Emerging Signaling Pathways in Hepatocellular Carcinoma

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Key Words
Cascade · Chromatin remodeling · HDAC · Hippo · Liver cancer

Abstract
Signaling pathways have become a major source of targets for novel therapies in hepatocellular carcinoma (HCC). Survival benefits achieved with sorafenib, a multikinase inhibitor, are unprecedented and underscore the importance of improving our understanding of how signaling networks interact in transformed cells. Numerous signaling modules are de-regulated in HCC, including some related to growth factor signaling (e.g., IGF, EGF, PDGF, FGF, HGF), cell differentiation (WNT, Hedgehog, Notch), and angiogenesis (VEGF). Intracellular mediators such as RAS and AKT/MTOR may also play a role in HCC development and progression. Different molecular mechanisms have been shown to induce aberrant pathway activation. These include point mutations, chromosomal aberrations, and epigenetically driven down-regulation. The use of novel molecular technologies such as next-generation sequencing in HCC research has enabled the identification of novel pathways previously underexplored in the HCC field, such as chromatin remodeling and autophagy. Considering recent failures of molecular therapies in advanced clinical trials (e.g., sunitinib, brivanib), survey of these and other new pathways may provide alternative therapeutic targets.

Introduction: Mainstream Pathway De-regulation in HCC

Hepatocarcinogenesis is a complex and multi-step process resulting from a combination of epigenetic and genetic alterations. In recent decades, much effort has been made to...
identify key molecules involved in the development and progression of hepatocellular carcinoma (HCC). Yet, our understanding of the molecular pathogenesis of this disease remains rudimentary. Several studies using high-throughput genomic technologies such as array-based gene expression profiling or parallel sequencing have facilitated the development of a molecular classification for HCC [1]. A meta-analysis including a total of 603 patients led to the identification of 3 robust molecular subclasses, characterized by de-regulation of specific signaling pathways [2]. Studies of mutations in key oncogenes and tumor suppressors in HCC have revealed that the most frequently mutated genes are TP53, and CTNNB1; this has been further confirmed by next-generation sequencing studies. However, the great variability in the occurrence of these mutations points to the possible impact of the underlying etiology on HCC molecular aberrations. Moreover, as recently highlighted in renal cancer [3], intra-tumoral molecular heterogeneity in solid tumors can further complicate the interpretation of molecular information generated from single biopsies.

Multiple signaling pathways that affect cell proliferation, angiogenesis, invasion, and metastasis are de-regulated in HCC (table 1), and have been extensively reviewed elsewhere [4, 5]. Among these, the most frequently reported pathways involve growth factors, such as insulin-like growth factor (IGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF). IGF signaling is essential for the regulation of growth and development, and has been shown to be involved in the pathogenesis of several malignancies, including HCC. Alterations of this pathway include allelic losses of the IGFR2 receptor (80%) and overexpression of the IGF2 ligand (16–40%) [5]. In addition, selective blockade of IGF signaling had antitumoral effects in experimental models of HCC [6]. Various components of the HGF/MET pathway have been suggested to contribute to HCC progression [7]. Furthermore, a gene signature suggesting MET activation was found in 40% of HCC patients [8], identifying patients with poor prognosis. EGFR signaling has also been shown to be present, with overexpression at both mRNA and protein levels [9]. De-regulation of EGF in cirrhotic tissue seems to impact HCC development, as shown in a gene signature able to predict prognosis in surgically resected HCC patients [10]. Moreover, a specific single nucleotide polymorphism in the EGF gene, which increases ligand half-life, correlated with the risk of HCC development [11]. On the hand, over-expression of both VEGF and its receptors has clearly been shown in HCC, which is significant considering the importance of angiogenesis in cancer in general and in HCC in particular [12, 13]. Moreover, high serum levels of VEGF have been associated with aggressive cancer behavior and poor prognosis [14]. Finally, there has been increasing interest in anti-fibroblast growth factor (FGF) therapy in HCC, based on evidence suggesting the importance of this system both in HCC progression and in acquired resistance to anti-VEGF therapy [15]. Moreover, a very recent publication has demonstrated that FGF19, which is amplified and overexpressed along with the known oncogene CCDN1 [16], acts as an oncogenic driver in HCC [17]. Unfortunately, the initial experiences of clinical trials with the FGF inhibitor brivanib as first and second line therapies have not yielded improved survival; however, in these trials patients were not selected based on the basis of FGF activation.

