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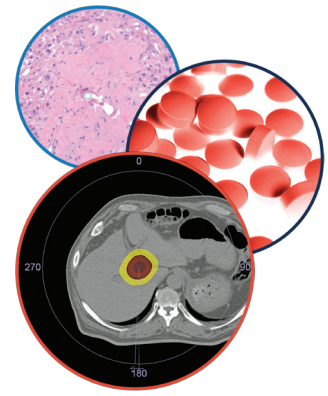

Hepatic Oncology

Molecular classification of hepatocellular carcinoma: potential therapeutic implications

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Practice points

- Hepatocellular carcinoma (HCC) is a growing, global medical problem with limited therapeutic options for advanced disease as a result of unsuccessful drug development.
- The epidemiology of HCC is shifting with an emergence of cases secondary to nonalcoholic fatty liver disease and new highly effective therapies for hepatitis C.
- Predictive biomarkers of response may resolve the challenge in HCC drug development by enriching potential responders in clinical trials to improve detection of therapeutic benefit.
- HCC can be reproducibly classified into several molecular subclasses with distinct phenotypes based on the transcriptome.
- Molecular subclassification of HCC tumors may serve as a universally applicable predictive biomarker of drug response and facilitate biomarker-enriched clinical trials.
- Molecular HCC subclasses are correlated with histopathological and clinical features.

Genomic profiling of hepatocellular carcinoma (HCC) tumors has elucidated recurrent molecular aberrations common or specific to disease etiology, patient race or geographic regions, allowing the classification of HCC tumors into subclasses sharing similar molecular and clinical characteristics. Previously reported transcriptome-based molecular subclasses have highlighted several common themes. Aggressive tumors are characterized by TP53 inactivation mutations and activation of pro-oncogenic signaling pathways, and further subclassified according to expression of stemness markers. The stemness marker-negative aggressive tumors display preferential TGF-β activation. Another group of less aggressive tumors contains a subclass characterized by CTNNB1 mutations accompanied with overexpression of liver-specific WNT targets such as GLUL. Molecular therapies selectively targeting features of the HCC subclasses have suggested their utility in enriching potential responders in clinical trials and guiding therapeutic decision-making for HCC patients.

Hepatocellular carcinoma (HCC) is a growing clinical problem, being the second leading cause of cancer deaths worldwide (GLOBOCAN). Although more than 80% of cases occur in Sub-Saharan Africa and Eastern Asia, the incidence has also been increasing in western countries [1,2]. In the USA, the incidence of HCC has nearly tripled in the past 30 years and HCC is currently the fastest rising cause of cancer-related deaths and even the third leading cause of cancer-related death in men aged 40–59. Potentially curative therapies such as surgical resection, transplantation

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and percutaneous ablation are only available to patients with limited disease, representing about one third of cases, and the majority then progress to advanced stage due to extremely frequent tumor recurrence [3]. Development of medical therapies in advanced-stage HCC has been a challenging task as evidenced by the series of failure in recent Phase III clinical trials [4]. Genomic molecular characterization of HCC has been expected to alter the situation. In this review, we summarize previous efforts to explore molecular classification of HCC tumors as well as commonly observed subclasses across patient populations, and discuss their potential utility in the clinical management of patients and for therapeutic development.

Changing landscape of HCC demographics

The majority of HCC arises on the background of underlying chronic liver diseases, often at the stage of cirrhosis. Worldwide, nearly 80% of HCCs has been attributable to chronic HBV or HCV infection [3], however the landscape of HCC demographics has dramatically changed in the past years. Nonalcoholic fatty liver disease/nonalcoholic steatohepatitis (NAFLD/NASH) associated with metabolic syndrome and obesity is increasingly recognized as an etiology of HCC, even without underlying cirrhosis in a significant proportion of cases [5,6]. NASH is the most rapidly increasing indication of liver transplantation for HCC in the USA [7]. It is still undetermined whether therapeutic outcome is comparable to other liver disease etiologies [8,9].

