Molecular Signatures in Hepatocellular Carcinoma: A Step Toward Rationally Designed Cancer Therapy

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Molecular characterization of hepatocellular carcinoma (HCC) has greatly improved our understanding of disease pathogenesis. Mutational analysis, RNA and microRNA expression profiling, and epigenetic characterization have revealed common aberrations in oncogenes and tumor suppressors that correlate with disease biology and serve as a guide for the rational design of targeted therapies. These approaches have also led to the discovery of novel targets, including mutations in isocitrate dehydrogenase and chromatin remodeling enzymes. With the advent of immunotherapy, RNA expression profiling of the tumor microenvironment has identified a subset of HCC with high lymphocyte infiltration that may benefit from checkpoint inhibitor therapy. Molecular signatures thus capture the biology of a tumor, providing a supplement to current staging schema, which are based on tumor size and number, for more accurate prognostication of recurrence risk and survival. Molecular signatures may also be used to guide interventional therapy by defining those most suitable for transplantation or locoregional therapy rather than surgical resection. Finally, a multiomics approach involves the aggregation and analysis of multiple signatures for a more comprehensive characterization of pathogenic mechanisms. This broader approach attempts to address issues with signaling pathway cross-talk and redundancy, which have greatly limited the potential value of targeted therapies to date.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer globally and the third leading cause of cancer-related mortality. The prevalence of HCC is expected to increase, presenting a major economic and health care burden for the 21st century. Surgical resection, tumor ablation, and liver transplantation remain the only options for potential cure, although most patients are diagnosed at an advanced stage, when these treatments are not an option. Even among patients who undergo hepatectomy, up to 70% may recur within 5 years after a curative resection. Similarly, recurrence rates after transplantation are near 20% to 30%. For all stages, the average 5-year survival in the United States remains below 20%, with even lower rates reported in less developed countries.

Multiple factors contribute to the poor prognosis in HCC, including shortcomings with surveillance strategies and the morbidity of liver failure, which is observed in the majority of patients. HCC also is a molecularly heterogenous malignancy for which the biologic underpinnings are poorly understood, limiting the development of more effective, targeted therapies. Conventional histologic evaluation supplemented with immunostaining of certain markers lacks the accuracy to distinguish meaningful patterns of prognosis and response to therapy. Although advances in sequencing technology and proteomic analysis have led to new insights into HCC pathogenesis, molecular characterization of HCC remains in an early stage. Most findings are retrospective in nature and lack prospective validation, limiting their mainstream clinical application.

This review focuses on molecular characterization of HCC and the potential implications for improved prognostication, personalized therapies, and new treatment development. We also review transcriptomic characterizations of cirrhotic liver tissue, which have potential implications for more selective surveillance and prescription of chemopreventive therapy. Finally, we propose several changes to current HCC management strategies based on molecular findings suggesting adverse biology.

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MOLECULAR CLASSIFICATION OF HCC

Most HCC staging systems are based on tumor burden alone, which is simple and moderately effective at stratifying disease by prognosis. However, these systems lack sensitivity to account for adverse biologic features that influence response to therapy and survival, particularly among similarly staged lesions. One consequence is wide variation in reported outcomes despite similar stages of disease. In this regard, attempts have been made toward more nuanced staging models. The refined seventh addition of the American Joint Committee on Cancer tumor-lymph node-metastasis (TNM) for HCC includes vascular invasion in tumor substaging. Other societies have incorporated HCC-specific serum biomarkers. The Japan Integrated Staging System now includes α-fetoprotein (AFP), lens culinaris agglutinin-reactive AFP (AFP-L3), and des-γ-carboxy prothrombin (DCP) in their staging system. However, the Barcelona Clinic Liver Cancer (BCLC) staging system and the Chinese University Prognostic Index (CUPI), 2 other commonly used HCC staging systems, do not take into consideration histologic or serum markers, because the added value of these markers remains questionable.

Molecular characterization of HCC describes the study of transcriptomic, mutational, microRNA (miRNA), immunologic, and proteomic changes that occur in certain patterns or signatures. Some molecular features correlate with relevant biologic behaviors, including cellular turnover rate, microvascular invasion (MVI), and the development of distant metastases. Therefore, molecular characterization may address multiple unmet needs for HCC treatment, including: 1) more accurate prognostication than current staging methods; 2) guidance for the development of targeted therapeutic regimens; 3) rationale for selective, intensive monitoring of patients with adverse biologic features; and 4) improved clinical trial design by narrowing the biologic heterogeneity among similarly staged lesions. To satisfy these clinical objectives, separate research groups have proposed different schema for HCC molecular subtyping. The sections below summarize key attempts to define HCC classification systems.

MUTATIONAL HCC SUBTYPING

Chiang et al proposed an HCC classification system with 5 subtypes based on changes in copy number and gene expression. To establish this molecular signature, they first analyzed tumor tissue from 103 patients with hepatitis C virus (HCV)-related HCC, measuring copy number alterations in 238,000 loci using mapping arrays. The most common gains were in chromosomal regions 1q, 8q, 6p21, 11q13, 5p, 7, 17q, and 20. The most common losses were in regions 8p, 17p, 6q, 4q, 13q, 16q, 14q, and 10q. The authors tested for oncogene expression in regions of gains and observed that vascular endothelial growth factor (VEGF) was located on 6p21 (4% of cohort). An approximately 2.8-fold increased VEGF expression was measured using quantitative polymerase chain reaction, suggesting clinical relevance for this subset of patients. Next, they measured gene expression profiles in 91 tumor samples, from which 5 HCC subtypes were derived: 1) catenin β-1 (CTNNBI) mutation, which is characterized by aberrant wingless-type mouse mammary tumor virus integration site (WNT) signaling with tumors measuring >3 cm on average; 2) proliferation, which is associated with high serum AFP levels, macrovascular invasion, chromosomal instability, and enriched activation of insulin-like growth factor receptor 1 (IGFRI), ribosomal protein S6 (RPS6), and protein kinase B (AKT) signaling; 3) interferon (IFN), which is associated with smaller tumor size, a low CTNNBI mutation rate, and overexpression of IFN-stimulated genes, including signal transducer and activator of transcription 1 (STAT1), IFN-stimulated gene 15 (ISG15), IFN-α-inducible protein 16 (IFI16), and IFI27; 4) polysomy 7, characterized by reduced gains in 8q and polysomy of chromosome 7, which is associated with met proto-oncogene hepatocyte growth factor factor receptor (MET) and epidermal growth factor receptor (EGFR) amplifications; and 5) a nonspecific class without identifying features. Given the small sample size, the authors were unable to correlate the subgroups to survival, only metastatic potential.

More recently, Schulze et al performed whole-exome sequencing of 243 HCC tumor samples to characterize their mutational landscapes. On average, there were 21 silent and 64 nonsilent mutations per tumor; and, the investigators identified a total of 161 known driver mutations that were associated with 11 different signaling pathways, which included: telomerase reverse transcriptase (TERT) activation of telomerase, WNT, phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR), tumor protein 53 (TP53) inactivation, MAPK, hepatic differentiation, epigenetic regulation, chromatin remodeling, oxidative stress, interleukin 6 (IL-6)/STAT, and transforming growth factor β (TGF-β). Nucleotide mutational patterns correlated with environmental risk exposures, including alcohol, tobacco, α-toxin, and aging. Alcohol-related HCCs were enriched for CTNNBI mutations, whereas hepatitis B virus (HBV)-related HCCs were enriched for TP53 mutations. IL-6 mutations were identified only in idiopathic cases. Finally, the authors were able to temporally categorize
mutations, characterizing the natural history of HCC progression. TERT mutations were identified early; whereas CTNNBI, cyclin-dependent kinase inhibitor 2A (CDKN2A), and TP53 mutations were observed in more advanced disease; and chromosomal abnormalities appeared later than gene mutations.

Despite their different methodologies, the findings from Chiang et al and Schulze et al have marked overlap, identifying common mutational drivers in HCC. TP53 and CTNNBI are frequent mutations; TP53 is associated with more aggressive disease, whereas CTNNBI mutation is associated with variable prognosis. Phosphokinase signaling (AKT and MAPK pathways) tracks with adverse biologic features, including vascular invasion, and is associated with decreased survival.

