Hepatocellular carcinoma (HCC) has emerged as a major cause of cancer-related death. Its mortality has increased in Western populations, with a minority of patients diagnosed at early stages, when curative treatments are feasible. Only the multikinase inhibitor sorafenib is available for the management of advanced cases. During the last 10 years, there has been a clear delineation of the landscape of genetic alterations in HCC, including high-level DNA amplifications in chromosome 6p21 (VEGFA) and 11q13 (FGF19/CNND1), as well as homozygous deletions in chromosome 9 (CDKNN2A). The most frequent mutations affect TERT promoter (60%), associated with an increased telomerase expression. TERT promoter can also be affected by copy number variations and hepatitis B DNA insertions, and it can be found mutated in preneoplastic lesions. TPS3 and CTNNB1 are the next most prevalent mutations, affecting 25%–30% of HCC patients, that, in addition to low-frequency mutated genes (eg, AXIN1, ARID2, ARID1A, TSC1/TSC2, RPS6KA3, KEAP1, MLL2), help define some of the core deregulated pathways in HCC. Conceptually, some of these changes behave as prototypic oncogenic addiction loops, being ideal biomarkers for specific therapeutic approaches. Data from genomic profiling enabled a proposal of HCC in 2 major molecular clusters (proliferation and nonproliferation), with differential enrichment in prognostic signatures, pathway activation and tumor phenotype. Translation of these discoveries into specific therapeutic decisions is an unmeet medical need in this field.

**Keywords:** Liver Cancer; Genomics; Signaling Pathways; Molecular Therapies.

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Our understanding of the molecular pathogenesis of hepatocellular carcinoma (HCC) has improved significantly in the last decade. Certainly, cumulative data from high-throughput analyses of large number of samples have provided an accurate landscape of HCC genetic alterations. This has enabled us to delineate some of the key events that might dominate tumor development and progression. Hopefully, translation of this knowledge into new targets and biomarkers might impact HCC decision making, and ultimately improve patient’s outcomes. There are, however, some challenges ahead. First, the most prevalent oncogenic mutations are currently undruggable. Second, in those cases where a molecular subclass or a dominant pathway can be identified, there have been minimal efforts to translating this knowledge into clinical trials. Third, the debate on the role of tumor heterogeneity and its assessment in advanced stages has fueled some skepticism on the future of single-biopsy stratified treatments. Finally, our ability to manipulate the immune system in the therapeutic arsenal against HCC is promising but remains to be evaluated. This review will provide an overview of the genetic changes involved in HCC development and progression, as well as discuss the role of molecular markers to stratify patients based on their prognosis and response to therapies.

**Abbreviations used in this paper:** AFB1, aflatoxin B1; EGF, epidermal growth factor; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HGDN, high-grade dysplastic nodules; LGDN, low-grade dysplastic nodules; SNP, single nucleotide polymorphism.
Genetic Predisposition, Environmental Factors, and Mechanism of Malignant Transformation in Hepatocellular Carcinoma

Gene–Environment Interaction in Hepatocellular Carcinoma Predisposition

HCC occurrence results from a complex interplay among genetic and nongenetic host factors, exposure to environmental carcinogens and virus, and development of an underlying chronic liver disease, which, at its ultimate stage (ie, cirrhosis), becomes a certain procarcinogenic field. Although cirrhosis is the “soil” where most of HCC grow, its development on noncirrhotic liver helps us to reappraise the risk factors and mechanisms that lead to HCC development without background liver damage (Figure 1).

Mendelian genetic predisposition to hepatocellular carcinoma. The liver is a very rare target of classical cancer predisposition syndromes. The most frequent monogenic syndromes that predispose to breast, ovarian, or colorectal cancers are usually not associated with development of liver tumors.1 Exceptionally, HCC can develop in patients with APC germ-line mutations.5 However, HCC predispositions are observed in several genetic metabolic diseases, mainly via development of cirrhosis. These rare monogenic diseases include iron (hemochromatosis, HFE1 gene) or copper (Wilson disease, ATP7B gene) overload, tyrosinemia type 1 (FAH gene), porphyria acute intermittent (HMBS gene) or cutanea tarda (UROD gene), and the α1 antitrypsin deficiency (SERPINA1 gene).1 In addition, genetic alterations of glucose metabolism leading to glycogen storage diseases (particularly type la or von Gierke disease, G6PC gene) or to specific maturity onset diabetes of the young type 3 (MODY3, HNF1A gene) can promote genetic liver adenomatosis occurrence and, in a second step, their rare malignant transformation to HCC without cirrhosis.2,5

Multifactorial genetic predisposition to hepatocellular carcinoma. Several single nucleotide polymorphisms (SNPs) were identified to be associated with HCC risk. Among them, many polymorphisms alter biologic pathways of carcinogenesis, including inflammation via TNFA, IL1B, or TGFB; oxidative stress through SOD2 or MP6; iron metabolism with a cooperation between alcohol intake and the HFE1 C282Y mutant variant; DNA repair (MTHFR or XRCC3); cell cycle with a major role of MDM2 and TP53; or growth factor with EGF.

