Progress and prospects of biomarkers in primary liver cancer (Review)

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Abstract. Tumor biomarkers are important in the early screening, diagnosis, therapeutic evaluation, recurrence and prognosis prediction of tumors. Primary liver cancer is one of the most common malignant tumors; it has high incidence and mortality rates and seriously endangers human health. The main pathological types of primary liver cancer include hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC) and combined HCC-cholangiocarcinoma (cHCC-CC). In the present review, a systematic outline of the current biomarkers of primary liver cancer is presented, from conventional blood biomarkers, histochemical biomarkers and potential biomarkers to resistance-associated biomarkers. The important relationships are deeply elucidated between biomarkers and diagnosis, prognosis, clinicopathological features and resistance, as well as their clinical significance, in patients with the three main types of primary liver cancer. Moreover, a summary of several important biomarker signaling pathways is provided, which is helpful for studying the biological mechanism of liver cancer. The purpose of this review is to provide help for clinical or medical researchers in the early diagnosis, differential diagnosis, prognosis and treatment of HCC.

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1. Introduction

Primary liver cancer is a common tumor worldwide, with a high incidence and mortality, and is a frequent cause of cancer death. Primary liver cancer mainly includes hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC) and combined HCC-cholangiocarcinoma (cHCC-CC), with incidences of 75-85, 10-15 and 1-4.7%, respectively worldwide (1,2). HCC is a malignant tumor that originates from hepatocytes and is the fifth most common cancer and the third leading cause of cancer-associated deaths (3). ICC, the second most common primary liver tumor, is a malignant tumor that originates from the biliary epithelium (4). cHCC-CC is a rare type of primary hepatic carcinoma that has the characteristics of bi-directional differentiation of hepatocytes and bile duct epithelial cells (2,5). Although these cancers have different biological behaviours, it is sometimes difficult to distinguish them by their biological characteristics. Therefore, the laboratory detection of liver cancer biomarkers, such as blood biomarkers and histochemical biomarkers, plays an important role in the early monitoring, pathological classification, treatment options and prognosis of patients with primary liver cancer. The most widely used biomarker of primary liver cancer worldwide is α-fetoprotein (AFP); however, its sensitivity and specificity are not very satisfactory (3,4). It is of great significance for the diagnosis, treatment effect observation and prognosis judgement of primary liver cancer to combine different tumor biomarkers according to different clinical conditions, and to develop new biomarkers. In the present review, current clinical and experimental studies have been summarized to highlight the progress in biomarkers, as well as some new promising biomarkers, for clinical and medical research to diagnose and guide the therapy of primary liver cancer.

2. Blood biomarkers

Blood biomarkers are of great significance in the early diagnosis of liver cancer and mainly include categories related to

proteins, cytokines, enzymes and isoenzymes as well as transcripts of associated genes (Table I). Regardless of the diverse acknowledged molecular indicators in liver cancer, each of them alone cannot be a specific biomarker in different types of liver cancer, and their combined application is important for the identification of liver cancer types.

AFP and AFP-L3. AFP is a ~70 kDa glycoprotein molecule consisting of 591 amino acids that is derived from fetal hepatocytes and the yolk sac. As a useful biomarker of liver cancer, AFP has been widely used in its diagnosis. However, AFP may also increase in hepatitis, liver cirrhosis and liver injury, especially in 10-20% of early-stage HCCs with elevated AFP-L3 levels (6). Moreover, serum AFP levels are not largely elevated in the 80% of HCC patients with small tumor sizes; the sensitivity of AFP in patients with HCC has been reported to be 52% for tumors >3 cm in diameter but only 25% for tumors <3 cm in diameter (3). The use of AFP in combination with a variety of serum biomarkers contributes to the early diagnosis of HCC, and can improve sensitivity and specificity compared with single use (3). AFP levels are not significantly changed in patients with ICC and, therefore, AFP may serve as a powerful biomarker to distinguish ICC from HCC (4). In one study, elevated serum AFP levels (>20 ng/ml) were detected in 58.3% of patients with cHCC-CC, which was slightly lower than that in patients with HCC (66.5%) and significantly higher than that in patients with ICC (13.7%) (5). Another study detected the elevation of AFP in 62.2% (28/45) of patients with cHCC-CC (7).

Due to the low specificity and sensitivity of AFP, other biomarkers are required to assist the diagnosis. AFP-L3 is a heteroplast of AFP, which is only derived from tumor tissue and is a specific biomarker for HCC. Clinical studies revealed a specificity of 90-95% and a sensitivity of ~51% for AFP-L3 in early-stage HCC detection (8). The AFP-L3 fraction is more sensitive than AFP for small-sized tumors or for patients with early-stage HCC, is highly specific for HCC and reflects tumor features such as poor differentiation or malignant invasion (9,10). AFP-L3 may serve as a supplementary test variable to improve the diagnostic value of HCC detection in patients with relatively low AFP levels (9). The specificity and sensitivity of a combination of AFP and AFP-L3 were found to be 79 and 87%, respectively, in the diagnosis of HCC (11). Serum AFP-L3 levels and the percentage of AFP-L3 in total AFP (AFP-L3%) can be efficiently applied to distinguish HCC from benign liver diseases and to diagnose HCC early in the clinic (12). The sensitivity of AFP-L3% has been reported to be 35-45% for HCC tumors with a diameter <2 cm, and 80-90% for HCC tumors with a diameter >5 cm, respectively, with variation according to clinical features (13).

