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Genomic Medicine and Implications for Hepatocellular Carcinoma Prevention and Therapy

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Abstract

The pathogenesis of hepatocellular carcinoma (HCC) is poorly understood, but recent advances in genomics have increased our understanding of the mechanisms by which HBV, HCV, alcohol, fatty liver disease, and other environmental factors, such as aflatoxin, cause liver cancer. Genetic analyses of liver tissues from patients have provided important information about tumor initiation and progression. Findings from these studies can potentially be used to individualize the management of HCC. In addition to sorafenib, other multikinase inhibitors have recently been approved for treatment of HCC and the preliminary success of immunotherapy has raised hopes. Continued progress in genomic medicine could improve classification of HCCs based on their molecular features and lead to new treatments for patients with liver cancer.

Author contribution

All authors made substantial contributions to conception and design of the review. All authors participated in drafting the article or revising it critically for important intellectual content. LRR and JZR gave final approval of the version to be submitted and any revised version.

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Keywords

HCC; epigenetics; tert; hepatitis; immunotherapy

Hepatocellular carcinoma (HCC) is the second-most common cause of cancer mortality worldwide [1]. HCC is most commonly caused by chronic hepatitis or cirrhosis, resulting from infection with hepatitis B virus (HBV) and C virus (HCV), as well as alcoholic or fatty liver diseases. However, the attributable risks from different etiologies vary significantly among regions. HBV is the most common risk factor for HCC in Southeast Asia and sub-Saharan Africa [2, 3, 4], whereas HCV infection is the most common risk factor in Egypt [5], Europe [6], North America [7], and Japan [8]. Despite the magnitude of the global burden of HCC, it is one of the least-understood cancers and has limited therapeutic options. Advances in genomic research have increased our understanding of HCC development, and could lead to new strategies for prevention and therapy. We review the genomic features of HCC, correlations between genotypes and phenotypes, progression of viral hepatitis-related HCC, and strategies to individualize treatment.

Genetics

Over the past decade the study of cancer has shifted from evaluation of variants of individual genes and pathways to analyses of gene expression patterns and epigenetic profiles of tumor tissues and cells. Advances in next-generation sequencing and computational data analyses can be credited for this shift. The genetic events that contribute to HCC initiation and progression can be classified as genomic (somatic mutations and genome structure changes such as gene fusions or copy number variations), epigenetic (changes in methylation, chromatin remodeling, microRNAs, and long non-coding RNAs [lnc RNAs]), and transcriptional (changes in gene expression) (Figure 1).

Somatic Genomic Events

Somatic mutations occur in somatic (non-germ) cells and are therefore not heritable. When these mutations occur in proto-oncogenes or tumor suppressor genes or in genes involved in regulatory pathways, they can lead to cell transformation and tumorigenesis. Whole-exome and whole-genome sequencing studies have identified mutations that contribute to development of HCC [9–19]. The well-characterized mutations in HCCs are in *CTNNB1* (which encodes beta-catenin), *TP53, AXIN1, RB1, ARID1A, ARID2,* and *NFE2L2*. Mutations in the catalytic telomerase reverse transcriptase (TERT) have been more recently recognized as frequent driver events detected in 40%–65% of HCC samples [20–24]. The first case of germline mutation in TERT was initially discovered in an analysis of data from the Cancer Genome Atlas (TCGA) of HCC, implying germline mutations in *TERT* might cause inherited forms of HCC [19]. *TERT* promoter mutations cause overexpression of telomerase, which allows cells to become immortal. Mutations in the *TERT* promoter that increase its expression appear to be early events in hepatocarcinogenesis [20, 21]. Furthermore, the *TERT* gene appears to be altered by HBV and HCV infection, via different mechanisms. Mutations in the *TERT* promoter have been more frequently associated with HCC resulting from chronic HCV infection and alcohol intake [20, 25] than with HBV-associated HCC. However,

in Hep B related HCC, telomerase expression can be activated by recurrent integration of HBV into the *TERT* promoter[26]. TERT alterations promote cell immortality and transformation also via interactions with transcriptions factors such as MYC [27], beta-catenin [28] and NF-KB [29], to alter expression of their target genes.

Mutations that disrupt the function of TP53 are detected in 12%–48% of HCCs, and with high frequency in advanced tumors, but no therapeutic strategies have been developed to restore TP53 function to cells. An analysis of HCCs in TCGA identified a TP53-regulated gene expression signature that can be used to identify HCC tumors with loss of TP53 function—even when the *TP53* gene is not mutated. The TP53-regulated gene expression signature was associated with clinical outcome and might be used as a biomarker to select treatment. HCCs have developed methods to reduce TP53 activity without mutating the *TP53* gene. For example, TP53 levels are reduced in liver tissues from patients with chronic HBV infection via direct repression of the *TP53* gene promoter by HBx [30].

Activating mutations of in *CTNNB1* have been found in 11%–37% of HCC samples, and inactivating mutations in *AXIN1* have been found in 5%–15% of HCCs. These mutations activate Wnt signaling, which promotes cell motility, de-differentiation, and proliferation [31]. Mutations in proteins that regulate chromatin remodeling, such as ARID1A, are detected in 4%–17% of HCCs; ARID2 mutations are found in 3%–18% of HCCs [9, 14, 19]. These mutations lead to transcriptional repression of genes regulated by the transcription factor E2F. In normal cells, these genes block cell proliferation by upregulating *CDKN1A*, which encodes the cyclin-dependent kinase inhibitor P21 [32].

Many HCC cells contain copy number alterations that result in either gains or losses of segments of genomic DNA. Genes with increased copy numbers amplifications in HCC include *FGF19* and *CCND1.* Amplification of *FGF19* results in increased expression of its product and FGF pathway activation [33, 17]. Brivanib, an inhibitor of VEGF and FGF, did not provide clinical benefit to patients with HCC. However, lenvatinib, another inhibitor of multiple tyrosine kinase receptors, including FGF receptors, increased survival times in patients with HCC in a phase 3 trial [34, 35]. Other highly potent or irreversible FGFR inhibitors are being evaluated in patients and these might be more effective and have better safety profiles[36]. Other oncogenes that are frequently amplified in HCCs include *TERT, VEGFA, MYC, CCND1,* and *MET* [10, 14, 19], whereas tumor suppressor genes such as *PTEN*[37,38] and *CDKN2A* (encoding P16INK4A) are frequently deleted in HCC samples [39, 40]. Loss of these genes leads to cell cycle progression and proliferation.

Epigenetic Changes

Epigenetic alterations also alter gene expression to affect cell and tissue phenotypes [41]. Epigenetic modifications occur via processes such as DNA methylation, covalent modifications to chromatin, alterations in nucleosome position, and changes in levels of micro-RNAs (miRNAs) and long noncoding RNAs (lncRNAs). Epigenetic and genetic events can co-operate to promote tumorigenesis or progression and metastasis. For example, *TERT* promoter mutations frequently co-occur with silencing of *CDKN2A* by promoter

hypermethylation [19]. The combination of telomerase overexpression and silencing of a cell cycle checkpoint inhibitor contribute to cell immortalization [42].