Polymorphisms modulate HCC risk at different steps of the disease, including predisposition to risk factors (eg, viral hepatitis, alcohol intake, or obesity), to the severity of the chronic liver disease and its evolution to cirrhosis, or to the malignant transformation and tumor progression.5 They could be used to stratify patients in personalized surveillance policies, and act as candidate targets for chemoprevention strategies.19 As an example, rs4444903 polymorphism in the 5′ untranslated region that activates EGF was associated with HCC on hepatitis C virus (HCV)-related cirrhosis and validated in independent studies.18 Epidermal growth factor (EGF) signaling is also involved in HCC development in mice and rat models that can be prevented by tyrosine kinase inhibitors targeting EGF receptor, such as gefitinib.20 In addition, EGF is among the top-ranked genes of a signature highly correlated with HCC development in HCV cirrhosis.21 An early clinical chemoprevention trial is currently evaluating whether erlotinib is able to revert this high-risk HCC signature into a low risk (NCT02273362). To date, no drug, including sorafenib, has been able to decrease HCC incidence in high-risk patients, or prevent tumor recurrence after curative treatments.22

Most of the polymorphisms associated with HCC development on chronic liver disease are related to specific risk factors. This observation highlights the close relationships among the nature of the exposure, the genetic background, and the mechanism of hepatocyte transformation/proliferation.23 The association between aflatoxin B1 (AFB1), hepatitis B virus (HBV), and SNP of GSTM1 and GSTT1 is the prototype of a strong interplay between specific exposure to genotoxic contaminant together with viral infection and common polymorphisms to dramatically increase HCC risk.24,25 A PNPLA3 polymorphism (coding for a lipase that mediates triacylglycerol hydrolysis) is strongly associated with fatty and alcoholic chronic liver diseases; it is also associated with HCC occurrence.26–28 In contrast, contribution of PNPLA3 polymorphism seems only minor in the risk of HCV-HCC.27–29

Recently, genome-wide association studies allowed the analysis of thousands of SNPs in HCC patients compared with controls. A genome-wide association study performed in HCV patients in Japan identified a polymorphism in MICA (gene involved in immune regulation),30 and another study in the same population identified a different polymorphism in DEPDC5 (gene of unknown function),31 associated with HCC development. In Chinese patients infected by HBV, SNPs were identified in STAT4 (a key protein of the inflammatory pathway),32 TPTE2 (a homolog of PTEN),33 DCL1 (a tumor suppressor gene implicated in HCC pathogenesis),34 and in a region containing the UBE2B, KIF1B, PGD genes in 4 different studies as being associated with HCC occurrence. All these polymorphisms require validation in patients with various etiologies and at different stages of the liver diseases.6

Early Genetic Alterations in Precancerous Lesions Involved in Malignant Transformation

Cirrhosis as a Cancer Field

There is a key event during malignant transformation in cirrhosis that involves damaged cells, possibly hepatocytes, surrounded by fibrosis and vascularized mainly by the portal system switching to highly proliferative cells, vascularized by arterial neovessels with an incremental invasive and metastatic potential.35 This is a multistep process defined by a precise sequence of lesions: cirrhosis → low-grade dysplastic nodules (LGDN) → high-grade dysplastic nodules (HGDN) → early HCC → progressed HCC and advanced HCC.36 Several lines of evidence underlined the
key role of telomere and telomerase in cirrhosis pathogenesis and tumor initiation. Teleomeres are repeat sequences of DNA essential to protect chromosome ends where they are located. They shorten during cell division leading to cell senescence and apoptosis. Telomerase is required for telomere synthesis and is composed of TERC, the RNA template, and of TERT, the enzyme at rate-limiting component of the complex. In human livers, telomerase is not expressed in mature hepatocytes; cirrhotic tissue exhibited telomere shortening with replicative senescence and germline TERT mutations, predicted to decrease telomerase activity, are associated with a higher risk of cirrhosis. Conversely, telomerase-deficient mouse with experimental liver injury develop cirrhosis, showing that telomere shortening fosters hepatocytes senescence. In contrast, this model enlightened that telomerase reactivation is required in a second step to ensure HCC development.

In humans, telomerase is reactivated in >90% of HCC due to somatic TERT promoter mutations (54%–60%), TERT amplification (5%–6%), or HBV insertion in the TERT promoter (10%–15%) (Figure 2). Interestingly, premalignant lesions exhibit TERT promoter mutations in 6% of LGDN and 19% of HGDN, and frequency of TERT promoter mutations dramatically increases in early HCC.
(61%) and remains stable in progressed and advanced HCC.\(^4\) In addition, whole-exome sequencing of early HCC and of premalignant lesions revealed no additional recurrent mutations in classical HCC driver genes.\(^{49}\) These results show that \(TERT\) promoter mutation is the earliest recurrent somatic genetic alteration, behaving as a “gatekeeper” during the transformation sequence. It also suggests that early HCC are monoclonal, genetically closer to LGDN and HGDN harboring \(TERT\) promoter mutations compared with progressed HCC that harbored mutations in many more cancer genes. Concordantly, there is a phenotypic proximity between these lesions, what frequently renders pathologic discrimination between HGDN and early HCC a difficult task, even among expert liver pathologists.\(^{50}\) This paradigm suggests that early HCC, LGDN, and HGDN harboring \(TERT\) promoter mutations are at high risk for full malignant transformation/progression to advance HCC, which also requires additional hits in other cancer genes.

