New Biomarkers for Hepatocellular Carcinoma

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Key Concepts

- Recent advances in genomic, proteomic and metabolomic technologies are enabling the identification of novel biomarkers for hepatocellular carcinoma (HCC). Most of these new markers are currently in phase 1 of biomarker development.
- HCC biomarkers are mainly used as screening tools in surveillance programs for early HCC detection, with the ultimate goal of reduction in HCC mortality. Currently, new biomarkers are also in development for individualizing the use of targeted therapies.
- Multiple marker-based approaches (e.g. a panel of genetic variants or a combination of several biomarker types or clinical parameters) will potentially be more useful than a single marker-based approach due to the complexity and heterogeneity of HCC biology and the complex interactions between host, viral and environmental factors contributing to hepatocarcinogenesis.
- Increasing understanding of the biology of HCC has yielded identification of highly sensitive and specific markers at the mRNA and protein levels that are being tested for their ability to detect, diagnose, predict prognosis, and tailor therapy.
- Improvements in the techniques for detecting and measuring biomarkers have also improved the utility of biomarkers.

- There is controversy about the use of alpha fetoprotein (AFP) in surveillance. AFP remains a useful HCC biomarker for diagnosis, monitoring treatment response and predicting recurrence and patient survival. Dynamic changes of AFP value over time, particularly changes in the standard deviation of AFP values, may be more useful than a single measurement in isolation as a biomarker.

Abbreviations: SNP - single nucleotide polymorphism, OR - odds ratios, CI - confidence interval, ROC - Receiver operating characteristics, ALT - alanine aminotransaminase

Narrative Summary

HCC biomarkers are used for predicting risk for HCC development, screening and surveillance, diagnosis, stratifying patients for targeted therapy, monitoring treatment response, and predicting HCC recurrence and patient survival. A number of SNPs have been identified as new biomarkers for HCC risk prediction. For HCC diagnosis, the serum AFP remains a useful biomarker, with a higher sensitivity than AFP-L3 and DCP. The combination of AFP with AFP-L3 or DCP may be better than using AFP alone as a biomarker for HCC diagnosis. Recent findings suggest that the combination of AFP with other variables, such as ALT, or patient and tumor characteristics (e.g. the MESIAH score), improves performance of AFP for early HCC detection and...
predicting patient survival. The variability of AFP over time, estimated by the standard deviation, performed better than AFP itself for early HCC diagnosis. The dynamic change in AFP levels is also helpful for predicting treatment response and patient survival. Although glypican-3 is very specific to HCC, the sensitivity of the currently available assays is not satisfactory for early HCC detection. Osteopontin is a promising biomarker for HCC diagnosis but is still in early phase biomarker studies. Currently available data on the performance of GP73 for HCC detection is controversial. Recently, blood and urine metabolomics have been proposed as potential biomarkers for HCC diagnosis. A number of novel biomarkers have been shown to be useful for guiding treatment and predicting prognosis. FGF3/FGF4 amplification and high MET expression in HCC tumor tissue have been shown to predict response to sorafenib and tivantinib, respectively. A number of microRNAs have been associated with treatment outcome and survival. The presence of EpCAM-positive circulating tumor cells in blood serves as a marker of poor prognosis. Although many new HCC biomarkers have been identified during the past few decades, none of them have shown broad utility in clinical practice. Several are currently under investigation to improve assay performance and to show proof of efficacy of the biomarker in clinical practice.

Definition and Scope of Cancer Biomarkers
Cancer biomarkers are indicators of the presence of cancer in the body or predictors of risk for developing cancer. They can be in the form of DNAs (e.g. gene fusion, genetic mutation), RNAs (e.g. non-coding RNA, microRNA), proteins and post-translational modifications, metabolites or antibodies. Biomarkers are not only detected in the serum or plasma, they can also be measured from other sources, such as cancer tissue, non-cancerous tissue and urine.

Applications of Biomarkers for HCC in Clinical Care
The traditional application of biomarkers for HCC is the use of serum AFP for diagnosis, as a screening or surveillance tool, and as a predictor of HCC risk. Recently, new biomarkers are being applied for stratification of patients for clinical trials, prediction of response to targeted therapies, prediction of recurrence and patient survival, and monitoring for HCC recurrence (Figure 1).

Challenges in the Use of Biomarkers in Clinical Practice
These challenges stem from the molecular heterogeneity of humans and cancers. The molecular heterogeneity of humans makes establishing the “normal” value of each biomarker difficult, whereas the molecular heterogeneity of cancers results in the observation that there are almost no unique biomarkers presented in all biospecimens of any particular cancer.

