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Liver Cancer Cell of Origin, Molecular Class, and Effects on Patient Prognosis

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Abstract

Primary liver cancer is the second leading cause of cancer-related death worldwide and therefore a major public health challenge. We review hypotheses of the cell of origin of liver tumorigenesis and clarify the classes of liver cancer based on molecular features and how they affect patient prognosis. Primary liver cancer comprises hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (iCCA), and other rare tumors, notably fibrolamellar carcinoma and hepatoblastoma. The molecular and clinical features of HCC versus iCCA are distinct, but these conditions have overlapping risk factors and pathways of oncogenesis. A better understanding of the cell types originating liver cancer can aid in exploring molecular mechanisms of carcinogenesis and therapeutic options. Molecular studies have identified adult hepatocytes as the cell of origin. These cells have been proposed to transform directly into HCC cells (via a sequence of genetic alterations), to dedifferentiate into hepatocyte precursor cells (which then become HCC cells that express progenitor cell markers), or to transdifferentiate into biliary-like cells (which give rise to iCCA). Alternatively, progenitor cells also give rise to HCCs and iCCAs with markers of progenitor cells. Advances in genome profiling and next-generation sequencing have led to the classification of HCCs based on molecular features and assigned them to categories such as proliferation–progenitor, proliferation–transforming growth factor β, and Wnt–catenin β1. iCCAs have been assigned to categories of proliferation and inflammation. Overall, proliferation subclasses are associated with a more aggressive phenotype and poor outcome of patients, although more specific signatures have refined our prognostic abilities. Analyses of genetic alterations have identified those that might be targeted therapeutically, such as fusions in the *FGFR2* gene and mutations in genes encoding isocitrate dehydrogenases (in approximately 60% of

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Conflicts of interest

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iCCAs) or amplifications at 11q13 and 6p21 (in approximately 15% of HCCs). Further studies of these alterations are needed before they can be used as biomarkers in clinical decision making.

Keywords

Liver Cancer; Molecular Drivers; Targeted Therapies; Prognosis

Liver cancer is the second most common cause of cancer-related death worldwide. It is one of the few neoplasms with a steady increasing incidence and mortality[1,2] and is the neoplasm with the greatest increase in mortality in the United States during the past 2 decades (Figure 1[3]). Liver cancer comprises a heterogeneous group of malignant tumors with different histological features and an unfavorable prognosis that range from hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA) to mixed hepatocellular cholangiocarcinoma (HCC-CCA), fibrolamellar HCC (FLC), and the pediatric neoplasm hepatoblastoma.[4,5] Among these, HCC and iCCA are the most common primary liver cancers; the other neoplasms, including mixed HCC-CCA tumors,[5] account for less than 1% of cases. The burden of liver cancer is increasing globally, and there could be 1 million cases by 2030.[6] It is not clear how direct-acting antiviral agents, which can cure hepatitis C virus (HCV) infection, will affect the burden of HCC. It has been estimated that curing more than 90% of cases of HCV infection would eliminate 15% of cases of HCC in the United States.[7] However, there is debate over the effects of direct-acting antiviral agents on progression of HCC.[8–11]

HCC alone accounts for 90% of all cases of primary liver cancer, with nearly 800,000 new cases annually.[2] The incidence is highest in Asia and Sub-Saharan Africa due to the high prevalence of hepatitis B virus (HBV) infection.[6] Unlike other cancers, the main risk factors associated with HCC are well defined and include viral hepatitis (B and/or C), alcohol abuse, and nonalcoholic fatty liver disease in patients with metabolic syndrome and diabetes. Other cofactors of HCC development, such as aflatoxin B1 and tobacco, increase the incidence of the disease if other common risk factors are present.[12]

The second most common liver cancer is iCCA, with the highest incidence in Southeast Asia (30–40 cases/10^5 inhabitants) and low incidence in Western countries (fewer than 5 cases/10^5 inhabitants).[13] Nevertheless, steady increases in incidence have been reported.[13,14] Risk factors for development of iCCA include primary sclerosing cholangitis (PSC), biliary duct cysts, hepatolithiasis, and parasitic biliary infestation with flukes, which is an etiology prevalent in Asia and linked to a specific molecular fingerprint.[13] More recently, shared risk factors with HCC have also been identified, such as HBV and HCV, particularly for iCCAs that develop in cirrhotic liver.[15]

HCC and iCCA have been considered to be independent tumors that originate from distinct cell populations. However, more recently, some have been recognized as tumor subtypes of a continuous spectrum of diseases. We review the theories behind the cell(s) of origin of liver cancer, describe emerging molecular classes, link these classes with their etiology and prognosis, and define pathways for future translation.

Cell(s) of Origin

Parenchymal (hepatocytes and cholangiocytes) and nonparenchymal cells (fibroblasts, stellate cells, Kupffer cells, and endothelial cells) form the basic hepatic structure (Figure 2); the existence of stem cells in adult liver has been heavily debated. Hepatocytes constitute 60% to 80% of the total liver mass. Architecturally, these cells are organized in lobules, which can be further divided in functional regions or zones.[16] Liver zonation is particularly relevant for hepatocytes, because it affects their function without altering their phenotype. Hepatocytes are frequently polyploid (4N, 8N, and so on); polyploid cells make up 50% of human liver and 90% of mouse liver.[17]

Liver tumors are heterogeneous in morphology, within the same and between different tumors. Some subtypes of HCCs and iCCAs have stem cell features,[18–20] such as HCCs with CK19-positive cells.[21] The rare, mixed HCC-CCAs have cells with a phenotype that is intermediate between hepatocytes and cholangiocytes.[22] These observations have led to several hypotheses about the cell(s) of origin of liver cancer. Hepatic progenitor cells might generate primary liver tumors because, during liver development, hepatocytes and cholangiocytes each arise from a common progenitor (hepatoblasts). However, HCCs and iCCAs might be distinct tumors that originate from mature hepatocytes and cholangiocytes, respectively.

Findings from recent studies indicate that adult hepatocytes are the source of HCCs because they can directly degenerate into HCC following sequential genomic insults, dedifferentiate into precursor cells that ultimately can transform into HCC cells with markers of progenitor cells, and transdifferentiate into biliary-like cells able to transform into iCCA[23–25] (Figure 3). In parallel, progenitor cells may also give rise to HCCs and iCCAs with progenitor-like features, whereas adult cholangiocytes, which lack the plasticity and transforming capacity of hepatocytes, can only give rise to iCCAs.[26]

Progenitor Cells and Hepatocytes in Liver Regeneration

Adult stem cells or somatic stem cells are defined as undifferentiated cells with limitless replicative potential. They can differentiate into all or some specialized cell types, and their primary role is to sustain physiological tissue turnover and direct tissue repair on different types of injury.[27] Stem cell compartments have been successfully identified in several adult tissues with fast turnover, including the gastrointestinal tract, skin, and bone marrow. Because of their potential for self-renewal and long life span, these cells are believed to be more prone to malignant transformation[28,29] and have been identified as the cell of origin of cancer in several organs (eg, skin and intestine).[30–32] Unlike these organs, however, cells of the adult liver have relatively slow turnover (a period of several months), with an average hepatocyte life span ranging from 200 to 300 days.[33,34] In this context, the contribution of stem cells to liver turnover is unclear (reviewed in Miyajima et al[35]).

