

Molecular Profiling of Liver Tumors: Classification and Clinical Translation for Decision Making

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Abstract

Hepatocellular carcinoma (HCC) is a complex disease with a dismal prognosis. Consequently, a translational approach is required to personalized clinical decision making to improve survival of HCC patients. Molecular signatures from cirrhotic livers and single nucleotide polymorphism have been linked with HCC occurrence. Identification of high-risk populations will be useful to design chemopreventive trials. In addition, molecular signatures derived from tumor and nontumor samples are associated with early tumor recurrence due to metastasis and late tumor recurrence due to de novo carcinogenesis after curative treatment, respectively. Identification of patients with a high risk of relapse will guide adjuvant randomized trials. The genetic landscape drawn by next-generation sequencing has highlighted the genomic diversity of HCC. Genetic drivers recurrently mutated belong to different signaling pathways including telomere maintenance, cell-cycle regulators, chromatin remodeling, Wnt/b-catenin, RAS/RAF/MAPK kinase, and AKT/mTOR pathway. These cancer genes will be ideally targeted by biotherapies as a paradigm of stratified medicine adapted to tumor biology.

Keywords

- ▶ hepatocellular carcinoma
- ▶ molecular profiling
- ▶ targeted therapies
- ▶ cirrhosis
- ▶ gene signatures

Liver cancer is the second leading cause of cancer mortality and hepatocellular carcinoma (HCC) is the 16th absolute cause of death world-wide.¹ In the United States, liver cancer mortality shows an increasing trend in contrast to what is observed for most cancer types.² Hepatocellular carcinoma accounts for 80% of all primary liver cancers worldwide. Besides the high prevalence of hepatitis C virus (HCV) infec-

tion as the main responsible for increasing HCC rates in Western countries,³ other etiologies have been identified increasing incidence of HCC (hepatitis B virus [HBV] infection, alcohol abuse, obesity, and metabolic syndrome). All these risk factors lead to chronic inflammation, hepatic fibrosis, and eventually cirrhosis. Although surveillance programs for patients at risk of developing HCC have improved significantly in

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the past years,⁴ only one-third of HCC patients are diagnosed at early stages, when they are still eligible for potentially curative therapies such as resection, transplantation, or local ablation.⁵ Patients with intermediate stages benefit from chemoembolization, whereas those at advanced stages would still have short survival expectancy despite the improved outcome obtained with sorafenib as the first systemic therapy.⁶ Advances in genomics, in parallel with the constitution of large patient cohorts encompassing exhaustive databases and referenced biobanks in clinical research units, have greatly facilitated better understanding of the molecular biology of this pathology. As is already the case for other types of cancers, molecular profiling may help to identify patients that will benefit from selective therapeutic targeting. Although molecular profiling is expected to be a valuable tool in clinical practice of HCC, it has not yet been integrated in the therapeutic decision-making algorithm and in the clinical management of HCC.

In this review, we describe the relevance of genomics as a novel prediction, diagnostic, and prognostic tool in HCC, and discuss how integration of this molecular data into the existing clinical algorithms might add valuable information to daily clinical practice leading to a more efficient treatment approach in HCC.

Contribution of Molecular Profiling for Risk Assessment of HCC

In Western countries, HCC arises on a cirrhotic background in up to 90% of cases.⁷ Hepatocellular carcinoma occurs in one-third of cirrhotic patients during their lifespan and is the main cause of death among them.⁵ Irrespective of the etiology, established cirrhosis serves as the factor that fosters initiation and promotion of carcinogenesis by facilitating genetic aberrations and cellular transformation, which is often referred to as “field effect.”⁸ Even after complete surgical resection or local ablation of early HCC tumors, nearly 70 to 80% of patients develop subsequent recurrence due to tumor dissemination or de novo tumors⁵ caused by the cancer-prone microenvironment in the surrounding cirrhotic liver. For HBV and HCV etiological agents, hepatocarcinogenesis is tightly associated with an inflammation scenario accompanied by immune-mediated destruction of infected hepatocytes followed by regeneration, oxidative stress, and DNA damage, which presumably leads to accumulating potentially oncogenic mutations.⁷ Furthermore, viral products such as HCV core protein may have direct carcinogenic effects by inducing activation of signaling pathways such as MAPK and activator protein-1 (AP-1).⁹ In fact, severity of the underlying cirrhosis is correlated with increasing risk of HCC,¹⁰ especially in HCV-infected patients.¹¹

Hepatocellular carcinoma is an attractive target of preventive intervention because at-risk individuals can readily be identified due to the presence of underlying viral hepatitis or other liver diseases. Ultrasound-based liver imaging is the most widely used and cost-effective screening strategy used for high-risk populations. Nonetheless, even with ultrasound-based screening programs, the development of HCC is 2 to 3%

annual rate in at-risk populations. Therefore, the identification of molecular biomarkers for defining populations at a very high risk of cancer development would be extremely useful for chemopreventive intervention.¹²