In order to have a perfect biomarker for each particular disease, two theoretical requirements must be satisfied. First, ideally, each individual must serve as their own control, i.e. each individual would provide blood, urine, tissue, or other biospecimens periodically and the dynamic changes of assay values over time would serve as biomarkers. Second, we will need highly sensitive and specific assays that are able to measure every potential biomarker, including DNAs, RNAs, proteins and post-translational modifications and metabolites.

Although attempts have been made to reach these goals, we are far from achieving them. The feasible alternative approach is to develop a strategy for the best use of combinations of currently available biomarkers to achieve the maximum test performance.
Phases of Biomarker Development for Early HCC Detection

Although cancer biomarkers can be used for several applications, the major role of HCC biomarkers remains as a screening tool for early HCC detection, with the ultimate goal of reducing HCC mortality. To achieve this goal, biomarkers need to progress through the following 5 phases of biomarker development shown in Table 1 (1).

Important Considerations for the Application of HCC Biomarkers in Clinical Practice

1) Positive and negative predictive value of a biomarker assay as a screening or diagnostic tool vary depending on the prevalence of HCC in each population. The assay should therefore be validated before routine use in that particular population.

2) The published sensitivity and specificity of biomarker assays in the literature are generally estimated from a single measurement at one particular time point. However, in real practice, practitioners usually consider changes in the levels of biomarkers over time to be more useful than a single isolated measurement.

3) It is important for new biomarkers to be assessed through all 5 biomarker development phases before they are accepted as screening tools in clinical practice, but full development can be very expensive or impractical.

Known HCC Biomarkers

1. Biomarkers for Prediction of HCC Risk:

Biomarkers associated with an increased risk for HCC development include viral hepatitis B (HBV) genotype C, baseline serum HBV viral load of ≥ 10^5 copies/ml, presence of hepatitis C viremia, serum aflatoxin B1-albumin adducts, urine aflatoxin B1 metabolites, and elevation of serum alpha fetoprotein (AFP), the Lens culinaris agglutinin-reactive glycoform of AFP (AFP-L3), or des-gamma-carboxy prothrombin (DCP) in cirrhotic patients.

2. AFP, AFP-L3, DCP and Glypican-3 as Biomarkers for HCC Screening and Diagnosis:

Alpha fetoprotein (AFP) is a glycoprotein that is expressed in high amounts by embryonic hepatocytes and the yolk sac. It is found in high levels in adults with embryonic carcinomas and hepatocellular carcinoma. AFP is more sensitive than AFP-L3 and DCP for detecting early stage HCC, with a sensitivity of 65% and a specificity of 82% at the cut-off of 10.9 ng/mL (2), although results of other studies have been variable. As compared to the AFP alone, the combination of AFP with AFP-L3 or DCP is only marginally more useful in detecting early stage HCC (2). In practice, clinicians use an increasing trend of AFP values over time as a trigger for further investigation for development of HCC. The combination of AFP and liver ultrasound performed every 6 months was studied in a prospective cluster randomized trial of HCC surveillance in Shanghai, China. HCC surveillance was associated with a 37% reduction in risk of death from HCC (3).

Des-gamma-carboxy prothrombin (DCP), also known as protein induced by vitamin K absence/antagonist-II (PIVKA-II), is an abnormal prothrombin that is produced as a result of defective post-translational carboxylation of the precursor protein to prothrombin. The performance of serum DCP for HCC diagnosis varies among studies. Marrero et al. found DCP at a cut-off of 125 mAU/mL had a higher sensitivity and specificity than AFP at a cut-off of 11 ng/mL in
distinguishing HCC from chronic liver diseases and cirrhosis (sensitivity of 89% vs. 77% and specificity of 95% vs. 73%) whereas Nakamura et al. found that DCP had a lower sensitivity and specificity than AFP for the diagnosis of small HCC (4) (5). DCP has some limitations. It is nonspecifically elevated in patients who are taking vitamin K antagonists such as warfarin or have vitamin K deficiency. Additionally, its ability to detect HCCs appears to vary substantially depending on tumor characteristics such as vascular invasion and metastases (6). These features may explain the variable performance of DCP in different studies.

