4 Molecular Mechanisms of Hepatocellular Carcinoma: Insights to Therapy

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CONTENTS

ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most frequently occurring human malignancies in the world and is associated with a high mortality rate. As such, understanding the molecular underpinnings of this cancer in order to identify novel diagnostic markers, therapeutic targets, and prognostic indicators that aid in patient care is a major goal for clinicians and researchers alike. Progress has been made on this front over the past several years resulting in the development of drugs that specifically target processes believed to propagate HCC cell transformation, growth, and metastasis such as cell surface receptor–ligand interaction and signal transduction, cell cycle and apoptosis progression, extracellular matrix remodeling, vasculogenesis, motility, histone modification, and others. Many of these agents

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have been assessed in pre-clinical animal models and are now being evaluated in human clinical trials in the United States and elsewhere. This chapter will discuss targeted therapies for HCC under study in humans as well as the pathways they intercept.

Key Words: Hepatocellular carcinoma; gene expression; clinical trials; targeted therapies; molecular mechanisms

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is an aggressive malignant tumor of the liver that accounts for about 80% of primary hepatic cancers in adults *(1)*. HCC is now the fifth most common type of malignancy and the third leading cause of cancer mortality worldwide *(2)*. The underlying determinants of HCC are diverse and include a variety of viral, toxic, and metabolic insults, most of which result in cirrhosis *(3)*. Populations from certain geographic regions such as Asia and Africa suffer disproportionately from HCC reflecting a high incidence of hepatitis B virus (HBV) infection and aflatoxin exposure *(3)*. However, the number of HCC diagnoses has been rising in low-incidence areas such as the United States, Western Europe, and Japan, likely due to an increase in hepatitis C virus (HCV) infection in these populations *(3)*. Over 20,000 new diagnoses of liver cancer in Americans were expected to be rendered in 2008 *(4)*. Without appropriate screening, HCC comes to clinical presentation late in its course when surgical intervention is no longer an option; at any stage, this tumor is notoriously resistant to standard systemic chemotherapy as a result of innate tumor resistance as well as underlying liver disease making it a difficult malignancy to manage clinically *(5)*. Due to HCC's aggressive behavior, insidious presentation, resistance to therapy, and general prevalence, a concerted global effort has been put forth over the past two decades to dissect the molecular mechanisms of HCC in order to reveal clues to diagnosis, therapy, and prognosis. Because of these Herculean efforts, novel diagnostic markers, therapeutic targets, and prognostic indicators for HCC have been discovered, and although the clinical utility of many of these newly identified molecular hallmarks must still be rigorously assessed, it has become evident that some discoveries may well constitute 'medical breakthroughs.'

Most major risk factors for HCC (such as HBV or HCV infection, aflatoxin exposure, alcohol abuse or metabolic derangements like hereditary hemochromatosis) *(6)* cause sustained hepatocyte damage by one mechanism or another and incite repair. However, the healing process in the liver may be incomplete rendering hepatocytes vulnerable to additional assault.

Cycles of hepatocyte death and replication promote fibrous deposition, cirrhosis, hepatic insufficiency, and outgrowth of pre-neoplastic and frankly malignant clonal cell populations *(1)*. DNA damage that accumulates in cell clones may result from replication errors inflicted by aberrant cell cycle transit, direct mutagenesis, oxidative stress triggered by inflammation, or a combination of these mechanisms *(7)*. Because HCC risk factors are so varied, each is capable of eliciting unique pro-tumorigenic alterations substantiating the notion that, despite falling under the same histologic classification, HCCs are, in fact, quite heterogeneous *(8)*. To this end, molecular 'signatures' reflecting the inciting cause or recurrence pattern have been identified *(9)*. However, it is also obvious that some molecular mechanisms are activated in the majority of liver tumors, regardless of the underlying risk factor. It is these candidates, in particular, that make attractive therapeutic targets.