Some genes that are silenced by promoter hypermethylation during hepatocarcinogenesis include the suppressor of cytokine signaling 1 (*SOCS1*)[43,44], hedgehog interacting protein (*HHIP*)[19, 45], *CDKN2A, CDKN1A, CDKN2B*[46], *APC*[47], carbamoyl-phosphate synthase 1 (*CPS1*, a urea cycle gene)[48], TIMP metallopeptidase inhibitor 3 (*TIMP3*)[49], and glutathione S-transferase pi 1 (*GSTP1*)[50]. HCV and HBV can induce epigenetic modifications that promote liver tumorigenesis. HCV induces overexpression of protein phosphatase 2 catalytic subunit alpha (*PPP2CA*), leading to deregulation of histone modifications, altered gene expression, and anchorage-independent growth[51]. In vivo and in vitro studies have shown that HCV can induce promoter hypermethylation and silencing of *GADD45B*, leading to aberrant cell cycle arrest and diminished DNA excision repair[52]. HBV infection also appears to lead to unique DNA methylation patterns that suppress genes including *MDM2, FGF4, FGF19,* and *HSP90AA1*[53]. HBV alters the epigenome via HBx protein[54,55]. HBx increases total DNA methyltransferase (DNMT) activity and promotes regional hypermethylation of specific tumor suppressor genes[54,56]. HBx also promotes recruitment and transactivation of co-activators of the CREB-binding protein CBP–P300 complex, leading to acetylation and thereby activation of cellular genes[57].

MicroRNAs are short (20–22 nucleotide) non-coding RNAs that pair with complementary 3'-untranslated regions mRNAs, inhibiting their translation or leading to their degradation[58]. A single microRNA can control levels of several mRNAs to regulate biological processes such apoptosis, differentiation, and metastasis. One of the most abundant microRNAs in the liver is microRNA 122 (MIR122), which is involved in regulating several genes in the cholesterol metabolism pathway and is also required for HCV replication[59]. Levels of MIR122 are significantly reduced in HCCs[19, 60], which is associated with metastasis and poor outcomes. MIR122-knockout mice develop spontaneous liver tumors resembling HCCs[61] and re-expression of MIR122 reduced tumor incidence and development in *Mir122a*$^{-/-}$ mice[61,62]. MIR375 is also downregulated in HCCs and appears to function as a tumor suppressor. Delivery of MIR375 into HCC cells, via MIR375 mimics on the surface of gold nanoparticles, reduced proliferation and induced apoptosis [63].

Several microRNAs appear to promote tumorigenesis, called oncomirs. Their levels are increased expression HCCs. MIR221 is one of the most highly expressed microRNAs in HCCs; transgenic expression in mice leads to liver tumor development [64]. Inhibition of MIR221 with an anti-sense oligonucleotide delayed tumor growth in *Mir221* transgenic mice[64]. The MIR17-92 cluster encodes at least 6 microRNAs that regulate cell survival, proliferation, differentiation, and angiogenesis. MIR17-92 is significantly overexpressed in HCCs, and its liver-specific overexpression promoted tumor development in transgenic mice [65]. Delivery of anti-MIR17 oligonucleotide via lipid nanoparticles was able to delay MYC-induced tumorigenesis in mice [66]. MicroRNAs might therefore serve as therapeutic targets and also as serum biomarkers. In a nested case-control study performed in China, expression patterns of 7 microRNAs (MIR29a, MIR29c, MIR133a, MIR143, MIR145, MIR192, and MIR505) could be used to identify patients with early-stage HCC [67]. So far, no serum microRNA-based tests have made it to the bedside, but results are promising.

LncRNAs are made of 200–300 nucleotides and regulate gene expression by various mechanisms, including recruitment of chromatin modifying enzymes or interaction with proteins to direct their binding to DNA[68,69]. Aberrant overexpression of lncRNAs like HOTAIR[70], HULC[71], HEIH[72], DREH[73], and MVIH[74] have been associated with HCC initiation and progression. Lau et al showed that integration of HBV DNA into the genome led to transcription of viral–human gene fusions that encode lncRNAs. These authors showed that the hybrid RNA HBx–LINE1 activated Wnt signaling to beta-catenin to promote tumor progression in transgenic mice expressing the viral-human chimeric fusion transcript[75]. Yang et al performed a comprehensive analysis of lncRNA expression levels in HCCs and found 917 recurrently deregulated lncRNAs whose levels correlated with clinical features [76]. Many of these lncRNAs were enriched in co-expressed clusters of genes related to cell adhesion, immune responses, and metabolic processes.

A different epigenetic mechanism of gene regulation in cancer cells is histone modification. Histones regulate gene expression by regulating access to DNA based on the open or closed state of chromatin [77]. Post-translational histone modifications such as methylation or acetylation can influence this process. Acetylation of specific lysine residues in histone tails reduces the affinity between histones and DNA, making DNA more accessible to transcription factors and polymerases, so acetylation generally promotes gene transcription. Transcription changes associated with histone methylation are more complex. Depending on the specific lysine residue, methylation can lead to activation or repression of transcription. Some examples of methylation changes in HCC which influence outcomes include trimethylated histone H3 lysine 4 (H3K4me3) and trimethylated lysine 27 (H3K27me3), whose overexpression correlates with reduced overall survival and poor outcomes of patients with HCC[78,79]. Another mechanism of epigenetic gene regulation is chromatin remodeling which involves dynamic changes in chromatin structure that regulate gene expression, apoptosis, and DNA repair[80]. *ARID1A, ARID1B,* and *ARID2* encode proteins that are part of chromatin-remodeling complexes and function as tumor suppressors which explains why they frequently undergo inactivating mutations in HCC[9, 19,81].

Many genomic and epigenetic events contribute to hepatocarcinogenesis, and viruses are directly or indirectly involved in several of these. What is the stepwise acquisition of genomic events during hepatic tumorigenesis?

Mechanisms of Hepatocyte Transformation and Genetic Alterations

Telomerase activation

Hepatocytes become transformed and form malignancies via a series of genetic and epigenetic alterations leading to genome diversification [82] (Figure 2). The specific mechanisms of tumorigenesis vary among patients with vs without cirrhosis, among patients with different liver diseases, and in patients exposed to different carcinogens. In patients with chronic hepatitis, non-alcoholic steatohepatitis, or alcoholic liver disease, persistent liver injury leads to cell proliferation in response to necrosis and telomere shortening due to the absence of telomerase activity in the adult liver cells[83]. Telomere attrition in senescent hepatocytes is characteristic of cirrhosis and could account for the reduced ability of cirrhotic liver to regenerate after liver resection[84]. Studies of mice have shown that telomere

attrition promotes cirrhosis development. In humans, rare germline inactivating mutations in *TERT* were associated with cirrhosis [85, 86].

During hepatocarcinogenesis, telomerase reactivation is required for malignancy, and is observed in more than 90% of HCC samples[87]. Mutations in the *TERT* promoter were observed in premalignant nodules of patients with cirrhosis, with a prevalence of 6% in low-grade dysplastic nodules and 19% in high-grade dysplastic nodules[21]. *TERT* mutations were detected in 60% of early-stage and progressing HCCs from patients with cirrhosis[20]. Mutations in the *TERT* promoter therefore associate with tumor initiation, whereas mutations in other genes, such as *TP53, CTNNB1* and *ARID1A* appear during later stages of HCC progression, to cause additional changes in the genome and transcription[20,88].