**Glypican-3 (GPC3)** is a heparan sulfate proteoglycan that is bound to the plasma membrane through a glycosylphosphatidylinositol anchor. GPC3 acts as a coreceptor and plays important roles in cell signaling by modulating the activities of various growth factors such as fibroblast growth factor and insulin-like growth factor, thus regulating cell growth and differentiation. GPC3 also upregulates the Wnt signaling pathway and therefore contributes to tumor proliferation and invasion (7). A recent meta-analysis showed the performance of serum GPC3 was comparable to that of AFP for the diagnosis of HCC (sensitivity of 57% vs. 52% and specificity of 85% vs. 97%, for GPC3 and AFP, respectively). The sensitivity for HCC diagnosis was significantly increased to 77%, with a specificity of 81%, when GPC3 was used in combination with AFP (8).

**Updates on AFP, DCP AND GPC3**

A recent study suggested that the variability of AFP values over time, determined by the standard deviation of AFP, may be more useful for detecting early HCC than the value of AFP itself (9). AFP can be used as a predictor of overall survival and outcome of HCC patients. An AFP level of >100 ng/mL before hepatectomy was a predictor of HCC recurrence after resection (10). In HCC patients treated with chemoembolization or radioembolization, a 3-month post treatment AFP ≥ 50% decreased from a baseline value of >200 ng/mL predicted better survival (11, 12). AFP is not useful as a predictor of response to sorafenib treatment (13).

In patients with HCC, DCP is typically identified using the MU-3 antibody, which reacts strongly with DCP molecules containing fewer gamma-carboxylated glutamic acid (Gla) residues. The serum DCP is also increased in patients with vitamin K deficiency due to obstructive jaundice or warfarin use, in whom DCP variants containing more Gla residues (designated NX-DCP) are also elevated and can be detected using the P-11 or P-16 antibody. Thus the DCP/NX-DCP ratio is significantly higher in HCC than in non-HCC cases. This novel DCP/NX-DCP ratio had a slightly lower sensitivity compared to conventional DCP, while having a substantially better specificity of 92 vs. 62% for conventional DCP (14).

Given that the performance of the original serum GPC3 assay for HCC diagnosis in clinical practice was unsatisfactory, a new sandwich ELISA assay has been developed which yielded a sensitivity of 40% and specificities of 87-99% for the diagnosis of HCC (15).

**Future directions:**

Combining AFP with ALT, DCP, or GPC3 may improve the performance of AFP for early HCC detection (16). Similarly, combining AFP with patient and tumor characteristics improved the performance of AFP in predicting patient survival in the Model for Estimating Survival in Ambulatory Hepatocellular Carcinoma patients (MESIAH; score calculator available at http://www.mayoclinic.org/meld/mayomodel10.html) (17). Validation of these results is warranted.

**Newer Biomarkers for HCC**

**1. Biomarkers of HCC risk prediction**

Using genome wide association studies (GWAS), a number of single nucleotide polymorphisms (SNPs) have been identified as susceptibility gene loci for HCC development. For HBV-infected subjects, the rs7574865 G allele at the STAT4 and the rs9275319 A allele at the HLA-DQ gene were associated with an increased risk for HCC with an OR (95%CI) of 1.21 (1.14-1.28) and 1.49 (1.36-1.63), respectively (18). For HCV-infected subjects, the rs2596542 A allele at the MHC class I polypeptide-related sequence A (MICA) gene was associated with an increased risk of HCC among Asian but not in Caucasian populations (19, 20). By contrast, the rs17401966 G allele at the kinesin family member 1B (KIF1B) gene was associated with a decreased risk for HCC in HBV-infected subjects with an OR (95%CI) of 0.81 (0.70-0.93), suggesting that KIF1B may play role as a tumor suppressor gene for HCC (21). There are limitations to applying SNPs for prediction of HCC risk in current practice, however. The ORs of these SNPs were all less than 1.5, the cut-off generally accepted as being clinically meaningful. The findings were also inconsistent across different populations. These limitations emphasize the fact that HCCs are heterogeneous and that using any one single SNP as a biomarker for HCC risk prediction may not be feasible. In the future, development of a panel of...
susceptibility SNPs or combining these SNPs with other biomarkers may improve the performance of tests for predicting risk for HCC development.