HCV is still the dominant HCC etiology in developed countries, accounting for 50–60% of newly diagnosed cases in the USA [1]. The emergence of highly effective direct-acting antivirals, reaching >90% sustained virological response (SVR) without serious adverse events, holds great promise in eventually eradicating the infection, although the high costs still limit their wider dissemination [10–12]. In addition, despite achieving SVR, the risk of HCC may persist for decades [13–15], in line with some modeling studies suggesting an increase of HCV-related HCC until 2030 despite improved SVR rates [16].

In line with the evolution of the demographics of HCC, future studies will have to focus on molecular characterization of NAFLD/NASH-related or post-SVR HCC.

Unmet needs for therapeutic interventions based on improved understanding of molecular pathogenesis

Therapeutic options for patients with advanced-stage HCC are still scarce: a multikinase inhibitor, sorafenib, is still the only approved systemic medical therapy for advanced HCC [17,18], and subsequent efforts of drug development have been unsuccessful [19]. To date, a number of mono or combination therapies have been tested as first- or second-line treatment in HCC. Phase III trials of sunitinib (a multikinase inhibitor), brivanib (VEGFR- and FGFR-inhibitor), linifanib (VEGFR and PDGFR inhibitor), erlotinib (EGFR-inhibitor) and ramucirumab (monoclonal antibody targeting VEGFR2) all failed to demonstrate superiority or noninferiority against sorafenib [20–24]. Clinically, a small fraction of tumors show dramatic response to molecular targeted agents such as sorafenib, suggesting that the response can be explained by on-target effects of the drug, modulating molecular aberrations specific to the tumors. It has been noted that enrolling patients in clinical trials irrespective of the presence of response-associated molecular aberrations, also known as the 'all comer' approach, likely obscured the therapeutic effect of investigational drugs in clinical trials [4], and therefore rationalizing the inclusion of patients positive for specific biomarkers predictive of response to detect therapeutic effects with smaller sample size as is the case for ALK-positive lung cancer [25].

Nevertheless, several new trials have been planned or initiated still using an 'all comer' approach. Regorafenib [26] and lenvatinib [27], both tyrosine kinase inhibitors, are currently being tested in Phase III trials. Axitinib, a VEGF inhibitor, achieved the primary endpoint in a Phase II trial and recruiting patients in a Phase III clinical trial [28]. Other drugs currently in the drug development pipeline include cabozantinib, a receptor tyrosine kinase inhibitor with activity against MET, RET and VEGFR, that showed clinical activity in multiple solid tumor types, currently tested in a Phase III clinical trial [29] and nivolumab, a PD-1 receptor blocking antibody mediating immune control of HCC, currently undergoing early clinical evaluation [30].

In addition to the 'all comer' trials, several trials with predictive biomarker-based patient enrichment have been planned in HCC. A recently completed Phase II trial of tivantinib,

an oral MET inhibitor, as second-line treatment showed survival benefit in a *post hoc* subgroup analysis of MET-high tumors, but not in all enrolled patients [31]. Based on this promising finding, a Phase III trial, enrolling only MET-high HCC by immunohistochemistry, was initiated [32]. Refametinib, a RAS inhibitor, is being tested in combination with sorafenib in *RAS*-mutated HCC in a Phase III trial (NCT01915602) after successful Phase II trial completion [33]. Ramucirumab, a VEGFR2 inhibitor, is currently in Phase III trial for HCC

Table 1. Molecular classifications and gene signatures of hepatocellular carcinoma tumors.

