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MINIREVIEWS

Liver cancer stem cell markers: Progression and therapeutic implications

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Abstract

Cancer stem cells (CSCs) are a small subpopulation in cancer, have been proposed to be cancer-initiating cells, and have been shown to be responsible for chemotherapy resistance and cancer recurrence. The identification of CSC subpopulations inside a tumor presents a new understanding of cancer development because it implies that tumors can only be eradicated by targeting CSCs. Although advances in liver cancer detection and treatment have increased the possibility of curing the disease at early stages, unfortunately, most patients will relapse and succumb to their disease. Strategies aimed at efficiently targeting liver CSCs are becoming important for monitoring the progress of liver cancer therapy and for evaluating new therapeutic approaches. Herein, we provide a critical discussion of biological markers described in the literature regarding liver cancer stem cells and the potential of these markers to serve as therapeutic targets.

Key words: Liver cancer; Cancer recurrence; Liver cancer stem cells; Cancer stem cell markers; Targeted therapy

Core tip: Liver cancer is the fifth most common cancer and the third leading cause of cancer-related mortality worldwide despite remarkable progress in understanding hepatocarcinogenesis and new therapeutic approaches. Recently, the presence of highly resistant cancer stem cells (CSCs) in liver cancer has been proposed to be responsible for tumor growth, invasion, metastasis and recurrence. CSC involvement in liver cancer pathogenesis also highlights them as preferential targets for therapy. This review specifically focuses on the markers used to define human liver

cancer stem cells, the therapeutic implications of the expression of these markers in patient's primary tumors, and the potential of the markers to serve as therapeutic targets.

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INTRODUCTION

Hepatocellular carcinoma (HCC), the most common liver cancer, is the third leading cause of cancer-related mortality worldwide[1], mainly due to its high rate of recurrence, which can be as high as 70% following conventional treatments, such as chemotherapy, arterial embolization, surgical resection, and radiofrequency ablation[2]. Some research studies have demonstrated that liver cancers are derived from liver stem cells that are present in adult liver tissue with endogenous or exogenous liver origin, where the former are oogonia located in the smallest terminal of the intrahepatic bile duct. However, the cellular origin of HCC recurrence remains poorly understood, and no specific treatment strategy has been developed that focuses on HCC recurrence. Although the cytological pathogenesis of HCC remains unclear, it has been proposed that HCCs are not created equally and display a great deal of heterogeneity[3,4], as there are abundant distinct tumor cell populations expressing different markers. Only a rare subset of cancer cells with stem cell properties, often referred to as liver cancer stem cells (LCSCs), are considered to be responsible for tumor growth, metastasis and recurrence of HCC as well as for the failure of chemotherapy and radiotherapy[5]. These findings indicate that liver cancer therapies, although killing a majority of tumor cells, may ultimately fail because they do not eliminate LCSCs, which survive to regenerate new tumors (Figure 1). Therefore, the cancer stem cell theory offers novel insight into tumor diagnosis, treatment and prevention.

Recent rapid progress in CSC research has encountered increasing challenges in which the identification of CSC-specific marker sets and targeted therapeutic destruction are the most frequently debated topics. CSC markers must be clearly defined for each tissue, and clarifying cellular and signaling functions of CSCs is key to conducting better identification and diagnosis based on CSC biomarkers for targeting CSCs, which will undoubtedly improve prevention and treatment for many types of CSCs. To achieve better understanding and treatment of LCSCs, we must better understand the markers of stemness and cell fractions associated with prognosis, metastasis, and resistance. These markers are necessary to isolate CSCs and analyze their biological characteristics to target them efficiently for therapeutic purposes. Therefore, we summarize here current knowledge on putative markers that define LCSCs, potential functional implications, and therapeutic targets of these markers and provide insights into new therapeutic approaches for more specific targeting and eradication of liver CSCs.

CELL SURFACE MARKERS OF LIVER CSCS

Current surface markers or a particular phenotype are used to identify CSCs. Several markers proposed in the literature to identify CSCs in liver cancer using cell surface antigens are enriched in LCSCs (isolated by FACS or Ab-conjugated magnetic beads). Additionally, cytokeratin 7 and 19 may also serve as relatively specific markers of LCSCs, playing significant roles in hepatocellular carcinoma[6,7].