Activation of the above signaling pathways leads to signal transduction involving cytoplasmic intermediates, mostly tyrosine kinases included in the RAS/MAPK and AKT/PI3 K/mTOR pathways. Unlike in other solid tumors (e.g., pancreatic tumors), RAS mutations are infrequent in HCC. Nevertheless, the RAS cascade is of special importance because it is one of the main targets of sorafenib, the only systematic therapy currently effective for advanced HCC [18]. Moreover, in resected HCCs, activated AKT correlates with increased recurrence risk of this cancer [19]. Robust evidence also indicates that the MTORC1 complex plays a role in HCC progression [9]. In fact, MTOR inhibitors such as everolimus are being tested in advanced clinical trials as first and second line therapy for HCC.
**Table 1.** Molecular alterations identified in multiple signaling pathways in hepatocellular carcinoma

| Altered Molecular Pathway | Relevant Molecule | Alteration | Molecular targeted therapies (Experimentally or clinically tested in HCC) | References |
|---------------------------|------------------|------------|---------------------------------------------------------------------|------------|
| **Known pathways in HCC pathogenesis:** | | | | |
| Differentiation and development: | | | | |
| Wnt/beta-catenin | CTNNB1 | Activating mutation/Overexpression | – | de La costa et al. Proc Natl Acad Sci USA 1998, Wie et al. Hepatology 2002, Taniguchi et al. Oncogene 2002, Cui et al. J Gastroenterol Hepatol 2003, Elmileik et al. J Surg Oncol 2005 |
| | AXIN1 | Inactivating mutation/Lost of heterozygosity (LOH) | – | Sato et al. Nat Genet 2000, Laurent-Puig et al. Gastroenterol 2001, Taniguchi et al. Oncogene 2002, Park et al. Liver Int 2005 |
| | APC | Inactivating mutation | – | Guichard et al. Nat Genet 2012 |
| Notch | NOTCH1 | Overexpression | Gamma secretase, DAPT | Giovanni et al. J Hepatol 2009 |
| | NOTCH3 | Overexpression | | |
| Hedgehog | SHH | Activating overexpression | GDC-0449/Vismodegib, cyclopomine | Patil et al. Cancer Biol Ther 2006 |
| | SMO | Activating overexpression | | |
| | HHIP | Down-regulated by LOH/ hypermethylation | | |
| Cell cycle regulation: | p53/cell cycle | | | |
| | TP53 | Inactivating mutation/LOH | Gene-therapy Ad5CMV-p53 gene | Hsia et al. Oncol Rep 2000, Xu et al. Proc Natl Acad Sci U S A 2001, Laurent-Puig et al. Gastroenterol 2001, Elmileik et al. J Surg Oncol 2005 |
| | CDKN2A (p16) | Inactivating mutation/Hypermethylation | Flavopiridol | Matsuda et al. Gastroenterol 1999 |
| | IRF2 | Inactivating mutation | – | Guichard et al. Nat Genet 2012 |
| Proliferation: | EGF | EGF/EGFR | Up-regulated | Erlotinib, Gefitinib, Cetuximab, Lapatinib | Motoo et al. Liver 1991, Heize et al. Anticancer Res 1999, Ito et al. Br J Cancer 2001, Vilanueva et al. Gastroenterol 2008 |
| | HGF/MET | HGF | Up-regulated | SU5416/11274 | Efinova et al. Eur Surg Res 2004 |
| | | HGFR (MET) | Up-regulated | Cabozantinib (XL184), Foretinib | Boix et al. Hepatol 1994, Ueki et al. Hepatol 1997 |
Emerging pathways:

- Chromatin remodeling:
  - Histone modification
    - HDAC
      - Up-regulated
        - LBH589/Panobinostat, vorinostat, romidepsin
  - Chromatin regulators
    - ARID1A/B
      - Inactivating mutation
    - ARID2
      - Inactivating mutation
  - ARIDIA/B
    - Inactivating mutation