Hepatocytes are quiescent in normal liver, but their turnover in the liver dramatically accelerates on hepatic injury or after a significant reduction of liver mass such as following partial hepatectomy. Under these conditions, adult hepatocytes have enormous proliferative potential (capacity to replicate more than 50 times), a feature that has enabled human liver

organ donor transplantation as a standard procedure to treat end-stage liver disease.[33–35] There is evidence that regeneration after partial hepatectomy relies predominantly on the replication of hepatocytes rather than stem cells. However, when the replicative capacity of hepatocytes is severely impaired, such as in patients with acute liver failure or chronic hepatitis, epithelial cells with intermediate hepatocyte-cholangiocyte phenotypes emerge and expand.[36–38] These cells, also known as oval cells, are believed to localize to the periportal area in the canals of Hering and are considered to be bipotential progenitors[35,39] (Figure 2). The capacity of these cells to repopulate the damaged liver has been shown in mice with massive liver damage, restoring more than 80% of hepatocytes.[40] Other studies from liver injury models have supported the liver stem cell hypothesis, showing proliferation of duct-like cells, frequently termed the ductular reaction, in the portal zone of the hepatic lobule.[41–43] However, this feature is controversial.[44,45]

Other cells have been implicated in regeneration, repair, and eventually carcinogenesis. HCV-related chronic injury can induce ductular metaplasia and proliferation, with mature hepatocytes transdifferentiating to bipotential oval cells via epigenetic reprograming.[25] These cells are different from the classic oval cells of the canals of Hering and may have a more relevant role in hepatocarcinogenesis (Figure 2). However, other sources of precursors have been identified. These include specialized pericentral liver cells, capable of self-renewal in the uninjured liver under the influence of endothelial Wnt signaling.[46] These pericentral cells express the early liver progenitor marker TBX3[47] and are diploid. Hybrid periportal cells express hepatocyte markers, along with low levels of SOX9 and several bile duct genes, and are able to repopulate the healthy and diseased liver mass.[48] It is not clear whether these cell populations are bona fide stem or progenitor cells or are distinct hepatocyte subpopulations and whether they contribute to tumorigenesis.[49]

Progenitor Cells as Precursors

Extensive experimental studies support the hypothesis that progenitor cells might originate liver cancer[50–52] (Table 1). For example, mice with genetic alterations that affect the Hippo pathway within the liver expand progenitor-like cells and subsequently develop HCC, iCCA, and mixed HCC-CCA.[53–55] Similarly, liver-specific deletion of the neurofibromatosis type 2 (*NF2*) tumor suppressor gene in developing or adult mice leads to expansion of progenitor cells without affecting differentiated hepatocytes; these mice develop HCC and iCCA.[56] Furthermore, any mouse hepatic cells, including hepatic progenitors, hepatoblasts, and hepatocytes, that express activated oncogenes (such as H-RAS or SV40LT) can undergo transformation to develop into iCCA or HCC[57] (Table 1). There could also be subpopulations of stem or progenitor cells in peribiliary glands, located throughout the biliary tree,[58] that give rise to iCCA[59] or FLC.[60] Proteins involved in stem cell renewal and cell fate decisions include MET,[50] which primarily induces hepatocyte differentiation, and EGFR and NOTCH, which promote cholangiocyte specification.[50,51] Activation of NOTCH in mice leads to development of HCC[61] and iCCA.[62] In addition, mutations in genes encoding isocitrate dehydrogenases, frequently detected in iCCAs[14] but rarely in HCCs, inhibit hepatocyte differentiation and promote oval cell proliferation and biliary transformation following liver injury, in cooperation with *KRAS* mutations[63] (Table 1). Similarly, *KRAS* mutations and homozygous *PTEN* deletion in embryonic bipotential

progenitor cells cooperate to induce onset of iCCA.[64] Overall, these experimental studies implicate progenitor cells in the development of the two most common primary liver cancers.

Adult Hepatocytes as Cell of Origin

There is significant experimental evidence implicating adult hepatocytes as the cell of origin in liver cancer.[48,65–69] Studies using fate-tracing systems have observed that HCC does not originate from progenitor cells but rather from hepatocytes in both hepatotoxin-induced[66,67] and carcinogen-free (Mdr2 knockout) models[65,67] (Table 1). In particular, these data indicate that Foxl1+ cells, which express the progenitor markers EPCAM, SOX9, and PROM1, do not contribute to HCC tumorigenesis,[66] in agreement with other studies showing that biliary cells do not give rise to HCC.[65] In addition, a recent study has shown that hepatocyte-specific p62 expression promotes c-MYC induction, mTORC1 activation, and HCC initiation.[70]

Adult hepatocytes, however, have additional properties consistent with a cell that originates cancer. These properties include their remarkable plasticity, which entails their capacity to dedifferentiate into a progenitor state and then restore the hepatocyte pool.[25] Mature hepatocytes can dedifferentiate following loss of the tumor suppressor *TP53*, which yields Nestin-positive progenitor-like cells that can expand and generate primary liver cancers after acquiring lineage-specific oncogenic lesions, such as mutations in WNT (in HCC) or NOTCH (in iCCA)[52] (Table 1). In addition, adult hepatocytes can also transdifferentiate into biliary-like cells (Figure 3)[24] that can degenerate into iCCA. Upon injury, NOTCH activation leads to reprogramming of mature hepatocytes into cells that closely resemble biliary epithelial cells[71] to induce iCCA. NOTCH can do this on its own[72] or in cooperation with AKT[73] (Table 1).

There is evidence that different cell types can serve as the cell of origin for primary liver cancers (Figure 3 and Table 1). Two complementary features of HCCs and ICCAs could account for their phenotypic complexity, their molecular features (pathways of activation, mutations, and so on), and the types of cells that become transformed. For example, development of HCC could involve specific molecular alterations in adult hepatocytes (Figure 3), whereas iCCA could originate from cholangiocytes, probably as a result of chronic biliary damage due to liver flukes, PSC, or other insults.

However, adult hepatocytes have also been implicated in the formation of other tumor types. First, tumors with a progenitor cell phenotype could derive from progenitor-like cells, either because they are bona fide progenitor cells or following dedifferentiation of adult hepatocytes. Transformation of progenitor cells could occur at any stage of hepatic development—from progenitor to adult liver cells—giving rise to tumors of different morphologies, ranging from HCC and iCCA with stem cell phenotypes to mixed HCC-CCA (Figure 3). Clinical and pathological analyses of mixed HCC-CCAs have indicated an origin from the progenitor cell compartment.[5,14,74] Finally, mature hepatocytes can transdifferentiate, generally as a result of viral infection, to biliary-like cells that give rise to iCCA.

Regardless of their cell of origin (mature hepatocytes vs progenitor cells), liver cancers with stem cell features have more aggressive clinical behavior and a worse prognosis than those without stem cell features.[19,21] However, they are difficult to study because of discrepancies between mice and humans. Studies are needed to determine under what circumstances adult hepatic cells, rather than progenitor cells, give rise to liver tumors and vice versa and to fill the gap between findings from animal models (cell fate tracing studies) and studies of patients with severe liver injury. It will also be important to determine the dominant reprogramming events involved in tumorigenesis in different species. Studies should explore which cells are most affected by oncogene mutations associated with HCC, such as those in the *TERT* promoter or *CTNNB1*.

Immune Response

Regardless of the cell type that becomes transformed to initiate tumorigenesis, the developing tumor also requires a specific microenvironment. This is particularly relevant to liver tumors, 90% of which develop under conditions of chronic inflammation.[2] Factors that contribute to tumor formation include changes in the extracellular matrix of the liver, signaling between parenchymal and nonparenchymal cells, and immune dysfunction.[75] An altered immune response is an important factor in carcinogenesis.[76] Cancer cells produce growth and angiogenic factors that promote tumor growth, invasion, and metastasis. iCCAs develop under conditions of chronic inflammation due to viral infections (in approximately 20% of cases), chronic fluke infestation, PSC, and hepatolithiasis.[13]

Chronic inflammation contributes to liver carcinogenesis through a cycle of cell death and regeneration, leading to production of cell survival and proliferation signals that promote formation of regenerative nodules, dysplasia, and cancer.[75] In this context, the phenotype of liver tumors could depend on interactions between oncogenes and the immune microenvironment. In a recent animal study, different types of liver tumors, such as HCCs, iCCAs, and mixed HCC-iCCAs, arose via activation of distinct oncogenes, such as AKT1 and CTNNB1, in combination with inflammatory microenvironment conditions such as carbon tetrachloride or 3,5-diethoxycarbonyl-1,4-dihydrocollidine.[77] Tumors that develop via activation of the same oncogenes can still have different transcriptomes, depending on the level of inflammation and features of the microenvironment.

Several mechanisms have linked the immune system with development of liver cancer. First, secretion of cytokines by immune cells, such as tumor necrosis factor and interleukin (IL)-6, can activate inflammatory signaling pathways in hepatocytes via JAK–STAT, nuclear factor κB, and so on to promote cell proliferation and survival.[75] A model of HCC tumorigenesis has been proposed in which leukocytes that infiltrate the liver organize into ectopic lymphoid-like structures, providing microniches that contain malignant hepatocytic progenitor cells.[78] The role of the adaptive immune system is becoming apparent from studies of animal models of liver cancer development with nonalcoholic steatohepatitis. Factors that activate inflammation, such nuclear factor κB and insulin receptor, contribute to carcinogenesis in patients with nonalcoholic fatty liver disease.[79] $CD8^+$ and $CD4^+$ T cells and natural killer cells[80,81] also promote oncogenesis.

