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Hepatocellular carcinoma stem cells: origins and roles in hepatocarcinogenesis and disease progression

Yi Shen[1], Deliang Cao[1]

[1]Department of Medical Microbiology, Immunology, and Cell Biology, Simmons Cancer Institute, Southern Illinois University School of Medicine. 913 N. Rutledge Street, Springfield, IL 62794, USA

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1. ABSTRACT

Hepatocellular carcinoma (HCC) is a treatment-resistant malignancy with an increasing incidence worldwide. More than 500,000 individuals suffer from this disease annually. Risk factors for human HCC include hepatitis B and C infections, dietary aflatoxin, alcohol abuse, smoking, and oral contraceptive use. Accumulating evidence suggests that liver stem cells play a critical role in HCC development and progression. Dedifferentiated hepatocytes, hepatic oval cells and bone marrow cells are the three major types of liver stem cells, and CD133, CD90, and EpCAM are identified as specific antigenic markers for HCC stem cells. Wnt, Hedgehog, and the angiogenic signalings are main pathways that regulate the HCC stem cell self-renewal and pluripotential, and may be potential targets for novel therapeutic strategies of this malignancy. This review article provides an update in the studies of live and HCC stem cells.

2. INTRODUCTION

Primary liver cancer is a global health concern with over 500,000 new cases diagnosed annually. This disease is the third leading cause of cancer deaths throughout the world, and is ranked at the fifth most frequent cancer in men and the eighth in women (1, 2). Primary liver cancer is comprised of two major types, hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC). HCC is a main pathological subtype, accounting for 80% of total primary liver malignancy. HCC incidence is highly correlated with geographical areas, and more than 80% of cases are claimed in South Asia, such as Japan and China (1, 3, 4). Although HCC is relatively rare in the United State, its incidence is almost doubled during the past 3 decades. Similar tendency is seen in Canada and Western Europe (5).

Dietary aflatoxin, excessive alcohol intake, cigarette smoking, and oral contraceptive use are identified

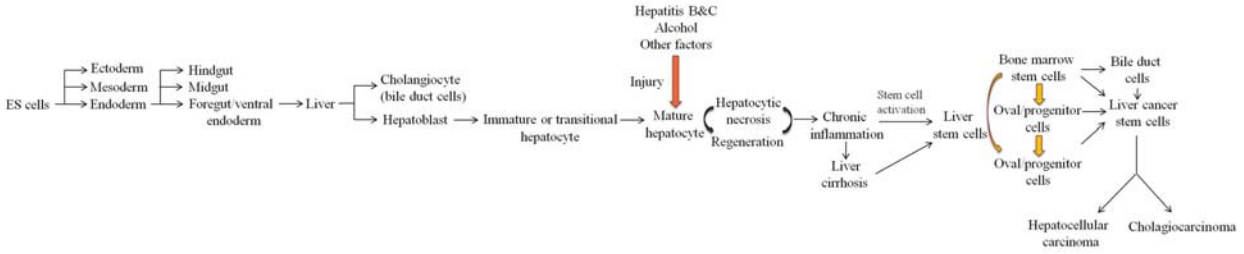

Figure 1. The lineage of hepatocarcinogenesis *in vivo*. Livers are derived from pluripotent embryonic stem (ES) cells that proliferate and differentiate to two major cell types, hepatocytes and cholangiocyte. The liver is vulnerable to various pathogens and toxic factors, such as hepatitis B virus, hepatitis C virus, alcohol, and dietary aflatoxin. Stem cells, hepatic-originated and non- hepatic-originated, participate in the hepatocarcinogenesis. Chronic inflammatory microenvironment favors the transformation of normal liver stem cells to cancer stem cells (CSC) through the deregulation of self-renewal pathways.

as risky factors for HCC, but in most prevalent countries, up to 80% of HCC arise in hepatitis B (HBV) or C (HCV) infections and cirrhosis (1, 4, 5). Globally, about three-quarters of liver cancer cases and half of mortalities are attributed to chronic hepatic viral infection (2). HBV and HCV are both prevalent in developing countries and are frequently transmitted through blood or body fluids. They are also passed from parental to filial generation during pregnancy.