**Malignant Transformation of Hepatocellular Adenoma**

In normal liver, HCC can sometimes arise from the malignant transformation of hepatocellular adenoma, a rare monoclonal benign proliferation of hepatocytes usually observed in young women taking oral contraception.\(^{51}\) Adenomas are classified in 4 molecular subgroups according to mutations in either \(HNF1A, CTNNB1\) coding for \(\beta\)-catenin, or

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**Figure 2. Mechanisms of malignant transformation in HCC.**

- **CIRRHOSIS**
  - Cirrhosis
  - Low-grade dysplastic nodule
  - High-grade dysplastic nodule
  - Early HCC
  - Progressed HCC
  - TERT promoter mutation
  - Somatic alterations
  - Chromosomal instability

- **NON-CIRRHOTIC LIVER**
  - Viral oncprotein
  - Insertional mutagenesis
  - Cancer gene
  - HBV DNA
  - Human DNA
  - Specific DNA mutagenesis
  - Aflatoxin B1
  - R249S
  - TP53
  - HNF1A, IL6ST, STAT3, GNAS, FRK, JAK1
  - CTNNB1
  - TERT promoter mutation
  - Female Oral contraception
  - Hepatocellular adenoma
  - Hepatocellular adenoma at risk of malignant transformation
in the genes activating the inflammatory pathway as **IL6ST, FRK, STAT3, JAK1, and GNAS**.\(^{51-53}\) Hepatocellular adenoma with exon 3 \(\beta\)-catenin–activating mutations have a higher risk of transformation, and **TERT** promoter mutation are involved at the last step of malignant transformation concomitantly with increased global hypomethylation, and chromosomal aberrations.\(^{54,55}\) However, the sequence of events differs in hepatocellular adenoma compared with cirrhosis: in normal hepatocytes, **CTNNB1** activating mutation is first and associated with monoclonal benign proliferation at risk of transformation, and in cirrhotic hepatocytes, **TERT** promoter mutation occurs earlier.\(^{38,55}\) Likely, cirrhotic hepatocytes might require early telomerase reactivation to proliferate and escape from senescence.

**Genotoxic Effect as a Cause of Hepatocellular Carcinoma Development**

HCC related to HBV can occur as a consequence of viral proteins, such as HBx with oncogenic properties or due to the viral genome itself with HBV insertional mutagenesis in cancer genes like **TERT, CCNE1, and MLL4**.\(^{23,46,56,57}\) Recently, the adenovirus-associated virus type 2 (AAV2), a DNA defective virus, was associated with HCC development on normal liver due to insertional mutagenesis in **TERT, CCNA2, CCNE1, TNFSF10 and MLL4**.\(^{58}\) Another layer of complexity emerged from the analysis of the cancer genome that carries the imprint of exposure to environmental carcinogens during a patient’s lifetime.\(^{59}\) Exposure to AFB1, a mycotoxin contaminating food in certain areas of Asia and Africa, is a well-known HCC carcinogen, a phenomena fostered by concomitant chronic HBV infection. AFB1-related HCC have a specific mutational signature due to AFB1-DNA adducts at guanine leading to a high rate of C\(\rightarrow\)A mutations and a specific hot spot of mutations R249S in TP53.\(^{25}\) In addition, null genotypes of **GSTM1 and GSTT1**, 2 genes important for carcinogen detoxification that belong to glutathione S transferase family, are associated with an increased risk of HCC development in Asian patients in cooperation with AFB1 exposure and chronic HBV infection.\(^{24}\) Analysis of the spectrum of nucleotide mutations in HCC enabled to identify signatures over the tumor genome specific of the AFB1 exposure.\(^{59}\)

Exposure to aristolochic acid, contained in a plant used in Chinese traditional medicine, is associated with urothelial cancer development that bears a highly specific high rate of mutations with T\(\rightarrow\)A transversion.\(^{60}\) Interestingly, the same mutational signature was identified in a subset of HCC in China, suggesting that aristolochic acid could be primarily involved in HCC pathogenesis in a subset of patients.\(^{60}\) In addition, next-generation sequencing identified a group of HCCs developed on noncirrhotic liver in France with an overrepresentation of T\(\rightarrow\)C at ApTpx with transcription strand bias, a pattern known to be strongly associated with genotoxic injury.\(^{49}\) Interestingly, these tumors developed in noncirrhotic patients with high alcohol and tobacco consumption.\(^{59}\)

All of these results showed that the analysis of the cancer genome is an identity card bearing the archeological features of exposure to carcinogens or perturbation of DNA maintenance. This could be useful to better understand the biologic events that shape HCC development and help identify new risk factors using a molecular-epidemiologic approach.\(^{61}\)

**Genomic Landscape and Driver Pathways in Liver Carcinogenesis**

Each HCC genome is the result of a unique combination of somatic genetic alterations with a mean number of mutations in coding regions varying from 35 to 80 per tumor.\(^{45,49,62-66}\) If most of the genetic alterations occurred in passenger genes without any predicted functional carcinogenic consequences, then a small numbers of mutations occurred in cancer driver genes that belong to key signaling pathways involved in liver carcinogenesis.\(^{67}\) An integrated view of recent whole-exome sequencing studies allows identifying the major pathways recurrently mutated in HCC (Figure 3, and Table 1).