TRANSCRIPTOMIC HCC SUBTYPING
Boyault et al established one of the first transcriptomic molecular classification systems for HCC composed of 6 groups, called G1 through G6.12 To characterize the mutational diversity of HCC, those authors performed genome-wide assessment for allelic loss and direct sequencing of previously documented HCC mutations on a set of 120 HCCs and 3 hepatocellular adenomas. They subsequently performed genome-wide transcriptome microarray analysis to assess for variation in gene expression. This approach yielded 6 distinct genetic groups. G1, G2, and G3 tumors were characterized by more frequent chromosomal aberrations compared with groups G4, G5, and G6. G1 tumors were associated with HBV infection, low viral DNA copy numbers, annexin A1 (AXIN1) mutation, and increased IGF-2 and AFP expression. G2 tumors also were associated with HBV infection, but with higher viral DNA copy numbers, PIK3CA mutation, and a clinical association with MVI and satellitosis. Both G1 and G2 tumors had increased AKT activation and downstream glycogen synthase kinase 3β (GSK3β) expression. G3 tumors were associated with increased allelic loss, including chromosome 17 deletion and TP53 mutation, CDKN2A hypermethylation, and increased expression of cyclins. The G3 subtype had the worst prognosis. G4 tumors were more heterogeneous at the molecular level but were associated with well differentiated tumors. G5 and G6 HCCs were associated with CTNNBI mutation (70% and 100%, respectively), WNT activation, and cadherin-1 (CDH1) hypermethylation; and they clinically displayed increased invasiveness and satellitosis. Thus, G1, G2, and G3 tumors, characterized by TP53 mutation, increased AFP expression, and aberrant phosphokinase signaling, were more aggressive subtypes. It is noteworthy that, similar to the work by Chiang et al and Schulze et al, CTNNBI mutation and aberrant WNT signaling independently capture a unique genetic phenotype, which has a less severe prognosis than subtypes with more enhanced proliferative features.

Hoshida et al used a similar approach to establish a transcriptomic HCC signature composed of 3 groups called S1, S2, and S3.13 Those authors analyzed publicly available sequencing data using multiple algorithmic gene clustering methods, followed by gene set enrichment analysis to functionally characterize the defined subgroups. Their subclasses were then validated using a set of 118 randomly selected, formalin-fixed, paraffin-embedded HCC samples. The subclasses were revealing in both clinical and biologic relevance. In S1 tumors, increased MVI and satellitosis were identified on histology and were associated with increased early recurrence (within 1 year). Molecularly, S1 tumors had aberrant WNT signaling mediated by abnormal expression of Transforming Growth Factor Beta 1 (TGFBI), which was associated with an invasive epithelial-to-mesenchymal (EMT) phenotype. S2 tumors were larger and had significantly greater AFP expression, and both S1 and S2 tumors were generally moderate to high-grade lesions. S2 lesions had increased AKT activation and downstream activation of epithelial cell adhesion molecule (EPCAM) and MYC target genes. It is noteworthy that S2 lesions also had decreased IFN target gene expression. In contrast, S3 lesions were smaller, more differentiated, and associated with a better prognosis, similar to the Boyault G4 subtype. S3 tumors had increased expression of normal hepatocyte functional genes and functional tumor suppressors p21 and p53, consistent with the clinically observed less aggressive biology.

The adverse biologic features captured by the Hoshida signature appear to correlate well with clinicopathologic observations. In a separate recent analysis, Murakata et al performed a transcriptomic evaluation of 275 patients who had nodular type HCC.14 Tumors were separated by morphology into 3 types: 1) single nodular, 2) single nodular with extranodular growth, and 3) confluent multinodular. Confluent multinodular types were associated with higher recurrence and worse survival. On multivariable analysis, these tumors were distinguished by increased EPCAM expression and elevated AFP, consistent with the Hoshida S2 signature.14

More recently, Desert et al performed a transcriptomic analysis specifically focused on well differentiated, nonproliferative HCCs, which are associated with improved outcome.15 They identified 2 distinct molecular signatures, referred to as periportal (PP) and perivenous
(PV) subtypes, which are CTNNB1 wild type and mutated, respectively. The PP subtype was enriched for gene expression associated with differentiated, perportal hepatocyte function, including gluconeogenesis and amino acid catabolism; whereas the PV subtype was associated with perivenous hepatocyte function, including fatty acid and bile salt metabolism. PP subtype HCCs were associated with low grade, low recurrence, and improved survival and had genetic overlap with the Hoshida S3 signature. In contrast, the PV subtype was more similar to the Boyault G6 and Chiang CTNNB1 signatures.

TRANSCRIPTOMIC SIGNATURES TO PREDICT HCC GROWTH, VASCULAR INVASION, MULTICENTRIC RECURRENCE, AND INTRAHEPATIC METASTASIS

The aforementioned methods for HCC subtyping involved stratification of patients based on differences in molecular patterns, which were then correlated to clinically relevant parameters, including recurrence, metastasis, and prognosis. However, a more commonly used approach involves the reverse analysis: stratifying patients by clinical parameters, then investigating for variations in molecular patterns. By using the latter approach, multiple research teams have designed transcriptomic signatures to predict MVI and recurrence after surgical resection or transplantation for HCC, although most have modest predictive capacity, in part because of small validation cohorts.16–23 Ye et al proposed one of the first signatures using gene expression profiling that could distinguish with 85% accuracy patients who had HBV-related HCC with and without intrahepatic metastases.24 Several interesting biologic insights regarding HCC pathogenesis arose from their work. First, the signature was independent of tumor size, encapsulation, and patient age. Although tumor size roughly correlates with adverse biology, these findings suggest that this association is imperfect. Moreover, the expression pattern was similar in the primary lesions and the metastatic sites, indicating that the changes necessary for metastasis occur in the primary tumor, likely through inherent differences in tumor biology or via acquired susceptibility to metastatic promoters, referred to as epigenetic switching. Second, multiple genes in the signature relate to cell disassociation and extracellular matrix degradation, processes that are essential for metastatic potential. The top hit was secreted phosphoprotein 1 (SPP1 [osteopontin]), which previously demonstrated the ability to induce cell disassociation and invasive phenotypes. This signature also was predictive of survival, as expected. Of note, SPP1 was not reported in the high-risk subtypes for either the Hoshida or Boyault classification systems.

A follow-up study using this same metastasis signature was performed on a larger sample of patients with HCC consisting of 2 cohorts: 247 patients with HBV-related HCC and 139 with HCC of mixed etiology.20 The signature was significantly predictive in all 386 patients for overall and disease-free survival, and predictive accuracy was further enhanced when the signature was combined with BCLC stage and AFP level.

More recently, Minguez et al proposed a 35-gene signature to predict vascular invasion in patients with HCV-related HCC.21 The signature was based on an analysis of 214 patients and had a negative predictive value of 77% in a training cohort. Combined with tumor size, the diagnostic accuracy for prediction of vascular invasion was improved. It is noteworthy that the authors were able to determine the signatures from both fresh-frozen and formalin-fixed, paraffin-embedded tissue, which has potential utility for preoperative biopsy assessment. Although this signature lacks sufficient accuracy for mainstream clinical application, it displays the strong potential of this technological approach for the prediction of phenotypic tumor features.

Finally, Villa et al proposed a 5-gene signature to predict tumor-doubling time in patients with HCC.25 In their study, 132 patients underwent 2 computed tomography scans 6 weeks apart to determine tumor growth. No treatment was received during this interval. The authors separated patients into quartiles based on tumor doubling times, which ranged from 30 to 621 days. In the fastest growing quartile, the authors identified 5 genes that were significantly upregulated compared with the other quartiles, which included angiopoietin-2 (ANGPT2); neuropilin tolloid-like 2 (NETO2); endothelial cell-specific molecule-1 (ESM1); nuclear receptor subfamily 4, group A, member 1 (NR4A1); and δ-like ligand 4 (DLL4). It was demonstrated previously that these genes played roles in endothelial cell activation and blood vessel development and thus may contribute to tumor neoangiogenesis. When applied to a validation cohort of 54 patients, the signature was predictive of both rapid tumor growth (area under the curve, 0.961; P < .0001) and mortality (hazard ratio, 3.987; P < .0001).

MICRORNA HCC SUBTYPING

MicroRNAs (miRNAs) are 18-nucleotide to 25-nucleotide molecules that regulate the translation of messenger RNA (mRNA), and are dysregulated in certain cancers, including HCC. After transcription, miRNA are processed by a nuclease called DROSHA and are...
subsequently transported to the cytoplasm, where they are cleaved by the DICER enzyme. Finally, the mature miRNA is functionalized by incorporation into the RNA-induced silencing complex (RISC). miRNAs bind to partially complimentary sites located near the 3’-untranslated region on mRNA, influencing translation or degradation. Previous studies have causally linked individual microRNA expression changes to adverse biologic processes in HCC, including rapid proliferation, epithelial-to-mesenchymal transition (EMT), local tissue invasion, and vascular invasion. A summary of recently published studies with purported miRNA mechanisms is provided in Table 1. For the majority of cases, miRNA loss correlates to induction of a target oncogene, and miRNA gain correlates to repression of a target tumor suppressor.

Toffanin et al proposed one of the first HCC classification systems based on an miRNA signature. To create this signature, the authors evaluated expression levels of 358 different miRNAs in 89 patients with HCV-related HCC who underwent liver resection or transplantation. By using unsupervised hierarchical clustering, they identified 3 different HCC subtypes based on miRNA expression variation, which were called clusters A, B, and C. Cluster A was enriched with samples that had low AFP levels and increased CTNNB1 mutations, similar to the Hoshida S1 and Boyault G5 and G6 subtypes. Cluster B tumors were smaller in size and were enriched for IFN response genes, similar to the Chiang IFN subtype. Cluster C was subdivided into 3 subgroups called C1, C2, and C3. In general, cluster C tumors were associated with adverse biologic features and worse prognosis and, accordingly, had marked overlap with the Chiang proliferation, Hoshida S2, and Boyault G1, G2, and G3 subtypes. They were enriched for aberrant activation of IGFR1 and PI3K/AKT signaling pathways. C1 tumors were associated with vascular invasion; C2 tumors were poorly differentiated and had an MET-positive signature with changes in gene expression related to oxidative stress response and angiogenesis; and C3 tumors had expression profiles similar to those of other high-risk signatures, including increased proliferation and invasive features, Ep-CAM positivity, TP53 mutation, high AFP levels, and reduced expression of microRNA-26a (miR-26a) and miR-26b. Downregulation of miR-26 was previously correlated with a unique transcriptomic pattern in HCC characterized by increased tumor IL-6 and nuclear factor κB (NF-κB) signaling, which were associated with a worse prognosis but an improved response to IFN therapy.