Type	Study (reference)	Patients (n)	Etiology	Patient race	Subclass, molecular feature	Clinical/biological phenotype	Ref.
Molecular classification	Hoshida	232 + 371	HBV, HCV, alcohol, cryptogenic	Asian, Caucasian	S1, S2, S3	Recurrence (S1)	[35]
	Lee	90	HBV, HCV	Asian, Caucasian	A, B	Death	[40]
	Boyault	57 + 63	Alcohol, HBV, HCV	Caucasian	G1, G2, G3, G4, G5, G6	High AFP (G1), high HBV (G2), death (G3)	[57]
	Chiang	91	HCV	Caucasian	Proliferation, CTNNB1, interferon, polysomy 7	Recurrence (polysomy 7)	[58]
	Chen	82	HBV	Asian	2 clusters	TP53 activation	[84]
	Breuhahn	39	HCV, HBV, alcohol	Not described	A, B1, B2	High IFN (A), high IGF2 (B1)	[51]
Gene signature of molecular feature	Coulouam	61 + 78	HBV, alcohol, HCV	Asian, Caucasian	TGF-β activation	Death	[46]
	Kaposi-Novak	61 + 78	HBV, alcohol, HCV	Asian, Caucasian	MET activation	Death	[37]
	Yamashita	40 + 238	HBV	Asian	EPCAM positivity	Death	[50]
	Andersen	Rat model + 53	HBV	Asian	KRT19 positivity	Death, recurrence	[38]
	Villanueva	73 + 164	HCV, HBV	Caucasian	KRT19 positivity	KRT19 positivity, early recurrence	[39]
Gene signature of biological phenotype	Lee	61 + 78	HBV, alcohol, HCV	Asian, Caucasian	–	Progenitor cell, death	[49]
	Woo	83 + 139	HBV	Asian, Caucasian	–	Cholangioma like	[47]
	Yamashita	156	HBV	Asian	–	Death, tumor initiating (cancer stem) cell	[52]
Gene signature of clinical phenotype	Minguez	79 + 135	HCV, HBV	Caucasian	–	Vascular invasion	[54]
	Ye	20 + 20	HBV	Asian	–	Intrahepatic metastasis	[85]
	Llovet	37 + 29	HCV	Caucasian	–	Early HCC	[86]
	Villa	78 + 54	HCV, HBV, alcohol, metabolic	–	–	Fast-growing tumor	[87]
Gene signature of clinical outcome	Iizuka	33 + 27	HCV, HBV	Asian	–	Early recurrence	[88]
	Kim	139 + 292	HBV	Asian	–	Death	[41]
	Roessler	20 + 386	HBV	Asian	–	Recurrence, death	[89]
	Kurokawa	60 + 40	HCV, HBV	Asian	–	Early recurrence	[90]
	Woo	65 + 139	HBV	Asian	–	Early recurrence	[42]
	Wang	23 + 25	HBV	Asian	–	Recurrence	[43]
	Yoshioka	42 + 97	HCV, HBV	Asian	–	Early recurrence	[91]
	Nault	189 + 125	HCV, HBV, alcohol, NASH	Caucasian, Asian	–	Death	[92]

n indicates number of patients used to define and validate molecular classification or gene signature.
HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; NASH: Nonalcoholic steatohepatitis.

with raised α-fetoprotein (AFP) as a surrogate marker of response (NCT02435433) [20,34].

Genome-based molecular classification of HCC

As functional readout resulting from various types of molecular aberrations such as somatic DNA mutations and copy number alterations as well as epigenetic changes, genome-wide transcriptome (i.e., gene expression) profiling has been extensively performed in diverse patient populations in the past decade. These studies have yielded a variety of molecular subclasses and gene signatures defined by a set of dozens to hundreds genes and associated with biological phenotypes and/or clinical outcomes (Table 1). Despite diverse results as well as heterogeneity in clinical demographics, several distinct themes are recurrently observed across the studies, indicating the presence of common HCC molecular subclasses. A transcriptome meta-analysis, involving 603 HCC tumors, depicted a molecular classification, onto which the subclasses, gene signatures and somatic DNA alterations can be mapped (Figure 1) [35,36]. HCC tumors can be first classified into two major subgroups. The first group is characterized by more aggressive biological/clinical features, including increased genetic instability, cellular proliferation, ubiquitination, activation of prosurvival signals such as E2F1 and MET pathways, impairment of tumor suppressor *TP53*, gene signature of *KRT19* positivity, larger and less differenced tumor, higher incidence tumor recurrence and poorer prognosis [35,37–45]. The remaining tumors are characterized by less aggressive features, including preserved hepatocyte function, smaller and more differentiated tumor and better prognosis.

