Molecular diagnosis of hepatocellular carcinoma: trends in biomarkers combination to enhance early cancer detection

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Abstract

Despite of the advances in clinical imaging and applied research in proteomic biomarkers, liver cancer, especially hepatocellular carcinoma remains detected at the very late and advanced stages when curable treatments are unavailable and ineffective. In this regard, there are still huge unmet medical needs in developing and clinically validating those high-potential protein biomarkers preferably in liquid biopsy samples. This review provides a glimpse of emerging biomarkers together with detection tools and techniques which are potentially commercially available to the markets. We also discuss several diagnostic biomarkers having therapeutic potential for developing first-in-class medicines.

Keywords: Hepatocellular carcinoma, biomarkers, targets, α-fetoprotein, cadherin-17, Yes-associated protein, AXL, Trop2

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related deaths worldwide[1]. The prognosis of HCC is generally poor, especially for late-stage malignancies, but a cure is possible if it is diagnosed at the early stages. In fact, 5-year survival for early stage HCC after curative treatments is as high as 70% [2]. This highlights the utmost importance of having a convenient, accurate and affordable diagnostic technique for early stage HCC.
The current clinical diagnosis of early HCC is mainly based on medical imaging, including ultrasonography, computed tomography (CT) and magnetic resonance imaging. Early stage HCC is classified by having less than 4 tumour nodules with less than 3 cm in diameter. The sensitivity and specificity for ultrasonography to pick up these tumour nodules are 60% and 97%, respectively, while CT has sensitivity and specificity of 68% and 93%, respectively. There have been several attempts to increase the sensitivity by combining imaging with α-fetoprotein (AFP) biomarker but with limited success so far, indicating the urgent need for new potential biomarkers. Many patients have no access to diagnostic imaging due to the lack of necessary equipment and imaging specialists in local and regional hospitals.

In this review, clinically approved biomarker, such as AFP, will be discussed in detail followed by updates on other diagnostic biomarkers under development, including AXL, thioredoxin and golgi protein-73 (GP73). A few promising HCC targets that can be used as both a diagnostic and a therapeutic biomarkers, such as glypican 3 (GPC3), Yes-associated protein 1 (YAP1), trophoblast cell-surface antigen 2 (Trop2) and vasorin (VASN), will also be described. Nucleic acids-based biomarkers, such as non-coding RNA, are beyond the scope of this review and are covered elsewhere in this special issue.

**DIAGNOSTIC BIOMARKERS FOR HCC**

**AFP**

AFP is the most well studied biomarker for HCC and is also the first biomarker approved for HCC detection in liquid biopsy. It is a 591 amino acids glycoprotein encoded by the *AFP* gene on human chromosome 4 (4q13). AFP transports a variety of molecules, including fatty acids and bilirubins, across the body. It is mainly produced by the visceral endoderm of the yolk sac and fetal liver during development. The highest AFP plasma concentration is detected during week 12 to week 16 of a fetal life and subsequently declines to virtually undetectable after birth. However, unusually high serum concentration of AFP is also detected in patients with HCC. Nonetheless, the use of AFP as a biomarker for HCC has been controversial ever since its discovery nearly half a century ago. One study that evaluated AFP as a standalone HCC biomarker on 5,581 men in China exhibited sensitivity and specificity of 55.3% and 86.5%, respectively. Although more early stage HCCs were reported in the test group than in the control group, there was no survival benefit in the test group. Another study that evaluated 18,816 patients with chronic hepatitis B demonstrated that combining ultrasonography with AFP test in a biannual screening scheme reduced the mortality of HCC in the test group by 37%. However, given the high false positive rate and additional costs, some argued the practicality of recommending such a biannual screening scheme. A systematic review of five trials conducted on patients with hepatitis C, a high risk group, between 1999 and 2002 concluded that AFP had limited ability to detect early HCC. The high false positive rate is not only because only 61% of HCC expresses AFP, but also the fact that AFP expression is detected in other liver abnormalities such as cirrhosis and acute hepatitis and other tumours, including endodermal sinus tumour and gastrointestinal malignancies.