CD133

One of the most commonly described surface markers in LCSCs is CD133. CD133, also known as Prominin-1, is a membrane glycoprotein encoded by the CD133/Prom-1 gene[8].It was first detected as a marker of hematopoietic stem cells and has since been shown to be a marker of CSCs in the prostate, colon, and ovaries[9-11]. $CD133^+$ HCC cells were first identified as a potential CSC subpopulation by Suetsugu *et al*[12]. They found that the isolated $CD133^+$ HCC cells from Huh7 HCC cell lines exhibited higher proliferative and tumorigenic potential and expressed lower levels of mature hepatocyte markers than those of the $CD133^-$ counterparts. Subsequent reports from Yin *et al*[13] demonstrated a similar result, where the $CD133^+$ population of HCC SMMC-7721 cells exhibits higher *in vivo* clonogenicity and *in vitro* tumorigenicity than those of the $CD133^-$ counterparts. We have also researched the enrichment and characterization of LCSCs through a sphere culture system and found that CD133 was significantly enriched in liver CSCs compared with that in MHCC97H cells. Additionally, liver CSCs proliferated significantly faster and induced more tumor colonies than those of MHCC97H cells[14]. Enhanced CD133 expression is also found to be an independent prognostic indicator for survival and tumor recurrence in HCC patients[15]. Furthermore, CD133-positive cells seemed to be increased with the loss of differentiation of the tumor[16].

Aldehyde dehydrogenase

Aldehyde dehydrogenase (ALDH) is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes, which is engaged in early differentiation of stem cells by retinol oxidation to retinoic acid[17]. ALDH activity has been found to be upregulated in murine and neural stem and human hematopoietic and progenitor cells[18]. ALDH is also widely used as a CSC marker in

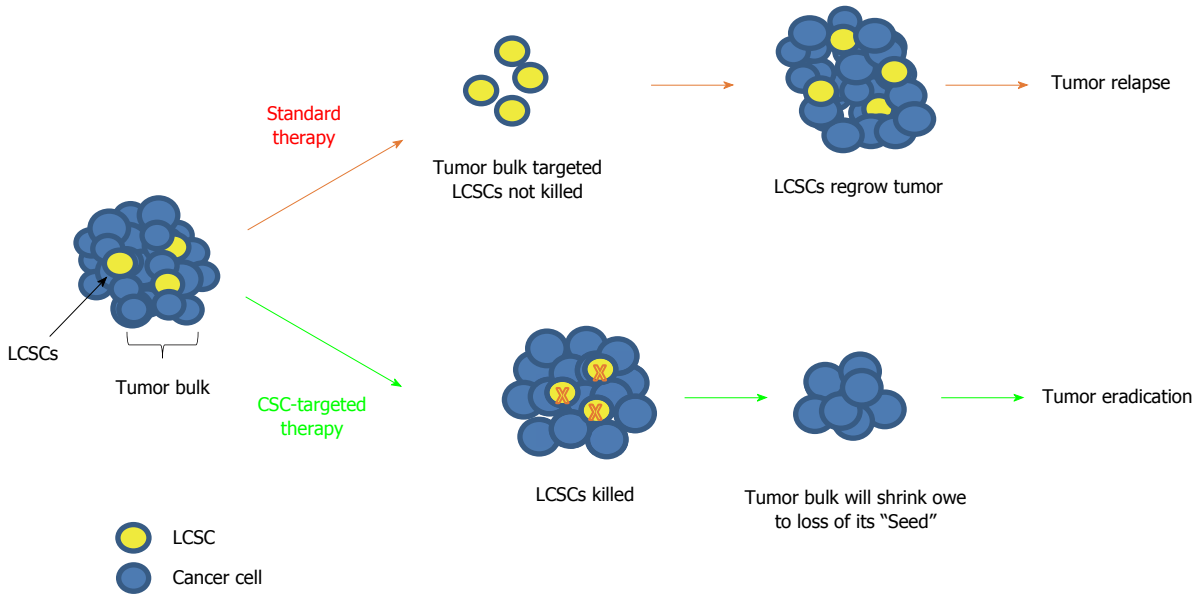

Figure 1 Targeting liver cancer stem cells is necessary to prevent tumor recurrence. LCSCs are resistant to the current standard of care: surgical resection, radiotherapy and chemotherapy. Therapeutic approaches and strategies to target LCSCs in addition to the differentiated tumor cells are necessary to effectively treat the entire cancer and prevent tumor recurrence. LCSCs: Liver cancer stem cells.