Although the etiology and pathogenesis of primary liver cancer remains unclear, recent studies have shown that liver stem cells play a critical role in hepatocarcinogenesis and disease progression. This review updates recent studies on normal and cancer stem cells (CSC) of the liver, in terms of their role and regulation in liver development and regeneration and hepatocarcinogenesis. Signalings that regulate the CSC in HCC are discussed and therapeutic approaches targeting CSC are reviewed. Notably, current efforts on CSC studies in HCC have significant clinical implications in its diagnosis, prevention, and treatment.

3. STEM CELLS AND LIVER DEVELOPMENT AND REGENERATION

3.1. Liver development

Liver development undergoes three key stages: specification, budding, and differentiation (6). Through liver embryogenesis, pluripotent embryonic stem (ES) cells raised from inner cell-mass differentiate into three principal germ layers: ectoderm, mesoderm, and endoderm (Figure 1). Anterior segment of definitive endoderm specifies into foregut endoderm, from which the endodermal cells start to proliferate and bud into the septum transversum mesenchyme (STM) (6, 7). By performing fate-mapping, it is understood that two parts of the embryonic endoderm give rise to the liver, i.e., the lateral domains in the ventral foregut and a small pack of cells along with the ventral midline (8). During the fusion of medial and lateral domains, the tissue-specific foregut endodermal stem/progenitor cells sense the developmental signals and specify to a hepatic fate. During the course of liver development, hepatoblasts are bipotential and able to differentiate into either hepatocytes or cholangiocyte (bile duct cells), through a process of immature or transitional hepatocytes to mature hepatocytes (Figure 1) (9, 10). Overall, the development of fetal liver is a systematic process that requires many cellular signals, which are crucial and may be derived from multiple cellular origins, including STM, cardiac mesoderm, hematopoietic stem cells (HSCs), and endothelial cells, as well as extracellular matrix (ECM) (11, 12).

Liver development also requires the participation of normal hepatic stem cells that are characterized with self-renewal and multilineage differentiation potential (11, 13). It has been reported that mouse primitive hepatic progenitor cells seeded in the recipient spleen can migrate to the liver and undergo differentiation into liver parenchymal cells (13, 14). Further evidence indicates that in the development of the liver, the differentiation of hepatic stem cells to hepatocytes and cholangiocytes provides cell materials for the reconstitution of the liver and bile ducts (13, 15).

3.2. Liver regeneration:

The normal adult liver plays an important role in governing physiologic homeostasis in the body and is widely involved in various metabolic processes, such as synthesis, storage and redistribution of nutrients. The liver is also an important detoxicant organ, protecting the body from various xenobiotic lesions by metabolic conversion and biliary excretion (16, 17). Therefore, the liver is featured with considerable self-regeneration capacity in response to hepatectomy and toxic/ viral infection damage (16, 18). In other words, the lost hepatic mass can be compromised by the proliferation of mature hepatocytes and/or other hepatic progenitor cells, such as hepatic stem/progenitor cells and bone marrow stem cells (7, 16, 18, 19).

In an adult liver, mature hepatocytes account for over 80% of the cell population, which remains quiescent and seldom proliferate in normal conditions. When a liver experiences partial hepatectomy or undergoes moderate toxic injury, hepatocytes re-enter cell cycle, undertake a serial growth and proliferation from dormant hepatocytes and cholangiocytes to hepatic stellate cells and endothelial cells, and eventually restore the original mass and functions of the liver (7, 17). Studies in rodent models have demonstrated that the restoration of the normal mass can be

accomplished within 3 days after standard partial hepatectomy. In the case of extensive two-thirds hepatectomy, remaining cells could reconstruct adequate numbers of preoperative cells within 10 days of post-resection (7, 17).