Des-γ-carboxyprothrombin (DCP). DCP is a new serological biomarker of HCC, which is also known as prothrombin-induced by vitamin K absence-II, and is produced in HCC cells due to a defect in the carboxylation of the prothrombin precursor after translation and is elevated in patients with HCC (14,15). In one study, DCP exhibited higher sensitivity than AFP; the sensitivity and specificity of DCP were 85 and 75%, respectively (15). In another study, DCP exhibited significantly improved results compared with AFP and AFP-L3 in the diagnosis of HCC, with sensitivity and specificity up to 86 and 93%, respectively, for distinguishing HCC from cirrhosis (16). DCP has served as an effective biomarker for the diagnosis of HCC in Japan, South Korea and India, particularly for judging intrahepatic metastasis and prognosis (17). Studies have demonstrated that elevated DCP levels are indicative of larger tumor size, greater tumor numbers, a later clinical phase, bile duct invasion, vascular invasion and a shorter median survival time (14,18). A meta-analysis suggested that DCP should serve as an indicator of HCC in the established guidelines of other countries and regions, especially those with a high incidence of hepatitis B virus (HBV) infections, such as East Asia (with the exception of Japan) and Africa (19). A combination of DCP and AFP enhanced the sensitivity of HCC diagnosis to >80 and 70%, respectively, in tumors 3-4 and 2-3 cm in diameter (20).

α-L-fucosidase (AFU). AFU is characterized as a lysosomal enzyme in all mammalian cells, enabling the degradation of a variety of fucose-containing fuco-glycoconjugates (21,22). AFU has been demonstrated to serve as a useful biomarker for HCC, and the serum level of AFU in patients with HCC has been found to be higher than that in patients with benign hepatic diseases (23). AFU is considered as an earlier biomarker, able to diagnose 85% of patients with HCC 6 months prior to detection by ultrasonography (24). Serum AFU activity can return to normal after liver transplantation and successful intervention for HCC, while AFU is also elevated with the recurrence of HCC; therefore, AFU can serve as a follow-up biomarker for patients with HCC (21). The preoperative AFU level has been suggested to be powerful in the prediction of tumor recurrence and mortality in HCC patients with low levels of AFP (21). The AFU level is positively associated with tumor size in patients with HCC and can be combined with AFP for the early diagnosis of HCC (8).

Golgi protein 73 (GP73). GP73 is a transmembrane glycoprotein of resident Golgi type II, with a molecular mass of ~70 kDa, and is expressed mainly in the epithelial cells of numerous human tissues (25). GP73 is hardly expressed in healthy subjects, but its expression is moderately increased in patients with cirrhosis and viral infection, and markedly increased in patients with HCC (25-27). A study reported that the sensitivity and specificity of GP73 for HCC were 74.6 and 97.4%, respectively, markedly higher in comparison with 58.2 and 85.3% for AFP (28). Another study revealed that the sensitivity and specificity of serum GP73 were each 95% for Egyptian patients with early HCC, and also suggested that GP73 was superior to AFP for the early diagnosis and examination of HCC (29). Furthermore, GP73 has been shown to be a powerful biomarker with a sensitivity, specificity and accuracy of 66, 96.2 and 84.6%, respectively, for the diagnosis of small HCC with negative AFP, which supports its potential contribution to the diagnosis of small HCC (30).

Osteopontin (OPN). OPN is a highly modified, phosphorylated and glycosylated protein of extracellular matrix that binds to integrin and is expressed in a variety of cells, such as those of the immune system, epithelial tissues, smooth muscle cells, osteoblasts and tumors (31,32). In one study, the prevalence of autoantibodies against OPN was found to be 12.8, 15.6 and

Table I. Blood and histochemical biomarkers used for liver cancer.

Biomarkers	B/T	Positive rate (%)	Sensitivity (%)	Specificity (%)	Type	Significance	(Refs.)
AFP	B	62.2	52 (>3 cm); 25 (<3 cm)	-	HCC, cHCC-CC	Diagnosis	(3,7)
AFP/AFP-L3	B	-	87	79	HCC	Diagnosis	(11)
AFP-L3%	B	-	35-45 (<2 cm); 80-90 (>5 cm)	-	HCC	Diagnosis	(13)
DCP	B	-	86	93	HCC	Diagnosis and indicator	(16)
DCP and AFP	B	-	80 (3-4 cm) 70 (2-3 cm)	-	HCC	Diagnosis	(20)
AFU	B	85	-	-	HCC	Early diagnosis	(24)
GP73	B	-	95	95	Early HCC	Diagnosis of Egyptian patients	(29)
		-	66	96.2	Small HCC with negative AFP	Diagnosis	(30)
AFP and OPN	B	-	65	-	HCC	Diagnosis	(32)
CA19-9	B	-	90	98	ICC	Diagnosis	(37)
GPC-3	T	-	-	97	HCC	Diagnosis, prognosis	(48,51)
Hep Par 1	T	-	80	80	HCC	Diagnosis	(56)
GS	T	-	100	90	HCC under a cirrhotic background	Diagnosis	(65)
Arg-1	T	-	-	100	HCC with TMA and FNA	Diagnosis	(67)
HSP70	T	-	-	-	Early and small HCC	Diagnosis	(57,58)
CK7	T	-	-	-	ICC	Diagnosis	(72,73)
CK19	T	-	-	-	ICC	Diagnosis	(72,73)

B, blood; T, tissue; AFP, α-fetoprotein; DCP, des-γ-carboxyprothrombin; AFU, α-L-fucosidase; GP73, Golgi protein 73; OPN, osteopontin; CA19-9, carbohydrate antigen 19-9; GPC-3, glypican-3; Hep Par 1, hepatocyte paraffin 1; GS, glutamine synthetase; Arg-1, arginase-1; HSP70, heat shock protein 70; CK, cytokeratin; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; cHCC-ICC, combined HCC-cholangiocarcinoma.

