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Clinical Implications of Cancer Stem Cell Biology in Hepatocellular Carcinoma

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Abstract

Solid tumors are thought to contain cancer stem cells (CSCs) as a distinct population responsible for tumor relapse and metastasis due to their abilities to self renew, differentiate and give rise to a new tumor in local or distant organs. CSCs have been identified in many tumor types, including hepatocellular carcinoma (HCC), the fifth most common and third most deadly malignancy with observable heterogeneity. Numerous studies have shown that hepatic CSCs could be enriched via different cell surface markers, e.g., CD13, CD24, CD44, CD90, CD133, EpCAM (CD326), and OV6. They could also be identified through functional assays such as isolating the side population cells by Hoechst dye staining or screening cells with a high activity of aldehyde dehydrogenase. Functional characterization of hepatic CSCs has revealed several deregulated signaling pathways, such as Wnt/ β -catenin, AKT, TGF- β , IL-6/STAT3 pathways to be critical in inducing “stemness” of HCC and in promoting self-renewal, tumorigenicity and chemoresistance. An increased understanding of the hepatic CSC biology shed light on the development of new diagnostic, prognostic therapeutic strategies in improving HCC clinical management. In this review, we summarized recent evidence including the identification of hepatic CSCs and its underlying biological mechanisms, and discussed potential clinical implications in HCC.

Malignant tumors are characterized by uncontrolled proliferation of abnormal cells with morphological and functional heterogeneity. Two models, i.e., the stochastic model and hierarchical model, have been proposed to explain tumor heterogeneity. The stochastic model suggests that all tumor cells within tumor bulk are biologically homogenous and, therefore, have an equal capacity to regenerate a tumor. In contrast, the hierarchical model, also termed as cancer stem cell (CSC) model, proposes that only a small subset of tumor cells within tumor bulk exhibit the capacity to initiate and sustain tumor growth. Recently, there has been growing support for the hierarchical model. In this review, we summarized recent evidence built upon the CSC model in hepatocellular carcinoma (HCC) regarding to the identification of CSCs and their underlying biological features, and discussed how one could apply these knowledge to HCC clinical management.

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1. Definition of hepatic CSCs

1.1. The CSC hypothesis

CSCs are cancer cells in tumor that possess characteristics associated with normal stem cells, i.e. self-renewal and differentiation, and the ability to give rise to a new tumor with the phenotype of original one in xenotransplant assays. They are considered to be responsible for tumor relapse and metastasis^{1,2}. About 20 years ago, two studies from Dr. Dick's group reported that the isolated CD34⁺/CD38⁻ leukemic cells are leukemic CSCs³. The authors established that the CD34⁺/CD38⁻ subpopulation is capable of initiating tumors in nonobese diabetic/severe combined-immunodeficient (NOD/SCID) mice, which are histologically similar to the leukemic CSCs donor. The tumors formed in mice are very heterogeneous and contain multiple cell types native to the host organ, indicating that isolated CD34⁺/CD38⁻ leukemic cells possess multidifferentiative potential, a classical hallmark of stem cells. The existence of leukemic CSCs prompted further research into other types of cancer. In 2003, isolation of CSCs from solid tumor was accomplished in breast cancer⁴. Recently, CSCs have also been identified in several other solid tumors, including brain cancer, lung cancer, liver cancer, colon cancer, prostate cancer and pancreatic cancer^{5–10}.

Accumulating evidence indicates that CSCs are tumor cells with the following characteristics: (i) self-renewal, (ii) differentiation, (iii) tumorigenicity, and (iv) chemo/radio-therapeutic resistance. Thus, compared to non-CSC, CSC in the tumor bulk may be responsible for metastasis and cancer relapse in local and distant organs due to its invasive and drug resistant abilities^{11,12}. These unique characteristics can be exploited as clinical applications, e.g., assisting cancer patients in diagnosis and prognosis prediction through detecting these CSC marker expressions, and developing the treatment by targeting CSCs.

1.2. Hepatocellular carcinoma

Liver cancer is the second most deadly cancer for men in the world and about 90% of liver cancers are HCC^{13,14}. Although HCC mainly occurs in Asia and Africa, a rising incidence and mortality have recently been observed in most industrialized countries including the United States in the past decade¹⁵. Resection or liver transplantation is the best option for a potential cure. However, only about 10%–20% of patients with HCC are currently eligible for surgical intervention. Moreover, patients with resection often have a high frequency of recurrence, and postoperative 5-year survival is only 30%–40%. For those inoperable patients, systemic chemotherapy is often offered with limited success. The overall survival rate for them is poor.