From hepatocellular adenoma to carcinoma

In rare instances, patients without liver cirrhosis can develop hepatocellular adenomas (HCAs), which are benign but can become malignant [89] (Figure 3). Development of HCAs has been associated with exposure to estrogen (such as in oral contraceptives), so they are most commonly detected in women[90]. Subgroups of HCA include: *HNF1A*-mutated; inflammatory; HCA with mutations in exons 3, 7, or 8 of *CTNNB1*; and sonic hedgehog HCA[90]. Genetic analysis of HCCs that developed in patients with HCAs reveal a sequence of genetic alterations that led to malignancy [91].

Increased Wnt signaling to beta-catenin has been associated with malignancy. Mutations in exon 3 of *CTNNB1* that activate its product, beta-catenin, have been associated with progression of HCAs to HCC whereas mutations in exons 7 and 8 that do not lead to beta-catenin activation have not been associated with progression to malignancy [92, 93]. Mutations in the *TERT* promoter also appear to be required for progression of HCA to HCC[91]. In patients with cirrhosis, mutations in the *TERT* promoter allow senescent hepatocytes to bypass telomere attrition, whereas in patients without cirrhosis but with HCA, overexpression of *TERT* occurs after hepatocyte proliferation is induced by beta-catenin activation. These observations are important, because the sequence of accumulation of mutations during different stages of tumorigenesis might be used to select preventative or therapeutic strategies.

Mutation signatures at the start of carcinogenesis

How do hepatocytes acquire DNA mutations that lead to transformation and malignancy? Researchers have categorized the types of nucleotide substitutions found in HCCs associated with different environmental factors (Figure 4). Mutation-inducing processes can occur at the same time or sequentially, during formation and development of a tumor[94]. Whole-exome and whole-genome studies have identified mutation signatures found in large and small proportions of HCCs [15, 17, 95]. For example, COSMIC signatures 1 and 5 are related to patient age, whereas mutation signature 4 has been associated with HCC from patients with exposure to tobacco or polycyclic aromatic hydrocarbons. Mutation signatures 12 and 16 have been observed only in liver tumors, including HCCs and HCAs[95], and might result from exposure to carcinogenic products of liver metabolism; this signature includes mutations in *CTNNB1*. Mutation signature 16 associates with HCCs from patients exposed

to tobacco and alcohol. These findings support results from epidemiology studies indicating that tobacco exposure increases risk of HCC [17, 95].

Mutation signature 24 is found in HCCs from patients exposed to aflatoxin B1. Aflatoxin B1 is a fungal mycotoxin that contaminates crops in Asia and Africa and increases risk of HCC in these regions, in synergy with chronic HBV infection[15, 17, 96]. Exposure to aflatoxin causes a unique mutation profile with a strong transcriptional strand bias for C>A mutations, indicating guanine damage that is repaired by transcription- coupled nucleotide excision repair. These mutations can lead to the R249S substitution in TP53. [97, 98]

Next-generation sequencing identified signature 22, characterized by sporadic mutations, in HCCs from patients exposed to aristolochic acid. This compound is derived from a medicinal plant used in Asia; [99, 100] it is used in traditional Chinese medicine and herbal supplements, and in weight-loss products in South East Asia. Exposure to aristolochic acid causes urothelial and liver cancers [98, 99, 101]. Signature 22 is characterized by predominance of A–T to T–A transversions at [C|T]AG tri-nucleotide motifs, resulting in tumors that have a significant enrichment in splice-site mutations [19, 99]. Aflatoxin B1 and aristolochic acid are banned in the United States by the Food and Drug Administration (FDA), and tests are available for high-risk food products such as peanuts and herbal supplements. Analyses of mutational processes have helped to identify risk factors for HCC that can be reduced by public health approaches.

Virus-induced mutagenesis

HBV can transform hepatocytes by integration of its DNA into the host genome. HBV is a 3.2 kb DNA virus found in a circular form (covalently closed circular DNA) in infected hepatocytes. Although HBV can promote HCC development indirectly, by promoting cell injury, inflammation, fibrosis, and cirrhosis, it also has direct carcinogenic effects. So, some patients with chronic HBV infection with normal liver still develop HCC. Virus oncoproteins, such as HBx or the preS2/S protein, alter cell signaling pathways to promote carcinogenesis [102]. Overexpression of truncated HBx protein increases hepatocyte proliferation and prevents apoptosis[103], regulating cell metabolism[104] and increasing invasiveness and metastasis [105]. HBV DNA sequences integrate into the human genome and can there serve as templates for viral DNA replication. [102] Insertion of HBV DNA near to or within oncogenes, or in cis, can alter expression levels to promote hepatocyte transformation [106–108]. Insertion of HBV DNA near the *TERT, CCNE1, CCNA2,* and *MLL2* genes has observed in HCC samples [108, 109]. HBV DNA was detected near the *TERT* gene in 15% to 20% of HBV-associated HCCs, independent of mutations in the promoter region. Also, integration of HBV DNA can lead to virus–human transcript fusions with functional effects. Asian patients were reported to have integration of HBV DNA in the *LINE* gene, resulting an HBV–LINE1 fusion transcript. Its product can activate Wnt signaling to beta-catenin [75].

The adeno-associated virus type 2 (AAV2), a DNA virus that inserts into human DNA, considered to be non-pathogenic in the general population, also causes mutations that have been detected in HCCs[110]. AAV2 DNA sequences were identified in the *TERT, CCNE1, MLL2,* and *TN6SF10* genes of HCCs from patients with normal liver, without inflammation or cirrhosis. These observations were confirmed in a Japanese study, indicating that AAV2

DNA integration can contribute to HCC development in patients with normal liver[9, 110]. There is controversy over whether HCV is a liver carcinogen—specific HCV proteins could have oncogenic properties. The HCV NS3, NS4B, NS5B, and HCV core protein can transform specific cell types. Mice that overexpress HCV structural proteins develop liver tumors [9, 111, 112]. However, in humans, HCV infection appears to primarily promote liver cancer via inflammation and cirrhosis [113].

Genome diversity and heterogeneity

All the mechanisms of malignant transformation lead to the acquisition of additional genetic alterations that result in the development of a complex genomic architecture during tumor evolution [114]. Tumor initiation, progression, and metastasis are associated with the acquisition of mutations and copy number variations in subclones, which result in considerable spatial and temporal heterogeneity in HCC. Mutations that promote HCC development, such as those in the *TERT* promoter or *CTNNB1*, have been identified in all parts of primary tumors and are therefore called core clonal alterations[88]. In contrast, several studies have confirmed the presence of spatial heterogeneity with mutations in subclones that are only present in specific regions of HCC tumors[115, 116]. There is an additional layer of complexity in liver carcinogenesis in patients with multifocal disease, who may have multifocal intra-hepatic metastases from the original tumor, along with inter-tumor heterogeneity due to the near simultaneous development of de novo tumors at multiple sites [117, 118].

