Chapter

Circulating Biomarkers for Early Diagnosis of Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors, which is also often fatal. An early and accurate diagnosis is a decisive step towards the survival of the patients. Molecular biology improved significantly the prognosis of liver cancers through learned use of tumor markers like proteantigens, cytokines, enzymes, isoenzymes, circulating RNAs, gene mutations and methylations. Nevertheless, much improvement is still achievable and needed in this area, which is crucial in order to make an early diagnosis and monitor the progression of the disease. We present in this review what we believe to be the most relevant data regarding tissue and serum biomarkers related to HCC.

Keywords: Biomarkers, Hepatocellular Carcinoma, Diagnosis, Liver Cancer

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in males, the seventh in females, and the third leading cause of cancer-related deaths. Each year there are approximately 800,000 fatalities [1–3]. In developing countries, morbidity and mortality rates are 84% and 83%, respectively [4]. HCC typically occurs in the context of chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, accounting for 85% of all HCC cases globally [3]. Lower risk factors include non-alcoholic fatty liver disease (NAFLD) and chronic alcohol consumption [4].

Tumor evolution is a complex process implying many stages and involving many factors, such as genetic and chromosomal changes. During tumor development, the number, type, extent, and distribution of markers and variants are closely related to the occurrence, progression, invasion, and metastasis of HCC. Therefore, diagnosis and early detection are highly important in management and treatment because it is only possible to cure the disease when the tumor when it is detected at a small size.

Advances in the understanding of tumor biology, combined with the development of molecular methods in looking for new biomarkers in the early detection of the disease, their invasiveness, likelihood of metastasis and recurrence, has led to the discovery and use of several new markers in this disease. In this review, we discuss the results of the studies that we consider the most relevant, and in particular their diagnostic performance for the detection of HCC at an early stage.

2. Embryonic antigen

2.1 Alpha-fetoprotein (AFP)

Alpha-fetoprotein (AFP) is a large serum glycoprotein that is synthesized in the liver that occurs during fetal life is repressed during adulthood [5]. Therefore, AFP levels often diminish rapidly after birth and remain low throughout adulthood. Since AFP was discovered in the serum of HCC patients in 1964 [6], it has been regarded as the most useful serum protein for patients at risk for HCC [7–9]. However, the sensitivity and specificity of using AFP for early HCC detection are widely variable as elevated AFP levels are also observed in many other cancers [10]. In addition, AFP levels are below the detection limit in small liver tumors, while it can be above the detection limit when the tumor is large, producing an AFP-negative HCC. AFP is considered to have a screening role in HCC but its role is limited since it does not allow to distinguish between cancerous lesions and some other benign liver damage pathologies, hence causing a high proportion of false positives and false negatives. Patients with hepatitis still have high AFP level even without liver tumors. The positive predictive value of AFP for detecting HCC is 70% for people with hepatitis viruses and 94% for those without. Therefore, AFP is more effective in detecting HCC in cases without hepatitis viruses.

According to the 2010 recommendations of the American Association for the Study of Liver Diseases (AASLD) for the diagnosis and treatment of HCC, the effectiveness of AFP as a test to diagnose HCC was lower than expected. AFP is also increased in biliary carcinoma in the liver or metastases from colon cancer. Biliary cancer in the liver is also quite common in cirrhotic patients, although the incidence of this disease is lower than that of HCC. The fact that these two liver cancers are common in cirrhosis makes it necessary to identify accurately the disease. Because AFP may increase in many cases other than HCC, it is no longer recommended to be used in Europe and the Americas for its diagnosis. The current diagnosis of HCC is based on imaging and histopathology [11]. The Asia Pacific Association for the Study of the Liver (APASL) also stated that AFP alone is not recommended to diagnose HCC. When combined with other methods, the diagnosis threshold of AFP was 200 ng/ml (**Table 1**) [12].

2.2 AFP heterogeneity

AFP exists as three glycoforms, each of them having a 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 early stages of liver disease progression, AFP-L2 has an intermediate affinity for lectin and is a major component during pregnancy because it is derived from the yolk sacs. AFP-L3 is only elevated in patients with HCC because it is solely produced by cancer cells, making it a specific biomarker for HCC [13, 14]. However, the drawback of AFP-L3 is that it can only be detected if AFP levels are >20 ng/ml.

AFP-L3 immunoassay sensitivity has been further improved by higher sensitivity analytical methods and advanced microfluidics-based separation science [15]. "Highly sensitive AFP-L3" (hs-AFP-L3) obtained significantly better results than conventional AFP-L3, even when patients had a single and/or small HCC tumor. The sensitivity and specificity of hs-AFP-L3 were 57% and 63.5%, and 40.4% and 81.1% for conventional AFP-L3 [16]. These results make hs-AFP-L3 a valuable biomarker for detecting early-stage HCC (**Table 1**).