Hepatocyte regenerative capacity could be substituted by liver facultative epithelial progenitor cells (hepatic stem/progenitor cells), referred to as "oval cells" in rodents, when the liver undergoes severe chronic injury and normal hepatocytes are inadequate to proliferate and regain organ function (20). Studies in injured rodent models have indicated that oval stem/progenitor cells are a reserved compartment that positions on the smallest branches of the intrahepatic biliary tree. These cells possess bipotential capability of differentiating into both small basophilic hepatocytes and biliary epithelial cells. It is understood that the differentiation level from oval stem/progenitor cells to mature hepatocytes is directly correlative to the degree of chronic inflammation and fibrosis in the disease liver (6, 7, 17). What is interesting is that when rodents are fed with peroxisome proliferators, certain carcinogens, or methionine-deficient diet, the differentiation potential of oval cells is not restricted to the hepatocyte lineage, but also to intestinal glandular epithelium or pancreatic-like tissues in the liver (21).

Numerous signaling pathways are involved in the regulation of wound healing processes during liver regeneration. For example, tumor necrosis factor (TNF)-α and interleukin-6 are key cytokines that trigger the signaling pathways for DNA synthesis of hepatocytes and initiate liver remodeling. Studies in the expression of immediate early genes during hepatocyte proliferation have demonstrated that IL-6 and TNF-α can restore the sensitivity of the liver to growth factors, such as hepatocyte growth factor (HGF), heparin-binding epidermal growth factor-like growth factor, epidermal growth factor, and transforming growth factor (TGF)-α (22). Interestingly, the rebuilding of liver is also contributed by Kupffer cells that participate in regeneration process with or without the regulation by the TNF-α pathway, and the preference is mostly dependent on the stages of the liver regeneration (23-26). In the initiation phase of liver regeneration, Kupffer cells are capable of stimulating hepatocyte proliferation via producing TNF-α. However, the increasing levels of TNF-α are compromised by TGF-β which induces a negative feedback to the regeneration process and lead to the termination phase of liver regeneration. It is believed that certain subpopulation of Kupffer cells may invoke the termination phase of liver regeneration through modulating the levels of TGF-β and IL-1β (23-25, 27).

3.3. Effects of liver regenerating process on tumor growth

Although liver regeneration is an important curative strategy for damage, animal studies suggest that molecular factors that facilitate the liver regeneration process may also favor tumor growth and metastases (28-33). Clinical data also shows that metastatic tumors have eight times higher growth rates in the patients who had liver hepatectomy than in normal liver parenchyma (34). As discussed above, Kuppfer cells participate in the liver regeneration process by producing pro-inflammatory cytokines and growth factors, all of which are also stimulators of metastases and growth of tumors in the liver remnant (35). For instance, HGF stimulates hepatocyte proliferation in normal liver regeneration, but it is also a promoter of angiogenesis and cell motility, inducing alterations of tumor cell matrix. It has been found that HGF overexpression is correlated to motility and invasive characteristics of malignant cells (36-38). It is noteworthy that the cytokines TNF-α and TGF-β may show an opposite function in cancer cell growth and proliferation, serving as tumor suppressors. It has been reported that TNF-α inhibits the liver cell proliferation and promotes apoptosis, and studies on Kupffer cells have proposed that the depletion of this cell type results in an immunosuppression via the TNF-α pathway in liver metastases (39, 40). The timing and dosage of TNF-α administration, as well as the stages of the liver remodeling process, significantly influences the progression of tumor metastases (39, 41, 42).

4. LIVER STEM CELLS AND HEPATOCELLULAR CARCINOMA

4.1. Cellular origins of hepatocellular carcinoma

The concept of cellular origins of HCC is controversial. In the early 1980s, scientists proposed that the de-differentiation of mature liver cells is the cause of liver cancer. In chemical-induced HCC rat models, investigators found that chemical exposures of animals led to the formation of abnormal foci of hepatocytes and preneoplastic nodules in the liver (43). This theory is further supported by studies on alpha-fetoprotein (AFP) and its correlation with hepatic cancer progress and prognosis (44, 45). AFP is a fetal-specific glycoprotein that is synthetically repressed in the normal adult liver. However, an increased serum level of AFP is observed in many HCC patients, and AFP-positive proliferating oval cells are successfully isolated from carcinogen-exposed liver tumors, suggesting the hepatic origin of HCC (43, 45, 46). Currently, AFP is used as a key diagnostic marker of HCC.