Contribution of Transcriptomic Signatures in Prediction of HCC Development

Many studies have established gene signatures based on array-gene expression profiling from cirrhotic and adjacent tissue of HCC.^{13–15} These signatures have been demonstrated to serve as a sensitive “readout” of the biologic state of the liver, reflecting molecular aberrations that may govern risk of HCC development and dissemination.^{8,16} However, most of these signatures are limited to identifying already established HCC lesions providing only an extra tool in HCC early diagnosis. In this sense, there are two gene signatures of particular interest able to select cirrhotic patients at the greatest risk for developing de novo HCC or late recurrence after surgical resection. First, a 17-gene signature from the tumoral adjacent tissue related to immune response was identified predicting metastasis, overall survival, and tumor recurrence.¹⁶ Then, a 186-gene “poor survival signature” from adjacent tumoral tissue was shown to have independent prognostic significance to predict overall survival and late recurrence in HCC patients.⁸ More recently, this poor survival signature—present in 20% of the universal cirrhotic population—was reported to have predictive potential for future risk of HCC development in patients with newly diagnosed hepatitis C cirrhosis.¹⁷ Interestingly, this molecular signature was derived from needle liver biopsy specimens obtained during routine clinical care and conserved as formalin-fixed, paraffin-embedded (FFPE) tissue. The easy availability of FFPE samples¹⁸ is a key factor in implementing these kinds of signatures in a routine clinical setting.

SNPs Predisposing to HCC

Genetic host factors play an important role in HCC development. The most common form of genetic variation between individuals are single nucleotide polymorphisms (SNPs), which are a variation at a particular nucleotide locus from the DNA. Although the vast majority of these modifications are situated in noncoding regions, some can alter either protein function or expression, affecting biological pathways. Single nucleotide polymorphisms, located in genes involved in carcinogenesis, may contribute to an individual's susceptibility to cancer, partially explaining the genetic heritability of this disease. Many studies have described associations between various SNPs and the presence of HCC, through two different approaches. “Candidate-gene” approaches are normally based on the hypothesis that a known identified variant located in a gene implicated in hepatocarcinogenesis might be associated with a higher risk of HCC occurrence. Nonhypothesis driven approaches use a genome-wide association study (GWAS) to compare genotypic distributions of thousands of SNPs in HCC patients and controls to reveal unsuspected variants associated with HCC.^{19,20}

Main pathways containing variants associated with HCC are oxidative stress and detoxifying systems, iron metabolism, inflammatory and immune responses, DNA repair mechanisms, and systems involved in cell-cycle regulation.²¹ One interesting example of a genetic variant detected through the candidate-gene approach is a SNP located in the epidermal growth factor (EGF) gene (rs4444903) and associated with increased EGF expression (by expanding the half-life of EGF protein) and elevated risk of HCC development in cirrhotic Caucasian patients.^{22,23} On the other hand, through GWAS several genetic regions were found to have statistically significant association with HCC in chronic hepatitis C patients. These are the 5' flanking region of MICA,²⁴ which is essential for direct immune system functions, and the isoform 1 of the DEPDC5 locus,²⁵ where deletion of the region containing DEPDC has also been reported in malignant brain glioblastomas. With this same approach in HBV patients, many other SNPs have been identified to be associated with HCC. An intronic SNP (rs17401966) possibly associated with altered expression and function of several potential tumor suppressor genes in 1p36.22 namely KIF1B, UBE4B, and PGD was described in HBV-related HCC patients.²⁶ Another study revealed the association of four SNP variants in the DLC1 locus (Deleted in Liver Cancer 1) with HBV-HCC risk.²⁷ Furthermore, an SNP associated with lower mRNA levels of STAT4 and HCC emergence was reported among HBV patients.²⁸ **Table 1** shows the most relevant SNPs reported to be associated with HCC in GWASs.

Although there are a huge number of publications in this field, most studies suffer from major methodological drawbacks such as poor selection of control samples (patients with other liver diseases), retrospective and single-center design, underpowered sample size, or lack of validation in distinct ethnic populations. As a consequence, external validation in well-characterized populations of different ancestry is needed before effectively translating these results to clinical practice.

Table 1 Relevant single nucleotide polymorphisms (SNPs) associated with hepatocellular carcinoma in genome-wide association studies

Main study	SNP locus	rs number	Etiology of liver disease	Ethnicity	Odds Ratio ^a	Cases/controls
Kumar et al ²⁴	MICA region	rs2596542	HCV	Asian	OR = 1.39	673/2596
Miki et al ²⁵	DEPDC5	rs1012068	HCV	Asian	OR = 1.95	710/1625
Chan et al ²⁷	DLC1	rs12682266 rs7821974 rs2275959 rs1573266	HBV	Asian	OR (combined) = 1.31–1.39	595/825
Zhang et al ²⁶	UBE4B-KIF1B-PGD region	rs17401966	HBV	Asian	OR = 1.63	1962/1430
Clifford et al ¹⁰⁶	TPTE2 region DDX18 region	rs2880301 rs2551677	HCV/HBV	Asian	OR = 3.70 OR = 3.38	206/336
Jiang et al ²⁸	STAT4 HLA-DQ	rs7574865 rs9275319	HBV	Asian	OR = 1.21 OR = 1.49	4319/4966

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; OR, odds ratio.

^aHighest reported value.