- Inflammation:
  - Lymphotoxin
    - LTalpha
      - Up-regulated

- Differentiation and development:
  - Hippo
    - MST1/2
      - Down-regulated
  - pYAP
    - Down-regulated

- Oxidative & reticulum stress
  - NFE2L2
    - Activating mutation

- PI3K/AKT/mTOR
  - PIK3CA
    - Activating mutation
    - BKM120
  - MTOR
    - Up-regulated
    - Down-regulated
  - PRKCA
    - Activating mutation
  - BRAF
    - Mutation/LOH
  - PTEN
    - Activating mutation
  - RAS/MAPK
    - P13K/AKT/mTOR
    - Overexpression

- IGF-1R
  - Up-regulated
  - DLL4
  - Up-regulated

- PDGF
  - PDGFRA
    - Up-regulated
    - Sorafenib, Sunitinib, Imatinib

- VEGF
  - VEGFR2
    - Up-regulating amplifications
    - Sorafenib, Brivanib, Sunitinib
  - Bevacizumab
    - Mise et al. Hepatol 1996

- FGF
  - FGF19
    - Up-regulated

- Angiogenesis:
  - FGF
    - Chiang et al. Cancer Res 2008
  - VEGF
    - Mise et al. Hepatol 1996
  - Misura et al. J Hepatol 1997

- RAS/MAPK
  - Sorafenib
    -KRAS
      - Activating mutation

- RPS6KA3
  - Up-regulated
  - Activating mutation

- Kras
  - Activating mutation

- MTorC1
  - Up-regulated
  - Down-regulated

- PI3K/AKT/mTOR
  - Overexpression
  - IGFl
  - Overexpression

- IGF-2R
  - Down-regulating mutation/LOH

- PI3K/Akt
  - Down-regulating mutation/LOH
Besides growth factor-related pathways, some data indicate aberrant activation of pathways involved in cell differentiation and development, such as WNT signaling. A number of studies show the presence of mutations in CTNNB1, mostly in Western HCC cohorts [5]. Gene expression studies have also identified activation of this cascade in roughly 25% of tumors [1]. A recent study showed two different patterns of WNT activation in HCC and a potential WNT-blockade effect of sorafenib in experimental models [20]. Unfortunately, various strategies have failed to develop drugs effective for selective abrogation of WNT signaling. Other pathways related to cell differentiation have also been studied in HCC, such as Hedgehog (Hh) and Notch, but their role in HCC pathogenesis appears to be less prominent than that of WNT.

Emergence of Novel Therapeutic Targets in HCC

Based on data from randomized trials, HCC seems to be highly resistant to conventional chemotherapy [21]. However, the positive results achieved with sorafenib [22] demonstrate that molecular therapies could play a prominent role in systemic therapy for HCC. In fact, a number of novel targeted therapies are currently under evaluation in different clinical trials [18]. Considering the recent failures of some of these (e.g., sunitinib and brivanib), the identification of novel oncogenic addition loops in HCC has become a research priority. In other tumors, selective blockade of these events resulted in significant increases in patient survival (e.g., vemurafenib in BRAF-mutated melanoma or crizotinib in lung cancer with ALK rearrangements). In this review, we summarize recent findings of emerging altered signaling pathways involved in HCC and their potential as candidate targets for future personalized molecular therapies (fig. 1). In addition, we provide an update on some of the previously less-characterized pathways involved in cell differentiation, such as Notch and Hh.

Epigenetic Regulation and Chromatin Remodelling

Chromatin remodeling is a core epigenetic mechanism implicated in the control of gene expression; it provides dynamic access of condensed genomic DNA to the transcription machinery proteins. Hence, it plays an essential role in a variety of cell processes, including proliferation, differentiation, and DNA repair. The principal mechanisms involved in this process are enzymatic covalent histone modifications (e.g., methylation, phosphorylation, and acetylation) and nucleosomal restructuring by ATP-dependent chromatin remodelling complexes. In recent years, there has been growing evidence for a tumor suppressor role for these complexes, due to identification of frequent inactivating mutations of these components in different malignancies [23].