**Telomere maintenance.** As telomerase reactivation is key during malignant transformation, 90% of human HCCs harbor an increased telomerase expression. The mechanisms of telomerase reactivation are mutually exclusive and include **TERT** promoter mutations (54%–60%).\(^{44}\) **TERT** amplification (5%–6%),\(^{45}\) and HBV insertion in **TERT** promoter (10%–15%).\(^{46,47}\) **TERT** promoter mutations are frequently associated with **CTNNB1** mutations, suggesting cooperation between telomerase maintenance and \(\beta\)-catenin pathway in liver tumorigenesis.\(^{44,45,49}\) Other mechanisms of telomerase re-expression remain to be discovered and the role of alternative pathways leading to telomere synthesis, such as alternative lengthening of telomeres, need to be specifically evaluated.

**WNT/\(\beta\)-catenin pathway.** The WNT/\(\beta\)-catenin pathway is pivotal in physiologic embryogenesis, zonation, and metabolic control in the liver. It is the oncogenic pathway most frequently activated in HCC by activating mutations of **CTNNB1** (11%–37%).\(^{68}\) and inactivating mutations of **AXIN1** (5%–15%),\(^{69}\) or **APC** (1%–2%). **CTNNB1** mutations, coding for \(\beta\)-catenin, are substitutions or in-frame deletions in a hotspot located in the domain targeted by the **APC/AXIN1/GSK3** inhibitory complex.\(^{68}\) Tumors mutated for **CTNNB1** have also a specific transcriptomic profile with overexpression of classical target genes like **GLUL** and **LGR5**,\(^{70}\) and a specific histologic pattern with intratumor cholestasis.\(^{71,72}\)

**P53 cell cycle pathway.** The P53 cell cycle pathway is altered in at least half of HCC patients with frequent **TP53** mutations (12%–48%).\(^{23,49,62,73,74}\) the tumor suppressor gene more frequently mutated in cancer. Except for the R249S mutation related to AFB1 exposure, no other recurrent **TP53** mutation hotspot has been identified.\(^{52,75}\) The retinoblastoma pathway that control progression from G1 to S phase of the cell cycle is inactivated in HCC mainly by homozygous deletion of **CDKN2A** (2%–12%)\(^{55,62}\) or **RB1** mutations (3%–8%).\(^{65}\) Interestingly, genetic alterations of **CDKN2A** and **RB1** were enriched in tumors with poor prognosis suggesting a role of P21 pathway inactivation in tumor aggressiveness.\(^{49,46}\) Finally, recurrent HBV insertions in **CCNE1** (cyclin E1, 5%)\(^{46}\) and amplification of the **CCND1/
**FGF19** locus (5%–14%),\(^{76,77}\) two key proteins involved in cell cycle progression, have been reported in HCC.

**Epigenetic modifiers.** Epigenetic modifiers are recurrently altered in HCC,\(^ {49}\) with inactivating mutations of ARID1A (4%–17%)\(^ {62}\) and ARID2 (3%–18%)\(^ {78}\) underlining the key role of SWI/SNF chromatin remodeling complexes (BAF and PBAF) as tumor suppressors. The physiologic role of these complexes is to modify chromatin structure and nucleosome position. Indirectly, they modify transcription fate of the cell. Recurrent somatic alterations in the histone methylation writer family mainly in **MLL** (3%–4%), **MLL2** (2%–3%), **MLL3** (3%–6%), and **MLL4** (2%–3%) genes by mutation or HBV insertions in **MLL4** (10%) are also frequent in HCC.\(^ {46,49,64}\) In physiologic state, these genes code for proteins that modify histone methylation by adding and removing H3K4 methyl. Altogether, the functional consequences of **ARID1A**, **ARID2**, and **MLL** genes mutations in hepatocarcinogenesis remain to be further explored.

**Oxidative stress pathway.** The oxidative stress pathway is altered by activating mutations of **NRF2** (coded...
NRF2 pathway activation was previously shown to protect mice against chronic oxidative stress and tumor initiation. In contrast, recurrent mutations identified in HCC revealed NRF2 activation as a driver event in tumor progression. In vitro study has demonstrated that NRF2 activation preserves tumor cells from toxic exposure to reactive oxygen species and subsequent death.

### Table 1. Candidate Drivers as Targets for Therapies in Hepatocellular Carcinoma

<table>
<thead>
<tr>
<th>Candidate oncogene addiction loop</th>
<th>Somatic aberration in human samples, %</th>
<th>Altered pathway</th>
<th>Experimental evidence of “driver” properties</th>
<th>Drug (clinical trials in any tumor type)</th>
</tr>
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<tr>
<td>DNA amplifications</td>
<td></td>
<td></td>
<td>GEM impact liver tumor development / progression</td>
<td>In vivo tumor response after selective induction/blockade</td>
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<tr>
<td>FGF19</td>
<td>5–14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>AKT/MTOR; RAS/MAPK</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CCND1</td>
<td>5–14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cell cycle</td>
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<td>Yes</td>
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<tr>
<td>VEGFA</td>
<td>7–11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>AKT/MTOR; RAS/MAPK; angiogenesis</td>
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<td>Yes</td>
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<tr>
<td>TERT</td>
<td>5–6&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Yes</td>
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<tr>
<td>MYC</td>
<td>4</td>
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<td>Yes</td>
<td>Yes</td>
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<tr>
<td>FAK</td>
<td>4</td>
<td>RAS/MAPK</td>
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<td>TERT promoter</td>
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<td>Yes</td>
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<td>Epigenetic modifiers</td>
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<td>AKT/MTOR</td>
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<tr>
<td>NFE2L2</td>
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<td>Oxidative stress</td>
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<td>30–50&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>PD1/PD-L1</td>
<td>NA</td>
<td>Immune checkpoint</td>
<td>NA</td>
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</table>