Contribution of Molecular Profiling for Diagnosis

Noninvasive diagnosis using the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases (EASL/AASLD) criteria is usually applicable in HCC tumors of more than 2 cm, developed in patients with cirrhosis; most of the time these tumors do not require percutaneous biopsies.^{29,30} However, there are frequent diagnostic difficulties in tumors smaller than 2 cm, particularly to discriminate between high-grade dysplastic nodules (HGDN) and early HCC in cirrhotic patients. As imaging is only accurate for defining the diagnosis of small nodules in half of cases, a liver biopsy is often indicated, though the pathological diagnosis can remain difficult even for expert pathologists.^{29,31–33} Moreover, in carcinogenesis developed in normal liver, differential diagnosis between very well differentiated HCC and HCA is challenging. Even if this situation is a rare event, it is important to diagnose benign or malignant tumors with a high degree of confidence; overall, the major diagnostic issue in primary liver tumors is related to the critical steps of malignant transformation from hepatic adenoma or HGDN into HCC.³⁴ Consequently, we need to identify new biomarkers with two major goals:

1. Identification of preneoplastic lesions at high risk of malignant transformation
2. Discrimination of HCC from preneoplastic lesions

Contribution of Immunohistochemistry in Pathological Diagnosis

In patients with cirrhosis, HCC occurs from a multistep process of carcinogenesis following the sequence: dysplastic nodules > very early HCC > small and progressed HCC.^{33,35} However, interobserver agreement for the diagnosis of very early HCC and HGDN is limited (kappa value from 0.30–0.49) despite international consensus and the introduction of stromal invasion as histological criteria for malignancy.³³

Identification of minute HCC is still a major issue because the treatment of precancerous liver lesions is not currently codified by guidelines. In contrast, detection of HCC at initial stages is clearly a major goal to propose curative treatment including liver resection, local ablation, or liver transplantation.^{29,31} A combination of three immunohistochemical markers including glypican 3, HSP70, and glutamine synthase help to discriminate early HCC from HGDN with a sensitivity from 46% to 72% and a specificity of 100%.³⁶ These three markers have been validated by an external group, although it was pointed out that they only slightly increase the diagnostic accuracy in an expert setting.³⁷ This combination of markers has been endorsed for diagnosis by the EASL guidelines.²⁹ Additional immunohistochemical markers have been proposed for early diagnosis, but they require further validation.³⁸

Transcriptomic Signatures

Molecular signatures derived from microarray experiments analyzing whole genome expression have been proposed to discriminate early HCC from HGDN. Two studies^{13,15} identified genes differentially expressed by microarray and established genes signatures (including 134 and 120 genes, respectively) for each step of the gradual process of carcinogenesis from cirrhotic tissue to HCC, in respectively HCV- and HBV-related cirrhosis. A more restricted approach combining

13 genes (TERT, IGF2, GJB2, TEK, TIAM1, CXCL12, TOP2A, A2M, PLG, CDKN2A, PDGFRA, MKI67 and THBS1) was proposed to distinguish dysplastic cirrhotic nodules and early cancer in cirrhosis.³⁹ Finally, we identified a combination of three genes including glypican 3, LYVE1, and survivin assessed by quantitative RT-PCR as a diagnostic tool for very early HCC.⁴⁰

This three-gene set has a sensitivity of 95% and a specificity of 94% for discriminating HGDN and early tumors less than 2 cm. A unified comparison of the accuracy of these signatures with the three immunohistochemical markers (GPC3, HSP70, and GS) is required.

Gene Mutations Profile

Recently, somatic mutations in the promoter of *TERT* that increase the expression of telomerase were identified in 59% of HCCs.^{41,42} Strikingly, 25% of cirrhotic preneoplastic lesions also harbored somatic *TERT* promoter mutations. This *TERT* promoter mutation is the first recurrent somatic genetic alteration identified in cirrhotic preneoplastic lesions.^{41,43} These results suggest that cirrhotic preneoplastic lesions harboring *TERT* promoter mutations might have a higher risk of malignant transformation in HCC. It also provides the rationale to test drugs targeting telomerase in chemoprevention or in a curative attempt in the field of hepatocarcinogenesis (► Fig. 1).