many types of cancer, including colon[19], breast[20], ovary[21], bladder[22] and prostate[23]. In liver cancer, Yin *et al*[13] suggested that ALDH is expressed in LCSCs and is positively correlated with CD133 expression. The combination of these markers can define LCSCs more accurately; dual-color FACS analysis found that the majority of $ALDH^+$HCC cells were $CD133^+$, yet not all $CD133^+$HCC cells were $ALDH^+$. A hierarchical organization of cells that differentially express CD133 and ALDH exhibit descending tumorigenic potential in the order of $CD133^+ALDH^+ > CD133^+ALDH^- > CD133^-ALDH^-$[13], which implies that ALDH express along CD133 can be used to characterize the tumorigenic liver CSC population more specifically.

CD90

CD90 is a 25-37 kDa heavily N-glycosylated, glycophosphatidylinositol (GPI)-anchored protein expressed in many cells, such as thymocytes, T-cells, neurons, endothelial cells and fibroblasts[24]. CD90 operates as an important regulator of cell-cell and cell-matrix interactions, apoptosis, adhesion, migration, cancer and fibrosis[25]. CD90 is also expressed in bone marrow-derived stem cells[26] and hepatic stem/progenitor cells from adult or fetal livers but not in adult hepatocytes[27-29]. It has been identified to be one potential marker in CSCs, including in HCC. Yamashita *et al*[30] investigated the expression patterns of three CSC makers (CD 133, EpCAM, CD90) in 15 primary HCCs with high viability, where EpCAM, CD90 and CD133 are positive in 3, 7 and 15 cell strains, respectively. Although strong correlation of $CD90^+$ proportions in tumor cells and liver cancer distant metastasis was suggested, the intrinsic mechanics still need to be determined. Additionally, the feasibility of eradicating cancer cells committed to mesenchymal endothelial lineages by imatinib mesylate, in which $CD90^+$ cells are believed to be chemosensitive, is proposed. Yang *et al*[31] found that the number of $CD90^+$ cells increased with the tumorigenicity and metastatic potential in a panel of HCC cell lines. Moreover, $CD45^-CD90^+$ cells were detected in all of blood samples from HCC patients, but none in normal subjects or patients with cirrhosis. The $CD45^-CD90^+$ subpopulation has the capacity to initiate and maintain tumor formation in SCID/Beige mice, whereas the $CD90^-$ and $CD45^-CD90^-$ cells do not. In conclusion, these results provide evidence of the tumorigenicity and stem cell-like properties of $CD45^-CD90^+$ and $CD90^+$ populations from HCC patients.

CD44

CD44 is a ubiquitous multi-structural and multi-functional cell surface glycoprotein involved in adhesive cell-cell and cell-matrix interactions, cell migration, cell homing, cell proliferation and angiogenesis[32]. All of these biological properties are essential to normal cell physiology, but under certain conditions they are associated with pathological activities, in particular, those of cancer cells[33]. Moreover, CD44 is the receptor for hyaluronic acid and has been identified as a CSC marker for several human cancers, including breast[34], gastric, colon, prostate[35], colorectal[36],pancreatic[37], and head and neck squamous cell carcinomas[38]. In human liver cancer, CD44 is also an important marker. CD44 and other markers were reported to more accurately define the surface phenotype of liver CSCs. The $CD90^+CD44^+$ cells showed a more aggressive phenotype than the $CD90^+CD44^-$ counterpart and formed metastatic lesions in immunodeficient

mice. CD44 blockade prevented the formation of local and metastatic tumor nodules, which showed that concomitantly expressed CD44 modulates the biological activity of the $CD90^+$ CSCs[39]. Another study demonstrated that CD44 was preferentially expressed in a$CD133^+$ population in four HCC cell lines, including Huh7, SMMC7721, MHCCLM3 and MHCC97L. Compared with $CD133^+CD44^-$ cells, $CD133^+CD44^+$ HCC cells showed more stem cell properties, including extensive proliferation, self-renewal, and differentiation into the bulk of cancer cells. Furthermore, cells double positive for CD133 and CD44 exhibited preferential expression of some stem cell-related genes and were more resistant to chemotherapeutic agents[40].