3.1% in the serum of patients with HCC, liver cirrhosis and chronic hepatitis, respectively, compared with 0% in the serum of healthy individuals, and when OPN was combined with AFP to diagnose HCC, the sensitivity increased to 65% (32). In another study, OPN levels were shown to be much higher in patients with hepatitis C virus-associated HCC compared with healthy subjects and patients with chronic liver diseases (33). Furthermore, levels of serum OPN were found to be elevated a year prior to HCC diagnosis, with OPN indicated to be superior to AFP in differentiating cases of HCC from those of liver cirrhosis (33). A study using an OPN-knockout HCC mouse model revealed that OPN induced chemotactic migration, alternatively activated macrophages, increased tumor-associated macrophage infiltration and upregulated the expression of programmed death ligand 1 in HCC through activation of the colony stimulating factor-1 (CSF1)-CSF1 receptor pathway in macrophages (34). OPN may also be considered for use in the monitoring of microvascular invasion in chronic hepatitis B (CHB)-associated HCC, as it has been found to be upregulated in patients who did not receive antiviral therapy compared with antiviral-treated patients, and exhibited an association with tumor aggressiveness and poor prognosis (35).

Carbohydrate antigen 19-9 (CA19-9). CA19-9 was originally identified in the culture medium of a human colorectal cancer cell line and is widely used to diagnose various adenocarcinomas, including cholangiocarcinoma (36); it is a significant serological biomarker in ICC. A study revealed that the sensitivity and specificity continuously increased with increasing CA19-9 serum concentration; when the serum level of CA19-9 was >632 U/ml, its sensitivity and specificity were 90.0 and 98.0%, respectively (37). In addition, the preoperative CA19-9 level can serve as an independent prognostic factor in patients with ICC (38). A study demonstrated that preoperative serum CA19-9 levels may be used to predict lymph node metastasis in patients with ICC (39).

At present, the triple detection of DCP, AFP and AFP-L3% is a common serum biomarker combination for the early diagnosis of HCC. The detection rate of HCC can be increased to 85.9% by the combined detection of these three biomarkers (40). In the prognostic evaluation of HCC, the higher the AFP, AFP-L3% and DCP levels, the lower the survival rate and the higher the recurrence rate (40). The increase in the three biomarkers is associated with the invasiveness of the tumors (40). DCP and AFP-L3% are positively associated with the size and stage of the tumors (40). A triple examination of cancer using DCP, AFP and AFP-L3% is helpful for the differential diagnosis of intrahepatic nodules. Authoritative guidelines and consensus, such as those of the Asia-Pacific Society of Hepatology (41), the Japan Society of Hepatology (42), Guidelines for the Prevention, Care and Treatment of Persons with Chronic Hepatitis B Infection (2015) (43) and Guidelines for Diagnosis and Treatment of Primary Liver Cancer in China (2017) (44), recommend DCP, AFP and AFP-L3% for the screening of high-risk populations, the auxiliary diagnosis of liver cancer, the monitoring of therapeutic effect and as a predictor of prognosis and recurrence. In conclusion, AFP is of great value in the diagnosis and prognosis evaluation of HCC. DCP and AFP-L3, as classical serological indicators in the diagnosis of HCC, are complementary to AFP.

3. Histochemical biomarkers

Pathological diagnosis is the gold standard of primary liver cancer, whose main detection methods include hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC). Tissue biomarkers are the true reflection of tumor tissue structure and cell morphology, and are more objective and accurate than various imaging examinations and chemical examinations (Table I). Therefore, tissue biomarkers serve an important role in the diagnosis, differential diagnosis, treatment and prognosis of patients with benign tumors, HCC, ICC and cHCC-CC (Table II).

Glypican-3 (GPC-3). GPC-3 is a membrane-bound heparan sulfate proteoglycan that belongs to the glypican family; it has an amino terminal protein and a membrane-bound carboxyl terminal protein (45,46). GPC-3 anchors at the glycosylphosphatidylinositol of the membrane by the C-terminus; can be enzymatically lysed, releasing a soluble form of GPC-3; and is released into the serum by a lipase called notum (46) (Fig. 1). GPC-3 is overexpressed in up to 80% of patients with HCC and is able to distinguish HCC from ICC and other malignant tumors (47). GPC-3 is a promising biomarker for HCC with a specificity of up to 97%; it modulates cell-cycle progression, and promotes cellular migration and invasiveness in HCC cell lines (48,49). GPC-3 is specifically expressed in the HCC tumor tissue and is widely used to distinguish HCC from ICC. GPC-3 expression is associated with differentiation grade, and exhibits higher expression in moderately differentiated and poorly differentiated HCC than in well-differentiated HCC (47). A study revealed that GPC-3 is overexpressed in HCC tissue and can serve as a sensitive and specific biomarker to diagnose early HCC (50). Clinicopathological analysis demonstrated that the overexpression of GPC-3 was associated with poor postoperative disease-free survival and overall survival (OS) and that it was an independent risk factor (51). GPC-3 is associated with HBV infection, TNM stage, periportal cancerous embolus and extrahepatic metastasis, and can serve as a prognostic factor (51). GPC-3 is not only a powerful histochemical biomarker but also a serological biomarker. A study showed that the serum GPC-3 level was >300 ng/l in 50% of early HCC patients, although their serum AFP level was <100 μg/l, revealing that the serum GPC-3 level can be used to monitor early HCC and may serve a role in the diagnosis of HCC (24). Recently, a simple 2D imaging probe with minimal background fluorescence and high binding affinity for GPC-3 was developed, which has been shown to sensitively and selectively image HCC cells and a normal cell line overexpressing GPC-3, as well as to effectively differentiate between HCC-positive and para-carcinoma tissue regions (52).

Hepatocyte paraffin 1 (Hep Par 1). Hep Par 1 was first identified in a formalin-fixed failed allograft liver and is a monoclonal antibody that is highly sensitive and specific for hepatocellular differentiation (53-55). Hep Par 1 is a mitochondrial urea cycle antigen associated with mitochondrial antigens from malignant and non-malignant hepatic

Table II. Histochemical biomarkers with clinicopathological associations.