AFP exists in three different glycoforms, namely AFP-L1, AFP-L2 and AFP-L3. Interestingly, AFP-L3 expression only increases in HCC but not in hepatitis or cirrhosis, suggesting that it could be a better HCC biomarker. However, a trial using AFP-L3 as the sole biomarker on 372 patients with hepatitis C virus demonstrated sensitivity of only 37%, despite a specificity of 92%. In the same study, combining AFP-L3 with another biomarker, des-gamma-carboxy prothrombin increased the sensitivity to 61% but sacrificed the specificity down to 71%. Another phase II study using the combo for early stage HCC reported sensitivity and specificity of 78% and 62%, respectively. The very low sensitivity is probably because AFP-L3 is minimally expressed and usually undetectable when the patients’ AFP level is below 20 ng/mL. Kagebayashi et al. utilized a microfluidic device in an attempt to detect low level of AFP-L3 in patient serum but reported
sensitivity and specificity of only 57% and 64%, respectively. Comparing AFP-L3 with AFP, Marrero et al. concluded that AFP was more sensitive than AFP-L3 for detecting early HCC. Taken together, these results suggest that AFP-L3, despite having higher specificity, is inferior than AFP as an HCC biomarker. The low sensitivity of AFP encourages combining AFP with other biomarkers that are significantly overexpressed in HCC [Table 1]. Three of these biomarkers that have performed extraordinarily when used in combination with AFP are AXL, thioredoxin and GP73.

**AXL**

AXL is a receptor tyrosine kinase that is expressed in a number of malignancies, including HCC\(^{[24]}\), lung cancer\(^{[25]}\), ovarian cancer\(^{[26]}\), colon cancer\(^{[27]}\), breast cancer\(^{[28]}\) and pancreatic ductal adenocarcinoma\(^{[29]}\) [Figure 1]. AXL is stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6. Stimulated AXL in turn activates the PI3K-AKT-mTOR, MEK-ERK, NF-κB and JAK/STAT signaling pathways that lead to tumour growth, immune escape and drug resistance\(^{[30-35]}\). AXL is also expressed in normal bone marrow stroma and myeloid cells to clear apoptotic material, suppress inflammatory responses and control natural killer cell activity\(^{[36,37]}\). Loss of AXL, therefore, leads to inflammation and autoimmunity\(^{[38,39]}\). AXL is a key downstream target that drives YAP-dependent oncogenic functions\(^{[40]}\). Knocking down AXL by RNAi decreased the ability of YAP-expressing MIHA and the primary HCC cell line to proliferate and invade. Furthermore, AXL also serves as a putative entry receptor for Zika Virus, Ebola Virus and West Nile Virus to infect the host cells\(^{[41]}\). Activated AXL undergoes proteolytic processing to yield a soluble protein that can be detected in the serum\(^{[42]}\). Detection of very early HCC (i.e., BCLC stage 0) by soluble AXL (sAXL)