CD13

CD13 antigen, a membrane-bound zinc-dependent type II exopeptidase, is widely distributed in many tissues of mammals, such as the intestine, kidney, and liver as well as the central nervous system[41-43]. CD13 participates in the final hydrolysis of nutrients and the degradation of bioactive molecules, such as enkephalin and endorphin. In addition, CD13 is highly expressed in many tumor cells and has been considered as a tumor marker that plays a crucial role in tumor cell growth, invasion, metastasis and angiogenesis. Importantly, CD13 is involved in angiogenesis-generating and -modulating signals and in the process of capillary tube formation and is a marker of angiogenic vessels[43-51].

Recently, Haraguchi *et al*[52] identified CD13 as a candidate liver cancer stem cell marker by a surface marker screen based on microarray analysis. $CD13^+$ liver CSCs were enriched in side population (SP) cells isolated from Huh-7, PLC/PRF/5 and Hep3B cells, which is known as the multi-drug resistant cell fraction with ATP-binding cassette (ABC) transporter expression, and also localized predominantly in G1/G0 phase.These results suggested that $CD13^+$cells represent the dormant or slow-growing population that is believed to account for the chemoresistant capacity in HCC. *In vivo* chemosensitivity assays indicated the high multi-drug resistant property of $CD13^+$ cells. Treatment of liver cancer cells with a CD13 inhibitor or CD13 neutralizing antibody efficiently induced cellular apoptosis *in vitro*, suggesting that CD13is a liver CSC target.

In mouse xenograft models, the combination of a CD13 inhibitor and the genotoxic chemotherapeutic fluorouracil (5-FU) more efficiently reduced tumor volume compared with either agent alone.CD13 inhibition suppressed the self-renewing and tumor-initiating abilities of LCSCs. In other respects, reactive oxygen species (ROS) have been found to be negatively correlated with surface marker CD13 in cells, where $CD13^+$ cells have relatively higher ROS than in their $CD13^-$ counterparts. Furthermore, CD13 inhibition is also able to increase ROS expression. These results suggest that chemo-resistant properties of $CD13^+$ cells are regulated by ROS, indicating a positive prospect of treating liver cancer with a CD13 inhibitor and ROS-inducing chemo/radiation therapy[52].

EpCAM

Epithelial cell adhesion/activating molecule (EpCAM) is encoded by the TACSTD1 gene, one of the first tumor-associated antigens identified[64]. EpCAM is highly expressed in a large variety of human adenocarcinomas and squamous cell carcinomas[53]. Yamashita *et al*[54] demonstrated that $EpCAM^+$ HCC showed a distinct molecular signature with features of hepatic progenitor cells, including the presence of known stem/progenitor markers, such as cytokeratin 19, EpCAM, c-Kit, and activated Wnt/β-catenin signaling, whereas $EpCAM^-$ HCC expressed genes related to mature hepatocytes. Because its expression is highly elevated in premalignant hepatic tissues and in a subset of HCC, EpCAM may serve as an early biomarker of HCC[55]. Similar results were observed by other researchers[56-58], reiterating the significance of EpCAM in HCC development. The $EpCAM^+/AFP^+$ subtype of HCC was significantly correlated with a poor prognosis for HCC patients. Functional analysis showed that $EpCAM^+$ HCC cells possessed CSC phenotypes, including the ability to self-renew, differentiate and initiate tumors[54]. Moreover, these cells demonstrated EpCAM enrichment by activation of Wnt/β-catenin signaling using the GSK-3βinhibitor BIO, suggesting that EpCAM is a downstream effect or of the Wnt/β-catenin signaling pathway. These data support therapeutic strategies targeting $EpCAM^+$ liver CSCs through the suppression of Wnt/β-catenin signaling. EpCAM knockdown by RNA interference (RNAi) was shown to cause significant inhibition of cell invasion, sphere formation, and tumorigenicity of HCC cells[55]. In addition, knockdown of EpCAM suppressed colony-forming ability in sorted $EpCAM^+$ HCC cells by EpCAM shRNA (shEpCAM) *in vitro*. $EpCAM^+$ liver cancer cells highly express the chromatin-remodeling enzyme CHD4, whose knockdown and overexpression separately increased chemosensitivity and chemoresistance to epirubicin *in vitro*[59]. Histone deacetylase and poly (ADP-ribose) polymerase are regulators of CHD4. The inhibitory effects of their inhibitors suberohydroxamic acid and AG-014699 were assessed by Nio *et al*[60], who proposed that either inhibitor alone reduced the number of $EpCAM^+$ liver cancer stem cells *in vitro* and that the combination of the two inhibitors successfully inhibited tumor growth in a mouse xenograft model.