The interaction between inflammation and development of iCCA has been shown in mice in which biliary epithelial cells that express transgenic *AKT* and *YAP* develop only iCCA on promotion of IL-33–mediated biliary tract inflammation.[82] Other inflammatory mediators that contribute to oncogenesis include IL-6 and inducible nitric oxide synthase (iNOS).[83] IL-6 is highly expressed in human cholangiocarcinomas and promotes cell survival in a STAT3-dependent manner.[84] Interestingly, a gene expression signature associated with IL-6–STAT3 signaling has been observed in a subset of human iCCAs.[85]

Normally, nascent transformed cells are eliminated by both the innate and adaptive immune systems in a process called immune surveillance. Cancer cells express antigens that induce adaptive responses mediated by T cells.[86] In the chronically inflamed liver, immune surveillance prevents proliferation and expansion of cancer cells. Some, but not all, recent reports have warned about an unexpectedly higher incidence of HCC occurrence and recurrence in patients treated with direct-acting antiviral agents for HCV infection.[8–11] If confirmed, the potential role of the immune system should be explored, because rapid clearance of HCV in patients with chronic infection for years might affect immune surveillance.

Immune Therapies

The immune microenvironment of HCC is characterized by the coexistence of immune responses leading to both immunogenicity and tolerance.[87] HCCs promote immunologic tolerance through the release of cytokines such as IL-10 and transforming growth factor beta (TGF)-β. The tumors and tumor-infiltrating immune cells also express immune regulators such as programmed cell death protein 1 (PD1), its ligand PDL1, and cytotoxic T-lymphocyte protein 4 (CTLA4), which suppress the antitumor immune response, allowing tumor growth and progression.[87,88] Tumors with immune infiltrates have been associated with longer survival times and reduced rates of relapse after resection and transplantation.[2,88] In addition, some HCC cases of spontaneous regression have been linked to immune-mediated mechanisms.[2] These observations establish the rationale for immunotherapy of HCC. Promising responses have been reported with nivolumab, a monoclonal antibody directed against PD1, in a phase 2 trial.[89] It appears that a T-cell response, features of inflammatory cells (PD1, PDL1 expression), number of mutations in tumor cells, and gene expression profiles might predict patient responses.[90–93] Using source separation techniques to dissect gene expression patterns of tumor cells from those of tumor-infiltrating immune cells, an immune-specific subclass has been identified in 25% of patients with HCC.[94] This subgroup is characterized by activation of immune cells, expression of PD1 and PDL1, and enrichment in signatures of response to immunotherapies. Furthermore, 2 robust microenvironment-based types have been identified, with either active or exhausted immune responses; these findings could be used to improve the design of clinical trials of immunotherapies.[94] Similarly, a subtype of iCCA characterized by high mutation load and increased expression of immune checkpoint genes has been identified that may predict response to immunotherapies.[95]

Classification of HCC Based on Molecular Features

Characterization of molecular subtypes and/or oncogene addiction loops has generated treatment algorithms for several types of cancer, including breast cancer, and they have improved patient outcomes. High-throughput technologies, including single nucleotide polymorphism arrays, combined with transcriptomic and exome sequencing analyses, have identified molecular subtypes of liver cancer, with distinct oncogene signaling pathways and recurrent mutations.[96] Even though molecular classifications have not affected decision making for patients with liver cancer, correlations between these subclasses and clinical and pathological characteristics (such as risk factors, outcome, and so on) have been proposed.

Reaching a Consensus

The development of a molecular classification of HCC has paralleled the progress in genomic profiling technologies and is extensively reviewed elsewhere.[97] Initial studies relied on differences in gene expression patterns determined by microarrays, whereas more recent sequencing-based studies used mutation signatures to classify HCCs.[3] Unsupervised clustering of gene expression patterns accurately identified 2 major subgroups (reviewed in Llovet et al[2] and Zucman-Rossi et al[97]). These 2 classes, broadly termed proliferation and nonproliferation, correlate with clinical and pathological features, etiology, and patient outcomes.[97] Tumors of the proliferation class are highly heterogeneous with significant enrichment in signaling pathways related to proliferation, such as insulin-like growth factor I[98] and mechanistic target of rapamycin (MTOR), or stem cell features, such as NOTCH.[61] This class also contains most gene expression patterns associated with tumor recurrence[99] and shorter survival time[100] (Figure 4). Epigenetic features, such as expression patterns of microRNAs[101] and DNA methylation patterns,[102] are also associated with these subclasses. Chromosomal aberrations are also frequent in HCCs, mostly in chromosomes 1 and 8, which correlate with transcriptome-based subclasses. High-level amplifications have been reported at Chr. 11q13 (at *CCND1* and *FGF19*) and 6p21 (at *VEGFA*); gains in 11q13 are enriched in tumors from the proliferation subclass (Figure 4). Tumors in the nonproliferation subclass tend to retain hepatocyte-like features; a subset has activation of the canonical Wnt signaling pathway, mostly via mutations in *CTNNB1*.[97] This class contains many less-aggressive, well-differentiated tumors with low levels of α-fetoprotein compared with the proliferation class. In parallel, molecular analyses of the adjacent nontumor tissues have provided valuable insights into mechanisms that promote hepatocarcinogenesis, including aberrant epidermal growth factor signaling and activation of inflammation pathways (involving nuclear factor κB and IL-6).[103,104]

Next-generation sequencing (NGS) has provided a more detailed picture of molecular alterations in HCCs.[105] This technique can be used to define mutation signatures, which are recurrent patterns of nucleotide substitutions across different samples. These data provide for an additional layer of stratification beyond transcriptome-based classes. Interestingly, mutation signatures can reflect the exposure history of a tumor. In HCC, specific mutation signatures are associated with environmental exposures such as smoking, alcohol, aflatoxin B1, or aristolochic acid[96] (Figure 4). Data from exome sequencing of large patient cohorts have confirmed the most prevalent mutations in HCC pathogenesis[106,107] (such as the *TERT*

promoter, *CTNNB1, TP53, AXIN1, ARID1A*) but also uncovered novel low-frequency events[106,107] and implicated mutations in noncoding DNAs.[108] In addition, NGS has provided a map of viral integrations in HCC, mostly related to HBV[109] and more recently for the adeno-associated virus type 2 (AAV2).[110] Recurrent clonal HBV integrations affect *TERT, MLL4,* and *CCNE1,* some of which may affect patient survival.[109] AAV2-mediated insertional mutagenesis has been reported in 5% of cases of HCC, mostly in noncirrhotic patients without a clear etiologic factor, predominantly affecting *TERT, KMTB2, CCNA2,* and *CCNE1.*[110]

Obstacles to Clinical Implementation

Ultimately, molecular classification of both the tumor and its microenvironment will translate into more effective clinical decision making and treatment selection. This means optimizing clinical staging systems, both in their ability to predict prognosis and in their capacity to stratify patients based on response to therapies. Despite substantial progress, clinicians cannot yet use molecular information to guide therapy, primarily because there are not yet biomarkers that predict the response to a given therapy, unlike other malignancies. For instance, patients with lung cancer who have *ALK* rearrangements have a significantly better response to ALK inhibition with crizotinib than those without *ALK* rearrangements.[111]

There are several reasons why molecular information on tumors is not incorporated into clinical practice. Unlike other solid tumors, the most frequent mutations in HCC are untargetable, including those in the *TERT* promoter (60%–70%), *TP53* (25%–50%), and *CTNNB1* (25%–30%). Furthermore, the few potentially actionable events uncovered have not yet been evaluated in clinical trials that include biomarkers. Only recently have some potential biomarkers been tested in early clinical trials, such as FGFR4 inhibitors in patients with high-level amplifications of Chr11q13, locus of *FGF19*.[112] Finally, a therapeutic response to blocking an oncogene in a few patients will be lost among many patients who lack a responsive phenotype if the trial has not been enriched to include those patients whose tumors harbor targetable defects. A recent report suggests that TSC2 loss predicts response to the MTOR inhibitor everolimus in different experimental models of HCC,[113] yet there is no clear biomarker of treatment response in the phase 3 clinical trial.[114] Indeed, the 2 systemic therapies that increase survival in patients with HCC in first-line (ie, sorafenib[115]) and second-line (ie, regorafenib[116]) regimens have been tested in all-comers with no patient enrichment strategy.