It has been proposed that transformation of a normal hepatocyte into one with malignant potential requires at least five or six individual genetic insults *(10)*. Numerous studies have been carried out comparing normal, cirrhotic, dysplastic, malignant, and metastatic liver tissues *(11,12)* in attempts to categorize the genetic mutations associated with each step leading toward malignancy. Due to HCC's inherent heterogeneity, however, this has been a difficult task. Depending on the type of molecular tool or test employed (e.g., classic cytogenetics *(13)*, CGH *(14)*, SNP *(15)*, expression *(12)*, or microRNA arrays *(16)*, or proteomic approaches *(17)*) and the type of tissue tested (i.e., normal vs. tumor, dysplastic vs. malignant, solitary vs. multifocal tumors, invasive vs. non-invasive tumors, HBV+ vs. HCV+ tumors, or mouse vs. human tumors), HCCs can be subclassified into a multitude of different categories. However, one intriguing HCC subclassification which has been further substantiated in human and rodent HCC *(18–20)* separates tumors into two groups: those with mutant p53 and genomic instability and those with beta-catenin aberrancy and cancer gene hypermethylation.

p53 is a multifaceted transcription factor that is crucial to inducing cell cycle arrest and eliciting apoptosis *(21)*. Dysregulation of p53 in HCC occurs through a combination of loss of heterozygosity (LOH) observed in over half of HCCs (57%–8/14) *(22)* and mutation detected in about 28% of HCC cases worldwide *(23)*. In addition, upregulation of cellular and viral factors that bind and sequester p53, such as mdm-2 *(24,25)* and hepatitis B virus X protein (Hbx) *(26,27)*, is seen in HCCs. Another key role of p53 is to maintain DNA integrity *(21)* which is commonly lost in human HCC. Genomic instability in HCC is characterized by non-random DNA losses on chromosomes 1p, 4q, 6q, 8p, 10q, 13q, 16p, 16q, and 17p and gains of genomic material on chromosomes 1q, 5p, 5q, 6p, 7q, 8q, 17q, and 20q *(28)*. Specific chromosomal losses and gains correlate with the underlying risk factor and tumor differentiation *(11)*.

Beta-catenin is a multifunctional protein, the ultimate purpose of which is to control gene transcription. It acts as a conduit linking signaling at the plasma membrane where beta-catenin normally resides with the nucleus where it transactivates a repertoire of gene targets, many of which are proto-oncogenes including c-myc and cyclin D1. Under normal conditions, soluble extracellular signals such as Wnt ligands, extracellular matrix interactions, Met transmembrane receptor, and other determinants control beta-catenin activation and localization *(29)*. However, in HCC, several molecular mechanisms leading to abnormal beta-catenin activation, such as beta-catenin gene mutation seen on average in about 22% of human HCCs *(23)*, downregulation of E-cadherin *(30,31)*, or PIN1 overexpression *(32)* bypass normal control steps thus leading to excessive gene expression driven by beta-catenin.

Aberrant DNA methylation is an epigenetic event usually described in the context of neoplasia: HCC is no exception. In hepatic and other tumors, DNA methylation alterations are characterized by a state of global demethylation and focal de novo hypermethylation of CpG islands in specific gene promoters. These changes can result in stimulation of proto-oncogenes and silencing of tumor-suppressor genes *(33)*. Interestingly, it has been postulated that DNA hypermethylation alterations in the liver may well reflect normal physiologic responses to aging and to inflammation. As compared to normal liver from younger patients, aged livers, livers with active hepatitis, and HCC tissues showed stepwise increases in DNA hypermethylation of a set of epigenetic markers *(34)*. Taken together, these findings regarding beta-catenin activation and global hypermethylation in HCC suggest that inhibition of key tumor-suppressor genes (via hypermethylation) in combination with mutation of oncogenes (like beta-catenin) can incite HCC development in the absence of large-scale genomic alterations.

In addition to p53 and beta-catenin, a host of factors have been linked to the malignant transformation, growth, or invasion of liver cancer. They fall into several categories such as cell surface receptors and their ligands, intracellular effector molecules, cell cycle and apoptosis regulators, extracellular matrix remodeling agents, vasculogenic factors, motility inducers, histone modifiers, and telomerases. As a wonderful testament to humankind's ingenuity and the power of scientific research, several targeted therapies have been designed to modulate the activity of some of these pathways. Many have since been assessed in pre-clinical models and are now being evaluated in human clinical trials across the world (Table 1). The remainder of this chapter will focus on those signaling pathways with agents showing therapeutic potential in HCC and engendering clinical enthusiasm.