NA, not available.<br><sup>a</sup>High-level DNA focal gains.<br><sup>b</sup>CDK4/6 inhibitor.<br><sup>c</sup>Target is MTOR (not TSC1/TSC2).<br><sup>d</sup>Frequency estimation not based on high-throughput RNA sequencing studies.<br><sup>e</sup>Data suggest that tivantinib might not be a specific MET inhibitor.<br><sup>f</sup>Target is IGF-IR.<br><sup>g</sup>Frequency estimation not based on high-throughput RNA sequencing studies.

by NFE2L2) or inactivating KEAPI in 5%–15% of the cases, preventing proteasome degradation of NRF2 physiologically induced by KEAPI/CUL3 complex ubiquitinylation. NRF2 pathway activation was previously shown to protect mice against chronic oxidative stress and tumor initiation. In contrast, recurrent mutations identified in HCC revealed NRF2 activation as a driver event in tumor progression. In vitro study has demonstrated that NRF2 activation preserves tumor cells from toxic exposure to reactive oxygen species and subsequent death.}

### PI3K/AKT/MTOR and RAS/RAF/mitogen-activated protein kinase pathways

The PI3K/AKT/MTOR and RAS/RAF/mitogen-activated protein kinase pathways are activated in around 5%–10% of HCC by amplification of the FGF19/CCND1 locus. Also, activating mutations of PIK3CA (0%–2%) and inactivating mutations of TSC1 or TSC2 (3%–8%) lead to activation of the AKT/MTOR signaling in a subset of HCC. In addition, homozygous deletion of PTEN, an inhibitor of the PI3K kinase, has been identified in 1%–3% of the HCC. However, some HCC with activation of the PI3K/AKT/MTOR cascade have no genetic alterations in this pathway. Indirect upstream activation through insulin growth factor pathway has been proposed as an alternative mechanism of AKT/MTOR activation.
Activating mutations of genes belonging to the RAS family are rarely observed in HCC (<2%), and inactivating mutations of \textit{RPS6KA3}, coding for the RAS inhibitor RSK2, were identified in 2%–9% of tumors.\textsuperscript{62} RSK2, is located downstream of mitogen-activated protein kinase kinase and extracellular signal-regulated kinase, and is a known negative control loop of RAS signaling.\textsuperscript{84} Inactivation of RSK2 released this negative feedback and induced a constitutive activation of the pathway.\textsuperscript{49} Experimental data also suggest that persistent RAS activation may be a mechanism of HCC resistance to sorafenib.\textsuperscript{85}

**Molecular Classes**

We have described the landscape of mutations and critical pathways involved in the development and progression of HCC. But 2 important questions emerge: Are we able to classify tumors according to the molecular events described here? And can we treat HCC patients based on those biomarkers? Molecular classifications are aimed at providing a molecular understanding of the different biologic events that drive tumor subclasses and also at defining specific biomarkers/targets for therapies. Development of whole-genome expression profiling in the late 1990s had a tremendous impact in biomedical research.\textsuperscript{86} The premise was that, despite being at the same clinical stage, tumors are significantly different at a molecular level. Implications are huge, because decision making in oncology relies mainly on clinical staging, and rarely considers tumor molecular singularities. Array-based technologies enabled the analysis of large numbers of transcripts in multiple samples, setting the basis for cancer classification based on expression patterns.\textsuperscript{87} These molecular classes reflected different biologic backgrounds with potential implications in patient selection for therapies and prediction of clinical outcomes. For example, gene signatures in breast cancer identified those women who would likely to benefit from adjuvant chemotherapy after resection.\textsuperscript{88} This could provide clinicians with additional tools to prevent patient’s exposure to unnecessary drug toxicity.\textsuperscript{49} There are 2 layers of information derived from molecular classification in HCC: first, its role as a prognostic or predictive biomarker, and second, its ability to increase our understanding of the molecular pathogenesis of the disease.

In HCC, many groups have reported molecular classification based on genomic profiling (Table 2).\textsuperscript{62,90–92} Most of these studies were conducted on resection specimens from patients with different etiologies for their underlying liver disease. Remarkably, despite that marker genes defining each subclass were different across studies, integrative analysis showed that most of them identified common genomic signals reflecting background biology.\textsuperscript{93} In other words, different investigators reported molecular subclasses that identified similar patients, probably by spotting core genomic alterations. Figure 4 summarizes our cumulative understanding of gene expression–based HCC subclasses and its integration with additional levels of molecular information. These studies along with a meta-analysis showed that, regardless of the specific nomenclature used for each class, HCC can be roughly divided into 2 major molecular subtypes.\textsuperscript{94} One is broadly characterized by an enrichment of signals related to cell proliferation and progression in the cell cycle (proliferation class) and is generally associated with a more aggressive phenotype, and the second class generally retains molecular features resembling normal hepatic physiology (nonproliferation class) (Figure 4).