Options to improve the current trial strategy could include conducting clinical trials with enriched populations based on biomarkers that predict response (FGF19,[117] MET) or investigating nonmutated deregulated pathways as targets, such as insulin-like growth factor II[98] or NOTCH[61] signaling. It might also be possible to use alternative antitumor approaches, such as immune checkpoint inhibitors, or strategies to antagonize the most prevalent genetic defects found in HCCs, such as mutations in *TERT, TP53,* and *CTNNB1.*

Molecular Subclasses of iCCA

There is no effective treatment for iCCA. Reasons include our poor understanding of its pathogenesis and the lack of studies conducted specifically in patients with iCCA, apart from patients with all biliary tract cancers[13,14]; this is important because iCCAs are characterized by a distinct set of mutations.[14] iCCAs were only recently recognized as a distinct entity with both its own unique staging system (see the 7th edition of the American Joint Committee on Cancer Staging Manual[118]) and ad hoc practice guidelines (see guidelines of the International Liver Cancer Association[13]). The classification of the distinct phenotype of iCCAs has led to the first studies that characterize its genomic landscape to generate an initial molecular classification. Other cholangiocarcinomas, such as perihilar and distal, are different molecular entities.[14]

Two main molecular subtypes of iCCA, proliferation and inflammation, have been proposed based on an integrative analysis of whole-genome expression patterns, chromosomal aberrations, mutations, and alterations in signaling pathways in a large cohort from the United States and Europe.[85] The inflammation subclass was characterized by activation of inflammatory pathways, overexpression of cytokines, and STAT3 activation, whereas the proliferation subclass was characterized based on activation of oncogene signaling pathways (KRAS, EGFR, and NOTCH) and HCC gene expression signatures associated with short survival times and earlier recurrence.[85] Although neither of the 2 subclasses displayed specific broad chromosomal gains and losses, the proliferation subclass is enriched in focal aberrations, including DNA amplifications at 11q13.2 (locus of *CCND1* and *FGF19*) and deletions at 14q22 (locus of SAV1). The proliferation class also includes a subtype with stem cell–like features,[119] chromosomal instability,[85] and mutations in isocitrate dehydrogenase genes[63] (Figure 5). Clinical characteristics such as moderate/poor differentiation, intraneural invasion, and poor outcome are significantly more frequent in the proliferation class. Interestingly, there is a genomic resemblance observed between the proliferation subclass of iCCA and HCC subtypes with poor prognosis (ie, cluster A,[19] G3[120]) and stem cell features (S2)[121,122] (Figure 5). This evidence further supports the presence of a common cell of origin in liver cancers.

An independent whole-transcriptomic analysis of a large cohort including iCCAs and extrahepatic cholangiocarcinomas also confirmed the existence of the 2 prognostic CCA molecular subtypes.[123] In this study, 2 main classes were reported: a poor prognosis group characterized by enrichment of *RAS* mutations as well as *MET* and *EGFR* overexpression and a good prognosis group characterized by signatures of immune response.[123] Genomic overlap between the poor prognosis group[123] and the proliferation subclass was observed.[85]

Similar to HCC, NGS studies have generated insights into the molecular subtypes of iCCA.[95,124–131] The most common genetic aberrations include *FGFR2* gene fusions (~25%) and mutations in *KRAS* (20%), *IDH1/2* (~20%), and chromatin remodeling genes (*ARID1A, BAP1, PBRM1,* ~30%) (reviewed by Moeini et al[14]). Other than *IDH1* and *IDH2,*[63] *KRAS,*[85,123] and *EGFR*[85] mutations, which are enriched in the proliferation subclass, no significant distribution of other molecular aberrations has been reported in any of the iCCA molecular subtypes.

The incidence of iCCA varies significantly among geographic regions but is highest in Asian countries, where infections with liver flukes such as *Opisthorchis viverrini* and *Clonorchis sinensis* are the major risk factor.[13] In non-endemic regions, other factors, including hepatolithiasis and PSC, likely contribute to risk. Associations between different risk factors and mutations detected in iCCAs were explored in 209 patients from Asia and Europe with cholangiocarcinoma. Mutations in *BAP1* and *IDH1/2* were found in a higher proportion of iCCAs not related to *O viverrini* (22% vs 3.2%), whereas mutations in *TP53* were found in a higher proportion of tumors from patients with *O viverrini* infection (45.2% vs 7.4%).[124] Although there have been other studies of large cohorts of patients, it has been difficult to draw conclusions from these studies due to the different types of neoplasms included (iCCAs, extrahepatic cholangiocarcinomas, and gallbladder tumors).[85,123]

Importantly, multiple *FGFR2* gene fusions have been discovered in approximately 25% of iCCAs[126,127,132–135] (reviewed by Moeini et al[14]); these are used to define tumor subtypes. Tumors with *FGFR2* fusions and *KRAS* mutations[126] or *BAP1* mutations[95] have been reported. No significant differences in patient age or outcome, or tumor differentiation or stage, were found when comparing patients with or without *FGFR2* fusion. One study reported a higher frequency of HCV infection in patients with *FGFR2* fusion,[132] although this finding has not been confirmed.[95,126,134] Early-phase clinical trials testing selective FGFR inhibitors in patients with advanced iCCA carrying alterations in *FGFR2* are under way (NCT02150967 and NCT01752920).[136,137] These studies have reported an objective response rate of approximately 15% (a signal of efficacy) and a manageable safety profile.[136] The discovery that *FGFR2* fusion can promote development of iCCA has been quickly translated to clinical trials. Fusion of the *FGFR2* gene might be used as a biomarker to select patients for treatment with these agents.

Molecular Features of Uncommon Tumors

FLC is a rare primary liver cancer with few treatment options that typically affects children and young adults without background liver disease.[60] FLC is now considered a unique molecular entity within primary liver malignancies.[138] FLC is caused by an approximate 400-kilobase deletion in chromosome 19 that incorporates the first exon of *DNAJB1* into all but the first exon of *PRKACA*.[139] This fusion is detected in 70%[138] to 100% of FLCs.[139] In addition, exome sequencing and RNA sequencing studies found deregulation of additional actionable candidates such as *ERBB2* and *AURKA*.[140] Unlike HCC or iCCA, there are no prevalent mutations in more than 10% of patients. Unsupervised clustering of gene expression data identified FLC molecular subclasses in a cohort of 58 FLCs, 2 of them associated with proliferative and inflammatory molecular traits. The same study provided a prognostic 8-gene expression signature.[138] FLC has less chromosomal aberrations compared with HCC or iCCA without recurrent high-level amplifications or deletions.

Hepatoblastoma is the most frequent primary liver tumor in children younger than 5 years of age. Like FLC, background liver disease is rare in these patients. Wnt signaling plays a major role; *CTNNB1* mutations (70%) are the most frequently reported molecular event. Integrative analysis of gene expression and chromosomal aberrations in 24 hepatoblastoma samples distinguished 2 classes, with different DNA gains, histological phenotypes, and

clinical outcomes.[141] The recent use of patient-derived xenografts in hepatoblastomas enabled the identification of novel therapeutic targets such as *NRAS* mutations.[142,143]

Mixed HCC-iCCA is a rare neoplasm accounting for less than 1% of all primary liver cancers.[5] Diagnosis is mainly based on histological examination and requires features of HCC and iCCA. According to the 2010 World Health Organization classification, there is a classic type, characterized by areas of typical HCC and iCCA with a transition area, and a stem cell–feature type.[144] The stem cell–feature type can be further divided into typical, intermediate, and cholangiolocellular carcinoma (CLC) subtypes. Gene expression profiling of small series of HCC-CCA samples indicated that this tumor type might share common characteristics with poorly differentiated HCC and iCCA with stem cell traits.[119,122,145] A comprehensive integrative genomic analysis of 18 mixed HCC-CCA subtypes defined 3 molecular types (CLC, stem cell, and classic subtype).[146] In particular, the CLC subtype, despite being grouped based on histology within the stem cell–feature subtype, has a unique molecular profile with biliary traits, low levels of chromosome instability, and activation of TGF-b and inflammation-related signaling pathways.[146]

Challenges in Tumor Heterogeneity

Liver tumors have a high degree of molecular heterogeneity, not only among patients with the same types of tumors but also within regions within the same tumor or tissue.[147] Expert recommendations and consensus definitions have been established to better address the extent and role of cancer heterogeneity in oncology research.[148]

From a clinical perspective, molecular heterogeneity should be considered within the concepts of trunk and branch as well as driver and passenger mutations. Trunk mutations (mutations that occur early in tumorigenesis) can be found in every cancer cell of a tumor.[148] Branch mutations are found in only a subgroup of tumor cells; these are acquired at later stages of tumor development, frequently following treatment, and are associated with resistance. Studies of other malignancies have indicated that trunk mutations promote tumor progression and are therefore the best targets for therapies. Trunk mutations identified in a single biopsy should be the first targets of molecular therapies.[2] Finally, passenger mutations account for the most common type of mutations, but their relevance to tumor development is marginal. However, they are often used to define the clonal features of a tumor and the antitumor response.