Table 1
Select Targeted Therapies for HCC Currently Under Evaluation in Clinical Trials (www.clinicaltrials.gov)*

Pathway	*Target*	*Agent*	*Class*
Growth factors/receptor tyrosine kinases	EGFR/ErbB2	*Gefitinib*	Kinase inhibitor
		Erlotinib	Kinase inhibitor
		Lapatinib	Kinase inhibitor
		Cetuximab	Anti-EGFR mAb
		Trastuzumab	Anti-ErbB2 mAb
	VEGF/VEGFR-1/-2/-3	*Cediranib*	Kinase inhibitor
		Sunitinib	Kinase inhibitor
		Brivanib	Kinase inhibitor
		Vandetanib	Kinase inhibitor
		Pazopanib	Kinase inhibitor
		ABT-869	Kinase inhibitor
		IMC-1211B	Anti-VEGFR-2 mAb
		Bevacizumab	Anti-VEGF mAb
	IGF1R	*IMC-A12*	Anti-IGF1R mAb
Intracellular signal transducers	Ras	*Lonafarnib*	Farnesyltransferase inhibitor
	Raf/Mek/Erk/MAPK	*Sorafenib*	Kinase inhibitor
		AZD6244	Kinase inhibitor
	mTOR	*Sirolimus*	Binds FKBP-12; inhibits mTORC1
		Everolimus	Binds FKBP-12; inhibits mTORC1
	Abl/Src	*Dasatinib*	Kinase inhibitor
Transcription factors	RAR-alpha	*TAC-101*	Inhibitor
Cell cycle modulators	p53	*Ad5CMV-p53*	Gene therapy
	CDK	*Flavopiridol*	Inhibitor
Pro-survival molecules	Survivin	*LY2181308*	Anti-sense oligomer

(*Continued*)

Table 1
(Continued)

Pathway	*Target*	*Agent*	*Class*
DNA modification enzymes	HDAC	*Belinostat*	Inhibitor
Inflammation modulators	Cox-2	*Celecoxib*	Inhibitor
Proteasome	26S Proteasome	*Bortezomib*	Inhibitor

Abbreviations used: CDK—cyclin-dependent kinase; EGFR—epidermal growth factor receptor; HDAC—histone deacetylase; IGF1R—insulin-like growth factor1 receptor; mAb—monoclonal antibody; VEGF(R)—vascular endothelial growth factor (receptor). *—at the time of writing.

2. RECEPTOR TYROSINE KINASES AND THEIR LIGANDS

In order to adapt to changes in the surrounding environment and sense the needs of the host organism, cells must be able to receive and act upon signals from the extracellular milieu. Such communication is facilitated by a variety of mechanisms; one of these is through receptor tyrosine kinases (RTKs) anchored in the plasma membrane. Through their extracellular domains, RTKs bind protein ligands with high specificity and affinity and, following engagement, emit potent intracellular cues that regulate cell division, motility, survival, and a number of crucial cellular activities. Because of their capacity to control cell growth, RTK signaling is tightly governed and short lived. Steps to ensure that RTK signal emission is of proper intensity and duration include limiting RTK–ligand interaction, promoting RTK internalization and degradation as well as activating phosphatases and other measures *(35)*.

A specific set of RTKs have garnered attention in the study of liver cancer. Some are activated by well-established hepatic mitogens. These RTKs and their ligands include the epidermal growth factor receptor (EGFR) and its family members which bind EGF, transforming growth factor alpha (TGF-alpha) and other EGF-related ligands, and Met, the RTK for hepatocyte growth factor (HGF). These particular RTKs and their ligands are often overexpressed in HCC and are thought to help drive malignant hepatocyte replication, invasion, and motility. Other RTKs are involved in tumor neovascularization such as the vascular endothelial growth factor receptors (VEGFRs). HCCs are highly vascular tumors and secrete factors like VEGF to promote vessel ingrowth in order to establish and maintain an oxygen-rich blood supply.