HCC has been known as a heterogeneous malignancy at multi-levels. First, it is triggered by various risk factors. Major risk factors include chronic viral hepatitis with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) infection¹⁶. Exposure to aflatoxin B1, hereditary diseases (hemochromatosis, α -1AT deficiency, tyrosinemia, and Wilson disease), and heavy alcohol intake are additional risk factors for HCC¹⁷. Furthermore, recent epidemiological studies suggest that metabolic syndrome such as obesity, type II diabetes, and non-alcoholic fatty liver diseases also increase the risk of HCC¹⁷. HCC is also heterogeneous in terms of its biological behavior and response to treatment^{10,18–22}. For example, primary HCC tissues with a propensity to metastasize or recur have a significantly different gene expression profile when compared to relapse-free HCC tissues^{23–26}. Combination of genomic and transcriptomic analysis identified six robust subclasses with distinct activation of biological pathways and therapeutic implications²⁷. Our recent findings indicate that primary HCCs with low level of microRNA-26 expression are biologically different from HCCs with high level of microRNA-26 based on gene expression profiling and signaling pathway analysis. Moreover, HCC patients with low level of microRNA-26 have a favorable response to

adjuvant interferon alpha (IFN α) therapy²¹. In addition, we also found that a subgroup of HCCs with EpCAM and alpha-fetoprotein (AFP) expression display CSC features such as a high rate of metastasis and poor outcome, as well as stem cell associated gene expression^{10,28}.

1.3. Hepatic cancer stem cells

Similar with other solid tumors, hepatic CSCs exist and contribute to the development of HCC (Figure 1). Several different markers, i.e., EpCAM, CD133, CD90, CD44, CD24 and CD13, have been used as specific cell surface markers to enrich CSCs in HCC. Although these hepatic CSCs are heterogeneous in marker expression and may represent different cellular origins, such as dedifferentiated hepatocytes, hepatic progenitor cells and bone marrow derived cells, they all retain similar “stemness” features, i.e., the abilities to self-renew and differentiate, and be tumorigenic and chemo-resistant. The existence of CSCs in HCC partially explains HCC heterogeneity, HCC metastasis/recurrence after resection and chemotherapeutic resistance of advanced HCC cells. Functional characterization of these hepatic CSCs further revealed that several deregulated molecular signaling pathways might confer and/or maintain features of hepatic CSCs. Moreover, more and more studies have begun to explore a potential utility of the hepatic CSC knowledge in HCC diagnosis, prognosis prediction, and in developing novel HCC therapeutic methodology.

2. Identification of hepatic cancer stem cells

Fluorescence-activated cell sorting and magnetic-activated cell sorting analyses are two common methods in isolating CSCs. Accumulating data have shown that hepatic CSCs could be isolated by several different cell markers, i.e., EpCAM, CD133, CD90, CD44, CD24, CD13, and OV6. They could also be selected through functional assays, such as isolating side population (SP) cells by Hoechst dye staining and screening cells with high activity of aldehyde dehydrogenase (ALDH) (Table 1).

2.1. EpCAM (CD326)

EpCAM is a cell surface molecule that is known to be expressed in almost all of carcinomas. In non-tumor liver tissues, it is expressed in embryonic liver, bile duct epithelium and proliferating bile ductules in the cirrhotic liver, but not in normal adult hepatocytes. It has been identified as a marker for stem/progenitor cells of adult liver and oval cells^{29,30}. Our research group has pioneered the work on the identification and characterization of hepatic CSCs using the EpCAM surface marker. We found that EpCAM is highly expressed in premalignant hepatic tissues, suggesting its role as an early biomarker for HCC³¹. We have developed a novel HCC classification system based on EpCAM and AFP expression, which can divide HCC into four subgroups with prognostic implication, where the HCC subgroup with double positivity of EpCAM and AFP has the worst prognosis²⁸. Gene expression profiling revealed that EpCAM⁺AFP⁺ HCCs and EpCAM⁻AFP⁻ HCCs resemble liver lineages. In particular, EpCAM⁺AFP⁺ HCCs display distinct features of hepatic stem/progenitor cells and have poor outcome, high metastasis, and stem cell-associated gene expression and signaling pathway activation. Meanwhile, EpCAM⁻AFP⁻ HCCs display features of mature hepatocytes with fair prognosis and gene expression associated with mature hepatocyte function^{10,32}. Moreover, isolated EpCAM⁺AFP⁺ HCC cells from HCC cell lines and clinical primary HCC specimens exhibit hepatic CSC-like traits. They possess not only the abilities to self-renew and differentiate but also highly chemoresistant to doxorubicin and 5-fluorouracil treatment. They are capable of initiating highly invasive HCC in NOD/SCID mice, even after serial transplantation. As few as 200 isolated EpCAM⁺ HCC cells from HCC cell lines and 10,000 isolated EpCAM⁺ HCC cells from primary AFP⁺ HCC tissues could generate tumor nodules in NOD/SCID mice. Yet EpCAM⁻ cells do not

show these properties. Of note, a majority of EpCAM⁺ HCC cells are also positive for CD133, another hepatic CSC marker.