OV-6

Hepatic oval cells are an important origin of liver stem cells. OV-6 has been found to be a useful marker for rat oval cells and is thought to be a hepatic stem cell marker[61-64]. Oval cells arise in the intraportal area of the liver after treatment with hepatocarcinogens

Table 1 Cell surface markers of liver cancer stem cells

Marker	Cell line/primary tumor	Characteristics of marker-positive CSCs	Inhibitors	Ref.
CD133	PLC8024, Huh7, Hep3B, primary HCC	Self-renewal, tumorigenicity, chemoresistance, invasiveness	Lupeol,Anti-CD133 antibody, antisense oligonucleotides	[10-14]
ALDH	Huh7, HPLC8024, Hep3B	Chemoresistance tumorigenicity	Diethylaminobenzaldehyde	[10,11]
CD90	HepG2, Hep3B, PLC, Huh7, MHCC97L, MHCC97H, Primary HCC	Tumorigenicity, metastasis, circulation	Anti-CD44 antibody	[28,29]
CD44	PLC/PRL/5	Tunorigenicity, invasiveness, chemoresistance, metastasis	RNAi interference, Anti-CD44 antibody, antisense oligonucleotides	[38]
CD13	PLC/PRL/5, Huh7, Hep3B	Tumor formation, cell cycle arrest, chemoresistance, self-renewal	Anti-CD13 antibody, CD13 inhibitor ubenimex	[51]
EpCAM	Huh1, Huh7, primary HCC	Invasiveness, Self-renewal, tumor formation, chemoresistance	RNAi interference, GSK-3β inhibitor BIO, Bispecific antibody EpCAMxCD3	[53,54,60]
OV6	Huh7, SMMC7721, primary HCC	Tumorigenicity, chemoresistance, invasiveness, metastasis	RNAi interference, targeting β-catenin	[70]
1B50-1	Huh7, HepG2Hep-12, SMMC7721	Tumorigenic, invasiveness self-renewal	RNAi interference ERK1/2 inhibitor U0126	[71]
SALL4	PLC/PRF/5, Huh7	Proliferation, chemoresistance, tumorigenic	ERK1/2 inhibitor U0126 RNAi interference	[76]
ICAM-1	Hep3B, Huh7	Tumorigenic, metastasis	RNAi interference	[78,79]

CSCs: Cancer stem cells.

or hepatotoxins in rats, these cells and their progeny have the ability to proliferate and differentiate into either biliary cells or hepatocytes[62,65-69]. Yang *et al*[70] showed that $OV6^+$ cells possessed a greater ability to form tumors *in vivo* and that these cells showed a substantial resistance to standard chemotherapy when compared with $OV6^-$ tumor cells. The $OV6^+$ population was enriched after Wnt pathway activation, whereas inhibition of β-catenin signaling led to a decrease in the $OV6^+$ population. $OV6^+$ HCC cells were more chemoresistant than the $OV6^-$ counterparts, but this characteristic was reversed upon lentivirus-delivered stable expression of a microRNA targeting β-catenin. This result suggested the importance of the Wnt/β-catenin pathway in the activation and expansion of $OV6^+$ populations within tumors. Therefore, therapies targeting Wnt/β-catenin signaling may be a promising approach to reverse the chemoresistant nature of $OV6^+$ liver CSCs[70].

1B50-1

In human HCC cells, mAb 1B50-1, which binds to $\alpha2\delta1^+$ isoform 5, selectively targeted LCSCs. Recent studies show that1B50-1^+ cells could initiate tumors.1B50-1 binds to a subpopulation of HCC cells, hereafter termed $\alpha2\delta1^+$cells, that display stem cell-like properties, such as the expression of stem cell-associated genes (OCT4, SOX2, NANOG, and BMI1), increased self-renewal, increased invasiveness and the ability to give rise to both $\alpha2\delta1^+$ and $\alpha2\delta1^-$ cells[71]. Interestingly, 1B50-1^+ cells overlapped with $CD133^+$, $EpCAM^+$, $CD13^+$, and $ALDH^+$ populations of Hep-12 cells. Although the majority of 1B50-1^+ cells were also positive for CD133, EpCAM, CD13, and ALDH in Huh7 cells, only a small fraction of $CD133^+$, $EpCAM^+$, $CD13^+$, or $ALDH^+$ cells were 1B50-1^+. A similar correlation between 1B50-1 and these reported liver CSC markers was also found in other HCC cell lines and patient-derived cells (Table 1). Thus, 1B50-1^+ cells represent fractions of $CD133^+$, $EpCAM^+$, and $CD13^+$ populations but not vice versa[71].