**Proliferation Subclass**

The main traits of this class, which account for around 50% of patients, are related to activation of signaling cascades involved in cell proliferation/survival, enrichment of signatures of poor prognosis, and association with clinical characteristics of aggressive tumors and poor outcomes. Activation of signaling pathway is remarkably heterogeneous at the genomic and phenotypic levels. These include AKT/MTOR,\textsuperscript{82} MET,\textsuperscript{95} TGFβ,\textsuperscript{96} IGF,\textsuperscript{83} RAS/mitogen-activated protein kinase,\textsuperscript{97} among others. Interestingly, this class is also enriched in genomic signals that identify markers of progenitor cells, such as epithelial cell adhesion molecule\textsuperscript{92,98} or hepatoblastoma-like.\textsuperscript{71} The cell of origin in HCC is a highly controversial issue, with recent experimental data supporting oncogenic reprogramming of different cellular lineages into cancer stem cells.\textsuperscript{99} A signature derived from hepatoblastomas, the most frequent primary pediatric liver tumor, as well as 2 signatures derived from cholangiocarcinoma, are also enriched in this class.\textsuperscript{100–102} as well as Notch,\textsuperscript{103} which acts as a master regulator of biliary differentiation. Altogether, these data further reinforce the notion of increased cellular plasticity in these tumors. Different factors might explain increased heterogeneity of the proliferation class, including higher rates of chromosomal instability or enrichment in aberrant epigenetic changes. For example, high-level DNA amplifications of chromosome 11q13 (locus for \textit{FGF19}, \textit{CCND1}, \textit{ORAOV1}, \textit{FGF4}) are enriched in this class, in addition to a DNA methylation-based prognostic signature that mainly correlates with signatures of progenitor cells.\textsuperscript{100} MicroRNA deregulation is also prominent in this class, with a clustered enrichment of a primate-specific family of miRNA located in chromosome 19 and microRNA/small nucleolar RNA on chromosome 14.\textsuperscript{105}

From a clinical standpoint, patients in the proliferation class have aggressive tumors, with higher α-fetoprotein levels, moderately/poor cell differentiation on histology, and frequent vascular invasion.\textsuperscript{81,93} HBV-related HCC are predominantly tumors belonging to this class. As predicted, patients with tumors from the proliferation subclass present higher risk of recurrence after resection and lower survival rates.\textsuperscript{93,106}

**Nonproliferation Class**

This molecular subclass contains at least 2 critical features: molecularly, it is dominated by activation of Wnt signaling in up to 25% of cases, and others are characterized by immune response; and from a functional perspective, the transcriptome of tumors resemble normal hepatic
physiology. In terms of aberrant signaling, genomic data suggest a dual modulation of WNT signaling in HCC, with differential representation in the proliferation and nonproliferation subclasses. Classical WNT signaling, as defined by up-regulation of well-known target genes, such as GLUL or LGR5, is significantly enriched in this class. These data are further confirmed when analyzing CTNNB1 mutations and nuclear translocation of β-catenin. A subset of tumors within this class is characterized by broad gains in chromosome 7, associated with overexpression of EGF receptor and male sex predominance. There are also data suggestive of immune signals within this class. Interestingly, a similar predominance in inflammation-related genomic traits was also found in the less aggressive molecular subclass of intrahepatic cholangiocarcinoma. From the clinical standpoint, tumors in this class show less aggressive phenotype, including better histologic differentiation, lower α-fetoprotein, and lack of enrichment in poor prognosis signatures. Regarding etiologic factors, HCV and alcohol-related HCC are more prevalent in this class.

The backbone of HCC classification relies on gene expression profiling. However, there have been recent attempts to enhance cancer classification with combined genetic and epigenetic features. Despite not yet being comprehensively applied in HCC, a curated selection of approximately 500 features, including copy number gains and losses, mutations, and methylation-enabled accurate cancer classification across 12 tumor types. Interestingly,
at the top of this hierarchical classification, 2 main tumor subclasses with significant differential prevalence of mutations (ie, M) and DNA copy number alterations (ie, C), which suggests that either mutations or chromosomal instability can act as drivers of progression in different tumors.

### Genetic Alterations as Biomarkers
#### Prognosis
HCC prognosis prediction is currently assessed using the Barcelona Clinic Liver Cancer algorithm, as endorsed by the American and European Associations for the Study of Liver Diseases. Barcelona Clinic Liver Cancer relies on a composite of tumor burden, degree of liver damage, and cancer-related symptoms. In terms of molecular-guided prognosis prediction, >40 prognostic gene signatures have been described, although none has become a tangible tool in clinical decision making. Many factors could contribute to this, including that molecular classes have been generated mostly from surgical specimens, limiting its theoretical applicability to patients at earlier stages. There are recurrent discussions on the impact of tumor heterogeneity, both intra- and internodular, on molecular class predictions based on single biopsies. Despite this, molecular signatures are slowly permeating clinical practice guidelines. A set of criteria has been proposed to determine formal inclusion of molecular-based biomarkers in GPC. The use of archived specimens collected in the context of high-end clinical trials has been suggested as a legitimate source for biomarker development.