Because RTKs and their relatives, the intracellular tyrosine kinases (such as src, abl, JAK, and others), are such powerful transducers of malignant transformation, growth, and invasion, they were among the earliest candidates to be explored for their therapeutic targeting potential. To that end, imatinib, an inhibitor with specificity for the bcr-abl oncogene product resulting from a t9;22 chromosomal translocation observed in human chronic myelogenous leukemia, was one of the first rationally designed targeted small molecule therapeutics approved by the US Food and Drug Administration (FDA) to treat cancer *(36)*. Since then, numerous inhibitors with specificity for other tyrosine kinases have been developed, and their efficacy in pre-clinical models and clinical trials for various types of cancer including HCC (Table 1) is under investigation.

2.1. Epidermal Growth Factor Receptor Family and Ligands

The EGFR family of receptors contains four members: EGFR (ErbB1 or Her1), ErbB2 (Her2 or neu), ErbB3 (Her3), and ErbB4 (Her4). Although ErbB2 is an orphan receptor with no known ligand, activation of its tyrosine kinase domain is facilitated by heterodimerization with and transphosphorylation by other EGFR family members *(37)*. Two of the four EGFR family members that bear relevance to HCC are EGFR and ErbB2, the most well characterized in hepatocyte biology and HCC being EGFR itself and its associated ligands, EGF and TGF-alpha. Ligand-activated EGFR promotes hepatoctye motility *(38)* and morphogenesis *(39)* and contributes to liver regeneration *(40)*.

With regard to liver cancer, TGF-alpha mRNA *(41)* and protein *(42–44)* are overexpressed in human HCCs, particularly in HBV+ cases, as compared to adjacent liver tissue. In addition, transgenic mice overexpressing TGF-alpha in the liver develop HCC after a year *(45,46)*. On the other hand, results of studies examining EGFR expression in liver cancer are conflicting with some showing increased EGFR expression in HCCs *(47,48)* while others not *(49,50)*. Perhaps a more relevant observation is that enhanced tyrosine phosphorylation of EGFR at residue Y845 was noted in 72% (13/18) of HCC tissues using Western blot *(51)*. However, two studies did not detect EGFR mutation in human HCC samples *(52,53)*.

Data supporting a role for ErbB2 in human HCC are limited. Ito et al. *(48)* demonstrated that 21% of HCCs expressed ErbB2, while others did not observe ErbB2 expression in liver cancers *(54)*. Mutation of the kinase domain in the ErbB2 gene (*her2/neu*) occurs in some solid tumors such as non-small cell lung carcinoma (NSCL) *(55)*. An analysis of human HCCs for ErbB2 mutation did not reveal the presence of those gene variants previously described by others in NSCL cancer but did identify a novel amino

acid change (H878Y) the authors propose could alter ErbB2 activity in 11% (2/18) of HCCs tested *(52)*.

Several targeted inhibitors of EGFR and ErbB2 are currently under study in clinical trials for HCC (Table 1). Results of Phase II clinical trials of erlotinib, an orally active inhibitor of EGFR, and cetuximab, an anti-EGFR monoclonal antibody administered intervenously, in patients with advanced liver cancer have been published. In the first of two reports of erlotinib efficacy, about a third (32%) of patients showed no disease progression at 6 months with erlotinib therapy while 9% of patients demonstrated a partial radiologic response. However, over a quarter (26%) of patients in the study required erlotinib dose reductions due to skin toxicity and diarrhea *(56)*. Patients in the second erlotinib study did not show evidence of radiologic response to the treatment but over 40% demonstrated progression-free survival at 16 weeks of therapy *(8)*. Phase II trials with cetuximab were less promising revealing that the median progression-free survival for patients on treatment was 1.4 months despite the drug being well tolerated *(57)*.

2.2. Vascular Endothelial Growth Factor Receptor Family and Ligands

Solid tumors require new blood vessel formation or neovascularization in order to enlarge *(58)*; this is clearly the case with HCC *(59)*. The portal circulation serves as the blood supply for early HCCs; however, as tumors expand, their oxygen demands increase. As a consequence, the oxygen-enriched hepatic arterial supply is tapped to feed the tumor *(59)*. The vascular endothelial growth factors consisting of six members (VEGF-A through -E and placenta growth factor [PLGF]) *(59,60)* and their receptors are essential to this process. Three tyrosine kinase cell surface receptors exist for VEGF including VEGFR-1 (flt-1), VEGFR-2 (KDR or flk-1), and VEGFR-3 (flt-4). The activities of VEGF-A, VEGF-B, and PLGF appear to be mediated primarily through VEGFR-1, while VEGF-A and -E utilize VEGFR-2, and VEGF-C and -D bind VEGFR-3 *(61)*.