Development of tumor heterogeneity is a dynamic process with complex timing. Exposure to carcinogens and acquisition of mutations by HCC clones and subclones changes with time. For example, early HCC clones can have a mutation signature associated with aflatoxin B1 among patients exposed in Africa during early life. However, if the patient develops HCC while living in a western country, HCC subclones that form later in life may no longer have the aflatoxin B1-associated mutation signature[95, 118]. So, tumor genomes accumulate alterations throughout life that reflect etiologic influences during the various periods of exposure. Moreover, the acquisition of chromosome or genome duplications appears to be a very late event during HCC evolution[95]. This spatial and temporal tumor heterogeneity of tumors is important to appreciate as it may explain the subsequent acquisition of primary or secondary resistance to targeted therapies.

Genotype and Phenotype Classifications

Interactions of genome alterations

Interactions among gene mutations, changes in transcription, alterations in epigenetic regulation, environmental factors, and histologic features should all be considered in classification of HCCs[82]. Whole-exome and whole-genome sequence analyses of HCCs identified 4 to 6 mutations in oncogenes per tumor; associations and exclusions among these mutations indicate redundancy and/or cooperation between factors in overlapping signaling pathways[9, 10, 15, 17, 19]. Mutations occur in groups of genes that are associated with specific signaling pathways. For example. tumors with mutations in *CTNNB1* do not usually have mutations in *TP53* or *AXIN1. CTNNB1* mutations are frequently associated with mutations

in the *TERT* promoter, *APOB, NFE2L2, ARID2,* and *MLL2*[19]. Mutations in *TP53* are frequently associated with mutations in *KEAP1, CCND1,* and *TSC2*. Mutations in *AXIN1* are frequently detected with mutations in *ARID1A* and *RPS6KA3*[15, 17].

Risk factors associated with molecular profiles

HCC risk factors associate with their genetic features. For example, HBV-related HCCs have a specific pattern of mutations that result from insertion of HBV DNA into the genome, as well as mutations in *TP53* and *AXIN1* and acquisition of stem cell features [15, 17, 119–121]. Some of these associations could result from the geographic risk factors, such as the coincidence of regions of high HBV prevalence with regions of high dietary aflatoxin exposure. Similarly, HCCs in patients with high alcohol intake frequently contain mutations in *ARID1A* and *CTNNB1*, whereas HCC of unknown etiology have fewer *TERT* promoter mutations and more frequent *IL6ST*-activating mutations [15, 17]. However, no HCC mutation pattern has been confirmed to be associated with HCV infection or metabolic syndrome.

Molecular alterations related to outcome

Tumor features identified from genetic, epigenetic, and transcriptome analyses have been associated with poor outcomes of patients treated for HCC. *RB1* and *TP53* mutations and *FGF19* amplification increase risk of tumor relapse and death [122–124] Interestingly, *TP53* mutations are a risk factor for poor survival and tumor recurrence in patients with HBV-related HCC but not in patients with HCC related to other etiologies [121, 125]. Transcriptome signatures from tumor tissues have been associated with tumor aggressiveness and tumor recurrence 2–3 years after surgery (early recurrence) [126–130], whereas transcriptomes of non-tumor liver tissues have been associated with carcinogenesis de novo, usually in patients with cirrhosis, and tumor recurrence after 3 years (late recurrence) [131]. Moreover, signatures from non-tumor cirrhotic liver have also been associated with severity of the liver disease and are consequently linked with HCC occurrence and decompensation of liver disease [132]. Expression levels of 5 genes (5-gene score) in tumor tissues, combined with an expression pattern of 186 genes in non-tumor liver tissue, were associated with early tumor recurrence and late recurrence, as well as overall survival [126]. However, most prognostic transcriptome signatures were derived from specimens obtained during resection of very early- or early-stage HCCs. These findings therefore require validation in studies of biopsies from patients with intermediate- and advanced-stage tumors who received different types of treatment. Prognostic transcriptome signatures are currently not used in clinical practice [82].

Molecular features and correlations with phenotypes

Classification systems developed to assess genome diversity identified different subgroups of HCC. One group is called proliferative HCC, characterized by chromosome instability (G1 to G3 subgroups, proliferative subgroup, cluster A, S1 and S2) and a second is considered to have less proliferative HCC cells, with chromosomal stability (G4 to G6, S3, cluster B) [120, 128, 133, 134]. Among the HCCs with less-proliferative cells, a subgroup was defined by somatic mutations in *CTNNB1*, leading to activation of genes regulated by Wnt signaling to beta-catenin (G5, G6)[120, 135]. Another subgroup, well-differentiated HCC, had a gene expression pattern close to that of mature hepatocytes (G4 subgroup, hepatocyte like, S3). Acquisition of progenitor cell characteristics and re-expression of fetal genes defined a

group of HCC with stem cells features (G1 subgroup, progenitor like, hepatoblast like, S2). Finally, a subtype of HCC with inactivation of *CDKN2A* and mutations in *TP53*, leading to dysregulation of cell cycle genes, was associated with poor outcome (G3 subgroup)[120].

HCC transcriptomes and mutation patterns were linked with specific histologic features[136, 137, 138]. The G1 to G3 subgroups of HCC are often poorly differentiated and have mutations in *TP53*. The G1 subgroup and tumors with *RP6SKA3* mutations were linked with the progenitor phenotype, with expression of stem cell markers such as CK19 or EPCAM, based on immunohistochemical analyses. The scirrhous histologic subtype of HCC was linked with mutations in *TSC1* and *TSC2* and expression of genes of the epithelial to mesenchymal transition[136, 137]. HCCs from patients with steatohepatitis are frequently classified in the G4 subgroup, characterized by immune cell infiltration and activation of the JAK-STAT signaling pathway [136]. In contrast, HCCs with steatosis and infiltration by inflammatory cells were associated, in a separate study, with the S1 subgroup, so additional studies are needed to classify these tumors [137]. Well-differentiated HCCs of the G5 to G6 subgroups are enriched in activating mutations of *CTNNB1* and are characterized by cholestasis, increased levels of glutamine synthase (determined by immunohistochemistry), and nuclear translocation of beta-catenin. A histological subtype called macrotrabecular massive is characterized by the G3 and S2 transcription profile, *TP53* mutations, and *FGF19* amplification[136,137]. This subgroup was associated with a higher rate of tumor recurrence in a large cohort of patients who underwent resection or radiofrequency ablation, so its identification in surgical samples or tumor biopsies can be helpful in clinical practice [139]. Another subtype, known as chromophobe HCC with abrupt anaplasia, characterized by nuclear atypia on a background of cells with bland nuclei, has been correlated with the presence of alternative lengthening of telomeres [140].