Histone deacetylases (HDACs) are responsible for the transcriptional control of many genes involved in diverse cellular processes through chromatin remodeling by histone acetylation or by functioning as transcriptional co-activators. There are 18 HDACs identified in mammals, which have been classified into four classes (Class I–IV) based on their DNA sequence similarity and function [24]. Currently, there is a strong body of evidence suggesting an important role for the HDAC machinery in cancer progression. Aberrant expression of several HDAC members (HDAC1–11) has frequently been shown to correlate with aggressive behavior of tumors and poor prognosis [25]. For this reason, HDAC inhibitors are increasingly being considered as one of the most promising anti-cancer drugs [26]. In fact, recently, two HDAC inhibitors (vorinostat and romidepsin) have received FDA approval for use in the treatment of cutaneous T cell lymphoma [27]. HDAC inhibitors exert their antitumor activity partly by means of histone hyperacetylation, resulting in reduced DNA–histone affinity, and thus facilitating access to transcription factors [28]. A recent study reported aberrant
de-regulation of 11 HDACs in a cohort of surgically resected HCC [29]. A subset of HDACs (HDAC1, 2, 3, 4, 5, and 11) was significantly up-regulated in HCC in comparison to normal liver tissue, and cirrhotic and dysplastic nodules. Furthermore, DNA copy number alteration analysis demonstrated significant DNA gains in HDAC3 and HDAC5, which correlated with their mRNA up-regulation. These findings partly correlate with those of a previous study in which HDAC expression levels were assessed through immunohistochemistry in a cohort of 43 HBV-related HCC cases [30]. A subset of HCCs presented increased expression of HDAC1 (51.2%), HDAC2 (48.8%), and HDAC3 (32.6%). Moreover, HDAC3 was determined to be an independent prognostic prediction factor for tumor recurrence following liver transplantation in HBV-related HCC.

Concomitantly, several studies have evaluated the efficacy of HDAC inhibitors, either alone or in combination with other agents, in preclinical HCC models. In particular, Lachmann et al. demonstrated that combination therapy with the pan-HDAC inhibitor (panobinostat) and sorafenib strongly potentiated treatment efficacy by significantly decreasing tumor volume and vessel density, and improving survival in HCC xenografts. This a proof-of-
concept study that support the evaluation of these compounds in early clinical developmental phases in humans [29, 31–36].

Recently, several studies in HCC have identified recurrent mutations in multiple chromatin regulators, including prominent members of the AT-rich interaction domain (ARID)-containing protein family. Gene-set enrichment (GSE) and functional analysis of whole genome sequencing data from 27 viral-associated HCCs identified recurrent mutations in a group of chromatin regulators (ARID1A, ARID1B, ARID2, MLL, and MLL3) in approximately 50% of the tumors [37]. This was further validated in an independent cohort of 120 HCCs, showing a mutation rate of 10, 6.7, and 5.8% for ARID1A, ARID1B, and ARID2, respectively. Interestingly, the frequency of inactivating mutations (non-synonymous mutations and indels) was significantly enriched in the chromatin regulator genes in comparison to other gene groups. Given that these mutations were marginally associated with liver fibrosis and hepatic vein invasion, it may be plausible that mutations in chromatin regulators contribute to aggressive behavior of HCC. Guichard et al. also identified chromatin regulators as the third most frequently mutated genes through whole-exome sequencing of 24 HCCs [38]. Interestingly, they reported that ARID1A mutations were more frequently associated with alcohol-related HCC.

ARID1A and ARID1B are two crucial and mutually exclusive subunits of the SWItch/Sucrose Non-fermentable (SWI/SNF) ATPase-powered nucleosome remodelling complex, which is involved in regulation of gene expression by controlling promoter accessibility. Interestingly, similar inactivating mutations in ARID1A, and its role as a tumor suppressor, has been reported in several malignancies, including ovarian, colorectal, and gastric cancer [39–42]. On the other hand, ARID2 is a subunit of the polybromo- and BRG1-associated (PBAF) remodeling complex, which is implicated in the control of ligand-dependent transcription by nuclear receptors. In addition to these studies, Li et al. also found mutations in ARID2 in 18.6% of 33 HCCs analyzed by exome sequencing [43], which was further validated in an additional set of 106 tumors. Interestingly, both ARID1A [38] and ARID2 [43] mutations have been associated with the presence of CTNNB1 mutations, which are some of the most prevalent mutations in HCCs and are a hallmark of WNT pathway activation. ARID2 mutations and TP53 mutations were also shown to be mutually exclusive. ARID2 mutations have been predicted to result in a loss of function of the corresponding protein, predominantly through alteration of the Zn-finger motif of the DNA-binding domain, thus suggesting its role as a candidate tumor suppressor gene in HCC. With the data available to date, de-regulation of ARID1/2 signaling appears to affect 6–18% of HCC samples.