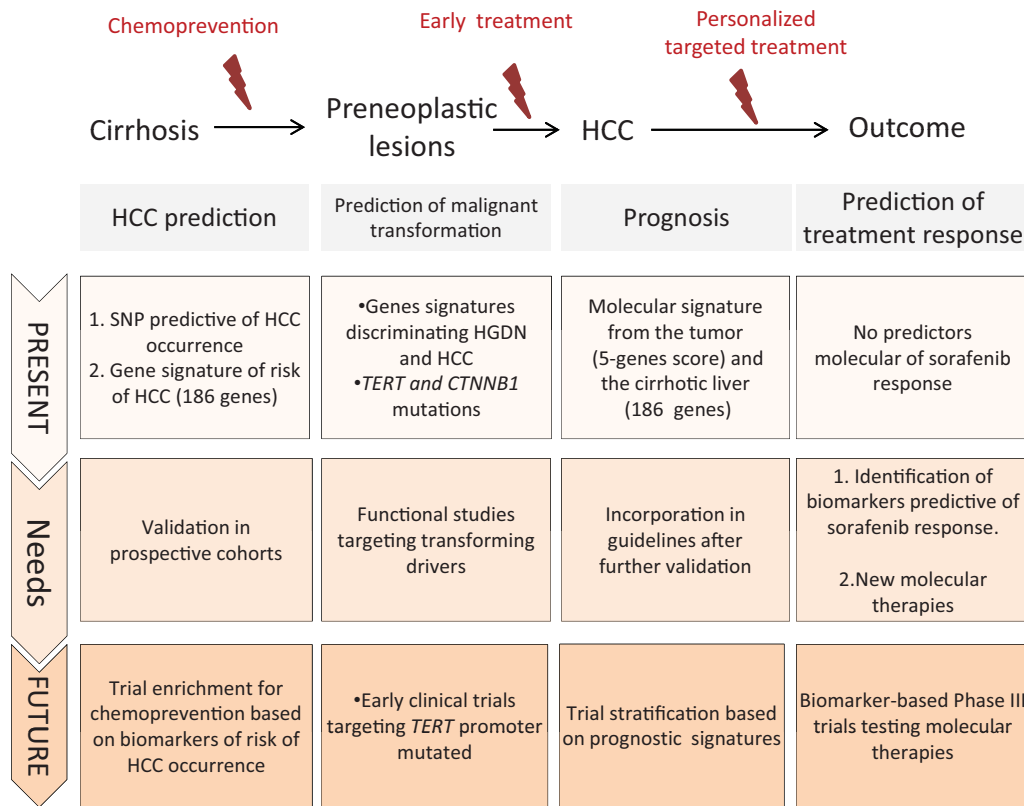


Fig. 1 Potential translation of molecular knowledge of hepatocellular carcinoma (HCC) in clinical practice. Potential uses of genetic and molecular knowledge in the prediction of HCC development in patients with cirrhosis, in HCC diagnosis, prognosis assessment, and stratification of targeted therapy according to the genetic defect of the tumor. It aims to develop tailored chemopreventive trial, targeted treatment of high risk preneoplastic lesions, stratified adjuvant therapies after curative treatment, and biomarker-driven randomized trial in advanced stages.

Malignant Transformation of Hepatocellular Adenoma

Hepatocellular adenoma (HCA) is a bona fide example of a translation of genomic studies in clinical practice.³⁴ Hepatocellular adenomas are rare benign hepatocellular tumors occurring in normal liver and mainly in young women taking oral contraception.³⁴ Malignant transformation of HCA in HCC is a rare event with a frequency estimated around 5%. Hepatocellular adenomas harboring *CTNNB1* mutations activating β -catenin have a high risk of malignant transformation in HCC and consequently should be treated by liver resection.^{44,45} More recently, we also identified somatic *TERT* promoter mutations as a late genetic event in the mutational process of malignant transformation of HCA.^{41,46} We showed that borderline lesions between HCA and HCC harbored *TERT* promoter mutations in 17% of the cases and HCA with overt transformation in HCC in 56% of the cases.^{41,46} In contrast, classical adenomas did not harbor *TERT* promoter mutations. Then, according to the aims of biomarker identification, *CTNNB1* mutations in HCA are able to identify lesions at high risk of malignant transformation and *TERT* promoter mutation is a potential biomarker of malignant transformation of HCA in HCC discriminating HCC from preneoplastic lesions.

Molecular Prognosis Markers and Consequences for Therapeutic Decision

The assessment of prognosis is a difficult and fine art in the field of HCC.⁴⁷ Numerous factors converge to influence the risk of tumor recurrence and the risk of death. In contrast to most other solid tumors, the risk of demise in patients with HCC developed on a cirrhotic background is related to primary tumor recurrence, de novo carcinogenesis and death derived from liver failure.⁴⁷ In addition, after curative treatment such liver resection and local ablation, the timeframe of tumor recurrence is roughly related to these factors. Early tumor recurrence, occurring in the 2 years following surgery, is usually related to intrahepatic metastasis of the initial tumor and consequently related to tumor biology.^{48,49} In contrast, late tumor recurrence, occurring after 2 years following a curative treatment, is more frequently due to de novo carcinogenesis in patients with cirrhosis and is related to microenvironment features. Intuitively, prognosis of patients with very early stage HCC (BCLC 0)²⁹ after successful removal is mostly related to de novo carcinogenesis and occurrence of liver dysfunction whereas prognosis of HCC at advanced stages (BCLC C)²⁹ is mostly related to tumor biology. However, prognosis of patients with HCC developed on a cirrhotic background and treated by resection depends on both tumor and cirrhotic biology.^{49,50} There is a continuum between very early stages and advanced stages and the magnitude of each effect (tumor biology vs. de novo carcinogenesis) in each stage have to be refined.⁴⁷ Consequently, several molecular signatures related to one of these events have been identified during the last decade.

Design of Prognostic Biomarker Studies

Prognostic biomarkers need to fulfill several criteria to assure their robustness before being implemented in a clinical

routine.^{29,51} The recurrent failure of biomarker translation in clinical practice is mainly due to the inadequate design of prognostic studies. The REMARK (reporting recommendations for tumor marker prognostic studies) and PROGRESS (prognosis research strategy) reports have paved the way for the identification and validation of prognostic biomarkers in translational research.^{52–56} The EASL guidelines²⁹ adopted strict criteria for assessing biomarkers in the HCC research field, summarized as follows:

- Identification of the new biomarker in a training and validation set mode
- Independent prognostic value compared with classical clinical, biological, and pathological features
- External and independent validation by other groups

Another issue is a technical one. Molecular signatures have been identified across different platforms and technics of microarray and quantitative RT-PCR. The robustness of molecular signatures should be tested across several platforms and methods. In addition, validation of these new molecular signatures should be performed from frozen to FFPE samples to facilitate their widespread use in clinical practice.¹⁸ When all these items are validated, the new biomarker could be suitable to be tested in clinical trial before implementation in routine practice.