VEGF expression is upregulated in most cases of human HCC *(62–65)*. Some studies indicate, however, that VEGF protein levels are elevated to a greater extent in non-tumorous adjacent cirrhotic tissue than HCCs *(66,67)*. Expression of both VEGFR-1 and -2 mRNA has been detected in human liver tumors; however, one study showed that, of the two, VEGFR-1 mRNA levels were greater in tumor tissues *(62)*, whereas another determined that VEGFR-2 transcripts were more abundant in HCCs *(68)*. Liu et al. *(69)* determined that human HCC cell lines express both VEGFR-1 and -2 by flow cytometric analysis and Western blot and that cell proliferation was augmented by addition of VEGF to the cultures. These findings point to the

possibility that, in addition to a paracrine effect of VEGF on endothelia to promote neovascularization in HCC, a VEGF/VEGFR autocrine circuit may also exist to stimulate growth of liver tumor cells.

A multitude of agents targeting the VEGF/VEGFR axis are available and in clinical trials for HCC. They include tyrosine kinase inhibitors, an anti-VEGF monoclonal antibody (bevacizumab), and an anti-VEGFR-2 antibody (IMC-1211B, see Table 1). Recently, a Phase II clinical trial assessing the efficacy of bevacizumab in combination with GEMOX (gemcitabine–oxaliplatin) in patients with advanced HCC was completed and results released *(70)*. In this study, CT perfusion scan was used to monitor tumor blood flow, blood volume, permeability surface area, and mean transit time as a means of tracking tumor vascularity pre- and post-treatment *(70)*; the degree of tumor contrast enhancement which can be assessed by CT has been shown to correlate with tumor neovascularization in HCC *(71)*. Bevacizumab therapy was significantly associated with longer mean transit time indicating increased tumor capillary leakiness. In addition, the results showed that the percent change in mean transit time following bevacizumab treatment correlated with patient outcome. Median progression-free survival was 5.3 months in this study *(70)*.

2.3. Insulin-Like Growth Factor Receptor-I and Ligands

The insulin-like growth factors-I and -II (IGF-I and -II) stimulate hepatocyte replication *(72)* and appear to be involved in human liver tumorigenesis. IGFs can engage three types of receptors: the insulin receptor (IR), IGF1R, and IGF2R/mannose-6-phosphate receptor. The first two are RTKs; the latter is not. Of the three, only IGF1R binds IGFs with high affinity and thus likely propagates most IGF-induced signaling *(73)*. The majority of evidence implicates IGF-II over IGF-I in human HCC. Several studies have shown that the human IGF-II gene is genomically imprinted in normal adult tissues *(74)* except in the liver: normal hepatic tissue expresses IGF-II from both of its alleles *(75)*. However, in HCC, biallelic IGF-II expression ceases *(76,77)*, and usage of a fetal-type IGF-II promoter recommences *(78,79)*. This is accompanied by increased IGF-II protein and mRNA expression in human HCCs *(78,79)*. To this end, a Phase II clinical trial to determine the efficacy of the anti-IGF1R monoclonal antibody known as IMC-A12 in those with advanced HCC recently began recruiting patients *(80)*.

3. INTRACELLULAR SIGNAL TRANSDUCERS

Numerous and diverse intracellular signaling molecules serve to receive and amplify cues emitted from cell surface receptors. They deliver them to intended recipients such as the mitochondria, the nucleus and other key

organelles, cellular structures, and proteins. In some cases, the signal 'hand off' between intracellular molecules occurs in a relatively orderly and predictable fashion—from one pathway member to the next, and so on; however, as more insight into these cascades is obtained, it is becoming clear that branch points, nodes, and various deviations along the signaling chain occur and complicate our understanding. The messages these cellular liaisons transport are certainly consequential to the well-being of the host; thus their pathways are highly regulated at multiple levels, in order to maintain normal cell function *(81)*. Because of their crucial role in governance of cell signal transduction, several of these signaling proteins and their respective pathways are mutational targets in cancer.