Marker	Cut-off value	Sensitivity (%)	Specificity (%)	Reference
AFP	>200 ng/ml	39–45	76–94	ADSSL
AFP-L3	AFP-L3/AFP > 15	55.3	93.9	Taketa [1]
GP73	85,5 mg/l	80	82	Schewegle [2]
GPC3	n.a.	55.2	84.2	Jia [3]
OPN	n.a.	86	86	Wan [4]
SCCA	n.a.	84.2	48.9	Gianneli [5]
DCP	+ AFP	72.7 74.2	90 87.2	Carr et al. [6] Bertino [7]
GGT	5,5 IU/ml	86	n.a.	Yao et al. [8]
hsGGT	n.a.	74	n.a.	Cui et al. [9]
AFU	n.a.	81.5	85.4	Wang et al. [10]
TGF-β1	800 pg./ml	95	n.a.	Song et al. [11]
TGF-β1 mRNA	> 1,2 μg/l	89.5	94	Dong et al. [12]
TSGF	62 IU/ml	82	n.a.	Yin et al. [13]
IGF-II	4,1 mg/l	63	90	Tsai et al. [14]
HGF	>1 ng/ml	100	n.a.	Vejchapipat et al. [15]

Abbreviation: *n.a.: not applicable; AFP, alpha-fetoprotein; GP73, Golgi protein 73; GPC3, Glypican-3; OPN, Osteopontin; SCCA, squamous cell carcinoma antigen; DCP, Des-γ-carboxyprothrombin; GGT, Gamma-glutamyltransferase; AFU, Alpha L fucosidase; TGF-β1, Transforming growth factor-β; IGF-II, insulin-like growth factor-II; HGF, Hepatocyte growth factor.*

Table 1.
Diagnostic performance of biomarkers for HCC.

3. Proteantigen

3.1 Glypican-3 (GPC3)

Glypican-3 (GPC3) is a member of the glycican family of heparan sulfate proteoglycans linked to cell membranes by glycosyl-phosphatidylinositol [17]. It is a fetal glycoprotein that exists on the cell surface to help regulate cell growth during pregnancy. GPC3 is associated with the malignant proliferation of cells but there are currently no studies to prove its association with healthy people and benign conditions. Quite a number of studies have proven the overexpression of GPC3 in malignant diseases such as breast cancer, ovarian cancer, or lung adenocarcinoma [18, 19]. With HCC, its expression is increased through the autocrine/paracrine regulator in conjunction with the Wnt signaling pathway [20]. Some studies have concluded that the sensitivity of GPC3 in HCC diagnosis ranges from 40 to 53%, which is interesting considering that in about 33% of cases, both AFP and DCP serum were within normal limits [21, 22]. GPC3 has been detected in HCC tumor but not in benign liver tissues, so it is likely a marker for early detection of HCC [23]. GPC3 expression does not depend on some clinical features such as tumor size, GPC3 sensitivity in early HCC diagnosis (size <3 cm) was 56% [23]. In a meta-analysis, the sensitivity and specificity of serum GPC3 to diagnose HCC were 55.2% and 84.2%, respectively [24]. A smaller analysis of the early-stage HCC group (BCLC 0 and A or TNM phase I) showed a sensitivity and specificity of GPC3 of 55.1% and 97%, respectively, which are higher than the those obtained with the AFP serum in

the same study, that were 34.7% and 87.6%. Combining GPC3 and AFP increased the sensitivity to 76% for early-stage tumors [24]. In short, GPC3 might be a marker for HCC, especially in the early stages, but GPC3 expression also increases in some other malignancies, so the specificity for HCC diagnosis is not high. It can still increase diagnostic sensitivity when combined with other valuable serum markers (**Table 1**).

3.2 Heat shock protein 70 (HSP70)

Heat shock protein (HSP) is an antiapoptotic protein whose overexpression allows cell survival. It protects cells and stimulates the reparation of tissue damage. A study indicated the positive rate of HSP70 and HSP27 in HCC tissues at 56.3% and 61.9%, respectively [25]. There was a correlation between the stained intensity of HSP70 and tumor size, portal vein invasion, and tumor stage, while HSP27 was only associated with hepatitis B virus (HBV) related HCC. In addition, the overexpression of HSP70 and HSP27 in HCC tumors may lead to increased tumor growth and metastasis (**Table 1**) [26].

Data suggest that HSP70 can be used as a prognosis indicator for HCC. Its expression was detected in 282 of 392 HCC cases (71.9%), compared to 14 of 115 non-neoplastic liver tissues [27]. The sensitivity and specificity in the detection of HCC have been measured at 57.5% and 85%, respectively [28]. The expression of HSP70 is also correlated with the differentiation and apoptosis of tumor cells. HSP70 promotes cancer cell growth by stabilizing cyclin D1 and suppressing apoptosis in cancer cells by inhibiting the p53 pathway [29, 30]. This information makes HSP70 and HSP27 potential markers of HCC that should be further investigated.

3.3 Golgi protein 73 (GP73)

Golgi protein 73 (GP73) is a type II Golgi-specific membrane protein, which is normally expressed in epithelial cells of many human tissue types, but not hepatocytes [31]. A study showed that serum GP73 levels of patients with HBV-related HCC were significantly increased compared to patients with HBV and healthy adults [32, 33]. The sensitivity of diagnosis of HCC (76.9%) was significantly higher than that of AFP (48.6%), suggesting that GP73 can be an effective serum biomarker for the diagnosis of HCC [34]. The combination of GP73 and AFP further increased the sensitivity and specificity to 89.2% and 85.2%, respectively, with an AUC of 0.96 (**Table 1**).

FC-GP73 further improves the HCC diagnostic performance made with GP73 from 65 to 90 to 90–100%, respectively. Even when GP73 is at a very low level or absent, FC-GP73 is still detectable [35]. These are encouraging data but there is still a lot of work to be done regarding the correlation between GP73 and tumor size, stage, recurrence, and prognosis before this marker can be used.