Features of mixed hepatocholangiocarcinoma tumors

The genetic features of cholangiocarcinomas differ from those of HCCs in that cholangiocarcinomas have frequent mutations in *KRAS, BRAF, BAP1, SMAD4, IDH1,* and *IDH2*; as well as fusion of *FGFR2, ROS1,* and *PRKACA* genes, but few *TERT* promoter mutations [141–143]. However, a continuum seems to exist among cholangiocarcinoma, mixed hepato-cholangiocarcinoma, and HCCs with stem cell features, indicating that similar early genetic alterations in different cell types results in different histologic and genetic subtypes of tumors[143]. Interestingly, next-generation sequencing analyses of specific areas of HCCs, cholangiocarcinomas, and hepatocholangiocarcinomas found common somatic mutations among tumor areas, indicating clonal origins for each part of these tumors[144]. Moreover, the proportions of tumors with *TERT* promoter mutations ranges from 59% in HCC, to 20% in hepatocholangiocarcinomas, to few in cholangiocarcinomas. Similar to HCC, amplifications in *CCND1* and *FGF19* were identified in some hepatocholangiocarcinomas. Some studies have reported gene dysregulation typical of cholangiocarcinoma in HCCs with stem cell features [145–147]. More studies are needed to determine how similar and different genetic alterations contribute to development of CCA, HCC and mixed tumors.

Personalized Medicine

The goal of personalized medicine is select specific treatments for each individual tumor based on its genotype or other features. This idea is not novel but is becoming a practical reality. Success stories in precision medicine include the use of imatinib mesylate for treatment of chronic myelogenous leukemia [148], BRAF inhibitors for treatment of melanoma with the *BRAF* V600E mutation[149], tyrosine kinase inhibitors such as erlotinib for lung adenocarcinomas with alterations in the epidermal growth factor receptor (EGFR), [150] and ALK inhibitors for lung cancer with *ALK* rearrangements [151].

HCC is relatively resistant to traditional chemotherapeutics such as 5-fluorouracil, cisplatin, doxorubicin, or gemcitabine. Until 2007, patients with advanced, unresectable HCC could receive only best supportive care. In 2007, sorafenib, a multi-kinase inhibitor that blocks signaling via vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor beta (PDGFRB), BRAF, and KIT, was the first systemic agent to increase survival times of patients with advanced HCC [152–154]. Although the drug increased patient survival time by only 3–4 months, it provided hope that additional targeted therapies could be developed for HCC.

Unfortunately, the approval of sorafenib was followed by a long period of failure of agents tested in phase 3 trials of patients with HCC, including sunitinib [155], brivanib [34], linifanib, [156] and erlotinib [157]. Nevertheless, there have been promising results for other kinase inhibitors, such as regorafenib, lenvatinib, and cabozantinib (Table 1). Regorafenib has been approved as a second-line therapy based on results from the RESORCE trial, which showed that this drug significantly increased survival times of patients with advanced HCC that progressed during treatment with sorafenib, compared to placebo (10.6 months vs 7.8 months)[158]. Lenvatinib, another multi-kinase inhibitor, was found to be non-inferior to sorafenib as first-line therapy for untreated advanced HCC in the REFLECT trial[35] and has recently been approved by the FDA. Also, Cabozantinib was reported to have met clinical endpoints, compared with placebo, in a phase 3 trial (CELESTIAL), as a second-line agent[159, 160]. Although studies of these multi-kinase inhibitors have produced encouraging results, there is much to be desired in terms of their efficacy and safety—most of these drugs only prolong overall survival by a few months and fewer than 10% of patients achieve the objective response.

One of the main reasons for failure of multiple targeted therapies in phase 3 trials was felt to be interpatient tumor heterogeneity and many solutions have been proposed to overcome this, including testing drugs in biomarker-stratified subpopulations. Tivantinib, a MET inhibitor, showed promising results in a phase 2 trial, especially for patients whose tumors had high MET expression[161]. This was followed by a biomarker-stratified phase 3 study, which included only patients with tumors that had high levels of MET, determined by immunohistochemistry. In this study, patients were randomly assigned to groups that were given tivantinib (n=226) or placebo (n=114). At a follow-up time of 18.1 months, median overall survival times were 8.4 months in the tivantinib group and 9.1 months in the placebo group (hazard ratio, 0.97). So, unfortunately, the encouraging results from the phase 2 study did not continue into the phase 3 trial [162]. In analyses of the disappointing results of this

phase 3 trial, researchers found that tivantinib did not act as a MET inhibitor after all, but instead as an anti-mitotic agent. So, MET overexpression is likely not a good biomarker of tumors likely to respond to tivantinib [163].

In the phase 3 REACH2 trial, ramucirumab, an inhibitor of VEGFR2, increased survival times, when given as a second-line agent, in patients with HCC and Child Pugh scores of 5 or 6 and serum levels of alpha-fetoprotein (AFP) above 400 ng/ml [164]. In a phase 3 trial of only patients with high baseline serum levels of AFP (NCT02435433), ramucirumab increased survival times compared to placebo. Apatinib, another inhibitor of VEGFR2, is being tested as a first-line therapy in a phase 2 trial (NCT03046979), based on promising results from smaller studies [165, 166].

Another challenge to development of therapies for HCC is that the somatic mutations associated with tumor development lie in genes whose products are not easily or safely targeted. Mutated forms of TERT, TP53, CTNNB1, and MYC are believed to be undruggable. Although our understanding of *TERT* promoter mutations has rapidly expanded, we do not have small molecule inhibitor of telomerase. A synthetic *hTERT* DNA vaccine, INO-1400, is being tested in a phase 1 trial of patients with solid tumors (NCT02960594) and some trials are using *TERT* promoter mutation as a biomarker for study enrollment (NCT02766270). New strategies might be developed to target these driver genes or their pathways, such as microRNA-based therapeutics. Advances in genome research should help identify events that can be targeted or used as biomarkers to select patients for specific therapies. Table 1 and Supp table 1 presents targeted therapies for HCC that are in phase 3 and phase 2 trials respectively. Most of the agents are being explored as second-line therapies for patients with advanced HCC who were failed by sorafenib, but this may change soon.

Immunotherapy

The combination of the immune-tolerant microenvironment of the liver, ability of HCV and HBV to evade the immune response, and the immune-modulatory effects of the tumor allow for growth and progression of HCC. Hence strategies to reactivate anti-tumor immunity can be used to prevent or treat HCC. Nivolumab was recently given accelerated approval for treatment of advanced liver cancer, based on promising results from a phase 2 trial (Checkmate-040)[167]. Approximately 20% of the patients had complete or partial responses to nivolumab and 40% achieved stable disease; the 12-month overall rate of survival was 59.9%. Although these responses are modest, they are more promising than previous systemic agents. Multiple clinical trials of immunotherapy agents are underway in patients with HCC and there is hope the treatment paradigm will improve. It is important to continue to investigate the effects of HCC on the immune system— especially in patients with viral hepatitis, to identify patients most likely to respond to specific therapies. This is being extensively discussed in another article in this issue [168].

Future Directions

Recent advances in genetic, genomic, and proteomic analyses have increased our understanding of HCC pathogenesis and our ability to classify tumors based on genetic and histologic features. We are learning more about the specific oncogenic effects of HBV, HCV, alcohol, fatty liver disease, and environmental factors such as aflatoxin and aristolochic acid. We have been identifying genetic alterations that contribute to liver carcinogenesis, learning the sequence of acquisition of these mutations, and discovering the chromosomal and epigenetic changes required for tumor development and progression.