Review Article

Jiang et al evaluated miRNA expression patterns to identify a 19-miRNA signature that could predict HCC prognosis. In general, patients who had poor survival exhibited downregulation of miRNAs, whereas those with improved survival had miRNA upregulation, consistent with the trend summarized in Table 1. Many of the targets associated with adverse biology were related to proliferation, including division, mitosis, and cell-cycle progression. Specifically, downregulation of miR-26b was present in the patients who had poor survival, consistent with previous reports. Wei et al performed a similar analysis in which they evaluated 110 patients with HCC and established a 20-miRNA signature to predict adverse prognosis. Patients in that analysis were divided into high-risk and low-risk groups, which had 3-year survival rates of 35.3% and 92.6%, respectively.

In a separate analysis, Budhu et al examined miRNA expression patterns to identify specific predictors of venous metastasis. They used unsupervised hierarchical clustering analysis to evaluate 240 patients with HCC, and they identified 20 dysregulated miRNAs with statistically different expression patterns between patients with and without metastases. This signature, as expected, was predictive of disease-free and overall survival. The most highly upregulated miRNAs included miR-219, miR-207, and miR-338; whereas miR-34, miR-30, and miR-148 had the greatest downregulation. Among patients with metastatic disease, 16 of 20 miRNAs in the signature were downregulated, consistent with a pattern of disinhibited oncogene mRNA translation. It was demonstrated previously that both miR-34 and miR-338 contribute to HCC pathogenesis. Yang et al demonstrated that miR-34 is downregulated in HBV-associated HCC by increased expression of TGF-β. One target gene of miR-34 is C-C motif chemokine ligand 22 (CCL22), a chemokine that recruits regulatory T cells (Tregs), which subsequently promote tumor immune evasion. This process supports the survival of micrometastatic disease, which is associated with decreased patient survival. It is noteworthy that miR-338 was upregulated in patients with metastatic disease, yet all prior studies associated upregulation with improved survival. Nie et al reported that the mineral corticoid receptor regulates miR-338 expression. One downstream target of miR-338 is pyruvate kinase, which is required for cytoplasmic glycolysis. The downregulation of miR-338 results in increased pyruvate kinase expression and enhanced glycolytic
TABLE 1. MicroRNA Aberrations and Associated Pathogenic Mechanisms in Hepatocellular Carcinoma

| MicroRNA | Reference          | Findings                                                                                                                                                                                                 | Adverse Prognosis |
|----------|-------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------|
| 9        | Han 2017\(^{29}\) | Associated with reduced survival; inhibits CDKN1A expression, promoting cell-cycle progression                                                                                                            | Gain              |
| 10       | Li 2012\(^{30}\)  | Inhibits expression of CADM1, inducing cell motility and invasion; associated with reduced survival                                                                                                       | Gain              |
| 21       | Meng 2007\(^{31}\) | Regulates expression (PTEN); upregulation associated with cell invasion and worse prognosis                                                                                                              | Gain              |
|          | Tomimaru 2012\(^{32}\) | Plasma miR-21 levels significantly elevated (\(P < .0001\)) versus normal controls and patients with cirrhosis, may be useful as a diagnostic biomarker                                                                                   | Gain              |
| 22       | Zhang 2010\(^{33}\) | Inhibits expression of HDAC4; downregulation observed in HCC, associated with reduced survival                                                                                                             | Loss              |
| 23       | Cao 2017\(^{34}\)  | Downregulation associated with decreased survival and increased metastasis; inhibits PYK2, which promotes epithelial-to-mesenchymal transition                                                                 | Loss              |
| 26       | Yang 2013\(^{35}\)  | Downregulation associated with recurrence and metastasis; inhibits IL-6 and downstream targets through STAT3, including BCL-2, MCL1, CCND1, and MMP2                                                                 | Loss              |
| 28       | Zhou 2016\(^{36}\)  | Downregulation associated with metastasis; inhibits IL-34 expression; IL-34 recruits tumor-associated macrophages, which inhibit miR-28 expression through TGF-\(\beta\) secretion                                                                 | Loss              |
| 29       | Parpart 2014\(^{37}\) | AFP inhibits miR-29 expression; miR-29 inhibits DNMT3A, thereby reducing DNA methylation, which is associated with AFP-positive tumors and reduced survival                                                                 | Loss              |
| 34       | Yang 2012\(^{38}\)  | HBV infection increases TGF-\(\beta\) expression, which suppresses miR-34a, leading to increased CCL22 secretion and recruitment of regulatory T cells that promote immune escape; downregulation associated with reduced survival | Loss              |
| 99       | Li 2011\(^{39}\)   | Inhibits expression of IGFR1 and MTOR, subsequently reducing cyclin expression, promoting cell-cycle arrest; downregulation associated with reduced survival                                                                 | Loss              |
| 101      | Wang 2014\(^{40}\)  | miR-101 is inhibited by PRC2 in a c-myc-dependent manner; miR-101 inhibits EZH2 and EED components of PRC2 in a double-negative feedback loop; EZH2 and c-myc associated with worse survival | Loss              |
| 124      | Zheng 2012\(^{41}\) | Inhibits expression of ROCK2; loss of miR-124 associated with decreased survival                                                                                                                                 | Loss              |
| 126      | Xiang 2017\(^{42}\) | Downregulation associated with HBV-related HCC metastasis; inhibits ADAM9, reducing cell migration and invasion                                                                                           | Loss              |
| 133      | Wang 2017\(^{43}\)  | Downregulation associated with decreased survival; inhibits EGFR and AKT/mTOR signaling                                                                                                                  | Loss              |
| 135      | Liu 2012\(^{44}\)   | Upregulated in patients who have HCC with portal vein thrombus; inhibits MTSS1, a tumor suppressor                                                                                                         | Gain              |
| 139      | Wong 2011\(^{45}\)  | Inhibits expression of ROCK2; loss of miR-139 associated with \(\beta\)-mediated HCC cell migration, increased satellitosis, and reduced survival                                                                 | Loss              |
| 140      | Yang 2013\(^{46}\)  | Downregulation associated with vascular invasion, multidulality, and decreased survival; inhibits TGF\(\beta\)1 and FGF9 expression, reducing TGF-\(\beta\) and MAPK signaling | Loss              |
| 145      | Law 2012\(^{47}\)   | Inhibits IGF pathway genes IRI\(S1\) and IRI\(S2\), promoting G2/M cell-cycle arrest; downregulation associated with reduced disease-free survival                                                             | Loss              |
| 155      | Han 2012\(^{48}\)   | Upregulation associated with reduced survival and increased microvascular invasion among patients with recurrent HCC after liver transplantation                                                                 | Gain              |
| 183      | Li 2010\(^{49}\)    | Downregulates PDCD4, thereby inhibiting TGF-\(\beta\)-mediated apoptosis                                                                                                                                 | Gain              |
| 187      | Dou 2016\(^{50}\)   | Downregulation associated with advanced TNM stage and metastases; targets S100A4                                                                                                                       | Loss              |
| 188      | Fang 2015\(^{51}\)  | Downregulation associated with multifocal disease; inhibits FGFR5, thereby decreasing cell proliferation and metastasis in vivo                                                                       | Loss              |
| 197      | Wang 2015\(^{52}\)  | Inhibited by IL-6/STAT3 signaling pathway; STAT3 activity associated with more aggressive biology                                                                                                | Loss              |
| 199      | Wang 2011\(^{53}\)  | Inverse correlation between miR-199 expression and HIF1A; downregulation of miR-199 associated with reduced survival                                                                                     | Loss              |
| 200      | Li 2017\(^{54}\)    | Downregulation associated with increased TNM stage; inhibits MAD2L1 to reduce proliferation and invasion                                                                                             | Loss              |
| 221      | Fornari 2008\(^{55}\) | Downregulates expression of CDKN1B and CDKN1C tumor suppressors; present in 71% of tested patients, associated with worse survival                                                                           | Gain              |
| 296      | Wang 2016\(^{56}\)  | Downregulation associated with reduced survival; inhibits FGFR1, which promotes cell-cycle arrest and apoptosis                                                                                     | Loss              |
metabolism, which have been associated with a worse prognosis.59

Several of the hits in these prognostic miRNA signatures, as expected, correlated with the Toffanin cluster C, which captures lesions with adverse biology. However, there was minimal overlap among the individual signatures. The signatures from Wei et al and Jiang et al shared only miR-26b downregulation. The signature from Budhu et al shared changes in miR-148 and miR-30 with Jiang et al and only changes in miR-15a with Wei et al. The lack of overlap was unexpected, because all signatures independently correlated with survival. Potential explanations include different microarray and in-silico analytic techniques, variation in patient demographics and etiology of liver disease, small sample size, and lack of external validation. However, this variability suggests that more robust validation studies are needed before this technology can be evaluated in a prospective clinical setting.