2. Biomarkers for Diagnosis of HCC

**Osteopontin**: Osteopontin is a highly phosphorylated secreted extracellular matrix protein that is a member of the Small Integrin-binding ligand N-linked glycoprotein (SIBLING) protein family. Osteopontin plays important roles in cell signaling pathways that control inflammation, as well as in tumorigenesis, invasion, and metastasis of various cancers, including HCC (22). Plasma osteopontin is a promising HCC biomarker. Osteopontin at an optimal cut-off of 91 ng/mL had a better performance than AFP at a cut-off of 20 ng/mL for early HCC diagnosis (23). The sensitivity and specificity for early HCC diagnosis were 74% and 66% vs. 53% and 93% with an area under the ROC curve of 0.76 vs. 0.71 for osteopontin vs. AFP, respectively. At these cut-offs, the combination of osteopontin with AFP performed better than either osteopontin or AFP alone, with a sensitivity of 85%, a specificity of 63% and an area under the ROC curve of 0.82, suggesting that osteopontin could be a complementary test to the AFP for early HCC diagnosis.

**Golgi Protein 73 (GP73)**: GP73 is an integral membrane protein localized in the Golgi of normal epithelial cells, including hepatocytes. Its expression is upregulated in chronic liver diseases, and it is found in high levels only in the presence of HCC (24). Two recent meta-analyses reported different results on diagnostic performance of GP73 as compared to AFP. One showed GP73 was comparable to AFP while another showed GP73 outperformed AFP (25, 26). It will be important to examine whether the combination of GP73 and AFP could improve the diagnostic performance of the test in a sufficiently large cohort of patients.

**Squamous Cell Carcinoma Antigen (SCCA)**: SCCA is a serine protease inhibitor that is found in the spinous and granular compartments of squamous epithelium. It has been shown to be overexpressed in epithelial neoplasms including HCC (27). A recent meta-analysis reported the diagnostic performance of SCCA was inferior to that of AFP (26). However, it may still be useful for detecting small HCCs as the SCCA level was inversely correlated with size of tumor. SCCA had a sensitivity of 56% and a specificity of 75% for the detection of HCC less than 3 cm (28).

**Sulfite oxidase (SUOX), aldo-ketoreductase family member B10 (AKR1B10), and hematopoietic progenitor cell antigen (CD34) expression**: A progressive increase in SUOX protein expression level in hepatocytes occurs during the process of hepatocarcinogenesis. By contrast, AKR1B10 protein and CD34 expression levels decrease progressively during hepatocarcinogenesis. The combination of these 3 biomarkers has been used as an immunohistochemical marker for differentiating well-differentiated small HCCs from pre-malignant high-grade dysplastic nodules with a sensitivity of 94% and specificity of 95% (29).

**Circulating microRNAs (miRNAs)**: A large number of miRNAs are dysregulated in HCC. The diagnostic utility of miRNA in serum, plasma and urine has recently been investigated. Serum mi-R16 had a better sensitivity than AFP, AFP-L3 or DCP for HCC diagnosis (sensitivity: 72%, 59%, 36% and 47%, and specificity: 89%, 93%, 97% and 98%, respectively). The sensitivity improved to 92%, with a specificity of 79%, when mi-R16 was used in combination with AFP, AFP-L3 and DCP (30).

**Blood and Urine Metabolomics**: Advances in liquid chromatography and mass spectrometry now enable us to profile small-molecule metabolites which are products of dysregulated metabolic pathways in hepatocarcinogenesis. These metabolites can be detected in blood or urine. Of a total of 13 serum metabolites identified as potential candidate biomarkers for early HCC diagnosis, canavaninosuccinate (CSA) was shown to be a promising biomarker. CSA is an organic acid metabolite synthesized in liver. The serum level of CSA increases significantly in HCC patients but decreases significantly in cirrhotic patients. Serum CSA had a better performance than AFP at a cut-off of 20 ng/mL for early HCC diagnosis in cirrhotic patients with a sensitivity of 79% vs. 74% and a specificity of 100 vs. 38%. The combination of CSA and AFP increased the sensitivity to 96% and the specificity remained 100% (31).

3. Biomarkers for Prognostication and Stratification for Therapy

**FGF3/FGF4 amplification**: FGF3 and FGF4 are proto-oncogenes that are amplified in less than 3% of HCCs. Despite the low frequency observed in HCCs, FGF3/FGF4 amplification has been shown to predict response to sorafenib. FGF3/FGF4 amplification was found in 3 of 10 (30%) HCC tissues of patients who had a partial or complete treatment response to sorafenib, but was not found in any of 38 HCC tissues of patients who did not respond to sorafenib (32).