Biomarkers	Type	Expression level	Associations	(Refs.)
GPC-3	HCC	High	Cell-cycle, migration, invasiveness, differentiation grade, HBV infection, TNM stage, periportal cancerous embolus, extrahepatic metastasis, poor postoperative DFS and OS	(47-49,51)
Hep Par 1	HCC	High	Hepatocellular differentiation	(53-55)
HSP70	Small and early HCC	High	Vascular invasion, high stage, low grade, poor differentiation, cell proliferation, lymph node metastasis, high Ki-67 index and larger tumor size and portal vein invasion	(58-60)
GS	HCC	High	β-catenin gene mutations, higher recurrence and lower survival	(63)
Arg-1	HCC	High	Hepatocellular differentiation	(69,70)
CK7	ICC	High	Aggressive tumor phenotypes and adverse OS	(73)
CK19	ICC	High	Aggressive tumor phenotypes and adverse OS	(73)
CK7/CK19 index	ICC	High	OS	(73)

GPC-3, glypican-3; Hep Par 1, hepatocyte paraffin 1; HSP70, heat shock protein 70; GS, glutamine synthetase; Arg-1, arginase-1; CK, cytokeratin; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; HBV, hepatitis B virus; DFS, disease-free survival; OS, overall survival.

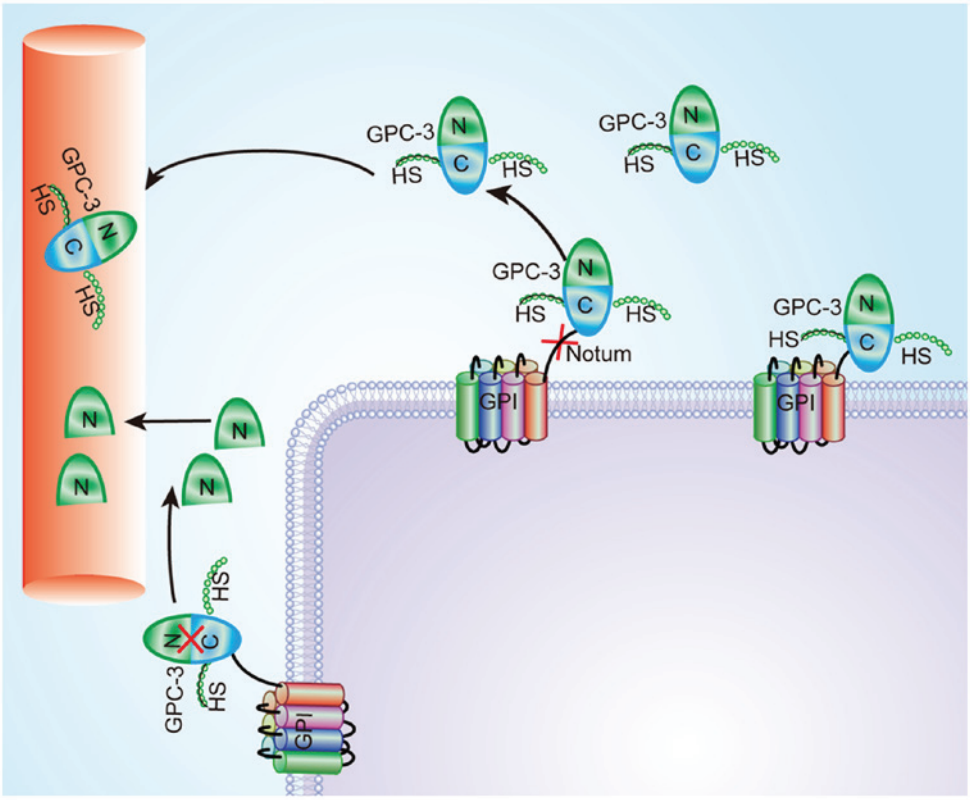

Figure 1. Secretion of GPC-3. GPC-3 is a membrane-bound HS proteoglycan that belongs to the glypican family; it anchors at the GPI of the membrane by the C-terminus; it can be enzymatically lysed, releasing a soluble form of GPC-3; and is released into the serum by the lipase Notum. GPC-3, glypican-3; HS, heparan sulfate; GPI, glycosylphosphatidylinositol.

cells (54). A study reported that the rate of Hep Par 1 expression was 100% in well-differentiated and moderately differentiated HCC (55). Another report revealed that Hep Par 1, whose sensitivity and specificity were both 80% in

Table III. miRNA biomarkers used for liver cancer.

Biomarkers	Type	Expression level	Associations	(Refs.)
miR-21 and miR-10b	HCC	High	Proliferation, migration, and invasion, advanced tumor stage, HIF-1α/ HIF-2α expression and disease-free survival	(82)
miR-122	HCC	Low	AFP, ALT, AST and ALP levels, and tumor size	(83)
miR-224	HCC	High	AFP, ALT, AST, ALP levels and tumor size	(83)
miR-1204	HCC	High	Proliferation, tumor growth, tumor size and advanced TNM stage, inhibited apoptosis	(84)
hsa-miR-210	HCC	High	AFP level, pathological grade, TNM stage, tumor stage and vascular invasion	(85)
miR-221	HCC	High	Clinical TNM stage, tumor capsular infiltration and poor. prognosis	(86)
miR-191	ICC	High	Proliferation, invasion and migration	(87)
miR-424-5p	ICC	Low	Invasion and migration	(88)

miR, microRNA; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; HIF, hypoxia inducible factor; AFP, α-fetoprotein; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase.