In summary, comparison of mutational, transcriptional, and miRNA-based HCC classification schema reveal common molecular themes that stratify disease by biologic behavior, providing a useful supplement to conventional TNM staging for more accurate prognostication. A summary of the different classification schema discussed is provided in Figure 1 to emphasize their commonalities with respect to defining genetic aberrations and the associated biologic features and survival outcomes.

### DNA METHYLATION SIGNATURES OF RECURRENCE AND PROGNOSIS

DNA methylation is an epigenetic modification that alters gene expression.72 Promoter methylation is associated with repression of target gene transcription, and global hypomethylation with focused regions of hypermethylation (CpG islands) is a hallmark of human malignancies. In HCC, it has been demonstrated that both methylation

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**TABLE 1. Continued**

| MicroRNA | Reference | Findings | Adverse Prognosis |
|----------|-----------|----------|-------------------|
| 302      | Chen 201757 | Associated with decreased survival; inhibits TGFBR2, a tumor suppressor, promoting cell migration and invasion in vitro | Gain |
| 326      | Hu 201758  | Downregulation associated with increased TNM stage; inhibits cell proliferation and invasion by targeting LASP1 | Loss |
| 338      | Nie 201559  | Downregulation associated with worse survival; miR-338 expression is controlled by the mineral coxid receptor; miR-338 inhibits pyruvate kinase, limiting glycolysis | Loss |
| 345      | Zhang 201760 | Downregulation associated with reduced survival; inhibits YAP1, decreasing cell migration and invasion in vitro | Loss |
| 486      | Fu 201761  | Inhibits NEK2 expression, which is associated with worse survival | Loss |
| 493      | Xu 201762  | Downregulation associated with reduced survival; inhibits ANTXR1 and RSP02, decreasing cell migration and invasion in vitro | Loss |
| 550      | Tian 201763 | Inhibits CPEB4, promoting cell migration and invasion; downregulation of CPEB4 associated with reduced survival | Gain |
| 622      | Liu 201564 | Downregulation associated with worse prognosis; miR-622 inhibits CXCR4, which is a driver of tumor proliferation and invasion; miR-622 is inhibited by EZH2-mediated promoter methylation | Loss |
| 638      | Zhang 201765 | Downregulation associated with decreased survival; inhibits SOX2, which promotes epithelial-to-mesenchymal transition | Loss |
| 1271     | Lin 201766  | Inhibits FOXK2, an oncogene associated with decreased survival on multivariable analysis | Loss |
| 1296     | Xu 201767  | Downregulation associated with increased metastasis; inhibits SRPK1, which promotes AKT signaling and cell invasion; miR-1296 inhibited by hypoxia | Loss |

Abbreviations: ADAM9, A disintegrin and metalloproteinase 9; AFP, a-fetoprotein; Akt, protein kinase B; ANTRX1, anthrax receptor toxin 1; BCL-1, B-cell leukemia 1; CADM1, cell adhesion molecule 1; CCL22, C-C motif chemokine ligand 22; c-myc, myelocytomatosis proto-oncogene; CDKN1B, cyclin dependent kinase inhibitor 1B; CDKNT1C, cyclin dependent kinase inhibitor 1C; CPEB4, cytoplasmic polyadenylation element binding protein 4; CXCR4, C-X-C chemokine receptor 4; DNMT3A, DNA methyltransferase 43A; EED, embryonic ectoderm development; EGFR, epidermal growth factor; EZH2, enhancer of zeste homolog 2; FGF, fibroblast growth factor; FGFFR, fibroblast growth factor receptor; FOXX2, forhead box k2; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HDAC4, histone deacetylase 4; HIF-1α, hypoxia-inducible factor 1α; HPV, human papillomavirus; IGF, insulin-like growth factor; IGF1R, insulin-like growth factor receptor 1; IL, interleukin; IRS, insulin receptor substrate; LASP1, LIM and SH3 protein 1; MAD2L1, mitotic arrest deficient 2 like 1; MAPK, mitogen-activated protein kinase; MCL1, myeloid cell leukemia 1; miR, microRNA; MMP2, matrix metallopeptidase 2; mTOR, mammalian target of rapamycin; MTSS1, metastasis suppressor 1; NEK2, NIMA-related kinase 2; PDCD4, programmed cell death 4; PCRE2, polycomb repressive complex 2; Pten, phosphatase and tensin homolog; PYK2, protein tyrosine kinase 2; ROCK2, p21-kinase 2; RSP02, R-spondin 2; S100A4, S100 calcium binding protein A4; SOX2, SRY-box 2; SRPK1, serine/threonine protein kinase 1; STAT3, signal transducer and activator of transcription 3; TGFBR, tumor growth factor β receptor; TGF-β, tumor growth factor β; TNM, tumor node metastasis; YAP1, yes-associated protein 1.
of tumor-suppressor genes and hypomethylation of oncogenes are relevant to carcinogenesis and prognosis.\textsuperscript{73,74} There is also a concept of epidrivers, which is similar in principle to the idea of driver mutations, suggesting that, among a multitude of aberrant methylation patterns, certain methylation sites have much greater influence on pathogenic phenotype. Epidrivers are thought to be crucial mediators of transcriptomic signatures that correlate with prognosis in HCC. It is important to note that methylation is potentially reversible, inspiring interest as a therapeutic target.

In one of the largest studies to date, Villanueva et al proposed a DNA methylation signature to predict HCC prognosis.\textsuperscript{75} They performed transcriptomic and methylocmic profiling on 248 surgically resected HCC samples, which they compared with 19 nontumor liver tissue samples. By using predefined thresholds for hypermethylation and hypomethylation between the 2 groups, the authors analyzed 11,307 CpG sites to select regions that were dysregulated in HCC. Among this set, they then used the random survival forest method to identify a 36-CpG signature (all sites hypermethylated in tumor tissue), which was used to establish a mortality index score. The methylome signature highly correlated with known clinicopathologic markers of poor prognosis, including vascular invasion, multinodularity, satellitosis, advanced BCLC stage, and elevated serum AFP levels. On multivariable analysis, the methylation score, along with platelet count and multinodularity, was independently predictive of survival. Moreover, the top 20% of methylation scores highly correlated with the Hoshida S2 and Ep-CAM–positive HCC subtypes.

The authors also identified new biologic insights into HCC pathogenesis. Consistent with previous reports, they observed hypermethylation of tumor suppressors, including Ras association domain family member 1 (\textit{RASSF1}), adenomatous polyposis coli (\textit{APC}), and neurofilament heavy (\textit{NEFH}), and hypomethylation of \textit{IGF-2}, a known oncogene.\textsuperscript{76-78} Decreased methylation of \textit{IGF-2} fetal promoters has previously been observed in HCCs with progenitor cell features and an aggressive phenotype, and most nucleic acid-based classification systems incorporate aberrant IGF signaling as a defining molecular feature (Chiang proliferation, Boyault G1, and Toffanin cluster C subtypes).\textsuperscript{79} Villanueva et al also identified the hypomethylation of epidrivers in HCC that had previously been described in other malignancies, notably \textit{NOTCH3} in leukemia, nuclear receptor-binding SET domain protein 1 (\textit{NSD1}) in glioblastoma, and zinc finger of the cerebellum (\textit{ZIC1}) in colorectal cancer.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{The hepatocellular carcinoma classification schema were based on gene expression profiling. AFP indicates \(\alpha\)-fetoprotein; AKT, protein kinase B; CTNNB1, catenin \(\beta\)-1; Ep-CAM, epithelial cell adhesion molecule; IGF, insulin-like growth factor; MET, met proto-oncogene (hepatocyte growth factor receptor).}
\end{figure}
Finally, they identified new candidate epidoiviers related to WNT, TGF-β, and fibroblast growth factor (FGF) signaling.75 Aberrant WNT signaling is a defining feature of the Chiang CTNNB1 subtype, Boyault G5 and G6, and Hoshida S1 subtypes. Therefore, this latter finding complements these subtypes by providing a novel mechanism other than CTNNB1 mutation for WNT activation. Further investigation is warranted to determine whether epigenetic therapies targeting these pathways would be of benefit.

More recently, Qiu et al recently demonstrated that aberrant DNA methylation also might be used to distinguish the risk of recurrence among patients with resected, early stage HCC.80 Those authors analyzed 66 tumors samples with an Illumina Methylation 450-k bead chip, from which they isolated 46 CpG sites that were associated with either a high or low risk of recurrence. They narrowed this set to 3 CpG sites near 3 genes (SCAN domain-containing protein 3 [SCAND3], SH3-containing GRB2-like protein-3 interacting protein 1 [SGIP1], and peptidase inhibitor 3 [PI3]), which were then used to create a risk score. They incorporated the methylation risk score into a recurrence risk nomogram that included other clinical variables, such as tumor differentiation, HBV antigen status, presence of cirrhosis, and history of antiviral therapy. The concordance index of the nomogram for 5-year recurrence-free survival was 0.693 when it was tested on an external validation cohort. Although the authors did not compare their recurrence nomogram with other known predictors of recurrence, the score may serve as a useful supplement to stratify postoperative patients for surveillance and adjuvant therapy.