| Table 1. Performance of various HCC diagnostic biomarkers and tools |
|----------------|----------------|--------|---|
| **Biomarker** | **Sensitivity (%)** | **Specificity (%)** | **Note** |
| Ultrasonography | 60 | 97 | Meta-analysis on 14 studies\(^{[4]}\) |
| CT | 68 | 93 | Meta-analysis on 14 studies\(^{[4]}\) |
| AFP | 55 | 87 | \(n = 5,581\)\(^{[14]}\) |
| | 66 | 82 | Early stage HCC, \(n = 836\)\(^{[22]}\) |
| | 59 | 89 | Early stage HCC, \(n = 1,100\)\(^{[47]}\) |
| | 35 | 88 | Meta-analysis on 19 studies\(^{[75]}\) |
| | 41-65 | 80-94 | Meta-analysis on 5 studies, cirrhotic patients\(^{[139]}\) |
| | 97 | 40 | \(n = 100\)\(^{[140]}\) |
| | 58 | 85 | \(n = 4,217\)\(^{[52]}\) |
| AFP-L3 | 37 | 92 | \(n = 372\)\(^{[21]}\) |
| | 57 | 64 | Detect using microfluidic device\(^{[23]}\) |
| DCP | 61 | 70 | Early stage HCC, \(n = 836\)\(^{[22]}\) |
| AFP-L3 + DCP | 61 | 71 | \(n = 372\)\(^{[21]}\) |
| AXL | 71 | 73 | \(n = 584\)\(^{[43]}\) |
| AXL + AFP | 84 | 92 | \(n = 584\)\(^{[43]}\) |
| Thioredoxin | 75 | 89 | Early stage HCC, \(n = 1,100\)\(^{[47]}\) |
| Thioredoxin + AFP | 83 | 94 | Early stage HCC, \(n = 1,100\)\(^{[47]}\) |
| GP73 | 75 | 97 | \(n = 4,217\)\(^{[52]}\) |
| GP73 + AFP | 89 | 85 | \(n = 4,217\)\(^{[52]}\) |
| GP73 | 55 | 84 | Meta-analysis on 19 studies\(^{[75]}\) |
| | 55 | 97 | Early stage HCC, meta-analysis on 19 studies\(^{[75]}\) |
| OPN | 75 | 62 | Early stage HCC, \(n = 312\)\(^{[126]}\) |
| SCCA | 84 | 49 | \(n = 961\)\(^{[141]}\) |
| Annexin A2 | 83 | 68 | Early stage HCC, \(n = 224\)\(^{[142]}\) |
| Annexin A2 + AFP | 87 | 68 | Early stage HCC, \(n = 224\)\(^{[142]}\) |
| suPAR | 76 | 90 | \(n = 267\)\(^{[143]}\) |
| MDK | 93 | 83 | \(n = 100\)\(^{[140]}\) |

CT: computed tomography; AFP: α-fetoprotein; GPC3: glypican 3; MDK: Midkine; OPN: osteopontin; SCCA: squamous cell carcinoma antigen; suPAR: soluble urokinase plasminogen activator receptor; DCP: des-gamma-carboxy prothrombin; HCC: hepatocellular carcinoma
showed an area under the curve (AUC) of 0.848 upon receiver operating characteristic curve analysis. The sensitivity and specificity of the detection was 76.9% and 69.2%, respectively. The accuracy of the detection was increased to 0.936 by the combined use of sAXL and AFP. Interestingly, sAXL combined with AFP could differentiate very early HCC from liver cirrhosis with an accuracy of 0.901, of which the sensitivity and specificity was 88.5% and 76.7%, respectively. Nevertheless, multicenter clinical studies are needed to validate these findings.

Thioredoxin
Thioredoxin, together with thioredoxin reductase, forms a ubiquitous oxidoreductase system that plays an important role in regulating intracellular redox environment, controlling cellular proliferation and providing defense mechanism against oxidative stress. Thioredoxin expression is detected in HCC, non-small cell lung cancer and colorectal cancer. It is generally associated with a more aggressive tumour phenotype, poor prognosis and a lower survival rate. As a sole early stage HCC biomarker, Li et al. found that thioredoxin (sensitivity, 75%; specificity, 89%) surprisingly outperformed AFP (sensitivity, 70%; specificity, 79%) in their study. When used in combination, they could detect early HCC with an impressive sensitivity and specificity of 83% and 94%, respectively. This supports the idea that an ideal combination of biomarkers can outperform a single biomarker in giving both lower false positive and false negative rates.

GP73
GP73 is a 400 amino acid, type II Golgi-specific membrane glycoprotein normally expresses on epithelial cells of liver and kidney. GP73 resides within the cis-Golgi complex but it can be secreted into the extracellular space by cleavage at the proprotein convertase site. In fact, soluble GP73 was detected in the medium cultured with HeLa, foreskin fibroblasts (HCA) and osteosarcoma (MG63) cell lines, suggesting that it may have some functions in the extracellular environment. A number of studies noted elevated level of serum GP73 in HCC patients. Mao et al. compared serum GP73 and AFP biomarkers in 4,217 subjects with a mixture of healthy adults, HBV carriers and patients with cirrhosis, HCC or others cancers. They found GP73 to be a superior biomarker (sensitivity: 75%, specificity: 97%) than AFP (sensitivity 58%, specificity 85%). The combination of both biomarkers improved the sensitivity further to 89% but with a drop-in specificity down to 85%.