Most prognostic signatures were generated using transcriptome data, such as the G3, 5-gene, epithelial cell adhesion molecule, or
hepatoblast-like.91 Others used epigenetic data to predict outcomes, like the 36-CpG DNA methylation signature,104 the 20-miRNA signature,116 or the identification and validation of miR-26 in HBV-HCC (Table 2).105,117 Most of them identify patients within the proliferation subclass, however, the 5-gene score is also able to predict prognosis of tumors classified in the nonproliferative subclass (Figure 4). In addition to those derived from the tumor, a number of studies have reported signatures derived from adjacent cirrhotic tissue able to classify HCC patients based on their prognosis. Presumably, these classifications capture oncogenic signals reflecting the so-called “field effect.” A 186-gene signature enriched in gene sets associated with inflammation, including interferon signaling, activation of EGF, nuclear factor κB, and tumor necrosis factor–α was able to identify HCC patients, mainly HCV-related, with poor survival after resection, as well as those cirrhotic patients at higher risk for HCC development.21,118 Differential inflammation patterns identified with genomic profiling were also determinant to predict intrahepatic metastasis in a cohort of HBV-HCC.119 More recently, a 233-gene signature identified in regenerating livers accurately predicts risk of de novo tumor formation (ie, late recurrence) in resected HCC.120 Patients with poor prognosis signatures from the tumor (eg, G3, Cluster A) were not significantly enriched in those signatures, conferring poor prognosis from the adjacent tissue (eg, 186-gene signature), as judged by an integrative analysis in a large cohort.93 In other words, and similar to prognosis prediction using clinical systems, genomic data from both components (ie, tumor and adjacent tissue) are complementary to maximize prediction accuracy (Table 2).

Recent refinements in these studies have enabled predictions based on individual risk assessments. For example, a nomogram that combines data from the 5-gene score in conjunction with Barcelona Clinic Liver Cancer stage and vascular invasion provides survival estimation on an individual basis.14 Similarly, the use of machine learning techniques for class identification and prediction of a DNA methylation-based signature provides a continuous gradient of risk, which ultimately might increase precision in outcome prediction.104 Signature-based prognosis predictions require tissue, which might constitute a limitation because HCC diagnosis can be confidently made with imaging techniques. The development of technologies that allow analysis of tumor byproducts in the blood, including cell-free DNA or circulating tumor cells, might facilitate the implementation of molecular-based biomarkers.121

Therapeutic

In oncology, survival benefits from molecular-targeted therapies might be derived as a result of treating all patients with very effective/broad therapies with manageable toxicity, targeting a specific, generally small, subgroup of patients whose tumors are addicted to an oncogenic aberration; and targeting a broad number of patients whose tumors present a frequent mechanism of activation (signaling pathway) or immune response. Studies in solid tumors have provided a broad picture of the mutational profile and identified a wide range of 5–150 mutations per tumor, depending on exposure to genotoxics (non—small cell lung cancer, melanoma-high mutation rate), tumors developed in infants or hematologic tumors (low-mutation rate122). In HCC, the mean number of mutations in coding regions per tumor is 35–80, among which it has been estimated that there might be 4–8 drivers of oncogene addiction.49 The oncogene addiction theory postulates that some tumors harbor certain somatic genetic alterations that dominate the malignant phenotype.123 Translation of this principle in cancer management provides the rationale for personalized care, commonly referred as precision medicine. There are many examples of survival benefits achieved after selective inhibition of aberrant molecular somatic events in solid tumors, such as vemurafenib in melanoma with BRAF V600E mutations,124 or crizotinib in lung cancer patients with anaplastic lymphoma kinase rearrangements.125 Effectiveness of this approach relies on the following factors: dominance of the candidate oncogenic addiction loop in tumor progression, existence of a biomarker able to accurately identify patients with this oncogenic loop, and a therapeutic agent able to selectively and effectively abrogate the loop. Ultimately, a well-designed, properly powered clinical trial enrolling patients with the activated oncogenic loop and treated with its antagonist will determine its clinical implementation.

In HCC, the only effective systemic agent is the BRAF/vascular endothelial growth factor receptor/platelet-derived growth factor receptor inhibitor sorafenib.126 No oncogenic addiction loop has been validated yet. In addition, none of the negative phase 3 trials in advanced HCC reported so far used biomarker-based patient enrollment.127 It is feasible that not all tumor types can harbor oncogenic addiction loops. In addition, inhibition of a single loop might not suffice to provide enough survival benefits at advanced stages, and could promote growth of resistant clones or metastatic sites. Also, a comprehensive signaling blockade might be needed to achieve clinical responses, as has been shown in breast cancer,128 and suggested in resistance models of HCC.85

Among the somatic alterations described previously and signaling pathway activation, we can define 2 areas where biomarkers can be used: targeting oncogenic addiction loops based on the 3 requirements mentioned here, based in human data and experimental models, and targeting signaling cascades or immune checkpoints (Tables 1 and 2).