**High MET expression**: Expression of c-Met, a proto-oncogene encoding the hepatocyte growth factor receptor, is associated with poor outcomes, and also has therapeutic implications as well. A randomized phase 2 study of
tivantinib, a MET inhibitor, demonstrated that response to tivantinib for treatment of HCC was better in patients with high tumor MET expression, as assessed by immunohistochemistry (median time to progression 2.7 months vs. 1.4 months) (33). This suggests that MET expression can be used as a biomarker for stratifying patients for treatment with MET inhibitors.

A 20-miRNA Signature, miR-185, and miR20-a Expression: Analysis of microRNA (miRNA) expression profiles has been used to subclassify various cancers and to assess associations with different prognostic clinical features. In HCC, a unique 20-miRNA signature in tumor tissues was identified to be significantly associated with venous metastasis (34). Low expression of miR-185 in HCC tissue was associated with higher recurrence after hepatic resection (35). Also, low miR-20a expression was the strongest independent predictor of worse survival and higher risk of recurrence in patients treated with liver transplantation (36).

EpCAM positive Circulating Tumor Cells: Stem cell-like, epithelial adhesion molecule (EpCAM)-positive circulating tumor cells (EpCAM+ CTC) are a subpopulation of tumor initiating cells. The presence of EpCAM+ CTC in blood was associated with tumor aggressiveness, i.e. higher BCLC stage, macroscopic and microscopic vascular invasion, and shorter overall survival (1.3 vs. 2.8 years) (37). As compared to other predictors of HCC recurrence (e.g. tumor characteristics, BCLC stage), the presence of ≥ 2 EpCAM+ CTC cells in 7.5 ml of blood obtained prior to hepatectomy was the strongest independent predictor of HCC recurrence after resection ,with a hazard ratio of 5.2 (95%CI: 2.7-10.2) (38). The presence of EpCAM+ CTC may have implications for stratifying patients for curative or systemic therapy.

Next Generation Sequencing: Next generation sequencing technologies are increasingly being used for genomic analysis of HCC. This approach has enabled confirmation of previously known mutations and identification of novel genetic alterations that are relevant to HCC tumorigenesis. These findings have the potential to further clarify the molecular landscape of HCC and facilitate the identification and development of novel biomarkers for diagnosis, prognostication, and management of HCC.

References

Table 2. Summary of HCC biomarkers

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Source</th>
<th>Current Phase</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP</td>
<td>Serum</td>
<td>5</td>
<td>Surveillance with AFP and ultrasound every 6 months decreased HCC mortality by 37% (3)</td>
</tr>
<tr>
<td>DCP</td>
<td>Serum</td>
<td>2</td>
<td>Combination of DCP and AFP superior to AFP alone for HCC diagnosis</td>
</tr>
<tr>
<td>Glypican 3</td>
<td>Serum</td>
<td>2</td>
<td>Comparable performance to AFP for HCC diagnosis (8)</td>
</tr>
<tr>
<td>GP73</td>
<td>Serum</td>
<td>2</td>
<td>Some studies report superior performance to AFP for HCC diagnosis; most are equivocal (25, 26)</td>
</tr>
<tr>
<td>SCCA</td>
<td>Serum</td>
<td>2</td>
<td>May be useful for detection of small HCCs (28)</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>Plasma</td>
<td>2</td>
<td>Superior to AFP in early HCC detection (23)</td>
</tr>
<tr>
<td>SUOX, AKR1B10 and CD34 expression</td>
<td>Tissue</td>
<td>1</td>
<td>Highly sensitive and specific markers for immunohistochemical HCC diagnosis (29)</td>
</tr>
<tr>
<td>Metabolites</td>
<td>Blood, Urine</td>
<td>1</td>
<td>Promising biomarkers for HCC diagnosis (31)</td>
</tr>
<tr>
<td>FGF3/FGF4 amplification</td>
<td>Tissue</td>
<td>1</td>
<td>Associated with better response to sorafenib (32)</td>
</tr>
<tr>
<td>High MET expression</td>
<td>Tissue</td>
<td>1</td>
<td>Associated with better response to tivantinib (33)</td>
</tr>
<tr>
<td>Circulating miR-16</td>
<td>Serum</td>
<td>1</td>
<td>Better performance that AFP, AFP-L3 and DCP for HCC diagnosis in small studies (30)</td>
</tr>
<tr>
<td>miR-185 and miR-20a</td>
<td>Tissue</td>
<td>1</td>
<td>Associated with worse outcome (35, 36)</td>
</tr>
<tr>
<td>EpCAM positive circulating tumor cells</td>
<td>Blood</td>
<td>1</td>
<td>Associated with more aggressive HCC (37, 38)</td>
</tr>
</tbody>
</table>