CLINICAL IMPLICATIONS OF NUCLEIC ACID-BASED SIGNATURES

Molecular signature characterization is still in early development and has been used primarily for retrospective prediction of prognosis in HCC. To date, there is limited evidence supporting the application of molecular signatures in the prospective design of personalized therapeutic regimens. However, several studies highlight the promise of these technologies for advancing HCC care. For example, Schulze et al performed a mutational analysis in which 28% of HCC tumors had mutations that were targetable with therapies already approved by the US Food and Drug Administration (FDA), supporting the assertion that sequencing may be a useful source of screening for individualized drug targets.11 Nucleic acid data also may predict drug resistance. Nishida et al analyzed sera from 16 patients with HCC from which they selected 5 miRNAs that differed significantly between responders and nonresponders to sorafenib therapy.81 Those authors observed that miR-181a-5p was significantly increased in patients who had disease control up to 3 months after treatment initiation. On multivariable analysis, serum miR-181a-5p was the only factor that independently predicted disease control and overall survival.

Clinical trials for HCC are currently limited by challenges of homogeneous patient selection. Heterogeneity in clinical trial patient enrollment may contribute to the failure to identify limited responses to therapies under study, such as those targeting EGFR, IGF receptor 1 (IGF1R), VEGF, MET, and mTOR.82-86 Mutational, transcriptomic and miRNA analyses performed on biopsied tissue may provide a method to select patients with specific pathologic molecular themes that are more likely to respond to targeted therapies under study.86 Tan et al tested the Hoshida S1, S2, and S3 signatures in this regard. Those authors used tumor biopsy samples from prior clinical trials to filter samples for molecular patterns that correlated with response to targeted therapies. Setting a predicted response rate threshold of 50%, they reduced the number needed to treat with subclass-targeting therapies by greater than 50%.87 The study provides one of the first examples of the potential utility of molecular subtyping for future trial design. In addition, the phase 1 clinical trial evaluating the safety and efficacy of BLU-554, a novel fibroblast growth factor receptor 4 (FGFR4) inhibitor, represents a current example of genetically targeted patient selection.88 In that trial, patients were selected based on FGF19/FGFR4 expression levels, thereby providing therapy to individuals with the greatest potential for response. Finally, the National Cancer Institute Molecular Analysis for Therapy Choice (NCI-MATCH) trial also recently launched, which represents an ambitious attempt to incorporate molecular profiling into targeted therapy.89 This trial includes multiple cancer types, including solid-organ malignancies. Patient tumor samples are screened for driver mutations, and this information is then used to match patients with therapy targeted for their particular abnormality.

Nucleic acid-based molecular signatures also provide evidence for the design of novel targeted therapies or clinical trials of off-label drug use. For example, miRNA therapeutics are a new class of drugs predicated on aberrant miRNA expression patterns.90 By using this approach, Kota et al developed an adenov-associated virus containing miR-26a, which is downregulated in HCC and associated with a poor prognosis.91 Administration and reconstitution of miR-26a levels in a murine HCC model inhibited
cancer cell proliferation and prevented disease progression, displaying the potentially utility of this therapeutic modality. Similarly, histone deacetylase inhibitors (HDACs) also may provide therapeutic value for the subset of patients who have HCC with aberrant DNA methylation. A recent phase 2 clinical trial evaluating belinostat (an HDAC inhibitor previously approved by the FDA for peripheral T-cell lymphoma) in patients with unresectable HCC showed disease stabilization with treatment, which was more pronounced in patients who were enriched for HDAC sensitivity (58% vs 14%; \( P = .036 \)) based on HR23B histoscores.92

HCC PROTEOMICS PREDICT PROGNOSIS AND IDENTIFY NOVEL TREATMENT TARGETS

Advances in mass spectrometry have allowed for expansion of proteomic profiling of HCC over the last decade, and recent findings have led to the discovery of potentially targetable pathogenic mechanisms. Morofuji et al evaluated expression levels of 192 apoptosis-related proteins from 80 surgically resected HCC samples to determine potential markers for early recurrence (within 2 years from the date of curative resection).93 They identified 6 proteins that were uniquely upregulated in early recurrence that included extracellular signal-regulated kinase 1 (ERK1), protein kinase G (PKG), apoptotic protease activating factor 1 (Apaf1), B-cell leukemia X (Bcl-X), Abelson murine leukemia viral oncogene (c-abl), protein inhibitor of activated STAT 1 (PIAS1), and PIAS2. It has been demonstrated that both ERK and Bcl-X correlate with a poor prognosis in HCC.94,95 Multiple ERK inhibitors are currently in early phase clinical trials for solid gastrointestinal malignancies and may be of potential value in the treatment of HCC.

In a separate analysis, Orimo et al similarly examined global protein expression profiles to determine adverse proteomic markers for HCC.96 They used 2-dimensional difference gel electrophoresis and mass spectrometry to evaluate 45 surgically resected HCC samples of various grades and 18 nontumor tissue samples. Twenty-six proteins were identified in association with HCC, of which 14 were linked functionally to c-myc, activator protein-1 (AP-1), hypoxia-inducible factor 1–\( \alpha \) (HIF1–\( \alpha \)), and the rat sarcoma (Ras) superfamily. Aberrant myc signaling and response to hypoxia are well documented in association with aggressive HCC behavior.97 Indeed, it was recently demonstrated that chemoembolization-induced hypoxia paradoxically may stimulate an aggressive progenitor phenotype in surviving HCC cells.98 Morofuji et al also observed that the APC-binding protein 1 (EB1), which is regulated by c-myc and RhoA, significantly correlated with high grade and was an independent predictor of decreased survival on multivariable analysis. The authors concluded that EB1 may be a useful novel proteomic biomarker for determination of prognosis.

Finally, multiple research teams have searched for proteomic biomarkers to predict response to sorafenib chemotherapy. Kim et al used proteomic profiling of HCC tissue samples to identify a panel of 3 proteins (cluster of differentiation 5 [CD5] molecule like [CD5L], immunoglobulin J chain [IGJ], and galectin-3–binding protein [LGALS3BP]) in which low protein expression was predictive of a poor response to sorafenib. Although the mechanisms of these proteins are not established in HCC, it has been demonstrated that they promote antitumor immunomodulatory effects in other malignancies.99 Zhang et al evaluated phosphorylated ERK (p-ERK) levels in multiple HCC cell lines as a predictor of sorafenib sensitivity.100 They observed that increasing basal p-ERK levels positively correlated with metastatic potential; however, low basal p-ERK levels conferred resistance to sorafenib-mediated growth inhibition. Because sorafenib has a significant side-effect profile, sparing patients unnecessary toxicity by using a response profile may improve quality of life.

THE PROGENITOR SUBTYPE OF HCC CORRELATES WITH POOR PROGNOSIS

For the majority of HCCs, it has been believed that damaged hepatocytes are the cells of origin. However, a subset of HCC was recently identified with markers of both progenitor and cholangiocyte-like features. Lee et al performed hierarchical clustering analysis of gene expression patterns from human HCC, human hepatocytes, mouse HCC, and rat fetal hepatoblasts and identified a subset of human HCC that had strong transcriptomic overlap with hepatoblasts.101 This subtype had increased expression of protein markers consistent with hepatic oval cells (progenitor cells), including cytokeratin-19 (CK-19) and vimentin (VIM). Pathway analysis revealed unique dysregulation of AP-1–related signaling networks, including JUN and FOS, compared with nonhepatoblast subtypes of HCC. On the basis of these findings, the authors concluded that hepatic progenitors might be the cells of origin for a subset of HCC. Of note, patients with progenitor-like HCC generally have a worse prognosis, and the expression of AP-1–related proteins was associated with decreased survival by the Orimo et al proteomic signature.96
Woo et al similarly used gene expression profiling to identify a subset of HCC with high expression of dedifferentiated markers, suggestive of a progenitor-like state, including CK-19, Ep-CAM, and CD133 (prominin-1). These tumors also uniquely expressed genes commonly observed in cholangiocarcinoma, including carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), mucin 1 (MUC1), and claudin 4 (CLDN4) and thus were labeled cholangiocarcinoma-like HCC. Progenitor and cholangiocyte-like features are commonly observed together in HCC, as indicated in this study, although the explanation for this occurrence remains unclear.

Tschaharganeh et al partly explain this relation in their seminal work demonstrating that the progenitor phenotype in HCC can also arise from injured hepatocytes. By using a conditional TP53 knockout mouse, they revealed that TP53 inactivation in the setting of oncogenic stimuli led to hepatocyte dedifferentiation characterized by nestin expression, which was essential for tumorigenesis in vivo. They also demonstrated that subsequent mutations in either WNT or NOTCH signaling led to the development of HCC or cholangiocarcinoma, respectively. Therefore, the overlap between expression of progenitor and cholangiocyte markers may be caused in part by the ability of TP53-mutated hepatocytes to dedifferentiate into progenitors followed by transdifferentiation into cholangiocytes.