2.2. CD133 (Prominin 1)

Human CD133, or prominin1, a 5-transmembrane domain glycoprotein, is an important cell surface marker for both stem cells and CSCs in various tissues including liver. The role of CD133 as a CSC marker has been documented in cancers of brain, prostate, pancreas, and colon. CD133⁺ HCC cells were first suggested to represent a potential CSC subpopulation by Suetsugu et al. Isolated CD133⁺ HCC cells from Huh7 HCC cell line were shown to have higher proliferative and tumorigenic potential and to express lower levels of mature hepatocyte markers when compared with their CD133⁻ counterparts³³. Similar findings were also reported in a CD133⁺ fraction isolated from the HCC cell line SMMC7721³⁴ and PLC8024³⁵. Moreover, the CD133⁺ cells could be induced to differentiate into nonhepatocyte-like lineages, supporting CD133 as a marker of hepatic CSCs. However, recent studies suggest that CD133 might be used with caution as a hepatic CSC marker due to the following issues. First, studies found that CD133 is also present in ductular reactions in both acute and chronically damaged livers³⁶ and it could be not only a hepatic progenitor cell marker but also biliary marker in vivo³⁷. Second, almost all CD133-related experiments carried out to date detect the expression of AC133 and AC141 epitopes, rather than total CD133 protein. Recent evidence suggests that CD133 mRNA and protein seem to be constant upon CSC differentiation in colon cancer, whereas the only AC133 epitope is lost following the differentiation³⁸.

2.3. CD90 (Thy-1)

Thy-1 or CD90 is a 25–37kDa glycosylphosphatidylinositol-anchored conserved cell surfaced protein. It is expressed in many cell types, including T cells, thymocytes, neurons, endothelial cells, and fibroblasts³⁹. It has been considered as a marker for various kinds of stem cells, such as hematopoietic stem cells, murine breast CSCs⁴⁰, and primarily cultured CD133⁺ glioblastoma cells⁴¹. Recent studies have suggested CD90 as another marker for hepatic stem cells and CSCs^{42–45}. Yang and colleagues identified a significant positive correlation of CD90 expression with tumorigenicity and metastatic potentials in the panel of HCC cell lines^{43,44}. Moreover, the authors combined CD90 with CD45, a lymphocytes marker, to isolate nonlymphoid CD90⁺ cells from tumor specimens and blood samples of HCC patients. CD90⁺ cells sorted from HCC cell lines and CD90⁺CD45⁻ cells isolated from primary tumor and blood samples of HCC could initiate HCC nodules when injected intrahepatically into SCID/Beige mice in the first and the subsequent secondary and tertiary transplantation experiments^{43,44}.

2.4. CD44

CD44 is a cell surface glycoprotein, which acts mainly as a receptor for hyaluronic acid. It is involved in cell-cell adhesion and migration and has been showed to be associated with tumor cell invasion and migration in liver cancer⁴⁶. CD44 has been identified as a CSC marker in breast, pancreatic, colorectal, and gastric cancer. In HCC, CD44 is an important marker used in combination with other CSC markers to enrich hepatic CSCs. Cells co-expressing CD133 and CD44, or CD90 and CD44, present a more aggressive phenotype than cells with a positive expression of either CD133 or CD90 alone. Yang et al. showed that CD44⁺ cells develop tumor nodules in immunodeficient mice faster than CD44⁻ cells, whereas lung metastases are only observed in immunodeficient mice transplanted with CD90⁺CD44⁺ cells⁴³. In another study, CD44 was found to be preferentially expressed in a CD133⁺ population in four HCC cell lines, including Huh7, SMMC7721, MHCC-LM3 and MHCC97L. Compared with CD133⁺CD44⁻ cells, CD133⁺CD44⁺ HCC cells are more

tumorigenic and chemoresistant, and express a higher level of “stemness”-associated genes⁴⁶.

2.5. CD24

CD24, a mucin-like cell surface glycoprotein, has been shown to be highly expressed in stem/progenitor cells and has been linked to CSCs derived from breast cancer, colon cancer, ovarian cancer, pancreatic cancer, and squamous cell head-neck cancer. Recently, in searching for markers elevated in self-renewing chemoresistant HCC cells, Lee and his colleagues identified CD24⁺ HCC cells are chemoresistant to cisplatin and capable of self renewal and tumor initiation⁴⁷. Compared to CD24⁻ HCC cells, CD24⁺ HCC cells could form larger and more hepatospheres. As few as 500 CD24⁺ cells from HCC cell lines are sufficient for consistent tumor development in NOD/SCID mice. 4000 CD24⁺ cells from primary HCC specimens could initiate tumors. They further demonstrated that CD24 expression is required for the maintenance of self-renewal, differentiation, and metastasis of tumors and to significantly impact patients' clinical outcome. Moreover, majority CD24⁺ HCC cells are also positive for CD133 and EpCAM.

2.6. CD13 (Aminopeptidase N)

CD13, Aminopeptidase N, is a membranous glycoprotein that plays an important role in cancer progression^{48,49}. Most recently, it was found as a marker for dormant or semi-quiescent CSCs in human HCC cancer cell lines and clinical samples. Haraguchi et al. demonstrated that CD13 is predominantly distributed during G1/G0 phase in HCC cells and commonly enriched in an side population sorted from HCC cell lines⁵⁰. Moreover, CD13⁺ HCC cells possess a high tumorigenic potential in NOD/SCID mice. Researchers also assessed tumorigenic potential of CD13⁺ cells co-expressing CD13 and CD133 or CD90. The results showed that CD13⁺CD133⁺ cells in Huh7 and CD13⁺CD90⁻ fractions in PLC/PRF/5 could initiate tumor formation effectively in limiting-dilution and serial transplantation assays. CD13⁺ cells have also been shown to be highly chemoresistant to doxorubicin and 5-fluorouracil treatment⁵⁰. These results suggested that CD13⁺ cells represent dormant hepatic CSCs in HCC.