SALL4

Sal-like protein 4 (SALL4) is a member of a family of zinc finger transcription factors that regulates embryogenesis, organogenesis, and pluripotency. SaLL4 is able to elicit reprogramming of somatic cells and is a marker of stem cells. Some research studies have shown that SALL4 is constitutively expressed in hematopoietic stem cells and is a potent regulator of their expansion[72,73]. SALL4 has also been identified as a novel molecule in reprogramming of somatic cells to become iPSCs[74,75]. Recent research shows that SALL4 is a novel therapeutic target for liver cancers. Bioinformatics analyses showed that elevated expression of SALL4in tumors is closely related to poor survival of HCC patients. *In vitro*, overexpression of SALL4 promotes cell proliferation and elevates the expression of EpCAM, cytokeratin 19 (CK19), and adenosine triphosphate (ATP)-binding cassette-G2 (ABCG2)[76]. In summary, SALL4 may be a prognostic marker of liver cancer and an indicator of stem cells, playing roles in 5-FU resistance and growth of cells, and tumors with suppressed SALL4 results in differentiation and delayed tumor growth.

ICAM-1

Intercellular adhesion molecule 1 (ICAM-1), a 90-kD cell surface glycoprotein of the immunoglobulin superfamily, is believed to be responsible for HCC metastasis[77]. Previous studies have shown that hepatocytes are negative for ICAM-1 in cancerous