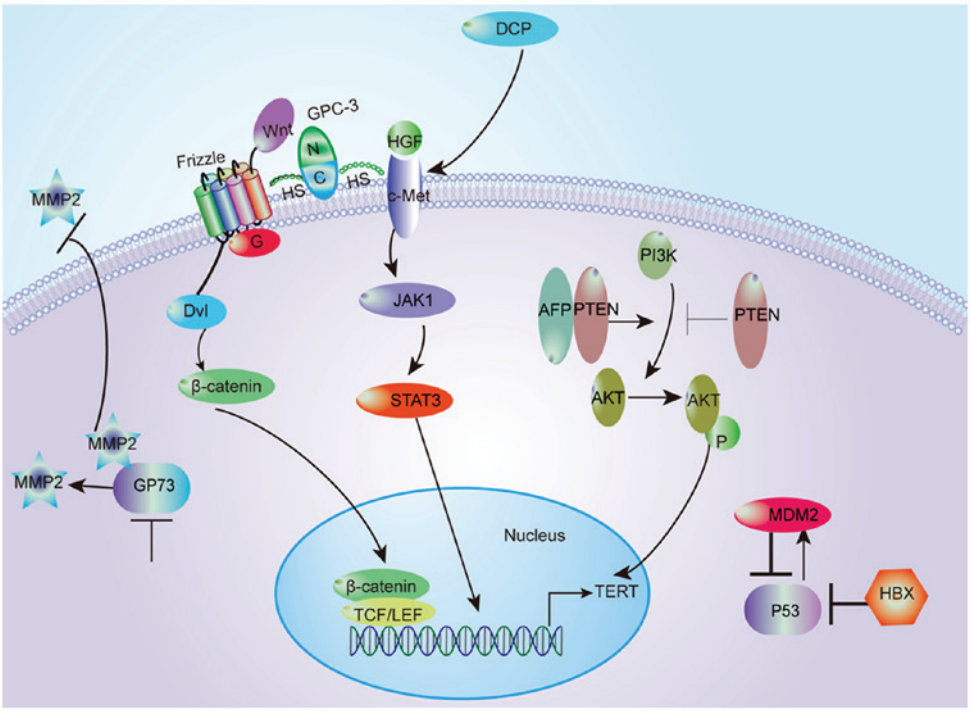

Figure 2. Biomarker-associated signaling pathways. GPC-3 can combine with Wnt to stimulate nuclear/cytoplasmic β-catenin. DCP activates the c-Met-Janus kinase 1-STAT3 kinase cascade. AFP interacts with PTEN to activate PI3K/AKT/mTOR signaling. PTEN downregulation also activates AKT. The dynamic balance between MDM2 and p53 is destroyed. The knockdown of GP73 suppresses the secretion of MMP2 by p53 signaling. GPC-3, glypican-3; DCP, des-γ-carboxyprothrombin; AFP, α-fetoprotein; PTEN, phosphatase and tensin homologue deleted on chromosome 10; PI3K, phosphatidylinositol 3-kinase; MDM2, murine double minute 2; GP73, Golgi protein 73; MMP2, matrix metalloproteinase-2.

well-differentiated HCC, was more likely to be negative in poorly differentiated and sclerosing HCC (56). Hep Par 1 was found to have low sensitivity in poorly differentiated HCC, and was not easily able to distinguish between HCC and adenocarcinoma (53). Therefore, Hep Par 1 can be combined with other biomarkers in morphologically difficult cases with poorly differentiated HCC and metastatic carcinoma of the liver to determine diagnoses.

Heat shock protein 70 (HSP70). HSP70 is a member of a highly conserved protein family, and is expressed at low levels under normal conditions but serves a significant role in response to heat shock, hypoxia, genotoxic agents and nutrient starvation (57,58). HSP70 is a useful histochemical biomarker that is nucleocytoplasmic and mostly focally stained in HCC by IHC (57). Clinically, HSP70 is considered an indispensable biomarker that is significant clinically in distinguishing

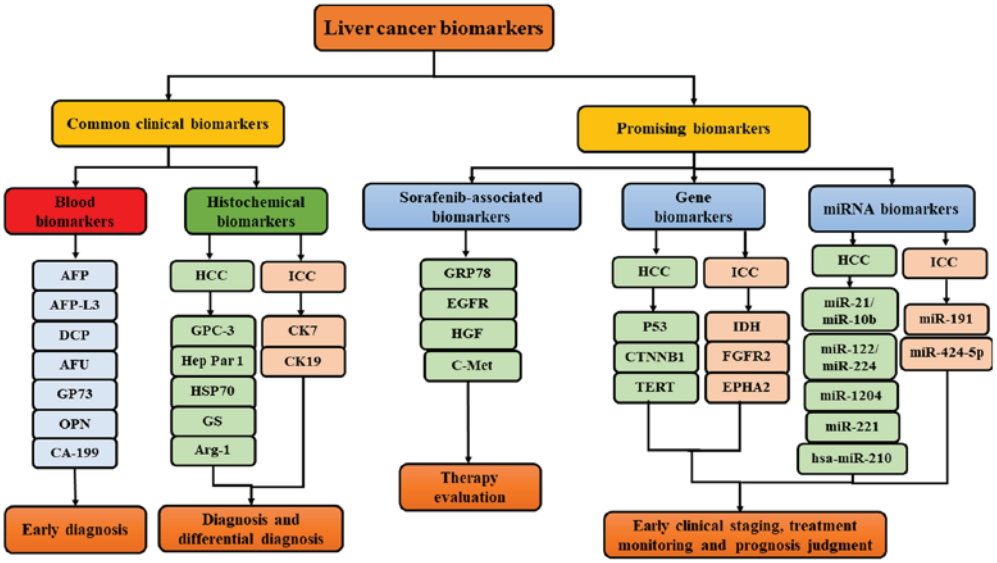

Figure 3. Biomarker profile of primary liver cancer. Liver cancer biomarkers are systematically summarized, and mainly include common clinical biomarkers, resistance-associated biomarkers and promising biomarkers, contributing to the diagnosis, differential diagnosis and treatment of patients with HCC and ICC. HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma.

the diagnosis of well-differentiated small HCC (sHCC) from that of high-grade dysplastic nodules in liver biopsies and surgical specimens (57). A study identified that HSP70 served as the most highly upregulated gene in early HCC tissue sections (58). HSP70 is associated with vascular invasion, high stage, low grade, poor differentiation, cell proliferation, lymph node metastasis, high Ki-67 index and larger tumor size in HCC (59). However, another study reported that the overexpression of HSP70 was associated with portal vein invasion, but could not predict the overall survival of patients with HCC (60).