2.7. OV6

OV6 is a monoclonal antibody raised against cells isolated from carcinogen-treated rat liver. Several studies have found OV6 positive cells in HCC and hepatoblastoma^{51,52}. Recently, Yang et al. demonstrated that OV6⁺ HCC cells possess greater tumorigenic ability and chemotherapeutic resistance when compared with OV6⁻ cells⁵³. In addition, the CD133⁺ population was significantly enriched in cells positive for OV6, indicating that OV6⁺ might also be a hepatic CSC marker.

2.8. Side population (Hoechst 33342 dye staining)

SP cell-sorting by Hoechst 33342 dye is also used to identify hepatic CSCs⁵⁴⁻⁵⁶. SP cells with low Hoechst staining are thought to be stem cells since adenosine triphosphate (ATP)-binding cassette (ABC) transporters are highly expressed in stem cells, which possess the ability to efflux Hoechst 33342 dye. SP cells isolated from two HCC cell lines (Huh7 and PLC/PRF/5 cells) have been identified as hepatic CSCs. Compared to non-SP population, sorted SP cells possess more stemness gene expression and higher tumorigenicity. As few as 1,000 SP cells could form tumors in NOD/SCID mice and tumorigenicity could be maintained in a serial transplantation⁵⁴. However, there are still some limitations in the use of SP cell sorting for defining hepatic CSCs. First, it is not technically feasible to detect SP cells in HCC samples in situ. Although examination of these ABC transporters, such as ABCG2, was used to localize CSCs, ABCG2⁺ cells seem to contain a larger range of cells

compared with SP cells^{57,58}. Second, transporter protein-expressing cells are likely to suffer from the toxicity of Hoechst 33342 dye. Consequently, SP cell might not grow normally, resulting in the apparent differential properties observed in these functional experiments.

2.9. ALDH

ALDH, a molecular metabolic mediator, was first identified as conferring resistance to cyclophosphamide in normal hematopoietic progenitor cells. Recent studies have suggested that isolated cancer cells with high ALDH activity display features of CSCs and the high ALDH activity can confer chemoresistance in CSCs^{59–61}. Ma et al. found that the expression and activity of ALDH1A1 positively correlate with CD133 expression in HCC cell lines. The combination of CD133 and ALDH is shown to more accurately define hepatic CSCs. A hierarchical organization of cells with the differential expression of CD133 and ALDH exhibit an ascending tumorigenic potential in the order of CD133⁺ALDH⁺ > CD133⁺ALDH⁻ > CD133⁻ALDH⁻⁶¹. It suggests that ALDH activity might be a more specific marker for the CD133⁺ hepatic CSC population.

3. Molecular signaling pathways in regulating hepatic CSCs

The development of methods to identify CSCs in HCC has paved the way to explore the biological signaling pathways in regulating hepatic CSCs. Although the key mechanism in regulating hepatic CSCs remains elusive, several signaling pathways, such as Wnt, AKT, transforming growth factor- β (TGF- β) and STAT3 pathways have been found to be frequently deregulated in hepatic CSCs (Figure 2). The difference in various marker expression and altered signaling pathways linking to marker-positive CSCs could be the results of CSC heterogeneity as these cells could be derived from different cell lineage or are de-differentiated tumor cells upon an activation of a particular signaling pathway.

The Wnt/ β -catenin pathway is commonly known for its fundamental role in development, cell growth, survival, regeneration, self-renewal, and cancer. Recent studies indicate that Wnt/ β -catenin pathway may play a key role in hepatic CSCs. Mechanistically it is known that Wnt binds to its receptors, which then activate β -catenin upon its release from the “destruction complex” that includes adenomatous polyposis coli (APC), Axin1, and glycogen synthase kinase-3 β (GSK-3 β), via a conformational change of Axin1. Consequently, stabilized β -catenin moves to nucleus and activates transcription of its target genes such as Myc and cyclin D1⁶². A number of studies have indicated that Wnt signaling is activated in HCC and plays an important role in the maintenance of hepatic CSCs^{32,63}. Our studies revealed that Wnt/ β -catenin pathway regulates the self-renewal of EpCAM⁺ hepatic CSCs^{10,28,32,64,65}. In particular, EpCAM acts as one of direct transcriptional targets of Wnt/ β -catenin signaling and activation of this pathway by inhibiting GSK-3 β significantly increases the population of EpCAM⁺ hepatic CSCs^{10,32}. Moreover, we found that microRNA-181s are highly expressed in EpCAM⁺ primary HCCs and isolated EpCAM⁺ hepatic CSCs⁶⁴. Wnt/ β -catenin could transcriptionally induce microRNA-181s' expression, which then suppress the expression of NLK (a Wnt/ β -catenin signaling inhibitor) by binding to its 3'-UTR. Our studies reveal a positive feedback mechanism between Wnt/ β -catenin and microRNA-181 for maintaining stemness in hepatic CSCs^{22,64,65}. In addition, high level of CD24 in HCC is associated with an activation of Wnt/ β -catenin pathway⁶⁶. The Wnt/ β -catenin pathway is found to be activated in both rodent oval cells and OV6⁺ tumor cells. Murine hepatic stem cells transduced with mutant β -catenin could acquire excessive self-renewal capability and tumorigenicity⁵³.