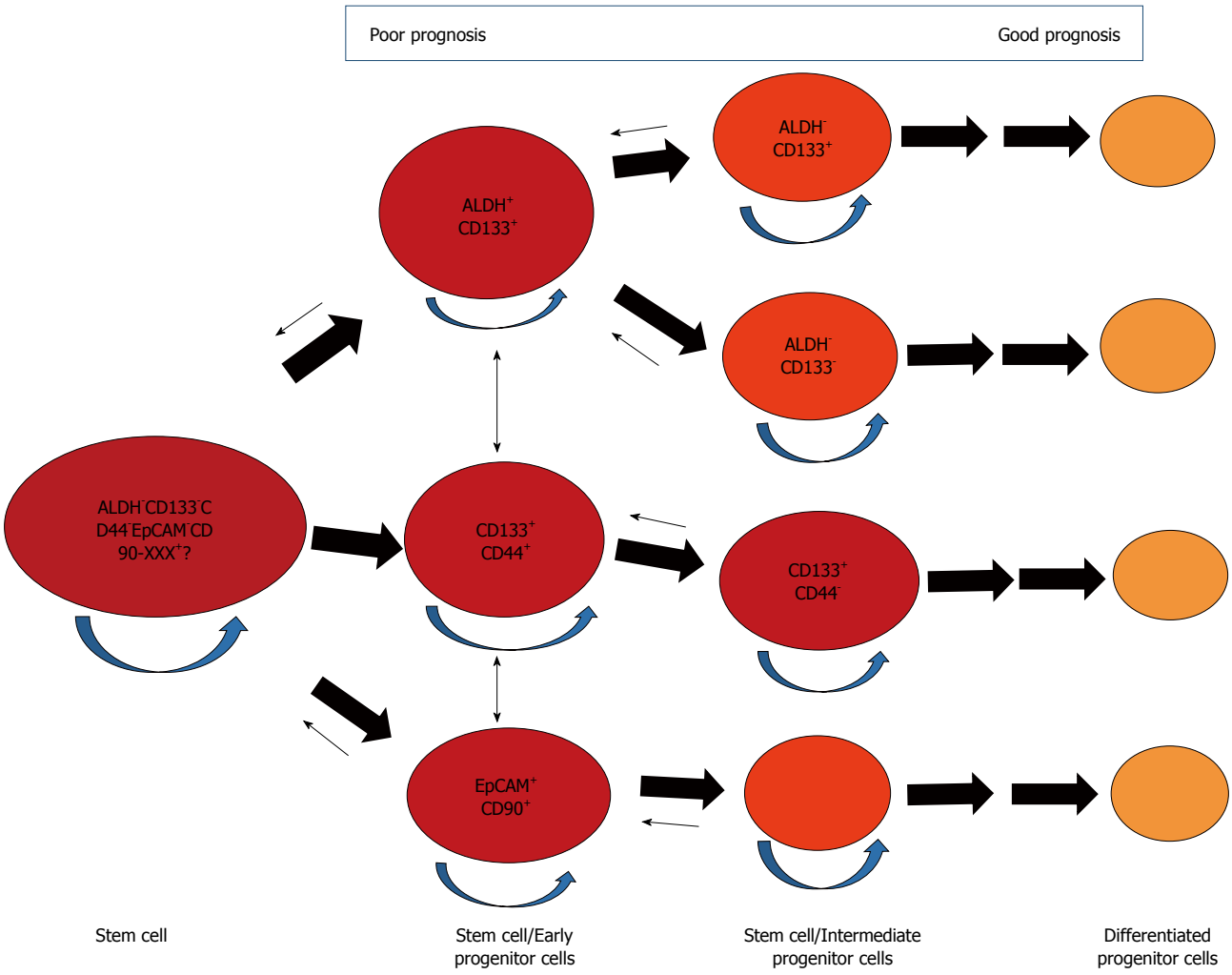

Figure 2 Proposed hierarchy of liver cancer stem cells according to the current literature. Liver cancer stem cell markers have been defined that can give rise to ALDH$^+$CD133$^+$, EpCAM$^+$CD90$^+$, CD133$^+$CD44$^+$ and other early progenitor cells, each of which can subsequently divide into more differentiated progenitor cells. Rounded arrows show cells with self-renewal capacity that have the potential to serve as CSCs. Small arrows show the unproven potential for "de-differentiation". CSCs: Cancer stem cells.

areas but not on hepatocytes in noncancerous areas. The expression of ICAM-1 has been reported to mediate adhesion-dependent cell-cell interactions and facilitate the movement of cells through the extracellular matrix, and it has been shown to be positively correlated with tumor size and poor prognosis in HCC[78]. Liu *et al*[79] indicated that ICAM-1 could be used as a potential CSC marker and may therefore be helpful in developing an effective treatment against cancer. ICAM-1$^+$ HCC have also been shown to be highly sphere-forming, have high tumorigenic capability and have increased expression of stemness-related genes in comparison with their ICAM-1$^-$ counterparts.

ESTABLISHMENT OF A HIERARCHY IN LIVER CANCER THROUGH LIVER CANCER STEM CELL MARKERS

Some research studies have indicated that different biomolecules are used as markers to identify and isolate cell populations with liver cancer stem cell properties, including the ability to generate tumors through the stem cell processes of self-renewal, ability to differentiate into multiple cell types, ability to undergo asymmetric division, and increased resistance to radio-/chemotherapy. One important task will be to confirm the relationship among these markers, as different markers of liver CSCs may be organized as a hierarchy of liver cancer cells (Figure 2). The establishment of such a hierarchy allows for the identification of which markers regulate CSC self-renewal, proliferation and differentiation. These abilities of LCSCs represent important therapeutic targets.

In the basic research field, Ma *et al*[80] found that ALDH and CD133 as CSC markers could be used either alone or in combination to identify different chemoresistant liver CSC populations and define a simple hierarchy. ALDH+ cells with CD133$^+$ or CD133$^-$ phenotype could initiate tumors in mice. The study also showed that the majority of ALDH$^+$ cells were