3.4 Squamous cell carcinoma antigen (SCCA)

Squamous cell carcinoma antigen (SCCA) belongs to the high molecular weight protease inhibitor family found in the squamous and granular layers of the normal squamous epithelium. It consists of two different isomers, encoded by two highly homologous genes: SCCA1 being neutral, and SCCA2 acid [36]. SCCA2 has been detected in many malignancies such as cervical, lung, head and neck carcinoma, and it has been used as a valuable diagnostic biomarker in clinical practice [37].

Giannelli et al. showed that SCCA expression was higher in the HCC group than in the cirrhotic group. The sensitivity of SCCA is 84.2%, but the specificity is low at

48.9%. In the small tumor group (≤ 3 cm) the sensitivity and specificity of SCCA were 56.1% and 74.9% with a cut-off of 3.2 ng/ml. In their study of SCCA expression in cells, using immunohistochemistry, Guido et al. demonstrated that SCCA expression in cancerous tissues and dysplasia nodules was much higher than that of newly formed nodules in early HCC diagnosis [38]. SCCA was highly sensitive, but its specificity was quite low. Its expression in early HCC tissue and in dysplasia nodules makes SCCA a valuable complementary marker for HCC diagnosis. An alternative biomarker is an immune complex between SCCA and IgM, SCCA-IgM, whose expression increases in early HCC. The immune complex SCCA-IgM has a higher diagnostic performance than the free SCCA and is also more relevant since it is not found in the serum of healthy people. However, the detection rate of SCCA-IgM immune complex is 18% for chronic hepatitis, 26% for cirrhosis and 70% for HCC [39]. Its sensitivity and specificity for HCC diagnosis are 89% and 50% [40]. The concentration of SCCA-IgM immune complex is constantly increasing in patients with cirrhosis who tend to progress to HCC. Sensitivity and specificity were of higher value than AFP in the studies of Pontisso et al. [37].

Increased serum SCCA in patients with liver disease can be considered a valuable marker for early diagnosis of HCC. Especially the SCCA-IgM immune complex, which is highly sensitive. However, since its specificity is quite low, it must be combined with other markers such as serum AFP or DCP to increase its diagnostic value.

3.5 Osteopontin (OPN)

Osteopontin (OPN) is known as a conversion protein and is a glycophosphoprotein associated with integrin, which is overexpressed in many types of malignancies such as lung, breast, and colon cancers [41]. OPN usually manifests in biliary epithelial cells, astrocytes and Kupffer cells, but not in liver cells [42]. However, increased serum OPN expression has been reported in patients with HCC, but not in those with cirrhosis, chronic hepatitis, or healthy controls [43, 44]. In a meta-analysis, the sensitivity and specificity of OPN were 86% for all HCC stages [45]. Shang et al. suggested that serum OPN concentrations at the cut-off level of 91 ng/ml were more sensitive than that of AFP (74% versus 53%) in the diagnosis of HCC. Combining two imprints with an OPN cut-off of 156 ng/ml and an AFP cut-off of 20 ng/ml increased sensitivity and specificity (95% and 96%). The sensitivity and specificity of OPN were 75% and 62% for early HCC, which means the sensitivity was higher than that of AFP, but the specificity lower (46% and 93%). When combined with AFP at the cut-off of 91 ng/ml for OPNs, sensitivity increased to 83% and specificity decreased to 63% [45] (**Table 1**). Based on such findings, OPN can be considered an important marker in HCC diagnosis, especially for tumors in the early stages, and when combined with AFP to significantly increase sensitivity. However, studies with larger sample populations are needed to confirm its relevance.

3.6 Tumor-associated glycoprotein 72 (TAG-72)

Tumor-associated glycoprotein 72 (TAG-72) is a macro-molecular glycoprotein complex, which is rarely expressed in normal tissues, but overexpressed in the majority of human adenocarcinomas, including gastric, colon, and pancreatic cancer. TAG-72 expression is significantly increased in HCC tissues compared to normal liver tissues [46], and it is suspected of promoting tumor invasion and metastasis. A correlation between overexpression of TAG-72 and poor survival in patients with HCC has been observed [46]. This makes TAG-72 a potential prognosis marker for HCC, and anti-TAG-72 monoclonal antibody has been used for tumors clinical detection [47].

3.7 Zinc-α2-glycoprotein (ZAG)

Zinc-α2-glycoprotein (ZAG) is a member of the class I major histocompatibility complex (MHC-I) family. It is considered a new adipokine because of its strong amino acid sequence homology with lipid mobilizing factor (LMF). ZAG is down-regulated in human obesity [48], but it is upregulated in different cancers such as breast, lung and prostate cancers, making it a potential biomarker for these. The serum proteome of the HCC, liver cirrhosis and healthy adult groups have been analyzed and it was found that the ZAG is overexpressed in the HCC patients suggesting a potential biomarker for the early detection of HCC [49].

3.8 Annexin A2

Annexin A2 is a calcium-dependent, phospholipid-binding protein found on the surface of endothelial cells and most epithelial cells [50, 51]. Annexin A2 serum concentrations in patients with HCC were often higher than those with benign liver disease, other malignant tumors, or healthy individuals [52–54]. High annexin A2 levels were observed in 83.2% of early-stage HCC and 78.4% of AFP-negative HCC patients [55]. Annexin A2 sensitivity and specificity were respectively measured at 83.2% and 67.5% in the detection of early-stage HCC, while HCC patients with normal AFP levels were 54.7% and 81.3%, respectively. The diagnostic performance of annexin A2 alone (AUC = 79%) was also greater than for AFP alone (AUC = 73%). As expected, the combination of annexin A2 and AFP further improved the overall diagnostic performance with a sensitivity of 87.4% and a specificity of 68.3%. This makes annexin A2 a potential independent biomarker for detecting early-stage HCC in patients with normal serum AFP.