Prognostic Markers in HCC

Molecular biomarkers have many applications in cancer. They can improve clinical decision making by helping to predict a patient's outcome, select patients most likely to respond to a specific treatment, and avoid treatments not likely to be effective. Unlike other cancers such as breast[149] and colorectal[150] cancers, HCC has no molecular markers that have been incorporated into clinical management.

As a general rule, clinical practice guidelines include recommendations based on a specified set of metrics, which rate the level of evidence and the strength of recommendations for each statement; the higher the evidence, the stronger the recommendation. For

example, sorafenib is recommended for treatment of patients with advanced-stage HCC; a strong recommendation was derived from a prospective, double-blind, randomized, placebo-controlled trial (the highest level of evidence).[151] These metrics are less clear when evaluating biomarkers for HCC. Initial recommendations for biomarkers were developed mostly to address technical aspects of the assay such as the Tumor Marker Utility Grading System.[152] This was later improved on by the REMARK guidelines for reporting prognostic biomarker in oncology,[153] which also addressed issues related to clinical utility. The PROGRESS initiative was the most recent attempt to provide a methodological framework for biomarker research.[154]

The highest level of evidence in biomarker research (level A) comes from randomized trials.[155] A large prospective study of 10,253 women showed that a 21-gene expression signature could identify patients with early-stage breast cancer at high risk for recurrence.[156] Level B evidence is provided by companion studies of biomarkers in randomized clinical trials, such as those reported for HCC in the Sorafenib HCC Assessment Randomized Protocol[157] and Everolimus for Liver Cancer Evaluation[114] trials. Neither of these studies identified markers associated with response to either sorafenib or everolimus, but they confirmed the prognostic role of levels of angiopoietin 2 and vascular endothelial growth factor in plasma of patients with advanced-stage HCC.[157] Level C evidence has been provided most frequently for markers of HCC, from studies of archived samples, with extensive validation (reviewed elsewhere).[97] Most level C studies have evaluated prognostic markers, identifying patients with the proliferation subclass of tumor, either the S1 (Wnt and TGF-β expression) or S2 (progenitor cell) subtype,[97] with or without amplification of *FGF19*.[106] The S2 subtype expresses many markers of progenitor cells and high levels of α-fetoprotein. Tumors in the S1 and S2 subgroups are poorly differentiated, and patients with these subtypes have worse outcomes than patients with other HCC subtypes.[97] Level D evidence comes from studies of convenience, in which specimens were collected for unknown reasons and happened to be available for assay.

Gene signatures associated with risk of development of HCC may have a more direct application (Table 2).[100,102,158–161] For instance, a 186-gene signature in liver tissues was able to predict occurrence of HCC[158] in patients with hepatitis C–related early-stage cirrhosis and might be used to select patients for trials of chemopreventive agents (Figure 4 and Table 2). However, chemoprevention studies are a challenge, because it takes a long time to collect data on a low number of events. Studies that included populations of patients at high risk for HCC may shorten trial time and identify effective agents more quickly. For predictive biomarkers in advanced HCC, ongoing clinical trials include populations selected based on high expression of MET in tumor cells (trial of tivantinib, NCT01755767) or high serum level of α-fetoprotein (trial of ramucirumab, NCT02435433), both as second-line therapies.

Molecular Markers of iCCA

Even fewer biomarkers of iCCA have been developed than for HCC; there are only level C data for the efficacy of a biomarker for this tumor type. iCCA is one of the few solid tumors for which no systemic molecular therapy has been approved by the Food and Drug

Administration.[13] Surgery is the only potentially curative option, although chemotherapy with gemcitabine plus cisplatin is the standard for patients with advanced-stage iCCA.[162] According to the International Liver Cancer Association practice guidelines, no studies have provided level A or B evidence for a marker of this tumor; some data from low-quality studies have led to weak recommendations.[13] These studies have been retrospective in design, without extensive validation in independent cohorts. Some gene expression and microRNA patterns have been associated with features of iCCA,[85,119,123] as well as mutations in *KRAS* and *IDH1* or *IDH2* and focal deletions at 14q22.1[85,128,129] (Table 2). Gene expression patterns have been reported to identify an iCCA proliferation subclass of tumor,[85] patients with a poor prognosis,[85,123] and tumors with a stem cell–like subtype,[119] who have aggressive disease and worse outcomes.

Features of cancer cells in a tumor not only determine the growth and progression of the tumor but also affect the tumor microenvironment (noncancer cells and supporting stroma).[76] Some studies associated features of the iCCA microenvironment with patient outcomes.[123,163–165] Two studies identified a stromal signature associated with shorter survival time by combining laser capture microdissection with gene expression profile analysis.[123,165] Although many studies have validated gene expression signatures associated with patient outcomes, the clinical significance of microRNA signatures is not yet clear due to the limited number of large comprehensive profiling studies.[119,166–168]

Mutations in oncogenes such as *KRAS* and *BRAF* can be used to determine prognosis for patients with pancreatic or colorectal cancers.[169–171] However, there is controversy over whether these mutations can predict outcomes of patients with iCCA. Some studies have reported significant associations between mutations in *KRAS*[128,129,131] or chromatin-remodeling genes (*BAP1, PBMR1,* and *ARID1A*)[125,172] and either shorter disease-free survival or shorter overall survival, respectively. It is not clear whether mutations in *IDH1* or *IDH2* are associated with overall survival.[125,129,173]

Most biomarker assays use cells or tissue collected by invasive biopsy procedures or from resection specimens, but these specimens are rarely collected from unresectable or metastatic iCCAs, which are the tumors of most patients in clinical trials for iCCA. Strategies should be developed to assess circulating markers (called liquid biopsy) from tumor cells and cell-free nucleic acids in blood.[174] Detection of molecular signatures or aberrations in the blood could significantly facilitate implementation of prognostic markers. Liquid biopsies might also be used to monitor response to treatment, especially in clinical trials of selective inhibitors of *IDH1* and *IDH2* (NCT02073994, NCT02273739) and *FGFR2* (NCT02150967).[14,136]

Future Directions

In the past decade, there have been tremendous advances in our understanding of the cellular and molecular complexity of liver cancers. Sophisticated functional studies and NGS-based research studies have provided information about the cells that form liver tumors and the proteins and pathways involved in hepatocarcinogenesis. These studies support a model of multiple cells of tumor origin. Most of the studies, using in vivo lineage tracing

models, revealed an unexpected plasticity of mature hepatocytes[24,25,44,52,72] that could be involved in their transformation into cancer cells. Hepatic progenitor cells might also become transformed to give rise to different subtypes of HCC, iCCA, and mixed HCC-iCCA with progenitor markers.

Studies of humanized mice to mimic the onset of liver cancers in multiple settings may help address existing challenges. In addition, cell- and lineage-tracing models should improve so that they recapitulate the severity of liver injury observed in humans and must clarify if hepatic progenitor cells can replenish the liver mass after marked liver injury, as shown in zebrafish models.[175] Finally, linking the main genomic hits at preneoplastic and early stages of human cancer development with the cell of origin is also an area in which further studies are required.

Identifying the cells that give rise to liver tumors and elucidating the different classes of tumors based on their molecular features could lead to new approaches to treatment and prognosis. Our knowledge of the molecular mechanisms of HCC and iCCA pathogenesis has not yet been translated into clinical tools, because few studies have linked specific molecular classes of tumors or aberrations to responses to therapy. It is important to incorporate biomarker studies into trials, as in the trials of patients with iCCAs with *FGFR2* aberrations.[136] The successes of immune therapies in different solid tumors, including HCC,[176] indicate the need to focus greater attention on the hepatic tumor microenvironment and interactions between the tumor and the immune response; we also need to identify markers of response to these therapies. Learning more about the effects of tumor heterogeneity and the use of liquid biopsies to determine the molecular and genetic features of tumors could overcome barriers to personalized treatment of liver cancer.[174]

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Abbreviations used in this paper:

CLC	cholangiolocellular carcinoma
FLC	fibrolamellar hepatocellular carcinoma
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCC-CCA	mixed hepatocellular cholangiocarcinoma

HCV	hepatitis C virus
iCCA	intrahepatic cholangiocarcinoma
IL	interleukin
MTOR	mechanistic target of rapamycin
NGS	next-generation sequencing
PSC	primary sclerosing cholangitis
TGF	transforming growth factor