Molecular Prognosis Marker Related to Cirrhotic Biology

Biological signals from cirrhotic tissue have emerged as a predictor of de novo carcinogenesis and late recurrence after HCC resection. As described in the section “Contribution of Transcriptomic Signatures in Prediction of HCC Development,” two molecular signatures derived from cirrhotic samples have been associated with overall survival and late recurrence in HCC patients treated by resection.^{8,16} Moreover, combining the molecular signatures derived from tumors and from cirrhotic tissues could refine prognosis prediction: Associating the five-gene score from the tumor and the poor survival signature from the cirrhotic tissue predicts prognosis more accurately than either signature alone.⁵⁰

Molecular Prognosis Marker Related to Tumor Biology

A huge effort has been performed by several teams to identify prognostic molecular signatures of HCC using transcriptomic assays. Now, more than 20 signatures derived from tumor tissues have been published. A seminal study has identified two subgroups of HCC named A and B with different prognosis.⁵⁷ The HCC subgroup A associated with poor prognosis was characterized by dysregulation in proliferative genes (the so-called proliferative subgroup). Then, six subgroups of HCC (G1–G6) were described closely related to clinical and genetic features.⁵⁸ A panel of molecular signatures related to signaling pathways encompasses roughly the same subtypes of tumor including the late TGF β signature,⁵⁹ the Akt/mTOR signature,⁶⁰ the metastasis signature,⁶¹ the hypoxia signature,⁶² or the MET signature.⁶³ Moreover, a subgroup of HCC of poor prognosis was characterized by re-expression of

stem-cell markers such as an hepatoblast signature associated with poor prognosis.⁶⁴ Other groups have confirmed the prognostic role of stem-cell signatures using microarray (the epithelial cell adhesion molecule [EPCAM] signature,⁶⁵ the CK19 signature,⁶⁶ the cholangiocarcinoma-like signature⁶⁷) or immunohistochemical markers (CK19 or EPCAM⁶⁸). However, other groups that analyzed HCC of early stages and not related to HBV infection failed to confirm these data,^{49,50} probably meaning that stem-cell markers could have a prognostic value restricted to HCC associated with specific etiologies or with advanced stages. Interestingly, a comparison of 18 different molecular signatures from tumor tissues have pinpointed that the G3 subgroup⁵⁸ characterized by mutations of *TP53*, inactivation of *CDKN2A*, and over-expression of genes controlling the cell cycle, was the most accurate signature to predict tumor recurrence after liver resection.^{49,58} Recently, we reported a five-gene score, based on the expression of *TAF9*, *RAN*, *RAMP3*, *KRT19*, and *HN1* genes, that could predict early tumor recurrence and survival after liver resection.⁵⁰ This five-gene score was externally validated in independent cohorts including 748 HCC samples treated by resection worldwide and encompassing a wide diversity of etiology and severity of underlying liver disease.⁵⁰

In addition to molecular information based on transcriptomic data, epigenetic features of the tumor and especially expression of miRNA have been recurrently linked with prognosis.⁶⁹ Several miRNA signatures have been reported including a 19-miRNA signature,⁷⁰ a let-7 family miRNA signature,⁷¹ a 20-miRNA signature,⁷² or a metastasis miRNA signature.⁷³ More thorough data linked low level of mir26 with a poor survival in HCC patients related to HBV infection and with high level of IL6 and NFKB.⁷⁴ Interestingly, a low level of mir26 was suggested to predict a better response to adjuvant therapy with interferon alpha (IFN- α).⁷⁴ However, validation of the prognostic and predictive value of mir26 is warranted in Western countries where IFN- α is not routinely used in the adjuvant setting.³²

Integration of Molecular Signature with Staging System and Pathological Features: Potential Consequences in Clinical Practice

In the future, integration of molecular signature from tumor and/or cirrhosis with staging system and pathological features will be useful to refine the patient's staging and stratification. As a proof of principle to overcome the traditional dichotomy between molecular features and clinical/pathological features, we proposed a composite nomogram that integrates the five-gene score, the BCLC staging, and the microvascular invasion to predict individually the risk of death.⁵⁰ **Fig. 1** summarizes the potential usefulness of molecular information in chemoprevention early treatment and trial stratification. In this line, the main utility of the molecular signature in clinical practice will be patient stratification according to their risk of relapse and death. Identification of patients with a high risk of de novo carcinogenesis through profiling of the cirrhotic tissues will help to identify a subgroup of patient candidates to a chemopreventive trial.⁷⁵ In the field of liver transplantation for HCC, identification of

HCC outside Milan criteria with a low risk of recurrence will help to refine and extend the criteria for liver transplantation.⁴⁷ In addition, the recent negative results of the adjuvant randomized trial comparing sorafenib against placebo after curative treatment of HCC have underlined the need to identify patients at even higher risk of relapse that will benefit from adjuvant therapies.⁷⁶ This strategy is currently used in adjuvant clinical trials for other cancers like breast cancer.⁷⁷ To ensure their full use in clinical practice, the different molecular signatures have to be validated in other curative treatments (i.e., local) and in advanced HCC, but also in biopsies where the small amount of materials could be challenging.