Glutamine synthetase (GS). GS is an enzyme of nitrogen metabolism that catalyzes the conversion of glutamate and ammonia to glutamine in the liver. GS is expressed in hepatocytes surrounding the terminal hepatic venues under normal conditions, but is diffusely located in hepatocellular tumors (61,62). Glutamine provides energy to tumor cells and so GS is diffusely expressed in HCC; the expression of HCC is closely associated with β-catenin gene mutations and the Wnt signaling pathway. GS positivity may imply specific epidemiological and genetic profiles for a subtype of HCC, which includes larger size, low grade, pseudoacini, hydropic changes, bile staining, lack of steatosis, and fibrosis, as well as tumor-specific and overall survival for HCC (62). GS can act as a detector of HCC recurrence, and its upregulation contributes to the increase in metastasis and markedly shorter disease-free survival time in HCC (63). GS and HSP70 positivity are more likely be observed in very well-differentiated HCC than in atypical neoplasms, and may be powerful biomarkers to distinguish between very well-differentiated HCC and atypical cases (64). Strikingly, in a study of cirrhotic and non-cirrhotic livers, the sensitivity of GS was 100% for HCC in cirrhotic livers, and the specificity was 90% (65). However, another study reported that HSP70 and GS immunoreactivity did not effectively identify tumor cell origin for ICC (66).

Arginase-1 (Arg-1). Arg-1, an enzyme associated with the hydrolysis of arginine to ornithine and urea, has been observed to be a highly sensitive and specific biomarker for benign and malignant hepatocyte (67). Arg-1 is a powerful immunohistochemical and biomarker for HCC, is an enhancer of zeste homologue 2, and is considered a useful biomarker for hepatocellular differentiation (68-70). Arg-1 has been shown to be a more sensitive biomarker compared with Hep Par 1 in poorly differentiated HCC, as the latter is sometimes expressed in other non-hepatic tumors (69). Similar results in another study showed that Arg-1 had a higher sensitivity than Hep Par 1 for moderately differentiated and poorly differentiated HCC (70). A comparison of different markers using tissue microarray and fine-needle aspiration specimens revealed that Arg-1 is a superior specific biomarker for HCC, and its specificity was 100% in a large series of surgical cases (n=1,222) (67).

Cytokeratin (CK)7 and CK19. CK7 and CK19 are members of the cytokeratin family, which are intermediate filament proteins that are mainly expressed in epithelial cells and belong to type II (CK1-8) and type I (CK9-20), respectively. CK7 and CK19 are primarily located in pancreatic ducts, mammary gland ducts and liver bile ducts but are absent from hepatocytes (71). Therefore, CK7 and CK19 can be used as immunohistochemical biomarkers to distinguish ICC from HCC (72), as well as biliary epithelial differentiation. A study found that CK7 and CK19 were highly expressed in patients with ICC, which was closely associated with aggressive tumor phenotypes and adverse OS (73). In addition, compared with the expression of either CK7 or CK19 alone, the CK7/CK19 index was indicated as a superior independent prognostic factor for ICC. The patients with both high CK7 and CK19 expression, heterogeneous high CK7 or CK19 expression, and both low CK7 and CK19 expression had the lowest, intermediate and highest 5-year survival rates, respectively. Furthermore, when combined with the CK7/CK19 index and clinical-pathological

risk factors, CK7 and CK19 were found to predict OS more accurately than traditional staging systems (73).

In summary, the commonly used hepatocyte markers are Hep Par-1, GPC-3, Arg-1 and GS, and the common bile duct cell biomarkers are CK7 and CK19. A reasonable combination of immunohistochemical biomarkers is needed to differentiate HCC from ICC and primary HCC from metastatic HCC.

4. Potential biomarkers

Although classical biomarkers of HCC have been routinely used, false positive results in their diagnosis of HCC occur, and their sensitivity and specificity are also insufficient. It is urgently necessary to develop more accurate and effective biomarkers for the early clinical staging, treatment monitoring and prognosis judgement of patients with HCC.

Gene biomarkers. The mutation of p53 is highly prevalent in human cancers, but its mutation is not always associated with evidently altered p53 protein expression in HCC (74). However, mutation of p53 is associated with tumor size, differentiation degree, TNM stage and vascular invasion, as well as to poor prognosis, angiogenesis, metastasis and resistance to standard therapies; therefore, the detection of the mutant p53 gene is significant for HCC (74,75). CTNNB1, encoding β-catenin, not only is one of the more commonly mutated genes for Wnt signaling activation in HCC but also is associated with mutations in telomerase reverse transcriptase (TERT) promoters (76). CTNNB1-mutated HCCs have been found to be well-differentiated, cholestatic, chromosomally stable and almost never steatotic, associated with an improved prognosis compared with other HCCs, and to usually display microtrabecular and/or acinar growth patterns (75). Mutation of TERT frequently occurs in HCC, suggesting that TERT overexpression is a significant risk factor in hepatocarcinogenesis (75,76). TERT upregulation with HCC has been found to be observably associated with intrahepatic metastasis but not markedly associated with other clinicopathological parameters, and therefore may only serve as a crucial prognostic indicator for late intrahepatic metastasis in HCC treated with curative resection (77). Isocitrate dehydrogenase (IDH) is the enzyme responsible for the conversion of isocitrate to α-ketoglutarate in the cytosol (IDH1) and mitochondria (IDH2). In one study, IDH1 and IDH2 mutations were observed in 10% of patients with ICC, were independently associated with a longer time to tumor recurrence after intrahepatic cholangiocarcinoma resection, and also exhibited an association with p53 overexpression in ICC (78). Fibroblast growth factor receptor (FGFR)2 is a member of the FGFR family that is stimulated by binding with fibroblast growth factors and functions as a serine/threonine kinase by activating the downstream Ras-MAPK-ERK pathway (79). FGFR2 gene fusion has been detected in 10-16% of ICCs, particularly in younger patients (≤40 years) with a non-obvious tendency for female sex; it affects the outcomes of patients with ICC, and contributes to the antitumor activity and manageable safety profile of derazantinib (80). Mutations of EPH receptor A2 (EPHA2), a member of the tyrosine kinase family, have been found to be frequent in ICC and exhibit a close association with lymph node metastasis, poorer differentiation, higher metastatic ability and angiogenesis in patients with ICC (81).