There is continued progress in identifying multi-kinase inhibitors of angiogenesis and other receptor tyrosine kinase signaling pathways in tumor cells and the tumor microenvironment that might slow tumor growth yet have an acceptable safety profile in persons with liver disease. Although we have not been able to use HCC subclasses to select the optimal therapy for patients, some trials have used biomarkers to identify the subsets of patients with highest rates of response to specific targeted therapies. As the key molecular drivers of HCC are identified, strategies are being developed to reduce levels of TERT, Wnt signaling to beta-catenin, MYC activation, P53 inactivation, and expression of chromatin modifying genes.

Studies are needed to determine the potential effectiveness of immunotherapies, to identify subgroups of HCCs that are most sensitive to checkpoint inhibitors or other agents, and to determine the potential of neo-adjuvant, adjuvant, and combination strategies to improve patient outcomes. It is important to continue to acquire and analyze intermediate- to advanced-stage HCC samples from participants in clinical trials of systemic targeted or immune therapies. Integrated molecular analyses of these samples will potentially identify the subsets of patients most likely to benefit from specific therapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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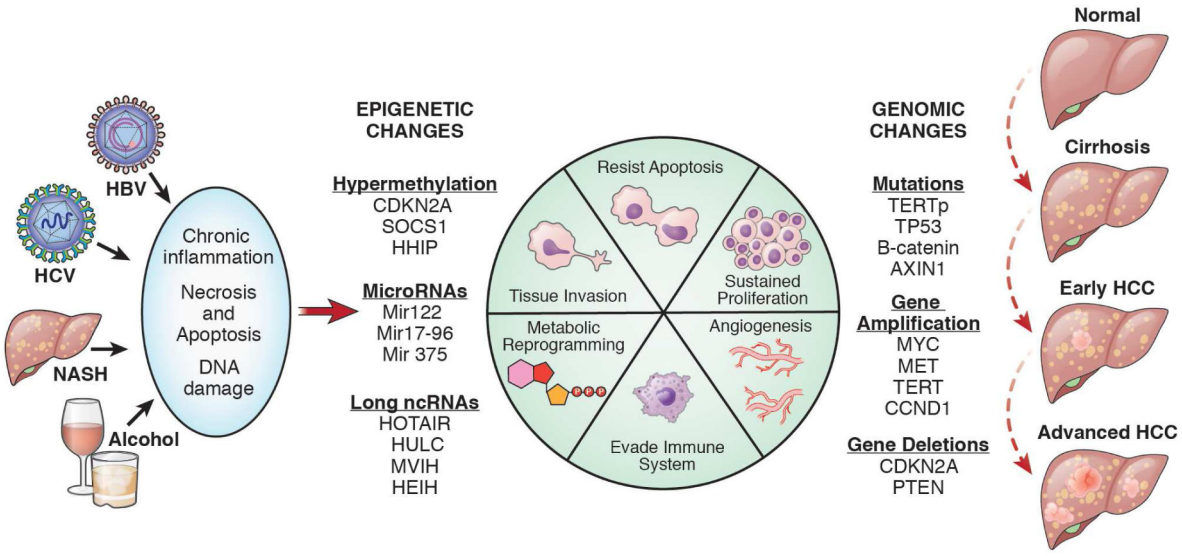

Figure 1. Pathogenesis of HCC

Liver cirrhosis is caused by chronic infection with HBV or HCV, alcohol abuse, or fatty liver disease. The chronic inflammation, necrosis, and regeneration that occur during development of cirrhosis cause genetic and epigenetic changes in liver tissue that lead to tumor formation.

NASH, non-alcoholic steatohepatitis

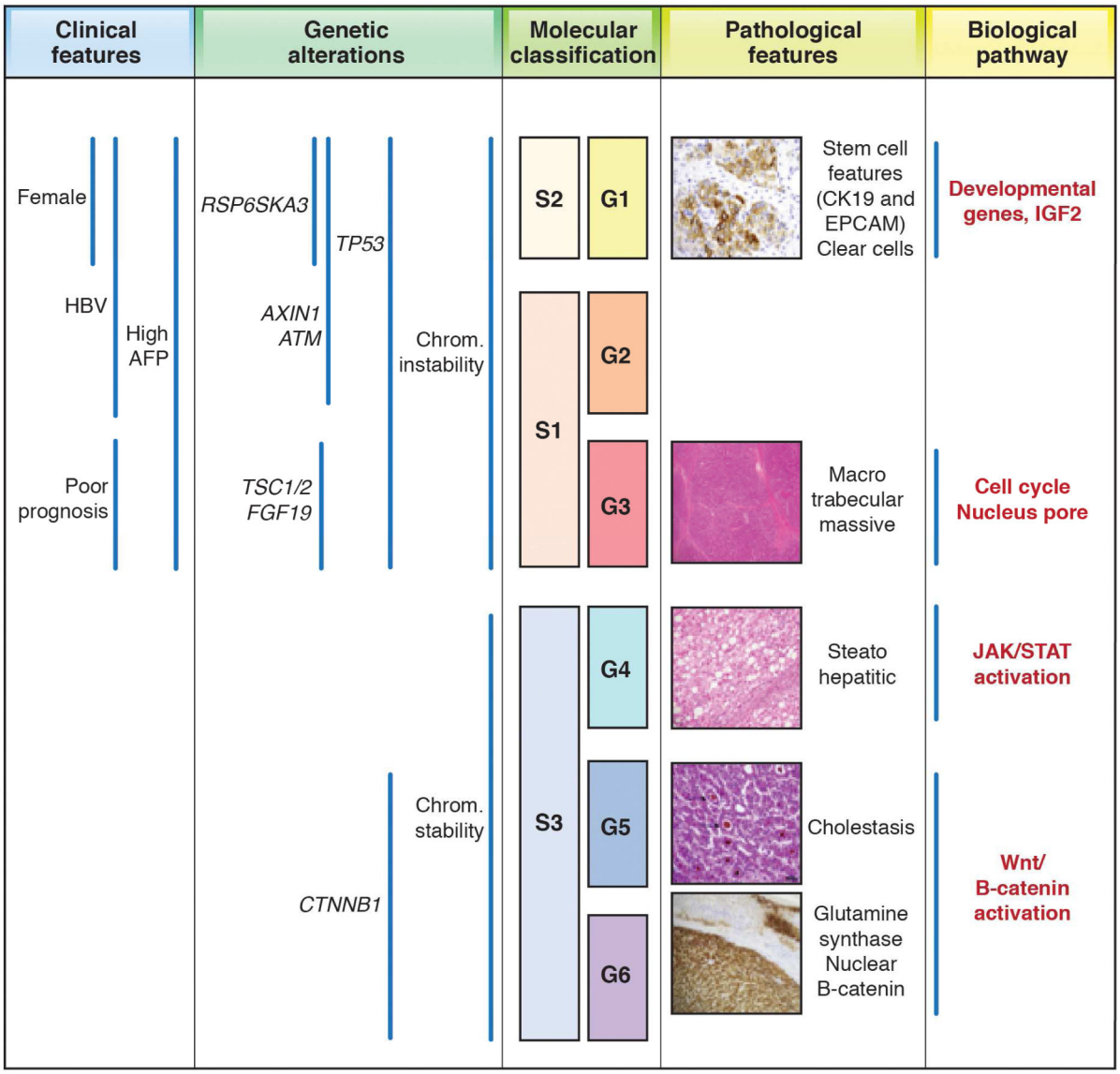

Figure 2. Genotype and Phenotype Classification of HCC

HCCs can be classified based on their genetic features, molecular features (S1 to S3, ref 134 and G1 to G6, ref 120) pathology features, signaling pathways activated, and clinical features of patients. Many of these subgroups overlap in their features. Chrom, chromosome.