The aggressive tumors are further subdivided into two groups: one (meta-analysis S1 subclass, common theme #1 in Figure 1) shows relatively higher activation of TGF-β pathway and cholangioma-like gene signature [46–49], and the other (meta-analysis S2 subclass, common theme #2) is characterized by positivity of stemness marker, EPCAM, high AFP (both serum protein and tissue gene expression levels) and GPC3, activation of IGF2 pathway, relative suppression of interferon target genes and hepatoblastoma-like gene signature [35,50–53]. A vascular invasion gene signature [54] is more strongly induced in the S2 subclass. As a tumor diagnostic marker, AFP has been criticized for its low positivity (generally 30–60%) [55]. However, these findings suggest that AFP can be regarded as a biomarker detecting a molecular subclass of HCC. A subset of the less aggressive HCC tumors (a subset of meta-analysis S3 subclass, common theme #3 in Figure 1) is characterized by somatic mutations accumulated in exon 3 of *CTNNB1* accompanied with induction of specific target genes, *GLUL*, *LGR5* and *SLC1A2* [56–58], but not with canonical WNT pathway target genes. Of note, slightly different sets of canonical WNT pathway target genes are more preferentially induced in either of the S1 or S2 subclass, suggesting biological context-specific induction of WNT target genes in HCC [59]. In addition to the transcriptome-based functional classification, DNA mutational signatures could provide another layer of classification capturing environmental exposures such as smoking and alcohol abuse [60].

Despite plausible distinct mechanisms of carcinogenesis according to the clinical background, all of the subclasses/themes are observed across different etiologies, patient race (i.e., host genetic variations) and environmental factors, suggesting that there is a certain commonality in affected molecular pathways irrespective of etiology. For example, *TERT* activation is most frequently and commonly seen across etiologies despite the wide variety of molecular alterations leading to the pathway activation such as HBV integration, genomic DNA amplification or promoter mutation [61]. Interestingly, the S2 subclass (common theme #2) is relatively more frequent in HBV-related HCC – also less associated with cirrhosis – suggesting that HBV genomic integration leads to enrichment of this molecular subclass and supporting the presence of etiology-specific bias in the affected pathways [57,62–66].

Molecular signature-based HCC tumor classification often identifies a small fraction of tumors with mixed subclass characteristics assumedly due to intratumor heterogeneity. Histologically, especially in larger tumors, intratumor heterogeneity is recognized as 'nodule-in-nodule' structure, where a less differentiated subnodule resides within a more differentiated parent nodule. In fact, distinct histological variants, which often co-exist within a tumor nodule, are associated with specific molecular subclasses [66]. For example, steatohepatitic HCC, a recently identified variant [67], was associated with S1 subclass, macrotrabecular/compact pattern and clear cell variant were associated with S2 subclass and microtrabecular pattern was associated with S3

subclass. Single cell-level characterization may enable molecular subtyping at a finer resolution beyond histological variants in the future.

HCC molecular subclasses can be experimentally modeled to study subtype-specific targets. HCC tumors *Myc*/*Tgfa* transgenic or Met-activated mice showed a gene expression pattern similar to human aggressive HCC tumors, whereas tumors in *E2f1*, *Myc* or *Myc*/*E2f1* transgenic mice showed profiles similar to the less-aggressive human tumors [37,68]. Hepatoma cell lines are classified into two distinct transcriptomic subgroups according to AFP expression status; interestingly, AFP-positive and negative cell lines exhibit S2- or S1-like gene expression patterns, respectively [35,69,70]. This may provide an opportunity to experimentally explore molecular subclass-specific response to molecular targeted agents [71,72].