Annexin A2
Annexin A2 (ANXA2), a member of the annexin family, is a 36-kDa calcium-dependent phospholipid-binding protein that plays a role in immune responses, phospholipase A2 regulation and anti-inflammation. The serum ANXA2 was found to be elevated in HCC patients (n = 50) as compared with patients with chronic disease (n = 30) or healthy subjects (n = 20) by ELISA. In the same study, follistatin, a potential serological HCC biomarker, was found elevated in both HCC patients and patients with chronic liver
BIOMARKER COMBINATIONS FOR HCC DIAGNOSIS

In addition to individual biomarkers and those combined with AFP, biomarker combinations for the diagnosis of early HCC have also been extensively studied. Cytokeratin-1 (CK-1) and nuclear matrix protein-52 (NMP-52) elevated in sera of patients with HCC. Combination of CK-1, NMP-52 and AFP showed an AUC of 0.9 for identifying HCC with 80% sensitivity and 92% specificity. More interestingly, this triple combination could differentiate HCC from liver fibrosis with an AUC of 0.94 with 80% sensitivity and 92% specificity.\(^{[54]}\) Epithelial membrane antigen and fibronectin, of which the serum levels were increased in HCC, when in conjunction with total bilirubin and AFP, could identify HCC from cirrhosis with an AUC of 0.92 with 89% sensitivity and 85% specificity.\(^{[55]}\) Combined use of plasma protein with immune cells in HCC diagnosis has been published as well. A combination of plasma Dickkopf-1, Tie2-expressing monocytes and AFP yielded an AUC of 0.833 for HCC diagnosis.\(^{[56]}\) The clinical utility of these biomarker combinations undoubtedly requires further validation in independent cohorts.

Diagnostic biomarkers with therapeutic potential

Although early diagnosis of HCC should translate into better overall survival, Chen et al.\(^{[14]}\) did not find this link in their study, citing lack of effective treatment as the main reason. Biomarkers that can serve as both a diagnostic tool and a therapeutic target would, undoubtedly, be more beneficial to the patients, as the diagnostic results can immediately assist physicians in planning treatment regimen. A number of promising biomarkers of this type are discussed below.

**GPC3**

GPC3 is a member of heparin sulfate proteoglycan family, which is bound to the cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor.\(^{[57]}\) A total of 6 glypicans have been identified to date, namely GPC1 to GPC6, and they are predominantly expressed during development.\(^{[58]}\) The amino acid sequence homologies amongst glypicans are low but the location of 14 cysteine residues are conserved, indicating that they may share similar high-dimension structures. The location of the heparin sulfate insertion sites of the glypicans appears to be restricted to the C terminus, putting the heparin sulfate chains near to the cell membrane.\(^{[58]}\) GPC3 is a 580 amino acid protein encoded by the *GPC3* gene located on human chromosome X (Xq26). Despite being a cell membrane protein, GPC3 is cleaved by the Notum lipase at the GPI anchor and released into the serum,\(^{[59]}\) making it easy for clinical detection. GPC3 is frequently upregulated in HCC and melanoma.\(^{[60]}\) By fixed tissue staining, GPC3 expression was detected in up to 72% of samples from patients with HCC, but not in healthy subjects or patients with benign liver diseases.\(^{[59]}\) The mRNA level of GPC3 was also upregulated in HCC.\(^{[61,62]}\) Moreover, at least three independent groups reported significant elevation of serum GPC3 in HCC but not hepatitis.\(^{[63-65]}\) Taken together, the data strongly suggest GPC3 to be an attractive serum and histochemical biomarker for HCC.