4. Enzymes and isozymes

4.1 Des-γ-carboxyprothrombin (DCP)

Des-γ-carboxyprothrombin (DCP) or Prothrombin induced by vitamin K absence II (PIVKA II) is a prothrombin molecule which is synthesized in abnormally high amount in HCC. During malignant transformation in liver cells, vitamin K-dependent carboxylase system weakens [56]. In essence, this is a carboxylation defect that leads to increased DCP synthesis [57]. Serum DCP levels in patients with liver cancer have differed from normal individuals [58]. In a comparative study of cases of chronic hepatitis and liver cirrhosis, DCP showed a sensitivity of 72.7% and a specificity of 90.0%, equivalent to AFP [59]. The combination of these two markers improves HCC diagnosis with a sensitivity and a specificity of 74.2% and 87.2%, respectively [60]. Although DCP has proven to have great potential as a biomarker for early diagnosis of HCC, it needs to be verified by further studies, especially in combination with AFP. In a large multicentre study, the sensitivity of DCP was 56% for early HCC diagnosis. Combining DCP with AFP increased the sensitivity from 65–87% 3 months before HCC diagnosis, but the specificity decreased from 84–69% [61].

Although the diagnostic value of DCP has been studied in Asian countries, its assessments in Western countries, especially in Europe, are still limited. A case–control study to evaluate the performance of serum AFP and DCP concentrations for early HCC diagnosis was conducted in France [62]. The cut-off threshold for serum DCP was 42 mAU/ml and 5.5 ng/ml for AFP, resulting in DCP being better than AFP for early diagnosis of HCC with a sensitivity of 77% compared to a 61%

one, and a specificity of 82% compared to a 50% one. The positive forecast value was 76% compared to 51%, and the negative forecast value was 83% compared to 62%. The combination of DCP and AFP improved diagnostic performance. These results further support the value of DCP as a marker for early HCC diagnosis. According to the 2010 recommendations of the Japan Society of Hepatology (JSH), the three biological markers AFP, AFP-L3 and DCP are checked by the state insurance for HCC screening, as a combination of two of the three biomarkers, or all three combined. These three markers help to increase sensitivity without reducing specificity in small liver cancer [63].

4.2 γ-Glutamyl transferase (GGT)

γ-Glutamyl transferase (GGT) is a membrane-binding enzyme, which appears in the development of liver cells during pregnancy, its concentration is high throughout pregnancy and decreases immediately after birth. The total GGT concentration increased in chronic liver diseases, HCC, and some extra-liver cancer diseases [63]. A study by Cui et al. on 90 patients with cirrhosis and 120 patients with HCC showed that the sensitivity of HS-GGT was 74%, irrespective of size, and 43.8% for small tumors (<3 cm) [64] (**Table 1**). The diagnostic value improves when combined with other biomarkers such as AFP, PIVKA II, or AFP-L3. This is a promising sign in the detection of small cancers and can be used in combination with AFP and AFP-L3.

4.3 Matrix metalloproteinases (MMPs)

Matrix metalloproteinase (MMP) is an enzyme belonging to the endopeptidase group, which helps regenerate tissue in various pathogenetic processes including tumor progression, and wound healing [65]. Kuo et al. showed that only cases of HBsAg-positive have high levels of MMP-2 expression [66], but the relationship between other markers of HBV and MMP was not clarified. Positive cases with HBeAg showed a high tendency for portal vein thrombosis along with high manifestations of MMP-7 and MMP-9. MMPs have a synergistic effect on HCC generation, proliferation and invasion, through ways that the study did not elucidate [67]. A significantly higher MMP-9/MMP-2 ratio was found in patients with advanced HCC compared to patients at an early stage [68]. The mRNA of MMP-14, MMP-15 and MMP-2 are highly expressed in most HCC cells suggesting an important role of MMPs in the growth, invasion, and metastasis of tumor cells. Selective inhibitors for these MMPs promise to be an effective mean of preventing the growth and metastasis of HCC [69].

4.4 Glutamine synthetase (GS)

Glutamine synthetase (GS) is an enzyme involved in catalyzing the synthesis of glutamine from glutamate and ammonia, it plays an important role in the function of ammonia metabolism and nitrogen balance of the liver [70]. Research by Haupt et al. demonstrated that GSmRNA increased its tissue and protein expression in the serum of HCC patients [71]. In addition, Osada et al. reported increasing GS expression correlated with cancer progression, suggesting GS can play a role in promoting HCC metastases [72].

4.5 Alpha L fucosidase (AFU)

Alpha L fucosidase (AFU) is a glycosidase responsible for hydrolysing fucoseglycoside bonds of glycoprotein and glycolipids and is found in all mammalian cell

lysosomes and is involved in the degenerative reaction of a series of fucoglyco-containing fucoglyco complexes [73]. Serum AFU levels are constantly elevated in cirrhotic patients who tend to progress to HCC. Deugnier et al. found that serum AFU had greater sensitivity and specificity than AFP and that it can be considered a marker for HCC diagnosis. However, the cause of this increased serum AFU activity is still unknown. The most likely explanation is that increased serum AFU activity is a result of an increase in tumor protein synthesis that increases fucoses [74]. Measuring the activity of serum AFU regularly during follow-up of cirrhotic patients provides very useful clinical data in monitoring cirrhosis progression to HCC. Although an increased serum AFU activity was not correlated with tumor size and was common in cases of early HCC, the HCC tumor would appear within a few years in 82% of patients with liver fibrosis if serum AFU activity exceeds 700 nmol/ml/hour. Serum AFU activity increased in 85% of patients at least six months before HCC was detected by a diagnostic imaging method [75]. AFU activity was significantly increased in HCC patients compared with patients with other liver diseases or other cancers. AFU sensitivity is 81.5% and its specificity is 85.4% in HCC diagnosis [76] indicating a promising specific marker for HCC diagnosis.