miRNA biomarkers. microRNAs (miRNAs) are endogenously expressed, highly conserved, small, noncoding RNA molecules, which are considered as promising candidates with non-invasive biomarkers for HCC and ICC (Table III). Exosomal miR-21 and miR-10b, hypoxia inducible factor (HIF)-1α and HIF-2α are activated via the acidic microenvironment of HCC, which further stimulates HCC cell proliferation, migration and invasion *in vivo* and *in vitro*. Furthermore, serum exosomal miR-21 and miR-10b levels exhibit an association with advanced tumor stage and HIF-1α and HIF-2α expression, which are also independent prognostic parameters for disease-free survival in early-stage HCC patients (82). A study found that plasma levels of miR-122 were significantly lower in patients with HCC compared with healthy controls and patients with chronic hepatitis C, while the expression of miR-224 was significantly higher (83). Furthermore, miR-122 and miR-224 were both directly associated with AFP, alanine transaminase, aspartate transaminase and alkaline phosphatase levels and the size of the tumor; therefore, they could be considered as noninvasive biomarkers for early diagnosis in the early stage of progressive HCC (83). In another study, miR-1204 levels were observed to be elevated in HCC tissues and cell lines, which promoted cell proliferation *in vitro* and tumor growth *in vivo* as well exhibiting an association with malignant clinical features, such as tumor size and advanced TNM stage, and inhibiting apoptosis *in vitro* (84). hsa-miR-210 has been identified as an independent prognostic factor, which was significantly overexpressed in venous metastasis positive HCC samples and associated with AFP level, pathological grade, TNM stage, tumor stage and vascular invasion (85). Recently, a study showed that miR-221 was upregulated in HCC tissues, cell lines and the blood of patients with HCC, and that miR-221 upregulation was associated with clinical TNM stage, tumor capsular infiltration and poor prognosis. The findings of the study suggested that combined serum miR-221 and AFP detection exhibits an improved performance than either alone for the early diagnosis of HCC (86). In patients with ICC, miR-191 is distinctly elevated in ICC tissue compared with adjacent normal bile duct tissues, and the overexpression of miR-191 has been shown to induce the proliferation, invasion and migration of cholangiocarcinoma cells *in vitro* and *in vivo* via the miR-191/TET1/p53 pathway (87). miR-424-5p has been shown to play different roles in the proliferation and metastasis of various tumors, acting as a suppressor or promotor (88). However, miR-424-5p has been found to be frequently downregulated in ICC tissues compared with adjacent normal tissues and in ICC cells compared with normal bile duct cells, with miR-424-5p knockdown inhibiting invasion and migration in ICC by targeting ARK5; therefore, the restoration of miR-424-5p expression has been suggested to be a promising strategy for ICC therapy (88).

5. Resistance-associated biomarkers

Sorafenib has been considered the standard of care for patients with advanced unresectable HCC since 2007; however, in a proportion of patients it is ineffective in clinical

application (89-91). Effective drug resistance biomarkers to guide clinical drug use are lacking. Glucose-regulated protein78 (GRP78) is an immunoglobulin heavy chain binding protein of the HSP70 family. A study found that autoantibodies against GRP78 may be promising diagnostic biomarkers for HCC, particularly when used in conjunction with AFP, with a sensitivity of 71.4% (90). In an *in vitro* study, GRP78 was found to increase the acquisition of sorafenib resistance in HCC, and GRP78 knockdown increased the efficacy of sorafenib-mediated cell death (91). Another study demonstrated that secreted GRP78 was associated with epidermal growth factor receptor (EGFR) via the EGFR-SRC-STAT3 signalling pathway, which conferred resistance to sorafenib (92). EGFR is a member of the HER/ErbB family of receptor tyrosine kinases, which is highly expressed in HCCs and associated with aggressive tumors, metastasis and poor patient survival significantly lower than that in patients with nonmetastatic HCC (93). EGFR may serve as a potential predictor of the resistance of HCC cells to sorafenib. A low expression level of EGFR or inhibition of the kinase activity of EGFR has been shown to increase the sensitization of HCC cells to sorafenib (94). EGFR is also overexpressed in ICC, with reported positivity rates ranging from 10 to 80% (95). Hepatocyte growth factor (HGF) is a member of the peptidase S1 family of serine proteases, and its receptor is c-Met, which is a single-pass tyrosine kinase receptor. c-Met has been observed to be overexpressed in >80% of patients with HCC, which is associated with poor prognosis and short survival (96). A study reported that, compared with sorafenib-sensitive HCC, patient specimens of sorafenib-resistant HCC exhibited increased levels of activated p-Met (97). Furthermore, c-Met expression was found to be expressed at markedly high levels in sorafenib-resistant HCC cells compared with their sorafenib-sensitive counterparts, and sorafenib treatment increased the production of HGF and the phosphorylation of c-Met (98). After 10 years of research, there are still no established drug resistance biomarkers to guide the clinical use of sorafenib in HCC. It is possible that the application of omics technology combined with bioinformatics to examine the association between patient outcome and resistance biomarker response to sorafenib could be an alternative approach in finding new biomarkers for HCC (89).