GPC3 can stimulate Wnt signaling through canonical and non-canonical pathways, which are initiated by Wnt ligands and Frizzled receptors.\(^{[66]}\) Given that Wnt proteins bind to heparin sulfate, it was suggested that GPC3 acts as a facilitator of the interaction between Wnt ligands and Frizzled receptors.\(^{[66,67]}\) GPC3 may promote tumourigenesis by facilitating canonical Wnt signal activation, which is frequently observed in HCC.\(^{[65,68,69]}\) In contrast, GPC3 expression is downregulated in breast and ovarian cancers, suggesting that the functions of GPC3 may be tissue-specific.\(^{[70-72]}\) Indeed, GPC3 is found to be a negative regulator of Hedgehog signaling pathway. Downregulation of GPC3 causes hyperactive Hedgehog signaling, which promotes ovarian and breast cancer progression.\(^{[73]}\) Filmus and Capurro\(^{[67]}\) proposed that GPC3 may exert different functions depending on cell types. In tissues that proliferate mainly via Hedgehog signaling, overexpression of GPC3 has an inhibitory effect on proliferation whereas in tissues where canonical Wnt signaling exerts a dominant influence, upregulation of GPC3 promotes cell proliferation.
As a HCC diagnostic biomarker, GPC3 outperformed AFP in a number of independent studies. Tangkijvanich et al.\textsuperscript{[74]} reported sensitivity of 56% for GPC3 while AFP stood at 33%. Interestingly, GPC3 overexpression did not correlate with AFP level, tumour size or stage of HCC but was significantly associated with the presence of viral hepatitis markers. A meta-analysis of 19 studies reported the superior sensitivity for GPC3 (pooled sensitivity, 55%; pooled specificity, 84%) over AFP (pooled sensitivity, 35%; pooled specificity, 88%)\textsuperscript{[75]}. Notably, the specificity for GPC3 was significantly higher if the analysis focused only on early HCC (pooled sensitivity 97%).

The potential of GPC3 goes well beyond being a diagnostic biomarker. GPC3 is an oncofetal antigen, a protein that is predominantly expressed in cancer and during fetal development. Murine model injected with GPC3 transgenic colon cell line showed that GPC3 was able to elicit T-cell mediated tumour rejection without autoimmunity\textsuperscript{[76]}. Similar results were reported using highly metastatic mouse melanoma\textsuperscript{[77]}. Importantly, the anti-tumour effects appeared to be mediated by both CD4\(^+\) and CD8\(^+\) T cells, which are essential for optimal anti-tumour response. CD4\(^+\) T cells have a broad role in orchestrating host anti-tumour responses, such as secreting cytokines to enhance cytotoxic T cell response, activating eosinophils and tumouricidal macrophages and secreting granulocyte/macrophage colony-stimulating factor. These results encourage development of immunotherapy targeting against the tumor-specific GPC3 isoform.

A phase I clinical study of a GPC3-derived peptide vaccine for 33 advanced HCC patients demonstrated that the vaccine was well-tolerated\textsuperscript{[78]}. Most patients had only grade I and grade II side effects. Four patients developed grade III hematological adverse events but were likely due to disease progression rather than the vaccine. In terms of efficacy, 1 patient showed partial response while 19 patients had stable disease 2 months after initiation of treatment. Given the favorable safety profile, it is rational to target GPC3 with other therapies, such as adoptive cell transfer. Along this line of thinking, there have been several Phase 1 clinical studies of chimeric antigen receptor (CAR)-T trials directed against the GPC3 antigen in HCC patients.

Recently, a phase I clinical trial of anti-GPC CAR-T cells was conducted on Chinese patients \((n = 13)\) with refractory or relapsed GPC3\(^+\) HCC. The 3rd generation CAR-T was engineered with CD28, 4-1BB and CD3\(\zeta\) downstream signaling domains\textsuperscript{[79]}. No dose-limiting toxicities were identified and only one patient experienced grade 3 fever\textsuperscript{[80]}. Without lymphodepletion, none of the five patients responded to the treatment but with lymphodepletion, 4/6 (67\%) clinical response was reported. The authors concluded that the anti-GPC3 CAR-T treatment is safe and tolerable.