In recent years, extensive animal modeling of chemical hepatocarcinogenesis raises a novel hypothesis that maturation arrest of liver stem cells may be the cellular founder of primary hepatic malignancies, such as HCC, teratocarcinoma, and cholangiocarcinoms (43, 47, 48). This idea was first articulated by Van Rensselaer Potter and colleagues in the early 1970s, who proposed that primary liver cancer would rather be due to blockage during the development of immature liver cells than de-differentiation of mature cells (49-51). However, this concept was challenged by the fact that in addition to HCC, fetal type liver enzymes are also present in preneoplastic nodules (52). Currently, the cells in nodules are no longer considered to develop cancer, but rather act as protectors to remove toxicity of carcinogens (43). Interestingly, chemical carcinogenic studies of hepatoblastoma suggest that other than ES cells, periductular oval cells and adult ductal liver progenitor cells give rise to HCC in adult animals.

Hepatoblastoma is most prevalent in young animals or human infants, characterized histologically with less differentiated cell phenotypes, which suggests an early proliferation stage of HCC developmental lineage, known as infant liver stem cells.

4.2. Malignant transformation of liver stem/progenitor cells

It is now accepted that liver cancer is a disease derived from malignant transformation of stem/progenitor cells. However, the identification of the founder cells for the two major liver cancers, HCC and CC, is a challenge because other than the continually renewing tissues such as gastrointestinal epithelium, hepatic progenitor cells (HPCs) and mature hepatocytes possess both longevity and longer repopulating potentials (47). Studies in hepatocarcinogenesis have shown that at least three distinct cell types, hepatocyte, oval cells (HPCs) and bone marrow cells, may 'inherit' the genotoxic injury and lead to neoplastic transformation in the liver (53). Animal modeling has indicated that the injury of mature hepatocytes can give rise to HCC, and oval cells are the target of highly risk carcinogens. Bone marrow-originated cells are more characterized in the process of periductular cell liver damage (54).

4.2.1. Hepatocytes

Hepatocarcinogenic studies have indicated the direct involvement of hepatocytes in HCC. In rat models, by tracing the β-galactosidase-expressing cells labeled by retroviral vector, Gourna and Bralet's groups (55, 56) have both noticed the β-galactosidase-positive hepatocytes at the completion stage of liver regeneration after a two-thirds partial hepatectomy. More specifically, in diethylnitrosamine (DEN)-induced HCC, Bralet and colleagues found that 17% of tumor cells were β-galactosidase positive, suggesting that the mature hepatocytes serve as a random colonial origin of HCC. In addition, animal studies have also shown that liver injury promotes the effect of genotoxic carcinogens, especially when liver tissues are undergoing a proliferation where 30-40% of hepatocytes are in S phase or during partial hepatectomy, necrogenic insult, or postnatal growth (57). It is noteworthy to know that hepatocytes are a major cell type that immediately responds to liver damage and therefore, it is more likely to become the origin of malignant transformation.

4.2.2. Hepatic progenitor cells (oval cells)

Increasing evidence suggests that hepatic progenitor cell (oval cells) activation (ductular cell reaction) is inextricably linked to hepatocarcinogenesis. Oval cells are known as the least bipotent cells among the three potential cancer stem cells (mature hepatocytes, oval cells, and bone marrow cells) for HCC, and are able to proliferate into hepatocytes and cholangiocytes (58). The oval cells may be a more plausible cell target for most HCC models because a mixture of mature cells and the cells phenotypically similar to oval cells is observed in many hepatic tumors (47, 48). Such cells include a population of small oval-shaped cells with OV-6, CK7 and CK19 expression and/or cells that undergo morphological changes, transforming from normal hepatic progenitor cells to malignant hepatocytes (59). In addition, oval cells are the major cell type that is infected by HBV during the chronic liver damage, which may increase the possibility of being a cellular target of carcinogens (55).

The key role of oval cells in the development of HCC is further illustrated by a CDE dietary (a diet deficient in choline and supplemented with 0.5% ethionine) mouse model. In this modeling, pre-treatment of animals with imatinib mesylate, an anticancer drug for *c-Kit* mutation cancer, reduces liver tumors and this may be ascribed to the blockage of oval cell expansion (60). This finding is consistent with the concept of stem cell maturation arrest (47, 48). Factually, a range of oval cells are found in HCC to be arrested in the 'transitional stage' with neoplastic phenotypes, not fully differentiated into hepatocytes (61).