Oncogenic loops. DNA copy number changes. VEGFA (gains in chromosome 6p21) is recurrently described, correlated with expression, and with an estimated prevalence based on fluorescence in situ hybridization of 7%–11%.81,129 Also, high VEGF plasma levels are correlated with poor survival130; there is solid evidence of anti-tumor effect after VEGF inhibition in experimental models. VEGFA could have a double pro-tumorigenic effect by promoting angiogenesis and inducing non–cell autonomous over-expression of hepatocyte growth factor.129 Early trials with VEGFA inhibitor bevacizumab in unselected populations show modest efficacy and raised some safety concerns.131
FGF19, CCND1 (gains in chromosome 11q13) are described in 5%–14% of HCC, and are also correlated with high expression levels and poor prognosis. FGF19 blockade with monoclonal antibodies inhibits clonal growth and tumorigenicity in vivo. Strikingly, transgenic mouse overexpressing FGF19, which binds to FGFR4 in skeletal muscle, develop HCC, which points toward a dual role of FGF19 in both tumor progression and initiation. In terms of FGF blockade, brivanib, a highly active FGFR1-3 inhibitor, failed to improve survival in unselected advanced HCC populations. CCND1 shares genomic locus with FGF19, and it is also frequently amplified in cancer. CDK4/6 dual inhibitors, such as palbociclib or abemaciclib, have shown anti-tumoral activity in experimental models, although data from breast does not confirm a particular benefit of CDK4/6 inhibition in CCND1 amplified tumors.

FAK, MYC (gains in chromosome 8q24) have broad gains described in 4% of HCC, with a single study reporting 26%. FAK inhibition induces tumor responses in experimental models of HCC, and selective inhibitors of FAK are currently under clinical development. MYC transgenic mice develop liver cancer and it has a central role during hepatocarcinogenesis. However, the development of effective pharmacologic strategies to inhibit MYC has been challenging.

**Gene mutations.** CTNNB1 is highly prevalent across studies (approximately 25%). Similar to MYC, difficulties developing selective and nontoxic WNT inhibitors have limited its clinical development, but clearly, CTNNB1 mutation is an appealing candidate for selective targeting.

TSC1/TSC2 are both negative modulators of MTOR cascade, and their inactivation promotes MTOR signaling. Mutations, as well as DNA copy number alterations are described in 2%–14% of HCC. Pathway-based analysis underscores the potential driving role of this cascade in HCC with a significant enrichment in Asian populations. In terms of MTOR inhibition, a phase 1 trial testing everolimus in combination with sorafenib show subtherapeutic concentrations for everolimus and precluded further development of this combination. When tested in second line, everolimus as a single agent failed to significantly improve survival in a phase 3 trial without biomarker-selected patients. Subgroup analyses suggested an increased survival in HBV patients, maybe in relation to a higher AKT/MTOR pathway activation in this population.

NRAS/KRAS/HRAS are mutated in <3% of HCC. Preliminary data from a phase 2 trial (ie, BASIL (Assessing BAY86-9766 Plus Sorafenib for the Treatment of Liver Cancer) NCT01204177)) in patients with advanced HCC receiving a combination of MEK inhibitor refametinib and sorafenib found mutations in approximately 6% of patients (4 of 69); analysis was conducted on circulating DNA. Three of these four patients had confirmed partial responses. Inactivating mutations in RAS inhibitor RP6SKA3 are more prevalent (2%–9%) and could also predict response to RAS inhibition. A phase 2 study testing the combination of refametinib and sorafenib in RAS mutant HCCs is ongoing.

**Supplementary Material**

Note: The first 50 references associated with this article are available below in print. The remaining references accompanying this article are available online only with the electronic version of this article. To access the supplementary frequency has been described up to 9% based on whole-genome sequencing data in HBV-HCC. This has not been confirmed in subsequent studies. Experimental evidence suggests that its blockage could have antitumor activity.

**Signaling Pathways or Immune Checkpoints**

**MET.** Overexpression at the messenger RNA and protein levels has been described in at least 40%–50% of HCC samples. A subanalysis of the phase 2b trial testing tivantinib vs placebo in second line suggested that the drug was particularly effective in those patients with high expression of MET on immunohistochemistry. There has been some concerns about tivantinib’s specificity as an MET inhibitor, but a phase 3 RCT enrolling patients with high MET is currently evaluating its role in second line (NCT02029157).

**IGF2.** Enrichment of high transcript levels of IGF2 (>20-fold) in approximately 10% of HCC samples suggest that it could act as a tumor driver. DNA methylation is a reported mechanism for IGF2 deregulation in HCC, and it has been described as an oncogenic partner of Notch-induced HCC. Functional validation indicate that IGF pathway inhibition could be an effective strategy in HCC, but results in early clinical trails using IGF-IR antagonists have shown limited efficacy and significant toxicity.

**PD1/PD-L1.** Both involved in the inhibitory signals to T cells, leading to suppression of anti-tumoral immune response. Data in other solid tumors show remarkable responses after selective inhibition with nivolumab, with a potential role of PD1/PD-L1 as a biomarkers of response. In HCC, intratumoral increased expression of PD1/PD-L1 correlates with poor outcomes, and its inhibition in orthotopic HCC models induces tumor responses. This represents a novel immune-base approach. Studies with checkpoint inhibitors are currently ongoing in phase 2 in HCC.

In conclusion, the studies reported during the last 10 years have been able to delineate the landscape of mutations and genomic alterations occurring in HCC development and progression. This has certainly changed our understanding of the disease. Nonetheless, we are at a point that there is a clear unmet need to translate this knowledge in actual advantages for patients, either in the arena of refining at-risk populations, patient prognosis, and responses to therapies. A huge translational effort needs attention. The research community in HCC has to link discoveries with actual survival benefits and, therefore, it is now about time to demonstrate that biomarkers defining subclasses of patients ultimately might lead to better therapies that change the decision-making process in this complex and heterogeneous disease.
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Supplementary References


Author names in bold designate shared co-first authorship.