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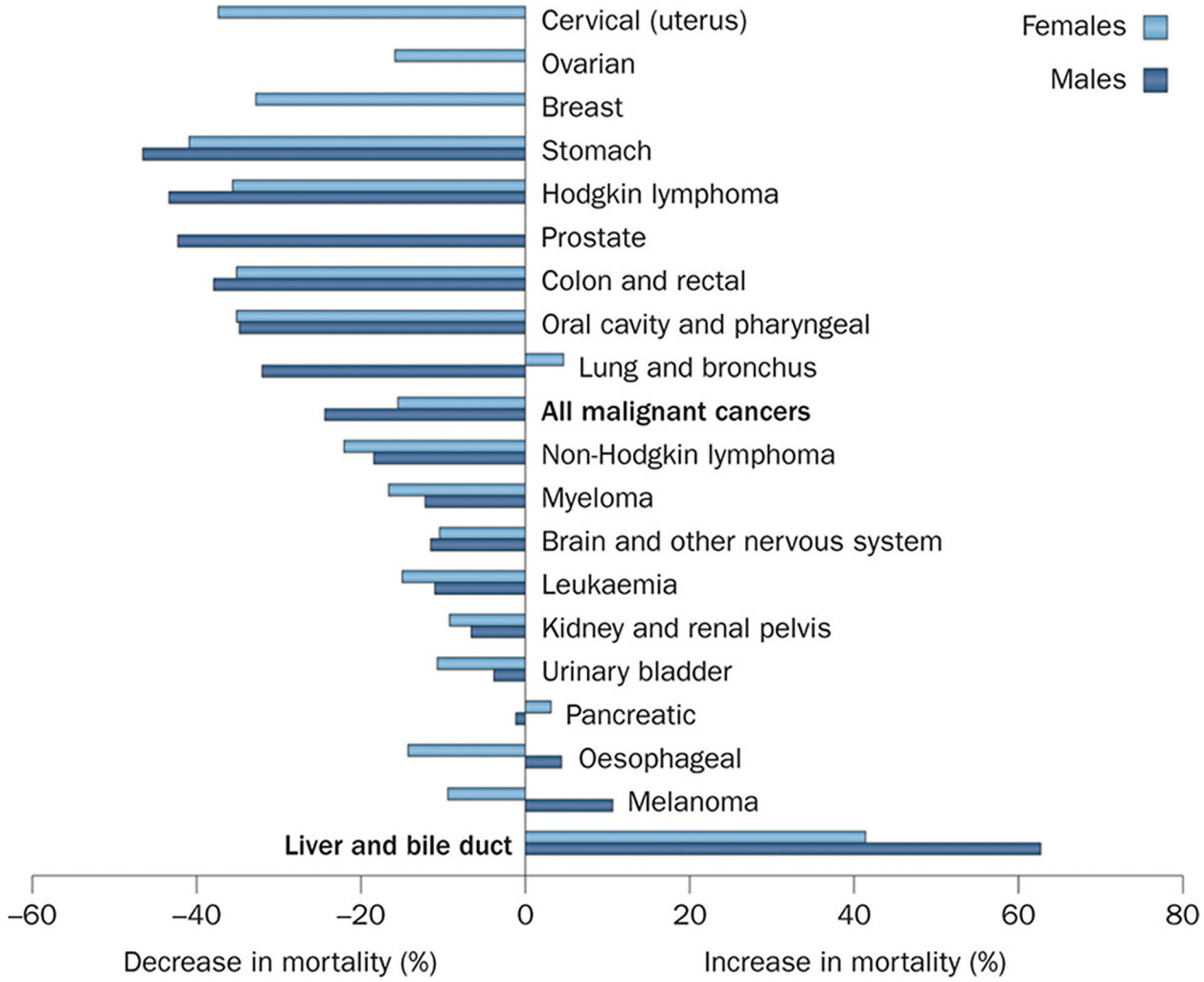

Figure 1.

Mortality trends of patients with different malignancies in the United States from 1990 to 2009 (reprinted with permission from Llovet et al[3]). Changes in cancer mortality among tumor types in the United States. Mortality from liver and bile duct cancers is increasing more rapidly than that from any other cancer in men and women. Data obtained from the 2013 American Association for Cancer Research Cancer Progress Report.

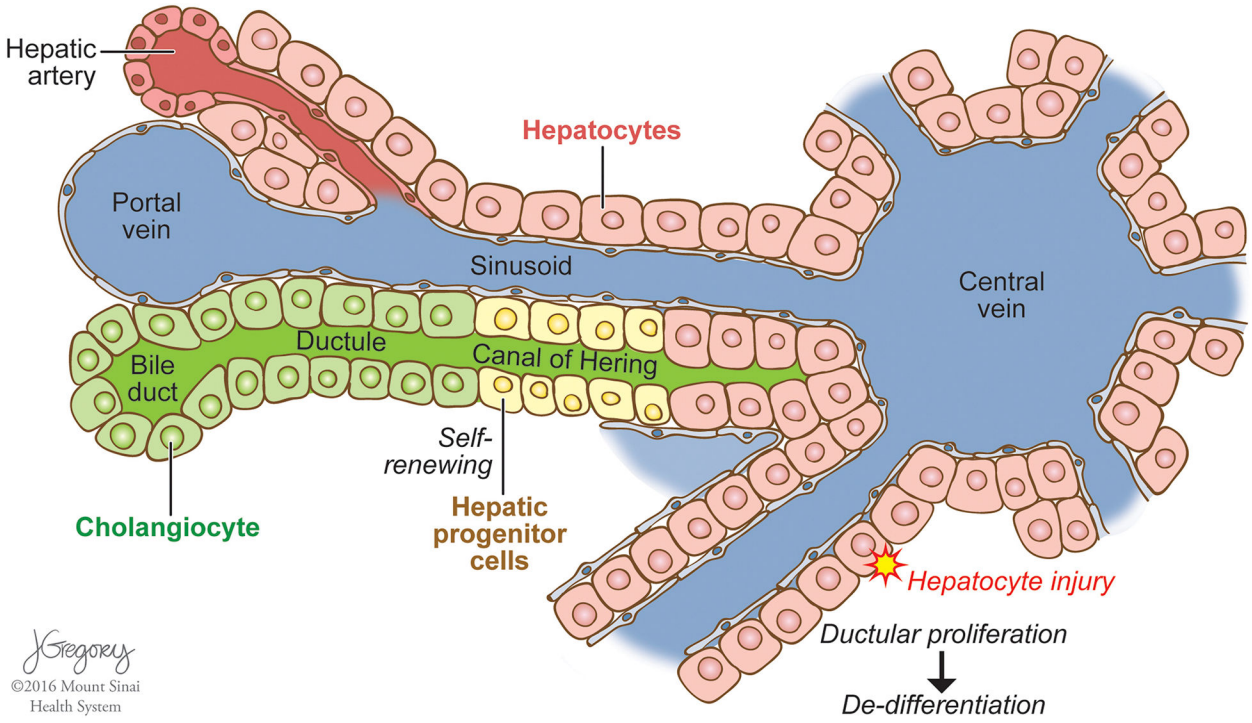

Figure 2.

Structure of the liver lobule and location of candidate cell of origin of liver cancer. Intrahepatic organization of the liver lobule and the localization of hepatic cells that form liver tumors. Different types of cancer (ie, HCC, iCCA, mixed HCC-iCCA) can originate in the liver, depending on the transformation event and the cell type undergoing neoplastic transformation. Hepatic stem or progenitor cells are believed to reside within the most terminal branches of the biliary tree (referred to as canals of Hering). Upon injury, hepatocytes can undergo reversible ductal metaplasia and dedifferentiate into hepatocyte-derived progenitor-like cells. Printed with permission from ©Mount Sinai Health System.