AKT signaling has been reported to be involved in regulating homeostasis and chemoresistance of hepatic CSCs. Under hypoxia, the AKT pathway significantly increases the expression of hypoxia-inducible factor-1 α (HIF-1 α), which further affects the

phosphorylation of AKT. The positive interaction between AKT and HIF-1 α leads to an over-expression of platelet-derived growth factor (PDGF)-B and vascular endothelial growth factor (VEGF), which are important for homeostasis and chemoresistance of CSCs⁶⁷. Moreover, AKT could induce the expression of ABCG2, which is important in drug efflux response to chemotherapeutic agents⁶⁸. It also has been shown that activated AKT pathway is associated with high level of cell survival proteins such as Bcl-2 in CD133⁺ hepatic CSCs⁶⁹. Thus, activated Akt signaling pathway is also important in hepatic CSCs.

Additionally, impaired TGF- β signaling with activation of IL-6/STAT3 pathway in hepatic progenitor cells could lead to the abnormal differentiation patterns and HCC development⁷⁰. It also has been reported that TGF- β could regulate the population of CD133⁺ hepatic CSCs⁷¹. Moreover, IL-6/STAT3 pathway is important for chemoresistance of EpCAM⁺ hepatic CSCs⁷². CD24 may functionally drive HCC tumor initiation through STAT3-mediated NANOG regulation⁴⁷. Taken together, these studies have improved our understanding about hepatic CSCs at a molecular level. It is conceivable that key components of these identified abnormal pathways might be utilized as the effective targets for eliminating hepatic CSCs.

4. Clinical potential of hepatic cancer stem cells

Following the identification of hepatic CSCs with different markers and the discovery of deregulated molecular pathways in hepatic CSCs, more and more studies have demonstrated that hepatic CSCs are responsible for metastasis and recurrence after HCC resection due to its ability of giving rise to a new tumor at a local or distant site (Figure 1). These shed lights on the potential utility of hepatic CSCs in assisting HCC clinical management. The markers used to isolate hepatic CSCs may contribute to diagnosis and prognosis prediction in patients with HCC. The development of strategies specifically targeting hepatic CSCs may provide new methods that could be used to improve HCC patients' survival.

4.1. Diagnostic potential

Some hepatic CSC markers might be useful for HCC early diagnosis. AFP is expressed in human hepatoblasts, fetal hepatocytes, but not in mature hepatocytes nor in hepatic stem cells^{30,73}. We have found that AFP could be used with EpCAM to define cancer cells with stem cell features¹⁰. Although there are no studies supporting AFP as an independent hepatic CSC marker, examination of serum AFP level remains a useful test for clinicians in HCC diagnosis. High level of serum AFP is a common feature for HCC. The test, when used with the conventional cut-off point of 500 ng/mL, has a sensitivity of about 50% and a specificity of more than 90% in detecting the presence of HCC in a patient with coexisting liver disease⁷⁴. Recently, Yang et al identified CD45⁺CD90⁺ hepatic CSCs in blood samples from HCC patients, suggesting that examination of CD45⁺CD90⁺ cells in blood might supply a novel diagnostic method for human HCC. In this study, CD45⁺CD90⁺ cells were identified in 31 out of 34 blood samples from HCC patients, but not in normal subjects (n=19) nor in patients with liver cirrhosis (n=19)^{43,44}. The existence of a CD45⁺CD90⁺ population in HCC blood samples suggests the presence of CSCs in the systemic circulation. The FDA-approved CellSearch system has been established for the use of EpCAM in the enrichment of circulating tumor cells using a magnetic ferrofluid approach and has been successful in predicting lung cancer prognosis⁷⁵. This method in combination with a serum AFP test might potentially be useful in subgrouping HCC patients and predicting their outcomes. More studies focusing on the identification of other hepatic CSCs populations in blood samples might significantly improve the current early diagnostic methods.

Sub-classification of HCCs based on the hepatic CSC marker expression might also assist in HCC diagnosis, which will further provide the guidance for certain adjuvant therapy. In our

group, we have identified four subgroups of HCC based on the EpCAM and AFP expression. These four subtypes display distinct gene expression patterns with features resembling certain stages of hepatic lineages. Among them, EpCAM⁺AFP⁺ HCC might not get benefit from the conventional chemotherapy due to the chemoresistance feature of EpCAM⁺ hepatic CSCs, but they could benefit from anti- β -catenin therapy due to its dominant activation in this type of tumors. These indicate that a molecule-guided secondary diagnosis defined by hepatic cancer stem cell markers may enable assessment of HCC patients with adjuvant therapy.