4.2.3. Bone marrow stem cells

Early studies of bone marrow stem cells in liver diseases have shown their potential in improving the fatal metabolic liver damage (62). Although the mechanism remains unclear, the role of bone marrow-derived multipotent adult progenitor cells (MAPCs) in the histogenesis of HCC has been evident experimentally. When cultured with grow factors, such as FGF4 and HGF, MAPCs differentiate into functional hepatocytes with expression of several liver-specific markers, such as epithelial cell adhesion molecule (EpCAM) and AFP (63-65). However, Lee and co-workers found that different from the normal hepatoblast-derived hepatocytes, bone marrow-derived hepatocytes have only the capability of uptaking low-density lipoprotein (LDL) (64). An interesting finding, however, was reported by Ong, *et al. (*66). Co-culture of human bone marrow mesenchymal stem cells (BM-MSCs) with rat liver slices derived from gadolinium chloride ($GdCl_3$)-treated rats led to alterations of hepatocyte function, such as albumin and urea production. This fact may suggest the benefit of some pro-inflammatory cytokines, such as TNF-α, in promoting differentiation. In fact, two separate reports exhibited the therapeutic effect of BM-MSCs in liver injuries induced by CCl_4 and N-nitrosodimethylamine (DMN) (67, 68). BM-MSCs improve the function of injured liver in rats, such as albumin and glutamic-oxaloacetic transaminase (GOT) production.

4.3. Precursor lesions in the evolution of hepatocellular carcinoma

Similar with the development of other types of cancer, hepatocarcinogenesis is a chronic process that always requires the progressive accumulation of genetic mutations and alterations, and is followed by angiogenesis and metastasis. Besides the normal tumorigenic routine, however, HCC may be derived from a serial malignant transformation of liver parenchymal cells. Clinical data shows that hepatocarcinogens, such as HBV, HCV and alcohol abuse with chronic liver inflammation, regeneration and fibrogenesis, could accelerate the cancerous progression (69). During this inflammatory process, morphological changes of liver tissues are significant and are widely accepted as 'preneoplastic lesions'. Two major

abnormal structures are prevalently observed in these lesions and referred as dysplastic foci and dysplastic nodules, respectively (70).

Dysplastic foci are microscopic lesions (<1mm) that are comprised by groups of deformed hepatocytes. Two major subsets of dysplastic foci are identified, known as small cell dysplasia (SCD) and large cell dysplastic foci (LCD), respectively (70, 71). The SCD is highly associated with HCC from cirrhotic liver diseases (72). The SCD and LCD are identified by morphology of hepatocytes that exist in the structure. For example, hepatocytes in SCD have relatively small volumes of cytoplasm and nuclear polymorphisms, but a larger nucleo-cytoplasmic ratio compared to the hepatocytes in LCD (70, 73). Both SCD and LCD are convinced of the preneoplastic lesions of HCC (3-54, 55), and induce cirrhotic liver damage through regulating DNA contents in the cell proliferation cycle (70, 74-76). It is understood that the DNA contents are decreased in SCD foci, but increased in LCD, and thus SCD may serve as early precursor lesions, while LCD are the direct precursor lesions of HCC (77).

On the contrary, dysplastic nodules are the moderated morphological changes during the development of HCC in cirrhotic liver tissues (78, 79) and are defined as macroscopic lesions in liver malignant progress. Dysplastic nodules are divided into low grade (LGD) and high grade (HGD) types (80). Similar to dysplastic foci, chromosomal abnormalities are directly involved in the nodular regeneration and progression (81, 82). In both animal and clinical studies, HGD has been proved to recapitulate the resemblance of vascular and metastatic features for HCC (83).

5. HEPATOCELLULAR CARCINOMA STEM CELLS

5.1. Cancer stem cells

In 1855, Rudolph Virchow first proposed the concept of “embryonal-rest” in the study of stem cell differentiation (84). However, not until the past decade, more compelling evidence has emerged in support of cancer stem cells (CSC) for carcinomas, including hematological malignancies and breast, liver, prostate, colon and brain cancers (85).