Clinical outcome & molecular classification

There has been great interest to identify molecular prognostic indicators from genomic profiling of HCC tumors, although tumor-based molecular information is not robustly associated with prognosis [73]. It is now well recognized that HCC clinical disease stage is the key factor substantially affecting prognostic performance of tumor-derived molecular biomarkers. HCC recurrence after radical therapies with curative intent can be classified into two types: dissemination of primary tumor cells clinically often recognized as 'early' recurrence, and *de novo* carcinogenesis from underlying liver diseases clinically manifesting as 'late' recurrence [74]. Tumor recurrence and prognosis in more-advanced HCC are more likely to be determined by 'early' recurrence, therefore tumor-derived molecular information is more prognostic. In contrast, less advanced, early-stage tumors are more likely curable, and *de novo* cancers independent of the profiled primary tumors as well as underlying liver diseases have more influence on patient prognosis. Historically, similarly inconsistent prognostic association was noted for an unambiguous histological variant, clear cell HCC, according to the disease stage [75,76]. Indeed, the variant is associated with S2 subclass [66], highlighting that the reason for inconsistent prognostic associations of molecular and histological observations is common. Studies have shown that molecular profiles of nontumor liver could yield prognostic information independent of disease

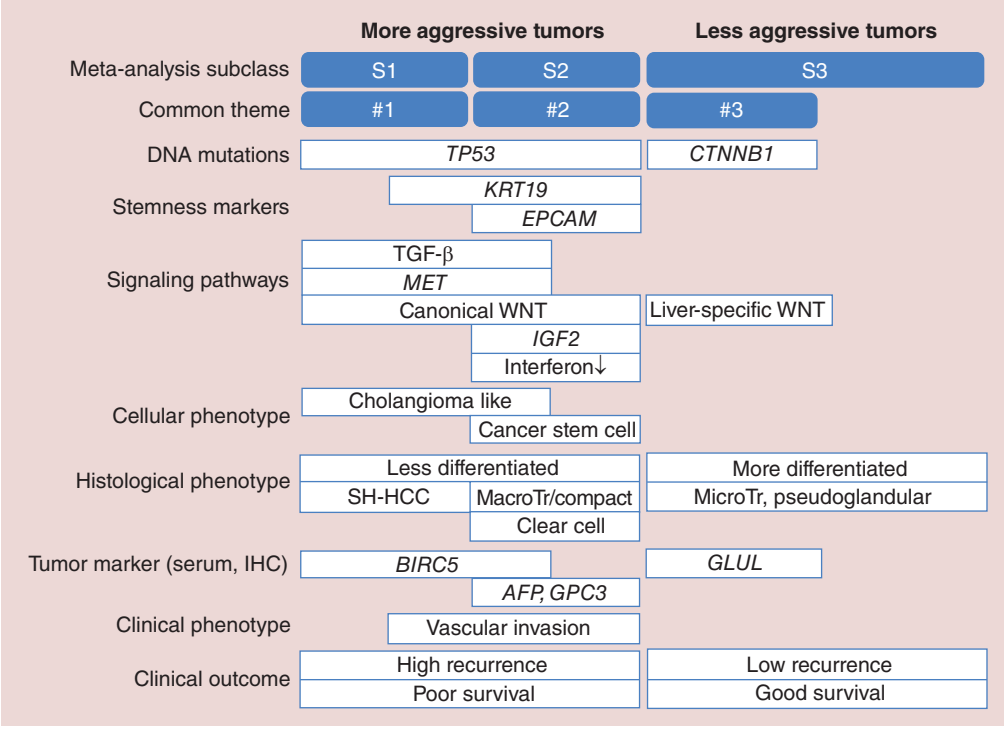

Figure 1. Molecular classification of HCC and molecular and clinicalcorrelates.
MicroTr: Microtrabecular pattern; SH-HCC: Steatohepatitic hepatocellular carcinoma.

stage, although these considerations are outside the scope of this review [73,77].