4.2. Prognostic potential

According to the CSC theory, CSCs could influence patient prognosis by promoting metastasis and recurrence. It is anticipated that the greater the size of the CSC population, the poorer the prognosis. Consistent with this hypothesis, recent findings show that the presence of CSCs could be linked with patients' survival. For example, increased CD133 expression is an independent prognostic factor for survival and tumor recurrence in patients with HCC^{76–78}. Overexpression of CD90 in HCC is also associated with poor prognosis⁷⁹. Expression of CD44 in HCC is related to a higher frequency of extrahepatic metastasis and shortened survival^{80,81}. CD24 overexpression in HCC correlates with more aggressive tumor behavior and poor clinical outcome^{47,66}. However, validation in independent cohorts as a diagnosis prediction model for these markers is required before their clinical utilization. In addition, the predictive range of a single marker is limited to a very small subpopulation, because of high degree of HCC heterogeneity. A combination of several hepatic CSC markers may provide greater specificity and reliability in predicting HCC prognosis. Our group has identified that EpCAM⁺AFP⁺ HCC with stem cell features have poor prognosis compared to EpCAM[−]AFP[−] HCCs, which has been validated in an independent cohort^{10,28}. Yang et al. further constructed a simplified predictive model using CD133, CD44, Nestin, and microvessel density (determined by CD34), which is found to be independent predictors of recurrence-free survival in multivariable analysis with clinic-pathological characteristics. High expression of these markers could classify HCC patients with a high risk of tumor recurrence after surgery⁸².

4.3. Potential in clinical intervention

Conventional chemotherapy (cisplatin/IFN- α /doxorubicin/capecitabine) and radiation for the treatment of patients with HCC in advanced stages have limited benefit in improving overall survival. The reason is that these anticancer therapies mainly kill rapidly growing differentiated tumor cells, thus reducing tumor mass. However, CSCs are potentially left behind, resulting in relapse and proliferation of therapy-resistant and more aggressive tumors. Since CSCs are involved in resistance and relapse to current anti-cancer therapies, a selective targeting and eradicating CSC is one of the most therapeutically important challenges. The current focuses for targeting CSCs towards clinical intervention are mainly related to three research areas: (i) impairing stem cell niche (homeostasis); (ii) inhibiting self-renewal and/or inducing cell differentiation; and (iii) disrupting chemoresistance (Figure 3).

4.3.1. Impairing hepatic cancer stem cell homing—Hypoxia influences microenvironment in HCC and hepatic CSCs^{67,83}. Lau et al found that a novel signaling pathway regulated by HIF-1 α confers cisplatin resistance in cancer cells and tumorigenic hepatic progenitors. Under a hypoxic condition, Akt and HIF-1 α are up-regulated, followed by an induced expression of PDGF-BB. Moreover, PDGF-BB stimulates the Akt pathway in a dose- and time-dependent manner, resulting in the formation of an autocrine signaling loop, i.e., Akt/HIF-1 α /PDGF-BB positive feedback. The activation of Akt/HIF-1 α /PDGF-BB autocrine signaling under hypoxia condition leads to cisplatin resistance in HCC cells

with a progenitor feature. Blockade of this signaling by inhibition of HIF-1 α activity could enhance the chemo-sensitivity of hepatic CSCs, and thus provide an effective therapeutic strategy for HCC⁶⁷.

4.3.2. Anti-self-renewal of hepatic cancer stem cells—Approaches in blocking self-renewal of CSC cells represent rational therapeutic strategies for cancer prevention and treatment. The Wnt/ β -catenin signaling pathway is important for self-renewal or maintenance of stem cells in many tissue types. We identified EpCAM and microRNA-181 as direct transcriptional targets of Wnt/ β -catenin in HCC^{32,65}. Moreover, microRNA-181 could maintain self-renewal of EpCAM⁺ hepatic CSCs through increasing β -catenin activity via targeting NLK, and through suppressing cell differentiation via targeting CDX2 and GATA6, two hepatocyte differentiation related transcriptional factors⁶⁴. Thus, the Wnt/ β -catenin/microRNA-181/EpCAM signaling pathway is critical in self-renewal of EpCAM⁺ hepatic CSCs. RNAi mediated EpCAM silencing has resulted in a decrease of self-renewal, tumorigenicity and invasive capacity of hepatic CSCs¹⁰. Suppressing microRNA-181 expression by delivering anti-miR181 oligos significantly inhibits self-renewal showing by a decreased spheroid formation and stem cell gene expression⁶⁴. Further exploration of the positive feedback regulatory loop between miR-181 and Wnt/ β -catenin signaling as a molecular target can lead to new therapeutic strategies to eradicate hepatic CSCs.

