (161). Although their discovery dates back to 1983, their potential as biomarkers for HCC has only been recently recognized (162). In rats, a combination of AFP, exosomes and circulating miRs (miR-10b, miR-21, miR-122 and miR-200a) detects HCC more accurately with an AUC of 0.943 as compared to AFP alone (AUC 0.826) (163). In humans, exosomal HCC-associated miR-21 increases in sera of HCC patients (164). Recently, another class of molecules has drawn attention in HCC research, namely long non-coding RNAs (lncRNAs). lncRNAs are longer than 200 nucleotides and are present in virtually all cell types, regulating gene expression via *cis*- and *trans*-acting mechanisms. In this regard, lncRNAs play significant roles in many cellular processes, such as chromatin remodeling, cell cycle control, apoptosis or cell fate specification. Importantly, lncRNAs were shown to be dysregulated in HCC, promoting cell proliferation and tumor progression (165). In this context, a recent study investigated the expression profile of lncRNAs in the sera of HCC patients for its suitability as a biomarker (166). The authors discovered a significant increase in lncRNA-UCA1 and lncRNA-WRAP53 in HCC vs. healthy or HCV controls. This suggests that lncRNAs may represent an important new class of HCC biomarkers, in addition to miR and protein biomarkers.

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