5. Cytokines

Cytokines are a heterogeneous group of proteins that play roles of mediators in cellular reactions and activities. They are the product mediating and regulating immune processes of immune cells. Some cytokines also act as potential markers for early diagnosis and treatment of HCC.

5.1 Transforming growth factor-β1 (TGF-β1)

Transforming growth factor-β (TGF-β1) is a versatile growth factor associated with proliferation, cell differentiation, embryogenesis, vascular proliferation, invasion and immune activity. One study found that serum TGF-β1 levels increased in the HCC group compared to the group with non-malignant liver disease and the healthy group. With a cut-off of 800 pg./ml, the specificity of TGF-β1 HCC diagnostic serum is above 95%. Taking the same value as the serum AFP at the cut-off of 200 ng/ml, the sensitivity of TGF-β1 is 68%, which is superior to that of AFP (24%). Moreover, in patients with serum AFP within normal limits, increased TGF-β1 levels can be observed in 23% of cases [77]. It has been shown that TGF-β1 and TGF-β1 mRNA can be used as a marker to diagnose and predict HCC due to HBV with a sensitivity and specificity of 89.5% and 94.0% with a cutting level of TGF-β1 > 1.2 g/l [78]. TGF-β1 mediates various biological effects through signal paths and manifestations of TGF-β1 polymorphism may affect tumor susceptibility. The TGF-β1 signaling pathway can be considered as a target for HCC treatment. The subject is currently under study to confirm its role and promises to bring new cancer treatments.

5.2 Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF) acts as an important factor in the process of tumor formation by forming new blood vessel systems that increase in size and promote invasion and metastasis. Studies have shown that angiogenesis is essential in tumor growth, including HCC, which is often characterized by the proliferation of blood vessels [79]. It has been demonstrated that VEGF expression in HCC tissues has a significantly higher incidence of portal vein thrombosis and

a lower average survival time than when VEGF expression is not present [80]. In the study of Xiang et al., VEGF was associated with lymph node metastatic characteristics in HCC. In addition, VEGF expression is closely related to relapse and prognosis. Notably, several manifestations of the VEGF receptor are related to some of the clinical characteristics and prognosis of HCC [81]. Inactivation of VEGF165 increases the expression of the *P53* gene that inhibits HCC development, invasion and metastasis.

5.3 Interleukin-8 (IL-8)

Interleukin-8 (IL-8) is a multifunctional CXC chemokine that is involved in the immune response of neutrophils in humans including kinetic phenomena, enzyme release and expression of surface adhesion of molecule. IL-8 also has a direct effect on tumor progression, including the proliferation of vascular endothelial cells and formation of new vessels. In addition, IL-8 increases the likelihood of metastases and new tumor formation in the liver [82]. A study showed that IL-8 serum concentrations increased in HCC patients compared to healthy subjects, it was positively correlated with tumor size (≥5 cm), portal vein thrombosis and advanced stage with lymph node metastases [83]. Therefore, it may be a biological marker that plays a useful role in HCC diagnosis and prognosis.

5.4 Tumor-specific growth factor (TSGF)

Malignant tumors have the ability to synthesize tumor-specific growth factors, releasing them into the capillaries surrounding the tumor and peripheral blood vessels during their development. Therefore, serum TSGF levels may be a marker of tumor survival. In one study, serum TSGF concentrations were used as a diagnostic marker for HCC with 82% sensitivity at 62 UI/ml [84]. Combined with other cancer markers, TSGF may yield higher diagnostic values with increased sensitivity. Theoretically, preeclampsia is highly expressed in many malignant tumors and HCC, but there are currently too few studies evaluating the role of TSGF in other malignancies to consider it as a potential factor. There are other markers, such as serum insulin-like growth factor-II (IGF-II), which can be used as diagnostic or prognostic markers for HCC. A cut-off of 4.1 mg/l of IGF-II obtained results of 63% sensitivity, 90% specificity and 70% accuracy in early HCC diagnosis with small tumor size. Moreover, the combination of IGF-II and AFP (cut-off value of 50 ng/ml) increases sensitivity up to 80% and accuracy up to 88% [85].

5.5 Hepatocyte growth factor (HGF)

Hepatocyte growth factor (HGF) is a multifunctional element produced in many organs in the body, it affects cell division, cell motility, intracellular invasion, and carcinogenesis [86]. In a study in Japan, serum HGF levels are increased significantly in the HCC group compared with cirrhosis, chronic hepatitis and healthy controls groups. With a cutting level of 0.6 ng/ml, its sensitivity can be up to 100% for any AFP or DCP concentration. The serum HGF concentration ≥ 1.0 ng/ml has a shorter shelf life, so it can be used as a prognostic marker for HCC [87]. The authors suggest that HGF causes proliferation and invasion of cancer cells through the expression of c-met receptors. In addition, increased HGF serum levels along with high expression of serum c-met protein after hepatectomy play an important role in predicting tumor recurrence and metastasis. This can be explained by the fact that HGF can increase the production and size of both normal and malignant liver cells after surgery, leading to tumor recurrence [88].