Cancer stem cells are regarded as the germinal center of tumor evolution, and possess similar features to normal adult stem cells, such as self-renewal capacity and differentiation potential (86). CSC isolation can be approached by their distinct immunogenic and functional properties from other cell types. Using antigenic assessments, several CSC markers have been identified for evaluating the involvement of CSC in cell morphological changes, anchorage-independent growth, asymmetric division, chemo-resistance, and pluripotency. However, the knowledge of CSC markers is still limited, and a single marker is not sufficient to characterize CSC, and both antigenic and functional properties need to be taken into consideration for the identification of CSC in different types of cancers.

5.2. Deregulation of cell cycle during hepatocarcinogenesis

To understand the role of CSC in hepatocarcinogenesis, it needs to be answered how the CSC are deregulated and eventually lead to the tumor initiation, metastasis and relapse. Studies in hepatic CSC have shown an increase in the expression of proliferative E2F factors during the priming phase of a cell cycle (87, 88). E2F proteins are key mediators for the G1/S progression of the cell cycle, and their transcription activity is regulated by binding with pocket proteins, pRb and p130, in early G1 phase and quiescent cells. Phosphorylation of pocket proteins by cyclin D1/ cyclin-dependent kinases (CDK) 4 and 6 or cyclin E/ CDK2 complexes releases the E2F proteins that sequentially activate their downstream gene expression, forwarding cell cycle (89). During liver carcinogenesis, this feed-forward loop is extremely activated in the early stage and the increased E2F in turn upregulates cyclin D1, forming a vicious loop (90, 91). Another protein that catches eyeballs of researchers is Foxm1b. This protein is a forkhead transcription factor controlling G2/M transition, and is also upregulated in human HCC (92). Recent studies revealed that Foxm1b disrupts the ongoing of DNA synthesis and mitosis in the late G1 phase, stabilizes p21, and reduces cdc25A and cdc25B (93, 94).

Genes associated with mitosis are also frequently attacked in HCC, leading to the de-regulation of mitotic spindle assembly, defects in chromosome segregation, and ineffectiveness of cell cycle checkpoints. Modern molecular bio-techniques have identified a cluster of transcriptional factors involved in the G2/M and S phases of the hepatocyte cell cycle, such as Aurora kinases, bul1b and survivin (95-97). Mutations or overexpression of these factors results in a cytogenetic insult called aneuploidy, due to inappropriate segregation of chromosomes during mitosis (98). This defect is specially characterized in human HCC (69, 95, 96), and an accelerated liver carcinogenesis is observed in the diethylnitrosamine-induced mouse model that is aimed to clarify the importance of accurate chromosome segregation (97-99).

Hepatocyte proliferation is self-terminated through a negative feedback when the liver reaches the size and sufficient functional capacity, and p53, p21, p27 and p18 are important suppressors to halt cell cycle progression (19, 90). It has been reported that p53 inactivation induced by hepatitis B x (HBX) protein stimulates hepatocarcinogenesis in an HBx transgenic mice (100). Recent studies have shown that p53 mutation may not only accelerate the tumor progression, but also stimulate the regeneration of nodules (101, 102).

5.3. Cell surface marker and tumorigenicity of hepatocellular carcinoma stem cells

Studies on liver CSC have identified CD133, CD90, and EpCAM as specific antigenic markers. CD133 was first discovered as a hematopoietic marker, but its value in liver CSC has been recently confirmed (103, 104). CD133 is positive in up to 65% of HCC cell lines, and may contribute to the tumor initiation. The $CD133^{+}$ cancer cells

exhibit many stem cell characteristics. They are capable of self-renewal and forming colonies *in vitro*, differentiate into anigomyogenic cells (a non-hepatocytic lineage), and sustain to high chemotoxic dosage (105). CD90-positive rate is much lower in human HCC cells compared to CD133. CD90 was proposed as mesenchymal stem cell marker in early studies, but tumorigenic property of $CD90^+$ HCC cells has been proved in recent studies (106-108). In addition, $CD90^+$ cells with or without co-expression of additional surface markers demonstrate more progressive phenotypes of HCC. For example, $CD90^+/CD45^-$ cells are prevalent in human HCC tumors and blood samples (69, 106), and $CD90^+/CD44^+$ cells induce more severe metastatic lesions (107, 108). Using EpCAM as a cell surface marker, Yamashita's group classified HCC into two subtypes with different expression levels of AFP and EpCam. Wnt/b-catenin signaling pathway participates in CSC-like characteristics and tumorigenicity of $EpCAM^+$ cells, and antibody-induced blockade of $EpCAM^+$ cells diminishes the formation of tumors and metastasis (109, 110).