Molecular Profiling to Define New Therapeutic Targets and Oncogenic Pathways

Recent successes in the treatment of cancer using molecular targeted therapies derived from the identification of genomic alterations have improved patient survival. Examples of these treatments include imatinib in leukemia patients with the gene fusion BCR-ABL, gefitinib or erlotinib for nonsmall cell lung cancer with mutations of the EGF receptor, or trastuzumab for breast cancer patients showing amplified HER2/neu receptor.^{78,79}

However, for HCC, so far only the multikinase inhibitor sorafenib has been shown to improve survival of advanced HCC patients.⁶ Because the median life expectancy of sorafenib-treated patients is one year and recently finished phase 3 clinical trials testing new biotherapies in nonselected patients have failed to improve patient survival,⁷⁶ there is an urgent need for additional efficient therapies for advanced HCC. Taking into consideration patients' genomic features in the design of clinical studies has been suggested to improve the trials' rates of success.⁸⁰

We provide here a comprehensive revision of some genomic features that have been proposed to drive HCC carcinogenesis, and thus provide preclinical proof of concept for selective molecular targeting in early clinical trials.

Among all tumor-driver genes, of special interest are those responsible for oncogenic addition. Tumors developed under oncogenic addition are largely dependent on a single activated oncogene, and when submitted to a treatment targeting this single gene these tumors have higher rates of curative response. In HCC, such targeted therapies are still a challenge. As described in the introduction, HCC may occur on very different liver backgrounds (HBV, HCV, metabolic syndrome in abnormal noncirrhotic livers or cirrhotic livers); this is reflected in the complexity of this malignancy at the molecular level.^{3-5,32} Each of these HCC scenarios is characterized by different genetic and epigenetic alterations, thus hindering the identification of relevant oncogenic additions. This is aggravated by additional levels of complexity. Development of solid tumors such as HCC is estimated to require (1) at least three signaling networks being simultaneously altered,⁸¹ or (2) five to eight alterations in driver genes. However, they present dozens of passenger genetic alterations (somatic

mutations, chromosomal translocations, amplifications, or deletions) that do not provide significant growth advantage.⁸² In contrast, hematological tumors typically present one single pathway altered, and exhibit far fewer somatic mutations.^{81,82} It is, therefore, not surprising that discovering the right HCC therapy turned out to be a complex task.

Understanding the mechanisms behind the pathogenesis, at the molecular level, have vastly contributed to the development of novel therapies. In this line of work, new-generation sequencing studies have contributed enormously. Genomic studies in multiple HCCs have confirmed previously identified mutated genes (TP53, CTNNB1 and AXIN1) and have also unraveled several novel HCC driver genes among which are TERT, ARID1A, ARID2, RPS6KA3, PIK3CA, IRF2, NFE2L2, and KEAP (►Table 2).^{41,83,84} Functional classification of these mutated genes suggested that the key signaling pathways for HCC are associated to Wnt/b-catenin signaling, chromatin remodeling, oxidative stress, and receptor signaling (EGF, PDGF, FGF, VEGF, and IGF) as well as to Ras-Raf-MAPK and PI3K/Akt signaling (►Table 2).

Targeting the Most Prevalent HCC Alterations

Therapeutic interventions based on TERT, CTNNB1 (b-catenin), or TP53—the most prevalent molecular alterations described to date in HCC (60%,⁴¹ 30%,⁸³ and 30%,⁸⁰ respectively)—have not yet been approved. Diverse drugs targeting telomerases and based on immunotherapy, small molecule inhibitors, or gene therapies are currently under evaluation, but to date have only exhibited modest success in phase II and phase III clinical trials.⁸⁵ The lack of approved targeted therapies specific for TERT, Wnt/b-catenin pathway or TP53, provides additional opportunities to identify HCC patients who may profit from alternative targeted therapies. However, additional efforts are warranted to decipher these signaling pathways and to identify new compounds that could target these driver genes with limited collateral toxic effects.

Targeting Raf

Mutations and other genetic alterations activating the Ras-Raf-MAPK pathway were soon of particular interest in HCC for therapeutic reasons. Despite the fact that mutations of the RAF/MAPK axis are uncommon in HCC (< 5%),⁷⁹ universal activation of RAF/MAPK signaling has been described in advanced HCC.⁸⁶ Activation of this critical pathway results from upstream signaling by EGF, IGF, and MET activation, and from the epigenetic silencing of tumor suppressors such as NOE1A and RASSF1A.⁸⁷ An exploratory pilot clinical trial is currently ongoing targeting patients with RAS mutations by the MEK inhibitor refametinib (NCT01915602; <http://clinicaltrials.gov/>).

Targeting IGF Signaling

One fourth of the HCC patients present an allelic loss affecting one of the IGF2 receptors (IGF2R).⁸⁸ In addition, 10% of the HCC patients show enhanced IGF2 expression, and the IGF binding proteins IGFBP2 and IGFBP3 have been shown to be deregulated as well.⁸⁸ Furthermore, the IGF1R was shown to

be activated in 21% of HCCs.⁸⁸ Thus, promising HCC-therapeutic strategies either targeting the IGF ligands or blocking the IGF receptors are currently being assessed (►Table 2).

Targeting mTOR

Another key pathway in HCC is mTOR. About 40 to 50% of the HCCs present disrupted mTOR signaling⁶⁰ and would be candidates for treatments based on mTOR inhibition such as everolimus (►Table 2). Everolimus failed to improve survival versus placebo in second-line treatment, but results in enriched populations were not explored or documented in this trial so far.