Potential implication of HCC molecular subclasses in therapeutic development

The series of failed Phase III HCC oncology trials of molecular targeted agents based on the 'all comer' approach has highlighted the need to incorporate molecular predictors of drug response to enrich potential responders and detect statistically significant on-target therapeutic effects. However, given that the majority of agents under evaluation are kinase inhibitors anticipated to elicit anti-tumor effect when activation mutations in the targeted kinases are present, the low frequency of such activation mutations (generally <5%) poses practical difficulty in conducting predictive biomarker-enriched clinical trial. In addition, the necessity to develop companion biomarkers assaying specific molecular alterations for each drug will be costly and may be practically infeasible in many cases.

By contrast, tumor subclass-based treatment, for example, hormone therapy for estrogen receptor-positive subtype in breast cancer, could enable more cost effective enrichment of potential responders because expected frequency of patients in the subclass of interest (>20%) is higher compared with the prevalence of somatic mutations (<5%) and development of a unique pan-HCC subclassification test may allow predictive testing for multiple drugs and avoid the development and validation of companion biomarkers each time a new drug is evaluated. This concept could be viable in HCC given the recent studies demonstrating that several hallmarks of the HCC subclasses could be therapeutically targetable. Epitope-optimized genetic vaccines targeting AFP, a marker of S2 subclass, successfully induced specific CD8 T cells and reduced HCC incidence in a murine model [78]. GC33, a humanized monoclonal antibody against GPC3, another marker of S2 subclass, showed promising anti-tumor effect and was well tolerated in Phase I studies [79,80]. LY2157299, a small molecule inhibitor of TGF-β, a pathway preferentially activated in the EPCAM/AFP-negative aggressive tumors (S1 subclass, theme #1) is now being tested in a Phase II trial [81]. The ramucirumab trial uses AFP, a marker of EPCAM/AFP-positive aggressive tumors (S2 subclass, theme #2), as a predictive marker of response. MET activation is used to enrich potential responders in the Phase III trial of tivantinib. Although subsequent molecular studies showed that the compound elicits nonspecific cytotoxic effect irrespective of MET activation status [82], MET activation, a characteristic of the aggressive HCC tumors (S1/S2 subclasses, theme #1/2), could still be useful in predicting response to tivantinib. This also highlights the notion that our understanding of the mechanisms of drug action may be superficial based on oversimplified assumptions and does not reflect reality. A Src/Abl kinase inhibitor, dasatinib, was tested in a panel of hepatoma cell lines, in which half of the cell lines expressing gene signature of the EPCAM/AFP-negative aggressive tumors responded to the compound [71]. Again the mechanism of action is still unclear, but this example also suggests the relevance of HCC molecular subclasses as universally applicable markers of drug response [72].

Conclusion & future perspective

Accumulating genomic profiles of HCC tumors over the past decade in diverse patient populations has revealed recurrent molecular themes that can be summarized in HCC molecular subclasses and linked to traditional histopathological and/or clinical features. The recent development of molecular agents selectively targeting some of the features associated with the molecular subclasses has led to an appealing hypothesis that HCC molecular subclasses could have clinical utility as a predictive biomarker of drug response and guide therapeutic decision as is the case for the breast cancer subclass. Implementation of molecular subclassification tests in clinically applicable assays will facilitate testing of this hypothesis. In addition, exploration of histopathological/clinical surrogate indices of the molecular subclasses may be a complementary approach for wider clinical translation of the molecular findings.

Although transcriptomic information is expected to capture the functional molecular status of the tumors caused by a variety of genomic/epigenomic alterations and environmental exposures at the time of sampling, further assimilation of somatic DNA alterations such as mutational signatures may provide additional information about history of tumor development, in other words, clonal evolution, and enrich clinically actionable information to better guide clinical management of the patients. It addition, it remains to be determined whether

the profile of infiltrating immune cells is associated with therapeutic response to immunotherapies and correlated with the HCC subclasses, host genetic features and/or environmental factors. In conclusion, it is expected that the characterization and classification of the molecular landscape of HCC tumors will facilitate the development of personalized/stratified/prioritized clinical management of our patients. In addition, it will enable more cost-effective drug development by enriching potential responders in clinical trials, and eventually contribute to the blossoming of precision medicine in HCC [83].

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