Some of the better known intracellular signaling molecules targeted in human liver cancer include beta-catenin (as described) and c-myc *(82,83)*. The ras GTPase, while historically a proven player in rodent hepatocarcinogenesis *(84)*, is now gaining significance as a mediator in human HCC as well. Additional factors recently linked to HCC are PI3K pathway constituents (p110alpha and PTEN) and members of the rho GTPase cascade. Pharmacologic inhibitors of several intracellular signaling pathways are now being tested in human HCC patients (Table 1). The following section will focus on a subset of the pathways with targeted therapies under clinical evaluation for liver cancer.

3.1. The Small GTPase Superfamily

Ras and rho are members of the small GTPase superfamily. Their localization to the inner plasma membrane is facilitated by farnesyl and palmitoyl lipid moieties attached to their protein backbone. Ras and rho are active when bound to GTP and, in this state, recruit signal transducers (such as raf in the case of ras). To become inactive, these small GTP_{ases} hydrolyze GTP to GDP. Several adaptor and regulatory proteins such as guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) positively and negatively regulate ras and rho. Some of these adapters, such as Grb2 and Sos (a rasGEF), link stimulated RTKs to the small GTPases leading to their activation thus initiating signaling cascades which influence cell proliferation, cytoskeletal rearrangement, and expression of genes such as cyclin D1, $p21^{WAF1/CIP1}$, and $p27^{KIP}$*(85)*.

A large body of evidence demonstrates that ras is involved in normal hepatocyte replication in culture *(86)* and in vivo *(87,88)*. The ras family consists of three major isoforms: H-, K-, and N-ras. Mutation of these isoforms has been detected in several types of cancer *(89)*. In the case of human liver cancer, about 33% (6/18) of vinyl chloride-associated HCCs

were found to harbor K-ras mutations. Incidentally, such mutations were also detected in surrounding non-tumorous liver tissue in two of the six cases *(90)*. Other than in the instance of vinyl chloride-induced liver tumorigenesis, K- or H-ras mutations have rarely been detected in human HCCs *(91–94)*. Despite a lack of data implicating ras gene mutation as a common cause of human HCC, the ras signaling cascade may be upregulated in this tumor through other mechanisms. One such mechanism may be due to suppressed expression of a ras effector molecule and suspected tumor suppressor known as ras association domain family 1A (RASSF1A). The gene promoter region of RASSF1A is hypermethylated in 93% of human HCCs (14/15). Aberrant methylation of RASSF1A was also seen in human livers with fibrosis (2/2) and cirrhosis (3/4), but not in normal liver (0/2) *(95)*, suggesting that ras pathway signaling provides a permissive environment promoting hepatocyte replication and accumulation of additional genetic alterations. Similar RASSFIA methylation differences were observed by others *(96,97)*.

The expression and activity of several factors involved in the rho cascade are also deranged in human HCC. These include rhoA, rhoC, and deleted in liver cancer-1 and -2 (DLC-1 and -2). The rho subfamily of GTPases can be subdivided into six smaller groups based on structural similarity: rhoA and rhoC, along with rhoB, comprise one of these six groups *(98)*. Recently, a study demonstrated that rhoA mRNA and protein levels were 2.0- and 2.7-fold higher, respectively, in tumor tissue than adjacent liver. These observations correlated with tumor invasion and poor histologic differentiation. The authors concluded that overexpression of rhoA is associated with a poor prognosis *(99)*, a finding supported by another group *(100)*. Wang et al. *(101)* examined human HCCs for gene mutation and mRNA expression of rhoC. They found no mutations in rhoC in any samples, but they did observe that intrahepatic and invasive/metastatic HCCs expressed 1.8- and 3.3-fold more rhoC mRNA, respectively, than adjacent liver tissues leading them to postulate that rhoC may be involved in liver tumor cell invasion and metastasis, an idea backed by others *(102)*.