5.4. Cancer stem cell signaling in hepatocellular carcinoma

Two predominate pathogenic events are involved in hepatocarcinogenesis. One stands for the cirrhotic lesions, and the other indicates important gene mutations. Hepatitis viral infections, toxins, and metabolic disorders induce cirrhosis and focal regeneration; and tumor oncogene or suppressor gene mutations lead to mitotic abnormalities and abnormal cell growth and proliferation (111-114). Both pathogenic mechanisms associate with disruptions in signaling pathways, ushering hepatocarcinogenesis. Among these growth factors that mediate angiogenic signaling, the tyrosine kinase receptor and Wnt/b-catenin pathways are most important in maintaining adult stem cells and liver CSC, which may serve as potential prognostic biomarkers and targets for new therapeutic strategies to HCC (7, 76, 115-118).

5.4.1. Angiogenic signaling

Tumor growth and metastasis highly rely on effective angiogenesis (119). Liver is the most vascular organ that requires sufficient angiogenesis for regeneration. Normal liver angiogenesis is maintained by a balance between pro- and anti-angiogenic factors, but this balance is interrupted in HCC (119-121). In addition, vascular microenvionment is remodeled through autocrine and paracrine interactions among tumor cells, vascular endothelial cells and pericytes (122). Angiogenic factors produced by these cells lead to vascular hyperpermeability that often associates with a serial processes, including reconstruction of cellular matrix, recruitment and activation of endothelial cells and pericytes, and formation and stabilization of new blood vessels (122).

Upregulated angiogenic growth factors in surgical HCC specimens includes vascular endothelial growth factors (VEGF-A), angiopoietins (Ang2), platelet-derived growth factors (PDGF), transforming growth factor (TGF)-α and β, and basic fibroblast growth factors (FGF) (120). These growth factors and cytokines activate cascades of angiogenic signalings, including ERK, PI3K, AKT, mTOR, RAF and Janus kinase (JAK) (123). It is understood that the expression of VEGF links with the disease relapse, massive vascular invasion and poor survival rate (124, 125).

5.4.2. Wnt/β-catenin signaling

Novel evidence suggests that Wnt/β-catenin pathway is not only involved in colorectal cancer, but also in HCC (126, 127). Wnt signaling abnormalities could be induced by mutational and non-mutational events, and result in the disruption of embryonic development (128). In the colon, abnormalities of Wnt pathway result from APC (adenomatous polyposis coli) inactivation and subsequent nuclear localization of β-catenin (129, 130). On the contrary, APC mutation is rare in HCC, whereas β-catenin mutation is more frequent (131, 132). Interestingly, increased Wnt/β-catenin signaling and its downstream mediators have been observed in $CD133^+/EpCAM^+$ liver CSC (103, 109), suggesting its fundamental role in hepatocarcinogenesis.

5.4.3. Hedgehog signaling

Hedgehog pathway is also involved in liver diseases. Like Wnt/β-catenin signaling, Hedgehog pathway was first identified as a critical signaling in controlling the homeostasis of gastrointestinal system (133), and the activation of this signaling was observed in $CD44^+/CD24^+/EpCAM^+$ pancreatic CSC, particularly at the invasive stage of the disease (134). The binding with Hedgehog receptor of ligand, Patched, favors the nuclear translocation and accumulation of Gli and induce transcription of genes that are involved in cell cycle, such as cyclin B1, D1, and E, insulin-like growth factor-2 (IGF-2), and β-catenin (133). Study on human HCC samples have shown that Gli is upregulated in more than 60% of tissues, and the blockage of this singling pathway downregulates the expression of Gli-related downstream genes (135, 136).

Clearly, signaling pathways play an important role in nearly every aspect of liver CSC and regulate their differentiation, proliferation and regeneration capacities. However, how to wisely take advantages of these cellular signalings in the clinical liver cancer treatment is a more serious challenge that needs to be overcome in future studies.