6. Biomarker-associated signaling pathways

Biomarker-associated signaling pathways contribute to revealing the mechanism of the occurrence and development of HCC. The present article summarizes the biomarker-associated signaling pathways involved in HCC progression, with a focus on the Wnt signaling pathway, c-Met signaling pathway, phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway and p53 signaling pathway (Fig. 2).

Wnt signaling pathway. Approximately 50% of HCCs are regulated by the Wnt/Frizzled/β-catenin pathway, and Wnt signaling is potentially activated in up to 95% of HCCs (99). GPC-3 can combine with Wnt and its receptor Frizzled to stimulate the Wnt signaling pathway, which is intimately associated with the nuclear/cytoplasmic localization of β-catenin (46). The activation of β-catenin and the aberrant expression of GS have intimate associations in HCC. A study found that a nuclear presence of β-catenin was associated with GS expression in all tumors, and 84% of HCCs showed the cytoplasmic presence of β-catenin associated with GS expression (100). In another study, Wnt activation was detected in HCC tissues and categorized into two subclasses, namely a CTNNB1 class and a Wnt-transforming growth factor (TGF)-β class (101). The CTNNB1 class was characterized by CTNNB1 mutations, nuclear β-catenin positivity, and tumor diameter >3 cm, while the Wnt-TGF-β class was characterized by TGF-β activation, cytoplasmic β-catenin positivity, vascular invasion, satellitosis and an increased risk of early recurrence after surgical resection (101).

c-Met signaling pathway. HGF/c-Met signaling is activated in HCC and promotes hepatocyte proliferation and regeneration. HGF and c-Met are upregulated in HCC, which is associated with early recurrence, metastasis and worse overall survival, as well as the inhibition of HCC apoptosis which facilitates HCC progression (102). Activation of the DCP-c-Met-Janus kinase 1-STAT3 kinase cascade stimulates HCC growth (13). Furthermore, GPC-3 has been shown to control the migration and motility of HCC cells via heparan sulfate chain-mediated growth combined with the HGF/Met pathway (103).

PI3K/AKT signaling pathway. The PI3K/AKT pathway is frequently activated in human cancer, including HCC, and regulates cell proliferation, metabolism, invasion, metastasis and resistance to various treatments (104,105). Phosphatase and tensin homologue deleted on chromosome 10 (PTEN), an inhibitor of the PI3K/AKT pathway in tumors, is often inactivated in HCC (105). TERT has been demonstrated to influence aberrant DNA methyltransferase 3B expression/aberrant DNA via PTEN downregulation and AKT overexpression to promote HCC development and progression (104). Furthermore, AFP suppresses autophagy and apoptosis and contributes to the promotion of proliferation, migration and invasion in HCC by interacting with PTEN and activating PI3K/AKT/mTOR signaling (105).

p53 signaling pathway. Mutations of the p53 signaling pathway are common in HCC, and the combination of p53 with hepatitis B virus X-protein in the cytoplasm has been shown to promote the development of HCC (106). p53 is principally controlled by the E3 ubiquitin ligase murine double minute 2 (MDM2). There is a dynamic balance between MDM2 and p53 in normal conditions, whereas in HCC cells, the balance is destroyed, and MDM2 is overexpressed and p53 is downregulated (106). Furthermore, a study demonstrated that GP73 knockdown promoted the accumulation of intracellular matrix metalloproteinase-2 (MMP2), suppressed MMP2 secretion and further inhibited invasion in HCC cells by inhibiting p53-p21 signaling pathways via a negative feedback loop (26).

7. Conclusions

The key to solving the difficulties in the treatment of primary liver cancer mainly lies in early diagnosis and treatment.

Biomarkers serve an important role in diagnosis, differential diagnosis and prognosis; they enable targeted therapy and also provide benefits for the progress of disease research. Early diagnosis is very important for the prognosis and treatment of patients with liver cancer, and the sensitivity and specificity of tumor biomarkers are of great significance for the early diagnosis of liver cancer. The combined application of several biomarkers is conducive to the early diagnosis of liver cancer. At present, AFP, AFP-L3 and DCP are common serum biomarkers used to diagnose liver cancer earlier in internal and external clinical settings. Recently, numerous studies have investigated novel serum biomarkers, such as AFU, GP73 and OPN, to provide accurate diagnosis and early treatment for patients. The final diagnosis and pathological classification of primary liver cancer have been achieved using H&E staining and IHC, and confirmed via histochemical biomarkers, including classical biomarkers (GPC-3, Hep Par 1, HSP70, GS, Arg-1, CK7 and CK19). These biomarkers indicate the differentiation degree, histological type, clinicopathological features and prognosis. Furthermore, a number of studies have shown that gene-targeted therapies have a good curative effect. Notably, integrated genomic studies have revealed potential therapeutic targets in HCC and ICC, including CTNNB1 and IDH, respectively. Therefore, genetic biomarkers in patients with liver cancer favor individualized gene-targeted therapy and improve prognosis. In the current review, various tumor biomarkers are summarized in different liver cancers, such as HCC, ICC and cHCC-CC (Fig. 3). Novel tumor biomarkers may be used in clinical practice in the near future, which brings new hope for the early diagnosis and accurate treatment of patients with liver cancer. Targeted drugs are being studied for some biomarkers, and individualized treatment is conducted for patients to improve their quality of life and survival. Further study of biomarker-related signaling pathways will contribute to the deep exploration of the mechanism underlying the occurrence and development of HCC. The development of biotechnology is also prompting researchers to use modern technology to improve the sensitivity of diagnostic biomarkers of HCC to achieve the early diagnosis of HCC, increase the cure rate and reduce mortality.

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YXG wrote the manuscript, searched literatures and made figures and lists . XNL and DXC provided article ideas, modified the lists and revised the manuscript.. TWY, JMY, PXY, BXK and MYC performed literature research and collected relevant articles. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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