A bispecific T cell-redirecting antibody that binds both GPC3 and CD3 is also under active development by Chugai Pharmaceutical (a Roche subsidiary). Early studies on animal models showed that the GPC3/CD3 bispecific antibody showed anti-tumour efficacy against various GPC3-positive xenografts including liver tumours\textsuperscript{[81]}. This bispecific antibody is now being investigated in a phase I clinical trial on patients with GPC3 positive advanced solid tumours (NCT02748837).

**Cadherin-17**

Cadherin-17 (CDH17) is a calcium-dependent cell adhesion molecule that belongs to the 7D-cadherin superfamily, characterized by the presence of 7 cadherin-like ectodomains followed by a short cytoplasmic tail\textsuperscript{[82]}. It is normally present in fetal liver and gastrointestinal tract during embryogenesis, hence the name liver-intestinal cadherin (LI cadherin). It is a peptide transporter and plays an important role during embryonic gastrointestinal development\textsuperscript{[81,84]}. CDH17 expression was reported in normal human colon, intestine and pancreas but not normal liver and stomach\textsuperscript{[85-89]}. However, the overexpression of CDH17 was observed in HCC as well as breast, ductal pancreatic, colorectal and gastric cancers\textsuperscript{[90-93]}. The upregulation was associated with malignant transformation of these cancers. Knock-down of CDH17 by RNAi inhibited proliferation of primary and metastatic HCC cell lines \textit{in vitro} and \textit{in vivo}\textsuperscript{[94]}. This anti-tumour effect was likely due to inactivation of Wnt signaling pathway because CDH17-knockdown HCC tumours...
showed re-localization of β-catenin to cytoplasm, concomitant reduction in cyclin D1 and increase in tumour suppressor retinoblastoma. In addition, CDH17 was reported as an useful diagnostic marker for adenocarcinomas of the digestive system\textsuperscript{[95]}. It was also associated with bone marrow metastasis of breast cancer\textsuperscript{[96]} and liver metastasis of colorectal cancer\textsuperscript{[93]}.

CDH17 expression was upregulated by 2.5 to 800 folds in over 80% HCC but not in healthy liver, making it an attractive diagnostic and therapeutic biomarker for HCC\textsuperscript{[88]}. Half of the CDH17\textsuperscript{+} HCC patients have gained genomic copy of this gene. Importantly, alternately spliced mRNA transcripts, characterized by loss of exon 7, were reported in roughly half of the HCC patient specimens. The splicing introduced a premature stop codon in the open-reading frame and resulted in a truncated CDH17 protein. It was speculated that overexpression of the truncated variant may act as a dominant inhibitor of wild-type CDH17, thereby enhancing tumour invasion. In consistent to this speculation, expression of this variant CDH17 was strongly associated with poorer overall survival, higher risk of relapse and venous infiltration after hepatectomy. The spliced transcripts were only detected in HCC samples but not normal liver samples, implying that the splicing is likely to be an aberrant cancerous event rather than a normal splicing phenomenon. Importantly, an antibody against the RGD motif of CDH17 has shown promising anti-tumour effects against metastatic colon cancer and melanoma, suggesting that it is likely to be effective against HCC\textsuperscript{[97]}.

YAP1

YAP1, also known as YAP or YAP65, is an oncogene encoded by the \textit{YAP1} gene located on human chromosome 11 (11q22)\textsuperscript{[98]}. It is a downstream nuclear effector of the Hippo signaling pathway, which is important for development, cell proliferation, repair and homeostasis\textsuperscript{[99]}. Given its importance in cell proliferation, YAP1 knockout mice showed development arrest and died prematurely\textsuperscript{[100]}. Studies on the \textit{Drosophila} Yorkie (Yki) protein, an ortholog of YAP1, suggested that YAP1 is negatively regulated by the Hippo pathway\textsuperscript{[101]}. Inactivation of Hippo pathways leads to accumulation of Yki proteins in the nucleus and upregulation of genes associated with cell survival and proliferation, including \textit{cycE}, \textit{diap1/thread} and \textit{bantam}\textsuperscript{[102]}. In mammalian cells, overexpression of YAP1 caused aberrant expression of genes associated with cell proliferation, anti-apoptosis, survival and migration, such as \textit{CTGF}, \textit{CCND1}, \textit{ITGB2} and \textit{BCL2L1}\textsuperscript{[103]}.