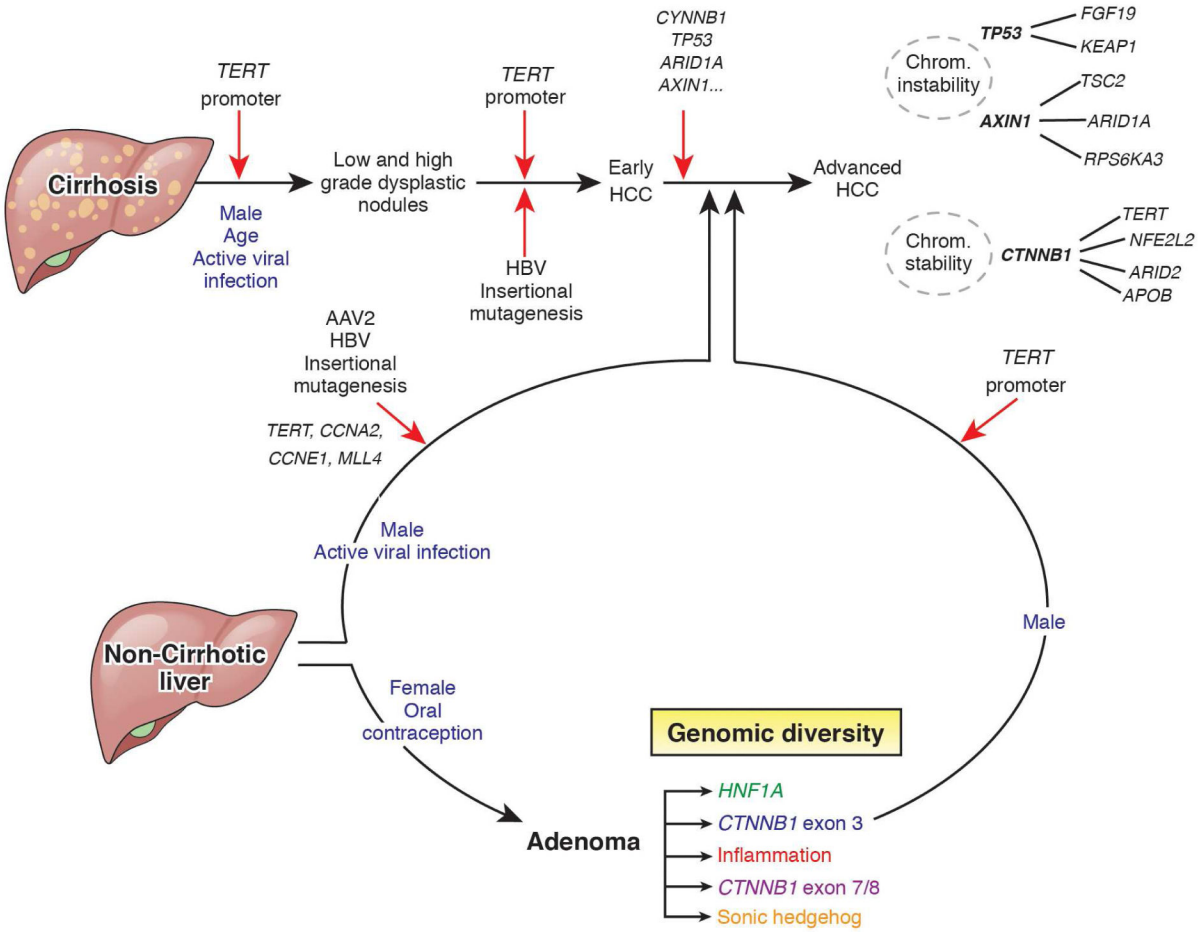

Figure 3. From Hepatocyte Transformation to Malignancy

Genetic changes that occur during transformation of hepatocytes in patients with and without cirrhosis. Mutations in the promoter region of TERT contribute to immortality and proliferation of hepatocytes, resulting in dysplastic nodules. Additional mutations, some induced by viruses such as HBV or AAV2, lead to early-stage HCC. Mutations in genes such as *TP53, CTNNB1,* and *AXIN1* lead to advanced HCC, with additional chromosome alterations. Patients without cirrhosis exposed to high levels of estrogen, such as through oral contraceptives, are at increased risk for hepatic adenomas (HCA), which are benign but can progress to malignant tumors if cells acquire mutations in the same genes that contribute to HCC development. Red arrows indicate genetic alterations believed to be required for heptocarcinogenesis.