Other work by Coulouarn et al supports this assertion that WNT dysregulation is a defining feature of progenitor-like HCC. Similar to the mechanism described in the Hoshida S1 subtype, WNT signaling was mediated by overexpression of TGF-β rather than CTNNB1 mutation. Currently, there are multiple clinical trials evaluating galunisertib (LY21557299), a novel, small-molecular inhibitor of TGF-β, for advanced HCC that may have particular efficacy in the progenitor-like subtype. Ji et al also demonstrated that miR-181 was highly upregulated in progenitor-like (Ep-CAM-positive) HCC. miR-181 target genes include hepatocyte differentiation regulators and the putative WNT inhibitor, nemo-like kinase (NLK), indicating a potential mechanistic association between miR-181 and the progenitor phenotype.

On the basis of these findings, the current consensus is that progenitor-like HCC might arise from either hepatic progenitor cells or differentiated hepatocytes. Nonetheless, unique pathogenic mechanisms associated with this phenotype serve as a guide for developing targeted therapy. For example, in addition to TGF-β inhibition, multiple, direct AP-1 small-molecular inhibitors are in development that may provide downstream inhibition of the dysregulated JUN and FOS signaling commonly associated with this phenotype.

Finally, the progenitor phenotype has prognostic value given its association with higher rates of recurrence and decreased survival. In a recent study, Miltiadous et al performed expression profiling of 132 HCCs outside the Milan Criteria in patients who underwent transplantation to determine markers of prognosis. After evaluating multiple previously published HCC molecular signatures, they observed that the progenitor phenotype (determined by either CK-19 expression or the Hoshida S2 signature) was most predictive of survival in this cohort. Patients without the presence of these signatures achieved survival rates similar to those reported in patients within the Milan Criteria, suggesting that characterizing tumor biology may add value in determining transplantation candidacy.

HCC IMMUNOLOGIC SIGNATURES: EXPLORING THE TUMOR MICROENVIRONMENT AND TAILORED SELECTION FOR IMMUNOTHERAPY
Chronic inflammation and the immune system are critical players in HCC tumorigenesis, response to therapy, and prognosis. Up to 90% of HCCs occur in the setting of chronic inflammatory disease states, and it has been demonstrated that proinflammatory signaling programs, notably those converging on NF-κB activation, produce antiapoptotic and proliferative stimuli that drive HCC formation. Inflamatory signaling also has been associated with promalignant epigenetic dysregulation and miRNA expression patterns. The HCC tumor microenvironment, characterized by desmoplastic stroma, plays an important role in coordinating the composition and function of various immune cells, thus determining the overall inflammatory state and immune phenotype of a tumor. It also has been demonstrated that variations in the tumor microenvironment change the behavior of tumors with similar genetic alterations. Desmoplasia contributes 2 critical components that support malignancy. First, fibroblasts, stellate cells, and certain immune cells provide an abundant source of growth factors and angiogenic factors that promote proliferation and invasion, respectively. Second, desmoplasia establishes an immune-suppressive ecosystem that supports cancer cell immune evasion. Much of this research was made possible only recently by advances in single-cell RNA sequencing technology. Seminal studies on HCC...
immunophenotyping have contributed to our understanding of desmoplasia and the immune phenotype.

Budhu et al performed one of the first genomic analyses of the tumor microenvironment in HCC, in which they defined an immunologic signature that accurately predicted venous metastases, recurrence, and decreased survival. Those authors evaluated hepatic tissue surrounding tumor in 115 samples from patients who had surgically resected, HBV-related HCC with and without known venous metastases. By using hierarchical clustering analysis, they identified 2 major clusters of genes that distinguished patients with venous metastases, which they refer to as cluster A and cluster B. Cluster A contained 38 genes that were under expressed in patients with metastases, whereas cluster B contained 68 genes that were overexpressed in patients with metastases. It is noteworthy that over 30% of these genes encoded proteins related to inflammation and immunologic regulation. For example, hits in cluster B included: human leukocyte antigens DR and DP1 (HLA-DR and HLA-DP1, respectively), 2 proteins related to major histocompatibility complex type 2 expression; programmed death ligand 1 (PD-L1), the immune-suppressive, T-cell coreceptor; annexin A1 (ANXA1), an anti-inflammatory protein that reduces leukocyte migration; and IL-10, an immune-suppressive cytokine. Histologically, there was a significant increase in staining for CD68-positive macrophages and HLA-DR–positive cells in the metastatic group. Moreover, in the nonmetastatic group, most HLA-DR–positive cells were macrophages, whereas the metastatic group also contained significant numbers of non-macrophage HLA-DR–positive cells, suggesting that other antigen-presenting cells were identified in the liver tissue of these patients. When analyzing specifically for type 1 T-helper (Th1) and Th2 cytokine expression profiles, the metastatic group had a significant increase in IL-4, IL-5, IL-8, and IL-10, consistent with a Th2 profile, whereas the nonmetastatic group had higher levels of IL1A, IL-1B, IL-2, tumor necrosis factor α (TNF-α) and IFN-γ, consistent with a Th1 profile. Taken together, the authors observed that the surrounding hepatic parenchyma in patients with metastatic venous disease was defined by an immunosuppressive microenvironment relative to patients with non-metastatic disease. On multivariable analysis, this signature was the most predictive measure of recurrence and metastasis in a validation cohort of patients. The only clinicopathologic factors that reached significance included the presence of MVI \((P = .071)\) and an absence of tumor encapsulation \((P = .079)\).

More recently, Zheng et al used single-cell RNA sequencing technology to deep sequence 5063 single T cells isolated from peripheral blood, tumor, and adjacent normal hepatic tissue from 6 patients with HCC. This novel approach led to the discovery of 11 T-cell subsets with unique molecular and functional properties variably present in the different tissues analyzed. The authors performed T-cell receptor (TCR) sequencing for each cell, which allowed for clonal tracing of T-cell development and activation status. Multiple interesting biologic insights were revealed in their study. In HCC tissue, relative to normal liver and blood, there were increased concentrations of Tregs and CD8-positive, exhausted cells. Specifically, layilin (LAYN), a Treg marker gene, was associated with CD8-positive T-cell exhaustion and poor prognosis. By using TCR-based clonal tracing, they observed that 82% of Tregs present in tumor tissue had unique TCRs, and only a small portion shared common TCRs with CD4-positive helper cells present in the tumor and adjacent liver tissue, suggesting that the main source of Tregs was recruitment from the periphery rather than clonal evolution in the microenvironment. Among the CD8-positive population present in tumor tissue, the majority had features of exhaustion (42.6%), as measured by IFN-γ expression. In contrast to Tregs, 37% of CD8-positive T cells shared TCR homology, suggesting clonal evolution from an active to an inactive state. For both CD4-positive and CD8-positive T cells, they observed a spectrum of activation among clonal populations, which they separated into effector (active), intermediate, and exhausted (inactive) states. Thus, one unique finding from their study is the possibility of exhausted T-cell rescue with immune-stimulating therapies. Finally, they reported significantly decreased levels of mucosal associated invariant T cells (MAITs), which have historically been associated with innate immunologic response to infection, in tumor tissue compared with surrounding hepatic tissue. Comparative analysis of HCC samples revealed that decreasing MAIT levels correlated with a worse prognosis.

Sia et al performed a separate gene expression analysis in 956 patients with HCC and reported findings similar to the work of Zheng et al. By using virtual separation analytic techniques, they were able to deconvolute gene expression profiles from immune infiltrates in HCC samples. The authors identified a new subset of HCC (in approximately 27% of patients) characterized by uniquely high levels of immune-cell infiltration, programmed death 1 (PD-1) and PD-L1 T-cell checkpoint expression, and active IFN-γ signaling. It is worth noting their
observation that mutational and neoantigen loads in HCC do not correlate with the immune phenotype or with PD-1 blockade response. They identified 2 unique subclasses within the immune phenotype, which they termed active immune response and exhausted immune response. The active cluster was defined by antitumor traits, including IFN-γ signaling, and the expression of adaptive immunity genes and was associated with better survival. The exhausted phenotype expressed protumor features, including markers of T-cell exhaustion and immunosuppressive signals (namely, TGF-β), and was associated with worse survival. These findings suggest that response to immunotherapy is a complicated, multifactorial process involving features of both the tumor and the microenvironment, and it warrants further investigation.

Finally, the authors trialed their immune-expression signature on patients with HCC who received treatment with nivolumab, a PD-1 monoclonal antibody inhibitor, from a recent phase 2 clinical trial. Only 16% of patients had a favorable response to therapy in this cohort, and the immune signature was only present among responders, who likely represented the active immune response subtype. Thus the immune signature proposed by Sia et al potentially may provide a means for accurately selecting patients to receive immunotherapy.