Lee et al found that Lup-20(29)-en-3 β -ol (lupeol), a triterpene found in fruits and vegetables, inhibits self-renewal ability of hepatic CSCs in both HCC cell lines and clinical HCC samples. Consequently, lupeol suppresses in vivo tumorigenicity. In addition, lupeol sensitizes HCC cells to chemotherapeutic agents through the phosphatase and tensin homolog (PTEN)-Akt-ABCG2 pathway. These results suggest that lupeol may be an effective dietary phytochemical that targets hepatic CSCs⁶⁸. It has been found that the polycomb gene product Bmi-1 plays an important role in self-renewal of SP cells in HCC^{46,84}. Bmi-1 is also highly expressed in CD133⁺ and CD90⁺ hepatic CSCs^{43,44}. The Bmi-1 knockdown abolishes tumor-initiating ability of SP cells in NOD/SCID mice through the impaired self-renewal of SP cells⁸⁴. miR-130b was found to be highly expressed in CD133⁺ cells compared to CD133⁻ cells and antagonizing miR-130b demonstrated a reduced potential of self-renewal, and decreased tumorigenicity partially through targeting TP53INP1⁸⁵. In addition, microRNA-150 was identified to be highly expressed in CD133⁻ cells compared to CD133⁺ cells. Overexpression of microRNA-150 could lead to a significant reduction of CD133⁺ cells and a reduced spheroid formation through inhibiting c-Myb⁸⁶. All these demonstrated that interrupting CSC self-renewal would lead to a reduction in tumorigenicity, indicating targeting self-renewal ability represents a potent strategy in HCC treatment.

Recently, CD24⁺ HCC cells are identified as hepatic CSCs and CD24 itself has been shown to functionally contribute to the traits of hepatic CSCs. Compared to non-target control groups CD24 knockdown could reduce self-renewal shown by fewer and smaller hepatospheres in vitro, and by fewer and smaller HCC nodules in vivo⁴⁷. CD13 was identified as a marker for semiquiescent CSCs in human HCC cells. In mouse xenograft models, combination of a CD13 inhibitor and 5-FU dramatically reduced tumor volume compared with either agent alone. CD13 inhibition suppresses self-renewal and tumor-initiating ability of dormant CSCs. These results indicate that combining a CD13 inhibitor with a conventional chemo/radiation therapy may improve HCC treatment⁵⁰.

4.3.3. Inducing differentiation of hepatic cancer stem cells—One approach to treat malignancies is to induce differentiation of CSC cells. Differentiation therapy could force hepatic CSCs to differentiate and lose their self-renewal property. Oncostatin M (OSM), an IL-6-related cytokine known to induce differentiation of hepatoblasts into hepatocytes, could

effectively induce differentiation and active cell division of dormant EpCAM⁺ hepatic CSCs. The combination of OSM and conventional chemotherapy with 5-FU efficiently eliminates HCC by targeting both CSCs and non-CSCs⁷². These findings indicate that combination of differentiation therapy and conventional chemotherapy may be an effective treatment of HCC.

Hepatocyte nuclear factor-4 α (HNF4 α) is a central regulator of differentiated hepatocytes. It has been shown to suppress tumorigenesis and metastasis by inducing HCC cell differentiation via a decreased “stemness” gene expression and reduced population of CD90⁺ and CD133⁺ cells⁸⁷. Arsenic trioxide could also induce cell differentiation, consequently sensitize hepatic CSCs to conventional chemotherapy in HCC⁸⁸.

4.3.4. Disrupting chemoresistance—Recent studies have shown that CSCs are resistant to conventional chemo- and radiation therapies, highlighting the need for the development of chemotherapy-sensitization strategies. Ma et al demonstrated that isolated CD133⁺ hepatic CSCs were resistant to conventional chemotherapeutic agents via a preferential activation of AKT/PKB and Bcl-2 cell survival response. The treatment of CD133⁺ HCC cells with an AKT1 inhibitor, specific to Akt/PKB pathway, abolishes the preferential survival of CD133⁺ HCC cells and sensitizes HCC cells to DOX and 5-FU treatment through reducing the expression of survival proteins including Bcl-2⁶⁹. Another study also revealed that inducing cell apoptosis could sensitize hepatic CSCs to chemotherapy. Researchers, who have examined potential benefits of targeting CD44 via a neutralizing antibody approach, found that treatment of CD90⁺ CSCs with anti-human CD44 antibody induces cell apoptosis in a dose-dependent manner. In vivo, The systemic administration of anti-human CD44 antibodies in immunodeficient mice suppresses tumor nodule formation in liver and metastatic lesions in lung^{43,44}. These studies suggest that a specific induction of cell apoptosis in CSCs may be a strategy to sensitize HCC to conventional chemotherapy.

Other studies have also shown potential methods to chemo-sensitize hepatic CSCs. Cheung et al. have found that a growth factor, granulatin-epithelin precursor (GEP), regulated chemoresistance in liver cancer cells through modulation of the expression of ABCB5 drug transporter. Specifically, chemoresistant HCC cells that expressed GEP had increased levels of ABCB5, CD133 and EpCAM. Suppression of ABCB5 could sensitize cells to doxorubicin treatment and apoptosis. Conversely, blocking ABCB5 reduces the expression of CD133 and EpCAM⁸⁹. In addition, the study of OV6⁺ hepatic CSCs also revealed that β -catenin signaling is required for protection of OV6⁺ progenitor cells from chemotherapeutics-induced cytotoxicity, and silencing β -catenin could lead to reduced chemoresistant colonies⁵³.