Differentiation and Development

Notch signaling pathway: The Notch pathway is a highly conserved signaling module present in most multicellular organisms. It plays an important role in cell–cell communications and participates in the regulation of multiple cell differentiation processes during embryonic development and stem/progenitor cell staging [44, 45]. In the liver, Notch acts in a temporal- and dose-dependent manner to coordinate biliary fate and morphogenesis [46]. Unlike most pathways, Notch signaling requires direct cell-to-cell interaction to ensure its proper activation. In human cancer, activating mutations and translocations affecting NOTCH1 is a characteristic feature of acute lymphoblastic leukemias [47]. In solid tumors, activation of Notch has been described in lung and prostate cancer [48, 49], although other reports also suggest the potential tumor suppressor activity of this pathway [50]. Thus, data regarding its role in cancer is contradictory. Two studies have demonstrated that NOTCH1 overexpression inhibits HCC cell growth by promoting cell cycle arrest and apoptosis [51, 52]. In contrast, there seems to be growing evidence suggesting an oncogenic role for Notch activation in hepatocarcinogenesis [53]. Significant over-expression of NOTCH1 and NOTCH3 was detected through immunohistochemistry in 60 HCCs [54]. In addition, the same study demonstrated
that depletion of NOTCH3 by specific shRNAs increased p53 expression and enhanced doxorubicin-sensitivity by promoting apoptosis. Moreover, considering the important role of HBV X protein (HBx) in the development of HCC, a recent study found that HBx upregulates key molecules in Notch signaling (NOTCH1, HES1, and JAGGED1) [55]. This finding suggests that Notch activation may be one of the mechanisms by which HBx promotes HCC progression. These contradictory data for the role of the Notch cascade in solid tumors may be due to a high context-dependency.

Hedgehog signaling pathway: Hh signaling is required for embryogenesis and regulation of a variety of essential functions, from differentiation to regeneration, as well as in stem cell biology, through control of cellular proliferation, apoptosis, and migration. It is activated by the interaction of Hh ligands (i.e., sonic, indian, and desert hedgehog) with the transmembrane Smoothened (SMO) receptor, resulting in nuclear translocation of Gli and transcriptional activation of target genes (fig. 1). Hh signaling targets include β-catenin, FGF, IGF2, EGF, Bcl-2, and different cyclins, along with Hh negative regulators such as PTCH and HHIP. In diseased livers, the Hh pathway promotes hepatic regeneration; however, excessive or continuous activation of Hh has been shown to halt successful regeneration and contribute to liver fibrosis [56]. In HCC, the initial findings demonstrated aberrant over-expression of GLI1 and SMO together with down-regulation of HHIP in a subset of HCCs [57]. Furthermore, inhibition of the Hh pathway by cyclopamine, a steroid alkaloid that binds to and blocks SMO, significantly inhibited cell proliferation, increased apoptosis, and repressed the expression of two of the Gli-related target genes, c-Myc and cyclin D1. Interestingly, a recent paper showed that Hh inhibition in a mouse model of HCC through the use of a selective antagonist, GDC-0449, led to the regression of both liver fibrosis and HCC, even in advanced stages of the disease [58].