In summary, these studies confirm the immunologic relevance of the tumor microenvironment in HCC prognosis and demonstrate that patients with findings of high immunosuppression are at increased risk of metastasis and decreased survival. They also shed light on potential therapeutic targets, such as Th1/Th2 switching, inhibition of Treg recruitment, and reversal of effector CD4-positive and CD8-positive T-cell exhaustion in high-immune-infiltrate tumors. Regarding this last point, Zhou et al very recently demonstrated that immune checkpoint-inhibitory molecules, including PD-1, T-cell immunoglobulin mucin 3 (TIM3), and lymphocyte-activation gene 3 (LAG3), restored HCC-derived T-cell response to tumor antigen in vitro. In that study, combination immune blockade therapy had a synergistic effect.

**GENE EXPRESSION PROFILING OF HEPATIC PARENCHYMA: PREDICTION OF DE NOVO HCC IN CIRRHOSIS**

Molecular characterization of noncancerous hepatic parenchyma offers potential value in risk stratifying cirrhotic patients for future HCC development. HCC occurs in a background of chronic liver disease in 70% to 90% of cases, although the lifetime risk of HCC varies, depending on the etiology of liver damage, the degree and duration of hepatic injury, and individual and other environmental factors. \(^3\) Clinical staging systems, including the Model for End-Stage Liver Disease and the Child-Pugh score, are only capable of differentiating mild from severe cirrhosis and the risk of mortality, without providing information on cancer risk. Attempts have been made to establish nomograms based on serum markers, with limited efficacy. \(^123\) Therefore, an effective method for stratifying patients based on HCC risk would offer several advantages. First, this would allow for selective, intensive screening of high-risk patients, potentially improving the rate of early stage diagnoses and subsequent outcomes. With current screening protocols, only 28% of patients are considered surgically resectable at the time of detection by BCLC staging criteria. \(^124,125\) Second, risk stratification would help focus chemopreventive efforts. \(^126\) Third, it would allow for precision-guided selection of patients for clinical trial design. Finally, it would provide a rational approach to more cost-effective use of health care resources among a growing cirrhotic population. \(^127\)

Application of novel proteomic and genetic techniques has led to the creation of signatures that have more prognostic accuracy than standard imaging and clinicopathologic data for the prediction of HCC development. Kim et al performed one of the first transcriptomic analyses of cirrhotic tissue to determine differential gene expression linked to hepatocarcinogenesis. \(^128\) They observed that various etiologies of cirrhosis could be separated into 2 molecular profiles based on a 556-gene expression signature. One group included HBV, HCV, Wilson disease, and hemochromatosis, which had expression profiles more similar to those of HCC and were at higher risk for de novo HCC development. The lower risk group included autoimmune hepatitis, alcoholic liver disease, and primary biliary cirrhosis. \(^129\)

More recently, Hoshida et al established a 186-gene signature that is predictive for HCC development across multiple etiologies of cirrhosis. \(^129\) The signature was originally created from gene-expression sequencing of cirrhotic liver tissue in patients with HCC using paraffin-embedded tissue biopsies. \(^130\) Those authors observed that surrounding hepatic tissue was more predictive of late recurrence and survival than expression patterns in tumors, likely because of de novo HCC development. \(^131\) The signature was subsequently validated in cancer-free patients who had HCV-induced, early stage cirrhosis (Child A) as a method of predicting HCC development. \(^132\) By using this model, they were able to differentiate patients into poor, intermediate, and good prognosis groups, with annual HCC incidences of 5.8%, 2.2%, and
1.5%, respectively. Moreover, they were able to predict HCC risk in patients who had a sustained virologic response more accurately than the normalization of hepatic serum biomarkers.

Gene set enrichment analysis allowed for identification of the lysophosphatidic acid pathway as abnormally active in high-risk patients. By using a diethylnitrosamine rat model for HCC, they were able to demonstrate a chemopreventive effect by inhibiting the lysophosphatidic acid receptor 1 (LPAR1) receptor.

Finally, the genes that represent high-risk and low-risk HCC in the Hoshida signature speak toward the mechanism of HCC carcinogenesis, which is thought to be a combination of mutagenic damage coupled with chronic inflammation. Low-risk genes are largely related to normal liver function (eg, plasma proteins, dehydrogenases, and reductases). In contrast, high-risk genes encode proteins involved more in inflammation and proliferation, including NF-κB, IFNs, EGF, and TNF-α. In this regard, Fuchs et al evaluated the role of EGFR in fibrogenesis and HCC development in multiple rodent models of hepatic injury. Erlotinib was both antifibrotic and chemopreventive, and the response to EGFR inhibition could be monitored with the Hoshida HCC risk signature. On the basis of this work, Tanabe et al have established a clinical trial evaluating the chemopreventive effect of erlotinib in patients with cirrhosis. Finally, the Hoshida poor survival signature has the potential for real-world application given that profiling could be performed on paraffin-embedded tissue biopsies.

HCC CLUSTERING: AGGREGATION OF MOLECULAR AND CLINICOPATHOLOGIC SIGNATURES

Over the last 2 decades, a plethora of signatures based on unique gene expression profiles and modifications of biologic macromolecules has been proposed, as summarized in Table 2. There is considerable overlap among these signatures, revealing common mechanisms of hepatocarcinogenesis and disease progression (Fig. 2). However, the complementary nature of these signatures has yet to be realized in a more robust manner. Therefore, the next major challenge in the field of HCC will be to combine different signature modalities into a unified multiomics system to guide comprehensive, personalized therapy.

In this regard, Villanueva et al performed one of the first analyses of multisignature clustering, in which they combined clinical, histopathologic, and gene-expression

| TABLE 2. Proposed Hepatocellular Carcinoma Molecular Signatures: Biologic Categorization and Prognostication |
| Signature Methodology | Reference | Classification |
|-----------------------|-----------|---------------|
| Transcriptomic        | Ye 200324 | Prediction of intrahepatic metastasis |
| Okamoto 2006136       |           | Prediction of multicentric HCC recurrence |
| Kaposis-Novak 2006136 |           | Characterize MET-induced expression signature |
| Boyault 200712        |           | G1-G8 subtypes |
| Chiang 200810         |           | CTNNB1, proliferation, interferon, polysomy 7, unannotated subtypes |
| Hoshida 200913        |           | S1-S3 subtypes |
| Hoshida 2013192       |           | Prediction of de novo HCC |
| Minguez 201121        |           | Prediction of vascular invasion |
| Villa 2016285         |           | Prediction of tumor doubling time |
| Makowska 2018137      | Clusters 1-3 |
| MicroRNA              |           |                  |
| Jiang 200870          | Prediction of survival |
| Tofannin 2011168      | Cluster A, B, and C subtypes |
| Methylation           |           |                  |
| Villanueva 201525     | Prediction of survival |
| Qui 201729           | Prediction of recurrence |
| Proteomic             |           |                  |
| Morofuji 201693       | Prediction of early recurrence (within 2 years) |
| Kim 201729           | Prediction of response to sorafenib |
| Progenitor            |           |                  |
| Lee 2006101          | Progenitor phenotype: CK-19, VIM, AP-1 signaling |
| Woo 2010102          | Cholangiocarcinoma-like HCC phenotype: CK-19, Ep-CAM, CD133, CEACAM6, MUC1, and CLDN4 |
| Coulouarn 2012104     | Progenitor phenotype: Aberrant WNT/TGF-β |
| Immune                |           |                  |
| Budhu 2006118        | Immune response signature to predict metastases, recurrence, and survival |
| Sla 2017120          | Immune phenotype with activation/exhaustion subtypes |

Abbreviations: AP-1, activator protein-1; CD133, cluster of differentiation 133; CEACAM6, carcinoembryonic antigen-related cell adhesion molecule 6; CK-19, cytokeratin-19; CLDN4, claudin 4; CTNNB1, catenin β-1; Ep-CAM, epithelial cell adhesion molecule; HCC, hepatocellular carcinoma; MET, met proto-oncogene; hepatocyte growth factor receptor; MUC1, mucin 1; TGF-β, transforming growth factor β; VIM, vimentin; WNT, wingless-type mouse mammary tumor virus integration site.
data to predict recurrence after surgical resection of early stage HCC (BCLC stage 0 or A) in 287 patients. These authors tested 22 previously published gene signatures, of which 17 accurately allocated patients to their predicted poor outcome subclass. They observed that signatures clustered into 3 main phenotypes: cell proliferation and cell-cycle progression (including the Boyault G3, Kaposi-Novak MET, and Hoshida S1 signatures), high-risk de novo HCC arising from surrounding cirrhotic tissue (Hoshida poor-prognosis signature), and progenitor-like (CK-19, Ep-CAM, and Hoshida S2 signatures). On multivariable analysis, only the presence of tumor satellites, the Boyault G3 signature, and the Hoshida poor-prognosis signature were significant predictors of recurrence in this cohort. It is noteworthy that the poor-prognosis signatures of tumors did not correlate with the Hoshida poor-prognosis signature (the latter being a measure of HCC risk based on surrounding cirrhotic liver), suggesting that these profiles capture different and unrelated aspects of recurrence, namely, residual microscopic disease versus de novo HCC. Finally, 39 patients (19.4%) lacked matching with any of the tested signatures. These lesions were generally well differentiated and had less vascular invasion and lower rates of recurrence.