5. Future Directions

To date, hepatic CSCs can be identified by several markers, such as EpCAM, CD133, CD90, CD44, CD24, CD13 and OV6, or by selecting for the SP cells and those with a high ALDH activity. Overall, the identification and functional characterization of hepatic CSCs have paved the way for aiding HCC diagnosis and prognosis prediction, assisting in HCC patient stratification with the potential for personalized adjuvant therapy, and developing the possible novel HCC therapeutic strategies. However, our understanding in hepatic CSCs still remains limited. Although various types of identified hepatic CSCs have similar “stem-like” characteristics, their similarities and differences in underlying molecular signaling pathways are not well defined. Whether these CSCs are lineage-restricted or representing different CSC groups remains unknown. Various combinations of known markers may be of value in identifying hepatic CSCs since there is no single marker being exclusively expressed by

CSCs in HCC. Thus, it would be good to isolate CSCs by different markers as well as various marker-combinations from the same HCC patients to investigate their heterogeneity and association by gene expression profiling and tumorigenic potentials. Moreover, identification and functional characterization of CSCs have been mainly performed in cultured cell lines. Additional validation studies are needed by using primary tumor specimens and circulating blood cells, which will provide further clinical relevance to support the exploration of CSC knowledge in HCC clinical diagnosis and prognosis. In addition, current understanding of hepatic CSCs is mainly focused on their stem cell features. The biological difference between normal and cancer stem cells has not been explored. The unique biological significance of CSCs compared to normal stem cells will likely lead to the development of the most effective strategies in eliminating CSCs without sacrificing any normal stem cells.

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Figure 1. Tumor initiation and metastasis in HCC based on cancer stem cell model

Hepatic CSCs could be derived from hematopoietic stem cells, hepatic stem cells upon somatic mutations of oncogenes and tumor suppressor genes to acquire a tumorigenic potential, or reprogrammed from differentiated hepatic cells to be de-differentiated tumor cells by mutations to acquire the stem cell-like capacity to self-renew and differentiate. These hepatic CSCs eventually expand to a HCC bulk with heterogeneity through self-renewal and differentiation. Moreover, the existence of hepatic CSCs is considered to be responsible to metastasis and recurrence after resection due to its resistance to conventional therapies and its ability of giving rise to a new tumor in local or distant organs.

Figure 2. Signaling Pathways Altered in Hepatic Cancer Stem Cells

Wnt/β-catenin, AKT, TGF-β and IL-6/STAT3 signaling pathways are deregulated in hepatic CSCs. Activation of the Wnt pathway results in β-catenin accumulation in the cytosol and translocation into the nucleus, where β-catenin transcriptional activates the expression of its targets, such as cyclinD1, EpCAM, and miR-181. AKT is activated by two phosphorylation sites Thr308 and Ser473. Phosphorylation of Thr308 is promoted by PI3K and suppressed by PTEN. Activated AKT protect hepatic CSCs from apoptosis through the induction of BCL-2 and ABCG2 under the pressure of chemotherapy. Activated AKT could also interact with HIF-1α to induce VEGF and PDGF-BB expression for regulating the homeostasis and drug resistance of hepatic CSCs. Impaired TGF TGF-β signaling together with activation of IL-6/STAT3 pathway is important to regulate the differentiation and chemoresistance of EpCAM⁺ hepatic CSCs. Studies have shown that these pathways are interconnected, which may further promote stemness and carcinogenesis in HCC.

Figure 3. CSC features, deregulated molecular signaling pathways in hepatic CSCs, and corresponding therapeutic strategies targeting CSCs in HCC

Similar to other tumors, HCC CSCs also possess three CSC features which include the abilities to self-renew and differentiate, tumorigenicity, and chemo/radio-therapeutic resistance. Functional characterization of hepatic CSCs have identified several signaling pathways including Wnt/β-catenin, AKT, TGF-β and IL6 pathways to be important in regulating hepatic CSCs. Further studies based on these identified molecular mechanisms have revealed potential therapeutic strategies by targeting the abilities of CSCs to self-renew, to be tumorigenic and to develop chemoresistance.

Table 1

Identification markers of hepatic cancer stem cells

Marker (Hepatic CSC)	Source (HCC Cell line/Primary HCC tissue/Blood)	Tumorigenicity (Minimal #)
EpCAM ⁺ AFP ⁺ ¹⁰	Cell line: HuH1, Huh7	2×10 ²
	Primary HCC	1×10 ⁴
CD133 ^{33–35}	Cell line: Huh7, PLC8024, SMMC7721	1×10 ²
CD133 ⁺ CD44 ⁺ ⁴⁶	Cell line: SMMC7721, MHCC-LM3, MHCC97L	1×10 ²
CD90 ⁺ CD45 ^{–43,44}	Cell line: HepG2, Hep3B, PLC, Huh7, MHCC97L, MHCC97H	5×10 ²
	Primary HCC	2.5×10 ³
	Blood	1×10 ⁴
CD24 ⁴⁷	Cell line: PLC/PRF/5, HLE	5×10 ²
	Primary HCC	4×10 ³
CD13 ⁵⁰	Cell line: Huh7, PLC/PRF/5	1×10 ²
OV6 ⁵³	Cell line: Huh7, PLC, SMMC7721, Hep3B, HepG2	5×10 ³
SP cells ⁵⁴	Cell line: Huh7, PLC/PRF/5	1×10 ³
ALDH ⁺ CD133 ⁺ ⁶¹	Cell line: PLC8024	5×10 ²