6. Circulating RNAs

6.1 AFP mRNA

AFP mRNA is a highly valuable marker only found in active cancer cells, which might be a sign of tumor metastasis. The non-recurrence time of HCC patients with high AFP mRNA expression after surgery was shorter than the group without this marker expression in liver cells (53% compared to 88% after 1 year; 37% compared to 60% after 2 years) [89]. In the advanced HCC stage, the AFP mRNA expression rate reaches 100%, and also acts as a predictor of recurrence after liver resection. However, the use of this marker in HCC diagnosis remains controversial, possibly due to the fact that it also manifests in many other malignancies and non-cancerous liver diseases [90]. Therefore, it could be used for diagnosis and prognosis when combined with other markers.

6.2 GGT mRNA

Gamma-glutamyl transferase mRNA (GGT mRNA) can be found in the blood and peripheral liver cells of healthy individuals, as well as in patients with benign liver disease, benign liver tumors or HCC. It has 3 types: A, B and C. Type A dominates in normal liver cases, non-cancerous liver diseases, benign tumors and secondary liver cancers, while type C is produced by the yolk during pregnancy. In contrast, type B predominates in HCC [91–93]. During malignant development, expression of GGT mRNA in liver tissues may change from type A to type B [93]. Patients with HCC and high type B expression will have a worse prognosis, with higher odds of a sooner and more serious relapse [94]. Therefore, hepatocellular expression of type B mRNA may be a valuable marker for HCC patients. As in liver tissues, peripheral blood type B expression has also been reported to be significantly higher in HCC patients than in healthy adults [91].

6.3 MicroRNA (miRNA)

MicroRNAs are small non-coding RNAs that inhibit or accelerate the translation process by attenuating or increasing the synthesis of target mRNAs or by binding to additional chains in the UTR region (3′-untranslated region). In recent years, the link between miRNA and tumor development has become a controversial issue. About 500 miRNA genes have been identified and contribute to control a number of cellular processes including proliferation, differentiation and apoptosis. In malignancy, the function of miRNA is determined to be carcinogenic and tumor suppressant [95]. miRNA can regulate many genes at the same time, they control the replication process and determine the characteristics of the cell. The variety in this functional role allows miRNA to be utilized as a diagnostic marker for early detection of cancer, risk assessment, prognosis and as a new therapeutic target.

Yamamoto et al. have used a global miRNA expression profile in mouse liver development and thus shown that miR-500 (miRNA) is a potential biomarker for HCC [95]. Their work showed that miR-500 is significantly associated with the regulation of liver development and thus is related to cirrhosis progression. The serum miR-21 levels were a valuable marker in distinguishing patients with HCC from those with chronic hepatitis with the sensitivity and specificity of 61.1% and 83.3%, respectively. Compared to the healthy group, the sensitivity and specificity

were 87.3% and 92.0%, respectively. Both values are higher than serum AFP concentrations, which have been confirmed as a very valuable biological marker for HCC [96]. Serum miR-15b and miR-130b concentrations are relevant miRNA markers that are highly expressed in HCC. miR-130b has 87.7% sensitivity and 81.4% specificity. In contrast, while the sensitivity of miR-15b is high at 98.3%, its specificity is low at 15.3%. Because the sensitivity of these two factors is rather high, it can be used as a valuable marker in HCC screening and early diagnosis with low AFP levels [97].

A group of markers including seven miRNAs (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801) has been shown to have a great diagnostic performance for HBV related HCC at an early stage [98]. Although its mechanism and signal path are still unknown, the expression of miRNA-29 may increase the susceptibility of cancer cells to apoptosis and reduce the expression of Mcl-1 and Bcl-2. Indeed, it has the ability to inhibit the formation and growth of cancer cells and is a potential marker in HCC prognosis and treatment [99]. MiR-122 is a specific miRNA found only in HCC, which concentration is inversely correlated with cancer growth and likelihood of invasion and metastasis. An analysis of miRNA markers revealed only tumor miR-21 expression and significantly higher serum miR-21 levels in HCC patients compared to those in chronic liver diseases and healthy control groups. Analysis of ROC curve between HCC and control group showed that sensitivity and specificity were 87.3% and 92% respectively, which is higher than that of serum AFP. Therefore, miR-21 is also a promising marker to support early HCC diagnosis [96].

Some of their features and expressions make miRNA particularly attractive as potential biomarkers. First, many miRNAs exhibit high stability and are easily detectable in peripheral blood of HCC patients. Secondly, miRNAs can be identified in urine, which will be a valuable non-invasive biological marker in detecting and managing HCC. The detection of the expression of some miRNAs in the urine (miR-625, miR-532, miR-618, miR-516-5P and miR-650) has been used for early detection of HCC [100]. However, more research is needed regarding miRNA before it can be used to detect HCC at an early stage.