Targeting c-MET

In addition, therapies targeting c-MET (mutated in 3% of the HCC, ►Table 2) are also under evaluation in HCC. Activation of MET signaling, through other mechanisms, in advanced HCC is estimated to be of 50%.^{59,89} Cabozantinib, a c-MET inhibitor, was proved to suppress tumor growth and metastasis in clinical studies.⁹⁰ A phase III second-line clinical trial is currently being performed in patients with high c-MET expression treated with tivantinib (a TKI targeting c-MET). The rationale for this clinical trial was provided by a phase II clinical trial with better outcome in patients with high expression of c-MET.^{80,89,91}

Targeting Chromosomal Alterations

To date, none of the above-described agents and none of the phase 3 clinical trials have exceeded the benefits of sorafenib, neither in first-line nor in second-line treatment.⁸⁰ Further efforts to discover new therapeutic targets through integrated genomic approaches are expected to improve this. Promising results from genomic studies assessing chromosomal alterations seem to be one of the tools to identify novel HCC target genes. In this sense, focal amplifications such as 7q31 (with c-MET),⁹² 11q13 (with FGF19),^{93,94} or 6p21 (with VEGF)⁹³ support the development of directed strategies. This finding provides the rationale for trials regarding FGF. Early-phase FGFR-targeted clinical trials are currently being performed in tumors presenting FGF alterations (Ref. NCT01948297 and Ref. NCT01004224; www.clinicaltrials.gov). Further details related to FGF-targeted therapies are reviewed in Cheng et al.⁹⁶ Following the discovery of focal amplifications of VEGFA, a recent study described the beneficial effect of sorafenib treatment in patients with HCC bearing VEGFA gains.⁹⁵

Additional chromosomal alterations have been recurrently identified in HCC (►Table 2). These include (1) the 8q gain that contains the c-myc proto-oncogene and for which quarfloxin, a VEGF TKI reducing c-myc mRNA levels, would be one of the candidate strategies to be tested⁹⁷; (2) the allelic loss affecting the IGF2R⁸⁸ that would support the above described IGF-therapies; and (3) the chromosomal gain harboring the well-established oncogene c-MET,⁹² which would suggest the candidate's selection toward c-MET-targeted treatments (►Table 2).

Additional genomic rearrangements expected to provide the rationale for improvement of HCC treatments are those

Table 2 Candidate oncogenes and tumor suppressor genes involved in hepatocarcinogenesis and related targeted therapies

Altered pathway/function	Genes involved	Somatic mutations		Chromosomal alterations (frequencies)	Targeted therapy
		Reported frequencies	Frequencies reported in COSMIC		
Telomere stability	TERT promoter	~ 60% ⁴¹		5p gain ⁹³ (33%)	Vx-001 (immunotherapy) BIBR1532 (telomerase inhibitor) GRN163L (antisense nucleotides) telomelysin (gene therapy)
	TERT		11%		
TP53 / cell cycle control	TP53	20–30% ^{83,84,107–109}	31%	17p loss ^{93,110} (39%)	Adenovirus p53 construct (gene therapy, phase I) RG7112 (inhibition of p53-MDM2 interaction, phase I)
	c-myc			8q gain ⁹³ (66%) Focal amplification ¹¹⁰ (4%)	
	CDKN2A	7.2% ⁸³	10%	9p homozygous deletion ¹¹⁰ (5%)	
	ATM	5% ^{84,110}	4%		
	RB1	3–10% ¹¹⁰		13q loss ⁹³ (19%) 13q13 homozygous deletion ¹¹⁰ (3%)	
	IRF2	~ 5% ⁸³	1%		
	CCND1			11q gain ⁹² (20%) 11q13 focal amplification ^{92,93,110} (5–14%)	
	CCNE1	~ 5% ¹¹¹	4%		
	CDKN1A	4% ¹¹⁰	1%	Focal amplification ¹¹⁰ (2%)	
	CTNNB1	9–41% ^{84,110}	19%	Focal amplification ¹¹⁰	
Wnt/beta-catenin signaling	AXIN1	4–15% ^{83,110}	12%	Homozygous deletion ¹¹⁰	
	APC	1.6% ^{83,110}	3%		
	ARID1A	10–17% ^{83,84,110}	14%		
	ARID1B	6.7% ⁸⁴	2%	6q loss ⁹³ (27%)	
Chromatin remodeling	HDAC family members		1%		Resminostat, vorinostat, belinostat (pan-HDAC inhibitors)
	ARID2	5–7% ^{83,84,108,110}	9%		
	MLL3	4–13% ^{84,107,110}	5%		
	MLL	1–6% ^{84,110}	2%		
	MLL2	6% ¹¹⁰	1%		

Table 2 (Continued)