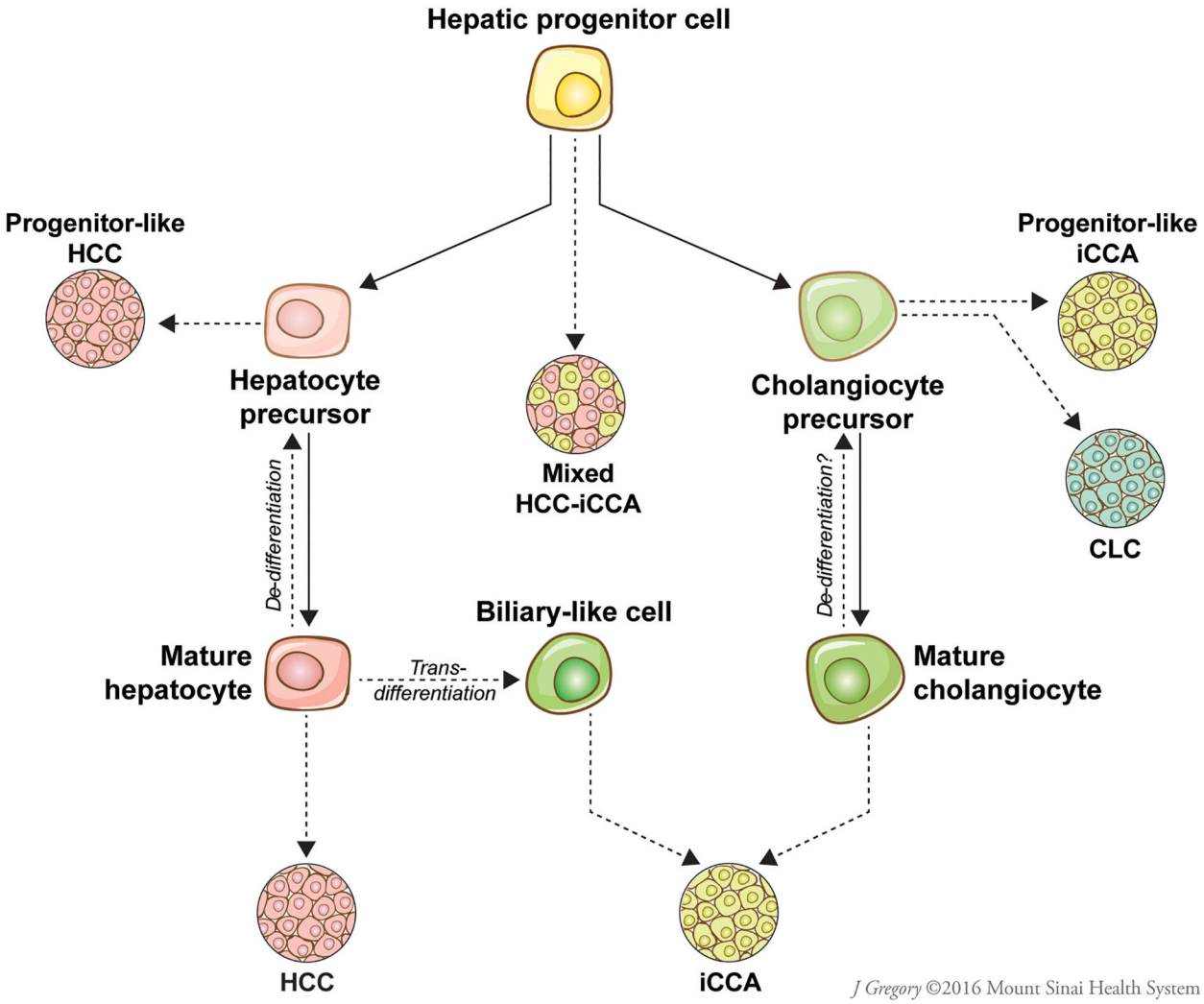

Figure 3.

Multiple cells of origin of primary liver cancers. HCC and iCCA can develop via transformation of mature hepatocytes and cholangiocytes, respectively. There is evidence that hepatic progenitor cells (HPCs), their intermediate states, or dedifferentiated hepatocytes can form liver tumors with progenitor-like features, including mixed HCC-CCA, such as CLC. Mature hepatocytes can also be reprogrammed into cells that closely resemble biliary epithelial cells and contribute to development of iCCA. Printed with permission from ©Mount Sinai Health System.

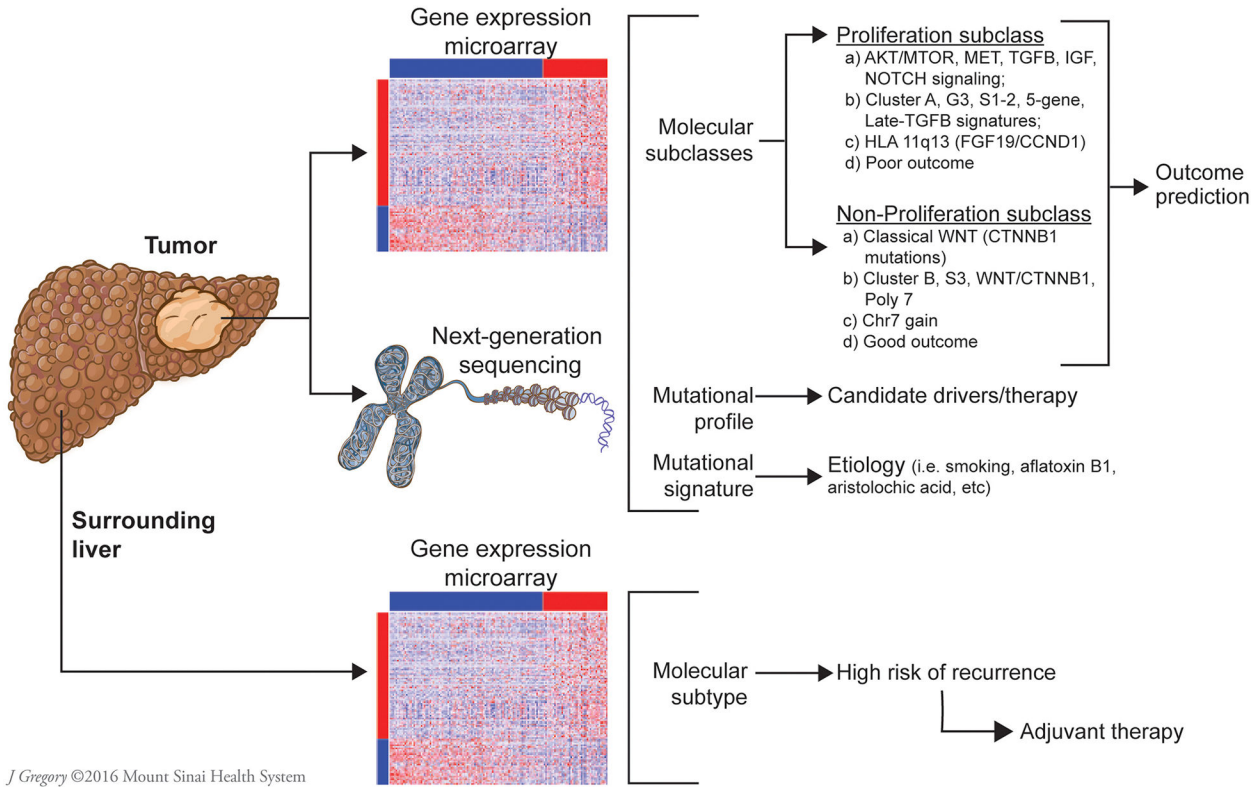

Figure 4.

Molecular biomarkers of HCC. Genetic features and gene expression of the tumor or its surrounding microenvironment correlate with clinical and pathological features, etiology, and patient outcomes. The proliferation subclass is characterized by proliferation signaling pathways, which involve proteins such as MTOR, NOTCH, and TGF-β and several gene expression signatures associated with poor outcomes of patients (such as the G3 and 5-gene signatures).[97] The nonproliferation subclass is characterized by mutations in *CTNNB1*, the S3 gene expression signature, and the classic Wnt signaling pathway. Specific mutations associated with etiology (smoking and aflatoxin B exposure) have also been described.[96] An expression pattern of 186 genes in the liver tissue surrounding the HCC can identify patients with HCC at higher risk for tumor recurrence after resection.[103] Printed with permission from ©Mount Sinai Health System.