Analysis on 177 HCC samples by immunohistochemistry, Western blot analysis and RT-PCR showed that approximately 62% of both YAP protein and mRNA were upregulated as compared to adjacent non-tumour tissues\textsuperscript{[104]}. The YAP proteins were mainly accumulated in the tumour nucleus. In an independent study, Zhao \textit{et al}\textsuperscript{[101]} also reported YAP overexpression in 63 of the 115 HCC samples tested by tissue microarray. Similar results were reported in non-small lung cell cancer, suggesting that YAP may have broad implications in different solid cancers\textsuperscript{[105]}. Importantly, YAP expression was associated with poorer tumour differentiation, high serum AFP level and lower overall survival rate, indicating that it may be used as an independent prognostic marker\textsuperscript{[104]}.

Both YAP and transcriptional co-activator with PDZ-binding motif (TAZ) are downstream effectors of Hippo pathway. Hayashi \textit{et al}\textsuperscript{[106]} reported that knocking down TAZ, under normal condition, inhibited cell growth in HCC. However, treating the TAZ knockdown cells with 5-fluorouracil induced YAP expression that conferred chemoresistance. The drug resistance was not observed when both TAZ and YAP were knockdown, suggesting that a shift to predominantly YAP expression when TAZ was depleted led to chemoresistance and tumourigenecity. The authors concluded that targeting both YAP and ZAP is essential for a complete anti-tumour response. Given that YAP expression is an early event in HCC tumourigenesis and its expression is critical to chemoresistance and proliferation of malignant hepatocytes, YAP is a promising HCC target for therapeutic intervention.

The oncogenic activity of YAP depends on its interaction with transcriptional enhancer activation domain family member 1 (TEAD1) that resides in the nucleus and therefore, disrupting YAP-TEAD1 interaction is
believed to have anti-cancer efficacy in YAP positive tumours\textsuperscript{[107]}. YAP-like peptides occupying the interface 3 on YAP/TEAD complex were shown able to block YAP-TEAD\textsubscript{1} interaction\textsuperscript{[106]}. Small-molecule inhibitors (SMIs) targeting the same interface were recently shown to suppress the expression of YAP target genes\textsuperscript{[109]}. Whether these peptides or SMIs would inhibit tumour growth in vivo however remains to be investigated.

**Trop2**

Trop2, also known as tumour-associated calcium signal transducer 2 (TACSTD2) or epithelial glycoprotein-1 antigen, is a calcium signal transducer encoded by the \textit{TACSTD2} gene located on human chromosome 1 (1p32.1). Trop2 is a cell surface glyprotein that is associated with regulation of cyclin D1 and protein kinase C levels. Trop2 stimulates the expression of cyclin D1 and cyclin E via the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway to promote cell proliferation\textsuperscript{[110]}. Numerous reports have confirmed that Trop2 is an oncogene associated with tumour development, progression and metastasis in various cancers, including pancreatic cancer\textsuperscript{[111]}, squamous cell carcinoma\textsuperscript{[112]}, gastric carcinoma\textsuperscript{[113]}, hilar cholangiocarcinoma\textsuperscript{[114]}, colorectal cancer\textsuperscript{[115]}, cervical cancer\textsuperscript{[116]}, ovarian carcinoma\textsuperscript{[117]}, gallbladder cancer\textsuperscript{[118]} and breast cancer\textsuperscript{[119]}. Unsurprisingly, Trop2 overexpression is often associated with poor cancer prognosis. A recent gene network analytic study found aberrant expression of Trop2 in HCC\textsuperscript{[120]}. Given that Trop2 is an oncogene in many cancers, it is speculated that it may be a potential biomarker candidate and a therapeutic target for HCC.