Altered pathway/function	Genes involved	Somatic mutations		Chromosomal alterations (frequencies)	Targeted therapy
		Reported frequencies	Frequencies reported in COSMIC		
PI3K/Akt/ mTOR pathway	RPS6KA3	2–10% ^{83,110}	7%		Everolimus, sirolimus (mTor inhibitor) MK2206 (AKT1–3 inhibitor)
	PIK3CA	< 5% ^{79,83}	6%		
	PTEN	~ 5% ¹¹⁰	4%	10q loss ⁹³ (11%) Homozygous deletion ¹¹⁰	
Chromosome segregation	TTN	4–15% ⁸⁴	9%		
Oxidative stress	NFE2L2	6–10% ⁸³	4%		
	KEAP1	3–10% ^{107,109}	3%		
JAK/STAT signaling	JAK1	0–9% ⁷⁹	2%		Baricitinib and AZD1480 (JAK inhibitors)
VEGF signaling	VEGFA			6p21 focal amplification ^{93,95} (4–7%)	Bevacizumab (monoclonal antibody against VEGFA) Ramucirumab (monoclonal antibody against VEGFR2, a VEGFA receptor) Cabozantinib (dual VEGFA/c-MET TKI) Brivanib (VEGFR2 and FGFR TKI) Sorafenib, regorafenib, sunitinib, lenvatinib, lenvatinib, axitinib (multi-TKIs)
FGF signaling	FGF19			11q gain ⁹² (20%) 11q13 focal amplification ^{92,93,110} (5–14%)	BGJ398 (pan FGFR inhibitors) FGFR4 inhibitors
	FGFR4		1%		
	FGFR2		1%	10q loss ⁹³ (11%)	Brivanib (VEGFR2 and FGFR TKI)
	FGF5		1%	4q loss ⁹³ (19%)	
	IGF2R			6q loss ^{88,93} (~26%)	MEDI-573 (monoclonal antibody against IGF1/IGF2) Cixutumumab, BIIB022, dalotuzumab (monoclonal antibodies anti-IGF1R)
IGF signaling	IGF1R	Receptor activation ⁸⁸ (21%)			Selumetinib (MEK/ERK inhibitor), regorafenib (MEK inhibitor) Sorafenib (multi TKIs), vemurafenib (BRAF inhibitor)
RAS/RAF/MEK/ERK pathway	KRAS/NRAS	~ 2% ⁸³	2%	Focal amplification ¹¹⁰	
	BRAF	< 5% ^{80,112}	4%		

(Continued)

Table 2 (Continued)

Altered pathway/function	Genes involved	Somatic mutations		Chromosomal alterations (frequencies)	Targeted therapy
		Reported frequencies	Frequencies reported in COSMIC		
MET signaling	c-MET			7q gain ⁹² (23%) chr7 gain ⁹³ (3%) 7q31 focal amplification ⁹² (3%)	Cabozantinib (dual VEGFA/c-MET TKI) Tivantinib (c-MET inhibitor)
PDGF signaling	PDGFRA		2%	4q loss ⁹³ (19%)	Sorafenib, regorafenib, linafinib, orantinib, sunitinib (multi TKIs)
EGF signaling	EGFR	< 5% ⁸⁰	1%	chr7 gain ⁹³ (3%)	Cetuximab (monoclonal antibody against EGFR) Erlotinib and gefitinib (EGFR TKIs)
Proteasome system	UBE3C	16% ¹¹³	1%	chr7 gain ⁹³ (3%)	Bortezomib (proteasome inhibitor)

Abbreviations: COSMIC, catalogue of somatic mutations in cancer; EGF, epidermal growth factor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; TKI, tyrosine kinase inhibitor.

involving gene fusions. Very recently, the first recurrent fusion has been reported in HCC.⁹⁴ The membrane receptor ABCB11 was found fused to multiligand receptor LRP2. This reported fusion opens a door to the generation of novel targeted drugs for HCC.

New Targeted Therapies

Finally, two other types of molecular therapies are currently under development in HCC. The first one takes into consideration epigenomic data. Because epigenetic modifications are one of the causes that have been proposed to drive HCC^{98,99} (Epidrivers⁸²), therapies targeting molecules such as DNA methyl-transferase or HDAC are nowadays an attractive approach.¹⁰⁰ In HCC, a phase-1 clinical trials using the HDAC inhibitor vorinostat is currently being developed (Ref. NCT01075113; www.clinicaltrials.gov). In addition to target directly the HDAC enzyme, peptide-based strategies blocking the interaction of HDAC with its substrates are also being investigated.¹⁰¹

The second type of targeted therapies takes into consideration miRNA data. Recently, miRNAs have been identified as important regulators of gene expression with association to HCC,¹⁰² and their value in clinical management, either as prognostic or diagnostic markers, has been demonstrated in several studies.¹⁰³ There is now accumulated evidence showing that molecular therapies based directed to miRNAs are a worthwhile alternative for cancer treatment.¹⁰³

Resistance to Targeted Therapies

As is the case for other systemic cancer therapies, molecular targeted therapies are liable to generate acquired drug resistances. In addition to the alterations targeted by the drug, tumor cells have additional alterations that may trigger drug-resistance mechanisms after the initial drug response.⁷⁸ This has been shown for crizotinib designed to inhibit the fusion protein EML4-ALK in nonsmall-cell lung cancer,¹⁰⁴ or for vemurafenib directed to B-Raf in melanoma.¹⁰⁵ Approaches to overcome this would be (1) merging strategies based on combining drugs specifically targeting drivers and broad spectrum drugs such as sorafenib, with strategies based on the molecular classification of the patients; and/or (2) generating second-generation drugs circumventing acquisition of resistance mechanisms. Moreover, targeting the microenvironment might be also fruitful in this sense.

The gold standard for future treatment approaches in HCC is expected to be based on combined molecular-targeted therapies aimed at blocking HCC key molecular pathways differently altered according with the molecular classification of each patient. To make this a clinical reality, several issues remain to be elucidated in the near future: (1) Identification of the subset of patients that tumors are driven by a molecular aberration, and (2) elucidate the impact of tumor heterogeneity in the management of the disease. The complexity of etiologies and landscape of mutations make this an important issue. It is unknown whether different tumors from the same patient share the main drivers or different drivers. This concept will have implications in obtaining biopsies that represent a readout for the landscape of mutations. It is

expected also that in the near future liquid biopsy might be a potential tool for capturing molecular heterogeneity.

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