Hippo signaling pathway: The hippo pathway is an evolutionary conserved cascade that plays an essential role in the control of organ size and cell contact inhibition by regulating cell proliferation and apoptosis. During recent years, growing evidence has pointed towards an oncogenic role for this pathway in human cancer, including HCC [59]. While the key components and upstream regulators of the hippo pathway (e.g., FAT, NF2, and FDM6) are mostly considered to participate as tumor suppressors, downstream mediators such as TAZ, YAP, and TEADs are mostly involved in oncogenic events. In general terms (fig. 1), the initial hippo kinases complex, formed by MST1, MST2, and its scaffold protein (SAV1), activates the Lats1/2-Mob1 complex, which, in turn, phosphorylates the transcription factors YAP and TAZ, preventing their translocation to the nucleus and consequent target gene transcription. Several studies have demonstrated that MST1/2 double knockout mice demonstrate liver outgrowth and HCC [60–62]. This is consistent with the results obtained in YAP-overexpressing transgenic mice, which also presented with a similar phenotype [63]. In addition, two other studies have demonstrated that MST1/2 plays an important role in regulating the liver progenitor/stem cell compartment in adult liver. Interestingly, approximately 30% of HCCs present with low levels of phospho-YAP and an inactive cleaved form of MST1 is present in the majority of these cases [62]. Moreover, a heterozygous deletion of YAP was able to suppress development of HCC caused by NF2 inactivation [64]. Altogether, these findings indicate that inhibition of YAP by MST1/2 can be considered as an important pathway for HCC suppression.

**Inflammation-related Pathways**

Solid evidence supports the causal connection between chronic inflammation and cancer. In HCC, close to 80% of patients develop tumors against a background of chronic liver inflammation; however, little is known about the specific molecular mechanisms underlying this process. Additional events also present in cirrhosis, such as persistent cell regeneration, may also contribute to malignant transformation in this context. A recent publication has revealed the promoting effect of translocation of intestinal microbiota to the liver through
activation of toll like receptor 4 (TRL4) in the advanced stages of HCC [65]. This study included numerous animal models (i.e., TLR4 genetic inactivation, gut sterilization and long-term treatment with low doses of LPS), where chronic liver injury was modeled using diethylnitrosamine and carbon tetrachloride. The authors demonstrated that once TLRs recognize microbial ligands, like lipopolysaccharide (LPS) and pathogen-associated molecular patterns (PAMPs), the NF-κβ pathway is activated, promoting the secretion of inflammatory molecules, such as TNF-α and cytokines. These molecules regulate multiple reactions in resident liver cells (e.g., hepatocytes, Kupffer cells, and particularly stellate cells), which are responsible for hepatic injury, a step that precedes HCC development. Furthermore, these results are consistent with previous data that showed that activation of NF-κβ mediates liver oncogenesis in liver-specific mice that over-expressed lymphotoxin [66]. This model developed chronic hepatitis, followed by HCC, at 12 months of age. However, once lymphotoxin expression was blocked, the chronic inflammatory hepatitis was reverted, also preventing the onset of HCC. These data further demonstrate the importance of an inflammatory environment in the initial steps of HCC development.

**Autophagy**

Autophagy is a conserved lysosomal degradation pathway responsible for maintaining cellular homeostasis through recognition and turnover of damaged proteins and organelles. This pathway has been implicated in different, and sometimes contradictory, processes capable of inducing both cell survival and death. Autophagy can be divided into constitutive autophagy, which is necessary for intracellular recycling and metabolic regulation, and stress-related autophagy, which is required for elimination of damaged intracellular components generated by cellular stress. Interestingly, numerous reports have described activation of autophagy by many anti-cancer therapies currently in use [67]. Hence, its role in carcinogenesis and its crosstalk with anti-cancer drugs is currently being evaluated. Constitutive autophagy is suppressed in many tumors (i.e., prostate, breast, ovarian); allelic losses of one of the genes essential to this process (*BECLIN1*) is one of the main molecular mechanisms known to underlie this abrogation of autophagy [68]; *BECLIN1* knockout mice (*beclin1* +/−) also develop HCC [69]. Conversely, another study demonstrated that sorafenib up-regulates autophagy by inducing autophagosome formation both in vitro and in vivo, thus protecting malignant cells from death [70]. Overall, these preliminary reports underscore a potential role for autophagy in the progression of HCC and its response to therapy. Further exploration of this cascade will delineate its relevance as a source of novel therapeutic targets.

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