Human chromosome 8p, in particular 8p21.3-22 *(103)*, is a deletion hotspot in HCC, and its loss is associated with metastasis *(104)*. The DLC-1 gene has been cloned from this region and encodes a novel rhoGAP *(105)*. About half of HCCs show LOH in the DLC-1 gene *(106)*. Others demonstrated loss of DLC-1 gene expression in about 20–67% of human liver tumors *(106,107)*. Decreased DLC-1 expression may be due to DLC-1 gene hypermethylation which has been observed in 24% (6/25) of HCCs as compared to adjacent liver *(106)*. The DLC-2 gene, encoding a rhoGAP related to DLC-1, has been cloned from chromosome 13q12.3 *(108)*. This region is also commonly deleted in HCC *(109)*. DLC-2 mRNA levels were reduced in 18% (8/45) of liver tumors compared to adjacent liver. Functional studies

demonstrated that DLC-2 preferentially regulates rhoA and another small GTPase cdc42 *(108)*. A Phase II clinical trial of lonafarnib, an orally available farnesyltransferase inhibitor that inhibits farnesylation of ras and rho, is underway for patients with primary liver cancer *(80)*.

3.2. Raf-Mek-Erk/MAPK Pathway

The raf, mek, and erk/MAPK serine/threonine kinases make up the core transducers in the mitogen-activated protein kinase (MAPK) signaling cascade, a network which controls cell proliferation, motility and survival *(110)*. Research has revealed that the once simple 'linear pipeline' concept of MAPK signal transduction is no longer valid. The observations that a variety of raf, mek, and erk/MAPK isoforms and regulatory molecules (such as Sprouty proteins) exist and that compartmentalization of various pathway constituents and effectors occurs complicate the scheme *(110)*.

The raf-mek-erk/MAPK pathway is activated in cultured hepatocytes after growth factor stimulation *(38)* and during liver regeneration *(111)*. Upregulation of the pathway is also seen in human liver tumors. Schmidt et al. observed that mek and erk/MAPK isoforms were significantly overexpressed in human HCC tissues as compared to adjacent liver tissue by Western blot; in addition, they determined that erk/MAPK protein levels correlated with increased erk/MAPK kinase activity in the tumor samples *(112)*. Elevated erk/MAPK expression *(113)*, phospho-erk/MAPK levels *(114)*, and erk/MAPK activity *(115,116)* in HCCs were also noted by others.

Sprouty (Spry) proteins and SPREDs (Sprouty-related proteins with an Ena/vasodilator-stimulated phosphoprotein homology-1 domain) are newly discovered negative regulators of the raf-mek-erk/MAPK pathway. Sproutys reside in the cytosol until they are recruited to the inner plasma membrane following RTK activation. There, they partner a variety of scaffolding proteins and signal transduction molecules, including raf itself, to control signal propagation of the raf-mek-erk/MAPK cascade *(117)*. SPREDs appear to function in a similar manner *(118)*. Recently, HCCs were examined for Sprouty-2 (Spry2) expression: 73% of tumors (8/11) expressed significantly less Spry2 mRNA than non-tumor liver tissue. However, neither LOH at the Spry2 locus nor hypermethylation of the Spry2 gene promoter was detected to account for the dampened expression *(119)*. Yoshida and coworkers *(116)* observed that mRNA expression of either SPRED-1 or -2 was downregulated in 84% (27/32) of HCCs as compared to adjacent liver. In over two-thirds of those cases (68%, 22/27), repression of both SPRED-1 and -2 mRNA levels was noted *(116)*.

Two small molecule inhibitors that target the raf-mek-erk/MAPK cascade are presently under clinical investigation for human HCC. The first, and least characterized, is AZD6244, an orally available drug that targets mek. Recruitment for a pair of Phase II clinical studies examining AZD6244 in advanced HCC is proceeding *(80)*. The second inhibitor of the raf-mek-erk/MAPK pathway to be studied in humans with HCC is sorafenib, a multikinase inhibitor with activity directed against raf and certain cell surface RTKs. Promising preliminary results of a randomized, double-blind, multicenter Phase III clinical trial examining the efficacy of sorafenib vs. placebo in patients with advanced HCC (the SHARP study) have been released *(120,121)*. These data prompted the US FDA to approve sorafenib use for HCC in late 2007 and to recommend it as a first-line therapy in patients with advanced, unresectable HCC with mild to moderate liver impairment (Child-Pugh class A or B) *(122)*, thus making sorafenib the first targeted therapeutic for HCC to obtain FDA approval.