6.4 Long non-coding RNA (lncRNA)

Like other cancers, HCC is characterized by a gradual accumulation of epigenetic changes. Among these changes, lncRNA has been found to play a significant role in the initiation and progression of HCC. Most lncRNAs express the characteristics of each species and the specific characteristics of the tumor. Increased or decreased expression of lncRNA has been found in cancerous tissues. Meanwhile, some lncRNA are found in urine, blood, and other body fluids. Moreover, the use of lncRNA as a marker for cancer pathology is superior to the coding RNA protein, due to the characteristic expression of lncRNA [101]. The sensitivity and specificity of lncRNA for HCC diagnosis found in some recent studies are quite high, while it has been demonstrated that JPX (just proximal to XIST) can have a sensitivity of up to 100% [102]. The 2-lncRNA signal has a high specificity of 90.62% but a low sensitivity of 60.65%, which could make it a potential marker to confirm an HCC diagnosis [103]. Recent findings suggest that lncRNA may be a potential marker for early diagnosis and monitoring of the risk of malignant progression in patients with chronic and highly specific chronic liver disease. These markers may contribute to the definitive HCC diagnosis without the need for histopathological diagnosis (**Table 2**).

miRNA/ LncRNAs	Diagnostic value	AUC	Sensitivity	Specificity	Reference
miR-21	Differentiate HCC from patients with chronic hepatitis		61.1%	83.3%	[16]
miR-21	Differentiate HCC from healthy individuals		87.3%	92.0%	[16]
miR-130b	Differentiate HCC from healthy individuals		87.7%	81.4%	[17]
miR-15b	Differentiate HCC from healthy individuals		98.3%	15.3%	[17]
2-lncRNA	Differentiate HCC from healthy individuals	0.764	60.56%	90.62%	Yu et al. [18]
DANCR	Differentiate HCC from cirrhosis and chronic liver	0.868	83.8%	72.7%	Ma et al. [19]
MALAT1 (plasma)	Differentiate HCC from patients with liver disease	0.66	51.1%	89.3%	Konishi et al. [20]
JPX	Distinguish HCC and control group	0.814	100.0%	52.4%	Ma et al. [21]
UCA1	Distinguish HCC and control group	0,91	91,4%	88,6%	El-Tawdi et al. [22]

Abbreviation: *DANCR, Differentiation Antagonizing Non-protein Coding RNA; MALAT1, metastasis associated lung adenocarcinoma transcript 1; UCA1, urothelial cancer associated 1.*

Table 2.
Diagnostic performance of miRNAs and lncRNAs for HCC.

7. Gene mutations

7.1 Mutations in *TP53* gene

P53 is an important protein in the P53 signaling pathway and mutation or loss of *TP53* gene function leads to abnormal cell growth [104]. Notably, the mutation rate of *TP53* varies by geographic area, reflecting the etiology and epidemiological changes of HCC [105]. Mutations in the *TP53* gene, commonly found in sub-Saharan Africa and Southeast Asia, has the highest incidence of HBV infection and Aflatoxin B1 exposure. In these areas, the most common mutation is TP53 R249S, which is associated with an exposure factor of Aflatoxin B1 [106].

TP53 mutation was identified as one of the common molecular alterations in HCC, of which, the *TP53* R249S mutation in exon 7 was found in HCC patients with a high incidence. Studies suggest that the *TP53* R249S mutation may occur relatively early in areas associated with Aflatoxin exposure and chronic HBV infection [107]. The *TP53* R249S mutation was an important factor in the carcinogenesis of HCC in Brazil, where Aflatoxin exposure is high [108]. In contrast, the *TP53* R249S mutation may not play a role in causing HCC in Egypt, where HCV infection is common [109]. These findings suggest that *TP53* mutations are involved in HCC pathogenesis in individuals with chronic HBV infection, especially in those exposed to high Aflatoxin B1.

Recent reports have shown that *TP53* mutation can be used as a marker to predict HCC in high-risk groups. TP53 mutation has been shown to be associated with significantly higher relapse rates and lower disease-free survival rates [110]. It is also documented that *TP53* mutation rate is about 30% and is associated with additional survival, non-recurrent survival and disease-free survival in HCC patients, with similar results observed in patients infected with HBV and HCV [111, 112]. However, a recent study showed that the *TP53* mutation was only associated with a shorter survival time only in HBV-related HCC, while the R249S mutation was not related to the survival rate in the European patients with HCV-related HCC [113]. Growing evidence suggests that the stability of the *TP53* mutation in tumors is important for its carcinogenic activities, decreasing the expression of the *TP53* mutation that reduces malignant growth of cancer cells. Therefore, the *TP53* mutation, especially at R249S position, can be considered as one of the early markers for HCC diagnosis and is an attractive therapy for cancer treatment.

7.2 hTERT gene mutation

The telomerase reverse transcriptase (hTERT) gene encodes an enzyme that maintains the telomeric DNA length and stabilizes the chromosomes [113]. hTERT is a major determinant of telomerase activity, which plays a key role in protecting cells from apoptosis and transforming into cancerous cells [114]. The reactivation of telomerase activity in cancer may be related to changes that occur during cancer development, including mutations and rearrangements of chromosomes [115].

The frequencies of *hTERT* mutations were observed in about 60% of HCC patients [116] but vary by geographical regions being the most common in Europe (59%) and less common in East Asia (20.7%) [117]. These data indicate that *hTERT* mutation is frequently associated with HCV-related HCC. *hTERT*-promoting mutations have been found with 6% of low-grade dysplasia nodules, 19% of advanced dysplasia nodules, 61% of early HCC and 42% of intermediate and advanced HCC [118]. Another study also found *hTERT* mutation in 57% of patients with chronic hepatitis and in 30% of those with early HCC [119]. Therefore, mutations in the *hTERT* promoter occur early in the course of malignant transformation and persists during tumor development. The regulation and expression of *hTERT* play an important role in the initiation and progression of HCC. *hTERT* mutation is one of the earliest gene mutations in cancer development and is also the most common gene mutation in HCC. Therefore, *hTERT* mutation is one of the most important markers in early diagnosis and may be a promising target for HCC treatment.