6. Therapeutic implications

Effective therapeutic strategies for liver diseases, including acute liver failure, cirrhosis and HCC, are still limited to liver transplantation, but the poor repopulation of new transplants in recipient liver enforces the development of more efficient curative strategies, particularly for end-staged patients. Due to the complexity, targeted therapy has become a plausible approach for cancer management. Up to date, several targeted therapies have been developed for HCC (Table 1). Among them, Apatinib, Bevacizumab, and Vatalanib have shown the capability of improving the progression-free survival time of HCC at an advanced stage (137-140), and Sorafenib is regarded as a new standard of

Table 1. Targeted cancer therapies

Compounds	Targets	Clinical Trials
Apatinib [a]	VEGFR-2	Phase II
Bevacizumab [a]	VEGF-A	Phase II
Cediranib [a]	VEGF	Phase II
Linifanib [a]	VEGFR, PDGF	Phase II/III
Vatalanib [a]	VEGFR (-1, -2, -3), PDGFR, c-KIT	Phase I
Brivanib [a]	VEGFR-2	Phase III
Cetuximab [b]	VEGFR (-1, -2, -3)	Phase II
Erlotinib [b]	EGFR	Phase II
Gefitinib [b]	EGFR	Phase II
Lapatinib [b]	EGFR, HER-2	Phase II
Brivanib [c]	VEGFR-2, FGFR-1	Phase II/III
Sorafenib [c]	VEGFR (-1, -2, -3), PDGFR (-α, -β), c-KIT, p38MAPK, FLT-3, RET	Approved for treatment of HCC
Sunitinib [c]	VEGFR (-1, -2, -3), PDGFR-β, c-KIT, p38MAPK, FLT-3, RET	Phase II/III
Gemcitabine	DNA replication	Phase II
Capecitabine	DNA synthesis	Phase II
Locoregional treatments	Metastasis progression	Phase III
AEG35156 (XIAP antisense)	XIAP (anti-apoptotic protein)	Phase I
LC Bead loaded with doxorubicin	Liver-dominant Metastases	Phase II
OSI-906	IGF-1R	Phase II
ARQ 197	c-MET	Phase I

[a]Anti-VEGF/VEGFR; [b]Anti-EGF/EGFR; and [c]Multikinase inhibitors. VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; EGF, epidermal growth factor; EGFR, EGF receptor; IGF-1R, insulin-like growth factor-1 receptor. Data are cited from www.clinicaltrials.gov

are in advanced HCC (141, 142). However, the clinical outcomes of the HCC patients remain poor, and novel effective therapies are needed.

The identification and investigation of hepatocellular carcinoma stem cells may provide a novel exploration of developing more clinically effective treatment of HCC (143-145). Via interrupting principal pathways regulating their self-renewal and radiochemoresistance, therapies targeting the tumor stem cells may successfully suppress the growth, metastasis and recurrence (146, 147). In fact, the CSC-specific markers have been tested for new therapeutic targets, and *in vitro* studies have shown that silencing of EpCAM using RNAi techniques significantly reduces CSC population, tumorigenicity and invasiveness of HCC cells, and in the case of EpCAM expression cells, the downstream signaling Wnt/β-catenin is also a 'hot spot' of cancer targeting therapies (109). Currently, therapies targeting the surface markers CD133, CD90, EpCAM and CD44, as well as their related signaling pathways, are being actively investigated (148).

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Abbreviations: AFP, alpha-fetoprotein; CC, cholangiocarcinoma; CSC, cancer stem cell; ECM, extracellular matrix; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HGF, hepatocyte growth factor; STM, septum transversum mesenchyme; TGF-beta, transforming growth factor-beta; and TNF-alpha, tumor necrosis factor-alpha

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Send correspondence to: Deliang Cao, Department of Medical Microbiology, Immunology, and Cell Biology, Simmons Cancer Institute, Southern Illinois University School of Medicine. 913 N. Rutledge Street, Springfield, IL 62794, Tel: 217-545-9703, Fax:217-545-9712, E-mail: dcao@siumed.edu

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