Findings from the SHARP study revealed that treatment with sorafenib was associated with an increased median time to progression from 2.8 months with placebo to 5.5 month with therapy. Over 60% of patients on sorafenib demonstrated progression-free survival at 4 months compared to only 42% in those receiving placebo. However, no complete responses were noted in the treatment group, and only 2.3% of those treated with sorafenib showed a partial response as compared to 0.7% of patients receiving placebo *(120,121)*, suggesting that sorafenib stabilizes, rather than cures, advanced HCC *(120)*. Several additional clinical trials of sorafenib in HCC are underway *(80)*.

4. OTHER THERAPEUTIC TARGETS: PRESENT AND FUTURE

Most targeted therapeutics under evaluation in human HCC have been developed against RTKs and their immediate downstream signal effectors; however, treatments directed toward other molecular targets are also being tested. Some of the more noteworthy include those which inhibit proteasomal degradation (bortezomib) and histone deacetylation (belinostat).

The proteasome comprises a large multi-subunit drum-shaped enzymatic complex that degrades damaged or excessively abundant proteins. Protein substrates destined for proteasomal degradation are tagged with ubiquitin, a small protein marker of about 8 kDa in size, by one of several ubiquitin E3 ligases. The relative abundance of a variety of proteins is managed by the proteasomal pathway. Evidence suggests that proteasomal blockade in cancer cells, including HCC, increases their susceptibility to undergo apoptosis *(123)*. One mechanism sensitizing HCC cells to apoptosis may be due

to upregulation of receptors for the death ligand, Trail, and to increased DISC formation *(124)*. The clinical efficacy of proteasomal inhibition with bortezomib was confirmed in the treatment of multiple myeloma *(125)*. Currently, Phase II trials of bortezomib in patients with advanced HCC are underway *(80)*.

Acetylation or deacetylation at specific terminal lysine residues in histones impacts chromatin structure, gene promoter access, and transcriptional regulation by promoting chromatin accessibility or condensation, respectively. Histone deacetylases (HDACs) are responsible for removing acetyl groups from terminal lysine residues in histones, thus allowing DNA to compact into heterochromatin repressing gene transcription. HDACs are increasingly recognized as important contributors to tumorigenesis; as such, HDAC inhibitors have been developed which, among other activities, lead to reactivation of pro-apoptotic gene expression and suppressed cancer cell growth in culture *(126)*. For example, in human HCC cell lines, exposure to the HDAC inhibitor trichostatin-A resulted in cell cycle arrest, apoptosis, and hallmarks of hepatocyte differentiation *(127)*. Upregulated HDAC expression has been observed in human liver tumors and correlated with a higher incidence of portal vein invasion and poor histologic differentiation *(128)*. One HDAC inhibitor belinostat completed a Phase I clinical trial in patients with advanced solid tumors and showed a favorable toxicity profile, a dose-dependent effect on HDAC activity and disease stabilization in over a third (39%) of patients *(129)*. Recruitment for a Phase II clinical trial of belinostat in patients with advanced HCC is ongoing *(80)*.

One molecular target in HCC primed for clinical assessment is the receptor tyrosine kinase Met, the ligand of which is HGF. Clinical trials with three different Met inhibitors are progressing, mostly for solid tumors including pancreatic and gastric carcinoma, but as of now, no trials specifically geared toward liver cancer have been initiated *(80)*. As mentioned earlier, the HGF-Met axis is a highly relevant hepatic signaling system. Its function is paramount to hepatic development *(130–132)*, hepatocyte replication, motility *(38)* and survival *(133,134)*, and to liver regeneration *(40)*. In addition, Met dysregulation is seen in most human HCCs *(135–137)*, and its overexpression is associated with the presence of intrahepatic metastases and poor patient outcome *(136)*. Met dysfunction in human HCC can also occur through activating mutations in the Met gene *(138)*. Overexpression of HGF in human liver tumors has not been a consistent finding *(137,139)*, but enforced overexpression of HGF in hepatocytes is oncogenic in a mouse model *(140)*.

The next decade should give the oncology community the necessary time to determine whether targeted small molecule therapeutics work well to stabilize or cure HCC. More likely than not, combination therapies, either as cocktails of molecularly targeted treatments or as mixtures of conventional

cytotoxic agents and targeted drugs, will yield the greatest clinical benefit for liver cancer patients with unresectable disease. The outcomes of these studies are eagerly awaited.

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