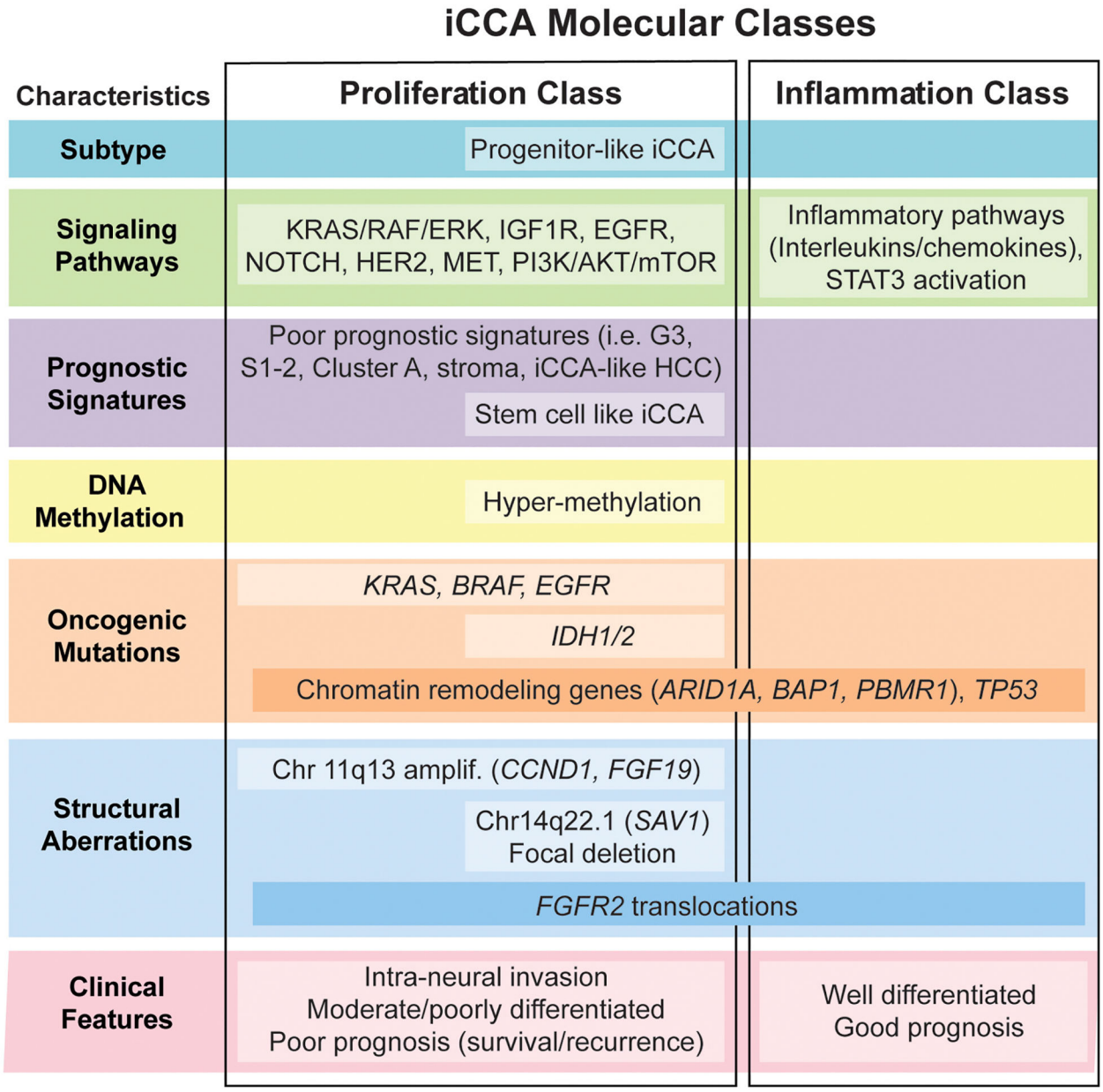

Figure 5.

Main characteristics of subclasses of iCCA. Analyses of gene expression patterns have shown that iCCAs can be assigned to the proliferation or inflammation subclasses. The proliferation class is characterized by chromosome instability, activation of pathways related to cell cycle progression, mutations in oncogenes, and shorter survival times of patients. Tumors of the inflammation subclass have gene expression patterns associated with activation of an immune response and less aggressive clinical behavior. *FGFR2* fusion events are evenly distributed between these subclasses.

Table 1.

Experimental Models Supporting the Different Hypotheses Regarding the Cell of Origin in Primary Liver Cancers

Hypothesis tested	Method	Animal models	Altered pathway	Tumor type	Proposed cell of origin	Reference
HPC/oval cells as cell of origin	Clonal analyses	—	BMI1, WNT	Mixed HCC-iCCA	HPCs	51
	GEMM	Sav1fl/fl, mst1fl/fl, and mst2fl/fl	HIPPO	HCC, iCCA	Oval cells	54
	GEMM	WW45flox/floxAlbumin-Cre	HIPPO	Mixed HCC-iCCA	Oval cells	55
	Multilineage differentiation	—	H-RAS, SV40LT	HCC, iCCA	HPC, hepatoblasts, hepatocytes	57
	GEMM	Alb-*Cre*;*Nf2*$^{lox/lox}$	NF2	HCC, iCCA, mixed	Oval cells	56
	GEMM	NotchIC::AlbCre	NOTCH	iCCA	HPCs	62
	GEMM	Alb-Cre;LSL-IDH2R172K;LSL-KrasG12D	IDH, KRAS	iCCA	Hepatoblasts	63
	GEMM	LSL-KrasG12D-Ptenflox	KRAS, PTEN	iCCA	Hepatoblasts, cholangiocytes	64
Hepatocytes as cell of origin	GEMM	AlfpCre^{+}Trp53$^{\Delta 2-10/\Delta 2-10}$	TP53, wnt	HCC	Hepatocyte-derived HPCs	52
	GEMM	AlfpCre^{+}Trp53$^{\Delta 2-10/\Delta 2-10}$	TP53, NOTCH	iCCA	Hepatocyte-derived HPCs	52
	GEMM	Mst1/Mst2 DKO, YAP KO, DKO Yap$^{+/-}$, and tTKO	Hippo	HCC	Hepatocyte-derived HPCs/oval cells	53
	Multilineage differentiation	—	H-RAS, SV40LT	HCC, iCCA	HPCs, hepatoblasts, hepatocytes	57
	Lineage-tracing system	Liver injury (BDL, DDC, MDA, CDE); GEMM (Mdr2$^{-/-}$)	MDR	HCC	Hepatocytes	67
	Lineage-tracing system	Liver injury (DEN); GEMM (Mdr2$^{-/-}$; PTEN$^{-/-}$)	MDR, PTEN	HCC	Hepatocytes	65
	Lineage-tracing system	DEN + TAA	—	HCC, HPA	Hepatocytes	66
	GEMM	Tsc1/Sqstm1Δhep and Sqstm1Δhep/MUP	p62	HCC	Hepatocytes	70
	Lineage-tracing system	Alb-CreERT2;R26RlacZ/+ and CK19-CreERT2;R26RlacZ/+	NOTCH	iCCA	Hepatocytes	72
	Lineage-tracing system	Hydrodynamic tail injection	NOTCH, AKT	iCCA	Hepatocyte-derived biliary-like cells	73
Cholangiocytes as cell of origin	Lineage-tracing system	Liver injury (TAA) and CK19CreER^{T}eYFPp53$^{f/f}$	TP53	iCCA	Cholangiocytes	26
	GEMM	LSL-KrasG12D-Ptenflox	KRAS, PTEN	iCCA	Hepatoblasts, cholangiocytes	64

HPC, hepatic progenitor cells; GEMM, genetically engineered mouse model; DKO, double knockout; KO, knockout; TKO, triple knockout; BDL, bile duct ligation; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; MDA, methylenedianiline; CDE, choline-deficient, ethionine-supplemented; DEN, *N*-nitrosodiethylamine; TAA, thioacetamide; HPA, hepatocellular adenoma.

Table 2.

Prognostic Signatures in Primary Liver Cancer

Signature	Cohort (etiology)	No. of patients tested Training/ validation set	Clinical end point	REMARK recommendations	Level of evidence Simon et al[155])	Reference
Hepatocellular carcinoma						
Messenger RNA based						
5-gene signature	HCC (alcohol, HCV, HBV)	189/434	Survival	OK	C	100
EpCAM signature	HCC (HBV)	40/238	Survival	OK	C	160
186-gene signature (adjacent tissue)	HCC (HCV)	82/441	Survival, HCC development	OK	C	103, 158
MicroRNA based						
20-miRNA signature	HCC (HBV)	131/110	Venous metastasis, survival	OK	C	161
Downregulation miR-26a	HCC (HBV)	241/214	Survival	OK	C	159
DNA methylation						
36 CpG DNA methylation signature	HCC (HCV)	221/83	Survival	OK	C	102
Protein markers (plasma)						
Ang2, VEGF	HCC (HCV)	491	Survival	OK	B	157
Cholangiocarcinoma						
Messenger RNA based						
Poor prognosis/ proliferation class	iCCA	119/104	Survival and recurrence	Further validation required	C	85
Poor prognosis group	iCCA and extrahepatic cholangiocarcinoma	52/52	Survival and recurrence	Further validation required	C	123
Stem cell–like subtype	iCCA and mixed HCC-CCA	23/68	Survival	Further validation required	C	119