7.3 Mutations in ARID1A and ARID2 genes

ARID1A and *ARID2* are two genes in the SWI/SNF complex (SWitch/sucrose non-fermentable) involved in chromosomal reconstruction. The mutation rate of the *ARID1A* and *ARID2* genes found in 10% HCC, depending on the cause. *ARID1A* mutation is associated with alcohol consumption while *ARID2* mutation is often associated with HCV infection [120]. Although the role of these mutations remains unknown, studies have shown that *ARID1A* and *ARID2* genes are associated with the growth of cancer cells through affecting several signaling pathways such as PI3K/AKT, beta-catenin and p53 mutation [121] and are thus potential markers for early HCC detection.

8. DNA methylation

In HCC, methylation can occur in two ways: total methylation and partial methylation. Total methylation affects the structural function of the nucleus by

promoting chromosome and genome instability, while partial methylation is associated with tumor suppressor genes [122]. Chronic hepatitis virus infections are the cause of DNA methylation aberrations in cancerous tissues. Although several DNA methyltransferase enzymes such as DNMT1, DNMT3A and DNMT3B have been shown to increase their expression in HCC related to hepatitis viruses, their mechanisms remain controversial and unclear [123].

p16 (CDKN2A), a tumor suppressor gene involved in cell cycle regulation, has been shown to be methylated and is related to clinical parameters in HCC [124]. A study has shown that the methylation levels of *p16* gene increased in tissue samples from cirrhosis to HCC [125]. The methylation level of *p16* gene is also associated with HBV infection, as the level of *p16* methylation is higher in patients with HBV than those without HBV, the *HBx* gene being especially involved in the methylation of the *p16* gene [126, 127]. A study on 64 HCC patients found that 77% of patients had *p16* methylation and that methylation levels were correlated to serum AFP levels [128]. In a meta-analysis on 272 HCC tissue samples, the methylation rate of p16 gene was 58.5%, much higher than those with cirrhosis and chronic hepatitis [129]. Therefore, methylation in the *p16* gene may serve as a promising molecular marker for HCC in patients with HBV infection.

Another potential marker for HCC prognosis is *SOCS1* methylation. *SOCS1* gene plays a role in modulating the JAK/STAT signaling pathway when methylation causes malignant cell proliferation. SOCS1 methylation correlates with tumor size and risk factors for HCC, it is more common in HCV and cirrhotic patients, but less common in HBV-infected groups. A study has shown that the methylation of *SOCS1* gene in peripheral blood accounted for 38% in the HCC group, 20% in the cirrhotic group and 23% in the control group without liver disease. Expression of methylation of *SOCS1* and *RASSF1A* genes in combination with serum AFP increased sensitivity to 86% and specificity to 75% for HCC diagnosis [130]. *SOCS1* methylation is quite common in HCC, and is correlated with a number of clinical parameters and other serum biomarkers like AFP. Therefore, *SOCS1* methylation in combination with serum AFP increases the sensitivity and specificity for early HCC diagnosis.

GSTP1 belongs to the Glutathione S-transferase family, which protects cells against carcinogens, regulates signaling pathways that control cell proliferation and cell death [131]. The methylation in the *GSTP1* gene promoter was observed in prostate cancer, HCC and other malignancies. GSTP1 has been shown to have a high methylation rate in HCC related to HBV or HCV infection. Interestingly, methylation of the *GSTP1* gene in HCC patients was 76.7% and those with high *GSTP1* expression had a shorter survival time [132].

Detecting the methylation status of genes in serum provides a promising method for diagnosis of HCC. A study found aberrant methylation in the *CCND2* gene in 39 out of 70 serum samples of HCC patients and methylation status was associated with a shorter disease-free survival time [133]. Yeo et al. showed that 17 out of 40 (42.5%) plasma samples of HCC patients had methylation in *RASSF1A* gene, and that methylation occurred mainly in patients with tumors ≥4 cm in size [134]. Methylation in *RASSF1A* in the serum of 85 HCC patients and found that 93% had methylation, it is associated with a shorter survival and disease stage [135]. The level of methylation in the *RASSF1A* gene of the HCC group is significantly higher compared to other liver disease groups and thus it is a promising independent marker for early diagnosis and prognosis of HCC [136].

9. Conclusion

A large number of markers have been studied and clinically applied for early diagnosis and monitoring of HCC treatment, of which serum AFP is a widely used

with a controversial diagnostic threshold. A number of protein markers such as AFP-L3 and DCP are also being applied to support HCC diagnosis with higher sensitivity and specificity compared to AFP. However, the available marker is neither specific for solely HCC diagnosis nor provides great diagnostic performance for HCC and thus a combination of several serum protein markers can improve the early diagnosis rate. With the development of molecular technology, biomarkers based on miRNA and lncRNA expression, gene mutation (*TP53, hTERT, ARID1A and ARID2*) and DNA methylation have a great potential to improve the rate of HCC diagnosis at an early stage, as well as predicting progression, metastasis and tumor recurrence. In addition, with the development of current cell technology, cancer pathways and the expression of genes specific for HCC tumor may be important markers for early detection and new targets for the treatment of HCC.

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Competing interests

All authors have no conflicts of interest to declare.

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