More recently, the Cancer Genome Atlas Research Network performed the most comprehensive HCC molecular analysis to date. Those investigators analyzed 363 HCC cases by whole-exome sequencing and DNA copy number analyses as well as 196 HCC cases by DNA methylation, RNA, miRNA, and proteomic expression profiling. They integrated these different analytic modalities using a joint multivariate regression approach to establish 3 main HCC subtypes, which they referred to as iClusters.

In their mutational analysis, the investigators identified 26 significant mutations, which had considerable overlap with previous mutational analyses performed by Schulze et al and Chiang et al. Common somatic mutations included: AT-rich interactive domain-containing protein 2 (ARID2) (5%), retinoblastoma 1
(RB1) (4%), ARID1 (7%), AXIN1 (8%), CTNNB1 (27%), TP53 (31%), and TERT (44%). They identified 28 recurring focal amplifications, including the known drivers FGFI19, MET (Chiang polysomy 7), MYC, VEGFA (Chiang 6p21), and myeloid cell leukemia 1 (MCL1). Frequent deletions included the tumor suppressors RB1, CDKN2A, ERBB receptor feedback inhibitor 1 (ERRFI1), and nuclear receptor corepressor 1 (NCOR1) (which suppresses β catenin expression). Multiple oncogene mutations that have been identified are targetable with drugs already approved by the FDA or in clinical trial, as previously reported by Schulze et al.11

Methylation profiling also revealed novel epigenetic signatures. The entirety of identified isocitrate dehydrogenase 2 (IDH1) and IDH2 gain-of-function somatic mutations was profiled to the same signature, which was characterized by diffuse hypermethylation, consistent with previous findings that IDH enzymes induce DNA methylation. Another signature group had a high proportion of HCV-related HCC cases and was defined by TERT promoter mutations, CTNNB1 mutations, and CDKN2A hypermethylation. In comparing mutational profiles with methylation patterns, the investigators observed that the inactivation of specific tumor suppressors was driven primarily by either somatic mutations or epigenetic silencing. For example, CDKN2A hypermethylation was identified in 53% of samples, whereas CDKN2A mutations were present in only 4%. Similarly, 23% of cases with TP53 inactivation were caused by epigenetic silencing of wild-type TP53. In addition to hypermethylation, a portion of patients with silenced TP53 had increased expression of double-minute 4 protein (MDM4), a known downregulator of TP53, for which inhibitors have been designed.140 Therefore, these findings illustrate the value synthesizing multiple forms of molecular characterization.

The Cancer Genome Atlas Research Network also compared each iCluster with previously established HCC signatures to identify for similarities. iCluster 1 was defined by tumors with high-grade, macrovascular invasion, low CTNNB1 and TERT mutations, high expression of miR-181, and silencing of miR-122. This cluster significantly overlapped with features of the Hoshida S2 and progenitor (Ep-CAM-positive/CK-19-positive) subtypes and was associated with decreased survival compared with iClusters 2 and 3 when tested on multiple validation cohorts. iCluster 2 tumors were lower grade with less microvascular invasion and were defined by hypermethylation, CDKN2A silencing, and frequent CTNNB1 mutation. iCluster 3 tumors were characterized by chromosomal instability, TP53 somatic mutation, and CpG hypomethylation and were correlated with the Hoshida S3 subtype.

Finally, the authors performed immunophenotyping by analyzing 66 immune markers that captured different cell-surface markers and immune-cell populations. Twenty-two percent of HCC samples had increased lymphocyte infiltration, similar to levels reported by Sia et al (27%).120 Among high-immune-infiltrate HCCs, they observed increased expression of PD-1, PD-L1, and cytotoxic T-lymphocyte–associated protein 4 (CTLA4). However, they did not correlate these observations with immune activation/exhaustion status.

FUTURE DIRECTIONS: CLINICAL APPLICATION OF MOLECULAR SIGNATURES FOR PRECISION-GUIDED CANCER THERAPY

Molecular characterization techniques greatly enhance our understanding of HCC pathogenesis and supplement standard clinicopathologic staging for more accurate prognostication, principally by elucidating adverse biologic factors that do not correlate with tumor size and multiplicity. This work may also serve as a foundation for the design of rationally targeted therapies. The molecular signatures summarized in this review reveal a multitude of potential drug targets, many of which are already FDA approved or are undergoing evaluation in clinical trial. In this regard, multiomics characterization provides 2 additional novel insights. First, signature analysis at different macromolecular levels (eg, mutational, RNA, miRNA, methylation) reveals that gain-of-function mutations, promoter hypomethylation, and downregulation of target miRNA all may contribute, and often simultaneously, to increased expression and function of a target oncogene. This observation suggests that multiple methods of drug targeting can apply to a particular driver. It also suggests an opportunity for targeting tumor suppressors. Epigenetic silencing of certain wild-type tumor suppressors, as reported by the Cancer Genome Atlas Research Network, is more common than somatic mutation and thus may allow for rescue by augmenting promoter methylation or target miRNA expression. However, it is worth noting that these observations are largely associative, and causality still must be established for the majority of proposed mechanisms.

Second, oncogenic drivers commonly affect shared signaling pathways, providing synergy and redundancy. Historically, we have lacked the resolution to address signaling cross-talk in a robust manner, which is a major
reason why many targeted therapies fail, either initially or after the development of rapid resistance. For example, aberrant MET signaling has been demonstrated in a subset of HCC (polysomy 7 subtype) that contributes to the tumor hypoxic response, cell proliferation and migration, and angiogenesis. Currently, cabozantinib, an inhibitor of MET, AXL, and VEGF receptors, is being tested in a phase 2 clinical trial for HCC. However, AKT is a downstream target of MET and also is dysregulated frequently in HCC. Unacknowledged aberrant AKT activation, if independent from MET, theoretically may thwart any attempt at MET inhibition. Fuchs et al also demonstrated the negative impact of redundancy in their analysis of HCC sensitivity to EGFR inhibitors. They observed that mesenchymal-appearing hepatoma cell lines, as opposed to epithelial-appearing lines, were enriched for increased AKT and STAT3 activation through integrin-linked kinase (ILK) signaling, which conferred resistance to erlotinib, gefitinib, and cetuximab. Inactivation of ILK reduced AKT and STAT3 phosphokinase activity, sensitizing mesenchymal cells to EGFR-inhibitor therapy in both in vitro and xenograft models. Multiomics network analysis, although still early in development, has the potential to identify such mechanisms of redundancy on an individual basis to support the rational design of personalized combinatorial therapeutic regimens.

Nucleotide-based signatures can be determined from formalin-fixed samples, including diagnostic biopsy. In this regard, Makowska et al recently proposed an HCC transcriptomic signature derived solely from diagnostic biopsy samples, which they observed was highly correlated with signatures derived from surgical specimens, including the Hoshida, Boyault, Chiang, and Lee signatures. This work indicates that diagnostic tumor biopsy samples may be directly comparable to surgically resected tumor specimens. Therefore, it may be possible to use gene expression profiling of biopsy samples to determine preoperative treatment course, which has several potential implications. First, patients who have high-risk lesions (Chiang proliferation, Hoshida S2, Boyault G3, Tofannin cluster C, Lee Progenitor, and Cancer Genome Atlas Research Network iCluster 1) that meet Milan criteria may be treated better by undergoing transplantation rather than surgical resection given their risk of microvascular invasion, intrahepatic metastases, and early recurrence. Those with high-risk subtypes should also be considered for adjuvant therapy and more aggressive surveillance after surgery or locoregional therapy. In contrast, patients who have low-risk lesions (Hoshida S3, Boyault G4, Tofannin cluster B, and Cancer Genome Atlas Research Network iCluster 2) are likely to do well with resection alone. In addition, as demonstrated by Miltiadous et al, patients who have low-risk lesions beyond Milan criteria have postoperative transplantation outcomes similar to those who have lesions meeting the criteria. This observation suggests that biologic characterization may be a better guide for determining treatment than standard staging by tumor size and number. However, the application of molecular signatures to guide therapy still must be tested in prospective clinical trials for validation.

Finally, immunotherapy has provided a major breakthrough in the treatment of cancer over the last decade. Expression profiling of the HCC tumor microenvironment has identified a subset of HCCs with high levels of immune infiltrate that may be responsive to checkpoint blockade. It is noteworthy that the immune phenotype does not appear to correlate with high neogen load or markers of adverse biology. Among high-infiltrate tumors, various degrees of lymphocyte exhaustion have been demonstrated, raising the prospect of T-cell rescue to improve drug response rates.

CONCLUSION
The application of molecular characterization techniques has greatly improved our understanding of HCC pathogenesis. Molecular subtyping will allow for improved prognostication and the rational design of targeted therapies. This work also will serve as the foundation of personalized care, influencing the decision to pursue more precise surgical interventions, drug therapies, and surveillance regimens.

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Kenneth K. Tanabe reports royalty revenue from UpToDate and consultation fees from Best Doctors and Advance Medical. Kenneth K. Tanabe and Bryan C. Fuchs report a patent for an epidermal growth factor single nucleotide polymorphism to identify risk for hepatocellular carcinoma and a patent for epidermal growth factor receptor inhibition to reverse cirrhosis and prevent hepatocellular carcinoma; they have not received any licensing or royalty revenues from these patents. Derek J. Erstad made no disclosures.

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