**VASN**

VASN is a cell surface and secreted protein that modulates the arterial response to injury by inhibiting the TGF-\(\beta\) signaling pathway\textsuperscript{[121,122]}. It was identified as a potential HCC biomarker using a subtractive EMSA-SELEX strategy from AFP negative serum of HCC patients with secondary metastasis\textsuperscript{[123]}. VASN expression can be detected in aorta, kidney, placenta, brain, heart, liver, lung and skeletal muscle tissues. It was highly expressed in HCC samples \((n = 100)\) but not in normal liver \((n = 97)\) or hepatitis samples \((n = 129)\), as verified by both Western blotting and quantitative PCR. This high VASN expression appeared to be negatively regulated by microRNAs miR145 and miR146a. Downregulation of these microRNAs led to overexpression of VASN, which promoted cell proliferation and migration and inhibited apoptosis. As a membrane protein, VASN has the potential to be a therapeutic target.

**Osteopontin**

Osteopontin (OPN), a matrix glycoprotein secreted by a wide variety of cell types, has also emerged as a biomarker with diagnostic potential\textsuperscript{[124]}. Plasma level of OPN in patients with HCC was significantly higher than in or healthy subjects or patients with chronic liver diseases\textsuperscript{[125]}. In a prospective study on 22 patients who developed HCC during follow-up, OPN was elevated in plasma one year before cancer diagnosis\textsuperscript{[126]}. A meta-analysis on 12 published studies showed that the sensitivity in HCC diagnosis was higher than that of AFP (OPN, 0.813; AFP, 0.639)\textsuperscript{[127]}. Plasma OPN could be used to differentiate HCC from other non-malignant liver diseases including chronic hepatitis C, cirrhosis, and nonalcoholic fatty liver disease\textsuperscript{[128]}. Importantly, serum OPN was associated with dismal overall survivals of patients with HCC with a hazard ratio of 2.38\textsuperscript{[129]}. In addition to its diagnostic value, OPN can be a potential therapeutic target for HCC treatment. Antiviral therapy suppressed early progression of hepatitis B-related HCC by modulating the expression of OPN in patients\textsuperscript{[130]}. Knockdown of OPN using RNA interference suppressed in vivo growth and lung metastasis of liver cancer xenograft in mice\textsuperscript{[131]}. Monoclonal antibodies (mAbs) against OPN have been reported to demonstrate anti-cancer effects in animal models. An antibody named AOM1, which abrogated the integrin binding of OPN, was illustrated to suppress the in vivo of Kras-mutant non-small cell lung adenocarcinoma in mice\textsuperscript{[132]}. Hu1A12, another OPN mAb that bound to the calcium binding domain of OPN, was demonstrated to inhibit primary tumor growth and spontaneous metastasis in a mouse lung metastasis
model of human breast cancer\textsuperscript{[133]}. There studies strongly support OPN as a potential target for the antibody-based cancer therapy, although the anti-cancer efficacy of OPN mAbs in HCC has remained to be studied.

CONCLUSION

HCC is an extremely difficult to treat cancer, which generally involves multiple pathologic complications including hepatitis, metabolic (NASH and diabetic), fibrotic and cirrhotic diseased conditions in addition to the notorious tumor burden. As a result, both the current diagnosis and treatments of HCC remain largely ineffective. Therefore, bringing new biomarkers and innovative treatments to the patients are in critical demand. With the recent advent of cancer immunotherapy, it is more than ever necessary to find tumour-specific biomarkers as therapeutic targets. One of the major challenges in immunotherapy on solid tumours is the extreme scarcity of highly specific targets\textsuperscript{[134]}. Failure to find such a target has led to not only ineffective treatment but also high toxicities and even deaths in clinical trials\textsuperscript{[135-138]}. Despite being extremely rare and highly challenging, fortunately, recent advances in next-generation sequencing and high-throughput technologies would, undoubtedly, accelerate discovery of such biomarkers and make progress for the diagnosis and treatment of HCC.

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