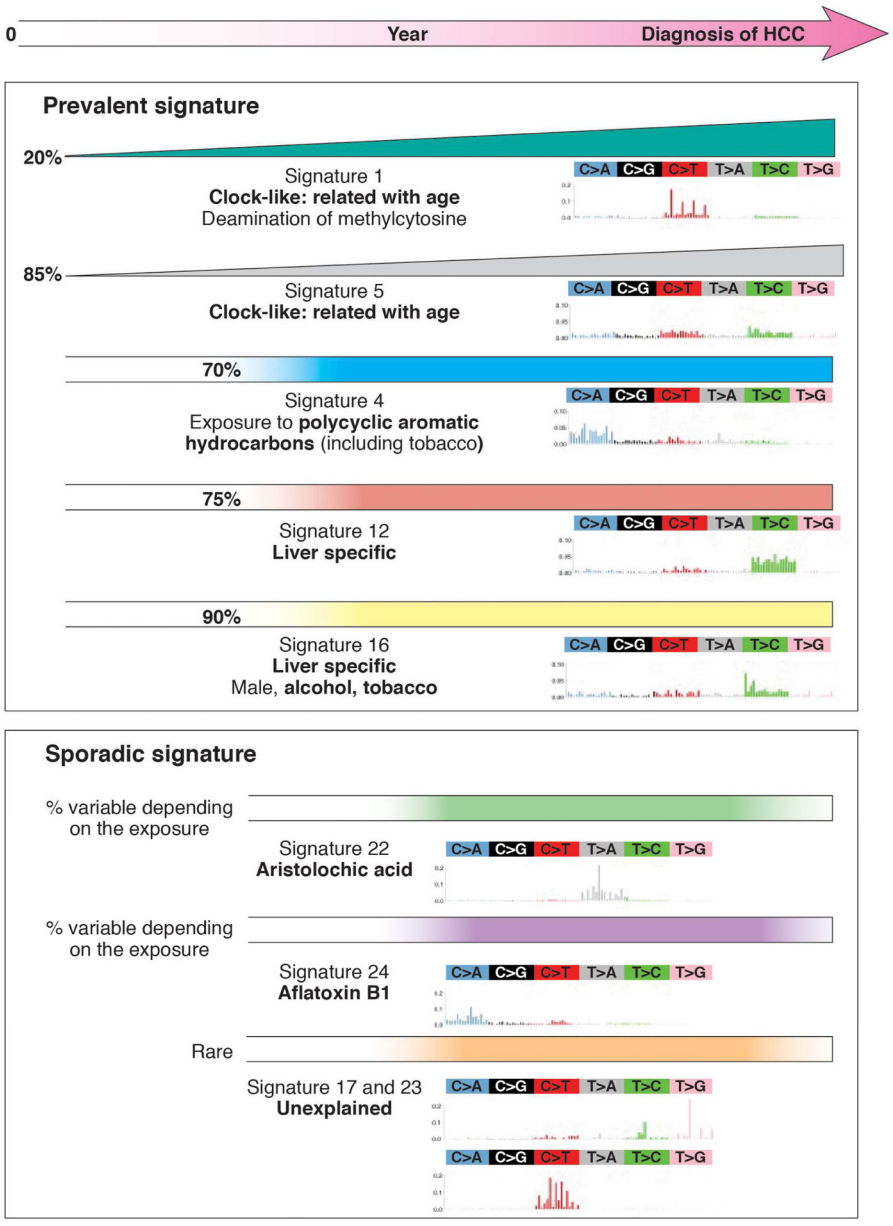

Figure 4. Accumulation of Mutations During Liver Carcinogenesis

Mutation signatures of different subgroups of HCCs. Some signatures are found in a large proportion of HCCs (percentage values given) worldwide, whereas others are found in small proportions of HCCs, related to specific carcinogen exposures or in sporadic tumors. Lines of increasing width from left to right indicate mutations that accumulate with time, whereas straight lines from left to right indicate mutations that do not increase with time.

Table 1.

Phase 3 Trials of Targeted Chemotherapy for HCC

BCLC, Barcelona Clinic Liver Cancer classification; ECOG, Eastern Cooperative Oncology Group; PS, Performance Status. mAb, monoclonal antibody. VEGFR2 -Vascular endothelial growth factor receptor 2; CDK- cyclin-dependent kinase; EGFR- Epidermal growth factor receptor. TGF- Transforming growth factor.

TRIAL NAME. DRUG	CLINICAL TRIAL ID	MECHANISM OF ACTION	TITLE OF STUDY	LINE OF THERAPY	INCLUSION CRITERIA	STATUS	
PHASE 3 COMPLETED. POSITIVE TRIALS							**Reference**
SHARP. Sorafenib	NCT00105443	Multiple tyrosine kinase receptor inhibitor	A Phase 3 Randomized, Placebo-controlled Study of Sorafenib in Patients With Advanced Hepatocellular Carcinoma	First	Child pugh A. BCLC B-C, ECOG PS 0-2.	Completed. Sorafenib improves overall survival over placebo.	152
ASIA-PACIFIC. Sorafenib	NCT00492752	Multiple tyrosine kinase receptor inhibitor	A Randomized, Double-blinded, Placebo-controlled Study of Sorafenib in Patients With Advanced Hepatocellular Carcinoma	First	Child pugh A. BCLC B-C, ECOG PS 0-2.	Completed. Sorafenib improves overall survival over placebo.	153
RESOURCE. Regorafenib	NCT02042144	Multiple tyrosine kinase receptor inhibitor	Randomized, Double Blind, Placebo Controlled, Multicenter Phase 3 Study of Regorafenib in Patients With Hepatocellular Carcinoma (HCC) After Sorafenib	Second	Child Pugh A, BCLC B-C, ECOG PS 0-1. Failed Sorafenib	Completed. Regorafenib improves overall survival over placebo in patients who progressed on Sorafenib.	158
CELESTIAL. Cabozantinib	NCT01908426	Multiple tyrosine kinase receptor inhibitor	A Phase 3, Randomized, Double-blind, Controlled Study of Cabozantinib (XL184) vs Placebo in Subjects With Hepatocellular Carcinoma Who Have Received Prior Sorafenib	Second	Child Pugh A, BCLC B-C, ECOG PS 0-1. Failed Sorafenib	Completed. Cabozantinib improves overall survival over placebo in patients who progressed on Sorafenib.	160
REFLECT. Levatinib	NCT01761266	Multiple tyrosine kinase receptor inhibitor	A Multicenter, Randomized, Open-	First	Child Pugh A, BCLC B-C, ECOG PS 0-1	Completed. Lenvatinib is	35

TRIAL NAME. DRUG	CLINICAL TRIAL ID	MECHANISM OF ACTION	TITLE OF STUDY	LINE OF THERAPY	INCLUSION CRITERIA	STATUS	
			Label, Phase 3 Trial to Compare the Efficacy and Safety of Lenvatinib (E7080) Versus Sorafenib in First-Line Treatment of Subjects With Unresectable Hepatocellular Carcinoma			non-inferior to Sorafenib in improving overall survival as first line therapy for advanced HCC.	
PHASE 3, ACTIVE							**Date of Completion**
REACH-2. Ramucirumab	NCT02435433	VEGFR2 inhibitor	Randomized, Double-Blind, Placebo-Controlled, Phase 3 Study of Ramucirumab and Best Supportive Care (BSC) Versus Placebo and BSC as Second-Line T reatment in Patients With Hepatocellular Carcinoma and Elevated Baseline Alpha-Fetoprotein (AFP) Following First-Line Therapy With Sorafenib	Second	Child Pugh A, BCLC B-C, ECOG PS 0-1 AFP>400ng/ml	Completed. Ramucirumab improves overall survival over placebo inpatients with advanced HCC with AFP>400 (164).	June 2020
Everolimus	NCT02081755	mTOR inhibitor	A 36 Month Multi-center, Open Label, Randomized, Comparator Study to Evaluate the Efficacy and Safety of Everolimus Immunosuppression Treatment in Liver Transplantation for Hepatocellular Carcinoma Exceeding Milan Criteria	First	Liver transplant for HCC and high risk for recurrence	Recruiting	August 2019
SATURNE. Sunitinib	NCT01164202	Multiple tyrosine kinase receptor inhibitor	A Double-Blind, Randomized, Phase 2/3 Study Comparing the Use of Chemoembolization Combined With Sunitinib Against	First	Child pugh A. BCLC B-C, ECOG PS 0-2.	Not Recruiting	July 2018

TRIAL NAME. DRUG	CLINICAL TRIAL ID	MECHANISM OF ACTION	TITLE OF STUDY	LINE OF THERAPY	INCLUSION CRITERIA	STATUS	
			Chemoembolization Combined With a Placebo in Patients With Hepatocellular Carcinoma (SATURNE)				
Apatinib	NCT02329860	VEGFR2 inhibitor	Study of Apatinib After Systemic Therapy in Patients With Hepatocellular	CSecond	Child pugh A-B. BCLC B-C, ECOG PS 0-1.	Not Recruiting	June 2018
Donafenib	NCT02645981	Multiple tyrosine kinase receptor inhibitor	Efficacy and Safety of Donafenib in Patients With Advanced Hepatocellular Carcinoma	First	Child pugh A-B. BCLC B-C, ECOG PS 0-1.	Not Recruiting	Aug 2019