$CD133^+$, yet not all $CD133^+$ HCC cells were $ALDH^+$. A hierarchical organization of cells that differentially express CD133 and ALDH exhibit an ascending tumorigenic potential in the order of $CD133^+ALDH^+$, $CD133^+ALDH^-$, and $CD133^-ALDH^-$. Similarly, Zhu *et al*[40] observed that CD44 was consistently preferentially expressed in $CD133^+$ cells at the mRNA level compared to the corresponding $CD133^-$ cells from HCC cell lines. Multimarker analyses by flow cytometry revealed similar preferential expression of CD44 in the $CD133^+$ cell population. Specifically, the majority of $CD133^+$ cells from the SMMC-7721, MHCC-LM3 and MHCC-97L cell lines also expressed CD44. For Huh7, although the percentage of $CD133^+$ cells was more than 60%, only 1.88% of cells co-expressed CD133 and CD44, more likely representing a minority of the CSC subset. $CD133^+CD44^+$ HCC cells showed stem cell properties, including extensive proliferation, self-renewal, and differentiation into the bulk of cancer cells. *In vivo* xenograft experiments revealed that, actually, the highly tumorigenic capacity of $CD133^+$ cells as previously described was primarily attributed to the $CD133^+CD44^+$ cell subpopulation instead of their $CD133^+CD44^-$ counterparts. Moreover, cells double-positive for CD133 and CD44 exhibited preferential expression of some stem cell-associated genes and were more resistant to chemotherapeutic agents due to the upregulation of ATP-binding cassette (ABC) superfamily transporters, including ABCB1, ABCC1, and ABCG2, further supporting that these cells are of HCC cell origin. These findings suggest that $CD133^+CD44^+$ cells might represent true cancer stem/progenitor cells in HCC, which could allow for a better understanding of HCC initiation and progression as well as establish a precise target for the development of more effective therapies[40]. Captivatingly, in the clinical research field, Yamashita *et al*[54] found that $EpCAM^+$ and $EpCAM^-$ HuH1 cells equally expressed CD133, but only $EpCAM^+$ cells developed large hypervascular tumors. In addition, these results suggested that EpCAM may be a better marker than CD133 for enriching HCC tumor-initiating cells from AFP^+ tumors. They also found that CD90 expression was limited to HCC cell lines that are $EpCAM^-AFP^-$, and Wnt/β-catenin signaling had little effect on $CD90^+$ cell enrichment. These results identified that the expression patterns of various stem cell markers in tumor-initiating cells with stem/progenitor cell features may be different in each HCC subtype, possibly due to the heterogeneity of activated signaling pathways in normal stem/progenitor cells where these tumor-initiating cells may originate. Consequently, it would be useful to comprehensively research the expression patterns of stem cell markers to characterize the population of CSCs that may correlate with the activation of their distinct molecular pathways.

From all of the observations described above, asymmetric division of liver CSCs gives rise to $CD133^+CD44^+$ or $CD133^+ALDH^+$ early progenitor CSCs, and additional tumor-forming progenitors with more differentiated histology could be produced by further asymmetric division of these early progenitor cells.

LIVER CSC MARKERS AS THERAPEUTIC TARGETS

CSCs are defined by several markers that could represent potentially important therapeutic targets. In addition, these markers may be functionally important for CSCs, making them even more attractive as therapeutic targets. Despite reports that some markers are useful in the isolation and study of liver CSCs, other tissues may share these markers with hepatocellular carcinoma because of histological variation. The CSC phenotype might not necessarily be universal in all cancer subtypes. It thus appears relevant to identify specific CSC biomarkers, including cell surface markers, to improve prognosis and ultimately patient survival. New treatment strategies involve the development of antibodies that can target these markers. Antibody therapies against tumor cell surface antigens have improved clinical prognosis through inhibition of specific signaling pathways or enhanced activation of direct immune effectors. In some cases, these antibodies are conjugated to a bioactive drug that enables selective targeting of chemotherapeutic agents. Additionally, they block a signaling pathway in which the marker may be involved. Antibodies may also act by an antibody-dependent cytotoxicity (ADCC)/complement-dependent cytotoxicity (CDC) mechanism, thereby enhancing the immune response against CSCs[81]. CD133-expressing cells have been suggested to be critical tumorigenic progenitors in HCC, conferring chemoresistance by preferential activation of AKT/PKB and Bcl-2 cell survival response[82]. The treatment of $CD133^+$ HCC cells with an AKT1 inhibitor, which is specific to the Akt/PKB pathway, significantly reduced the expression of survival proteins. In addition, suppression of CD133 by a murine antibody to human CD133 conjugated to a potent cytotoxic drug reduced the proliferation rate of Hep3B cells *in vitro* and delayed tumor growth in a SCID mouse model[83]. Potential drug-resistant cell subpopulations can hopefully be eliminated in many cancers, such as liver cancer, retinoblastoma, ovarian cancer, prostatic adenocarcinoma, pancreatic cancer, or colorectal cancer, through the development of $CD133^-$ targeting antibodies. Multimarker methods have been applied in the characterization of CSCs in breast[34] and pancreatic cancers[37]. In liver cancer, $CD133^+/CD44^+$ HCC cells were more tumorigenic than those of $CD133^+/CD44^-$ cells *in vivo*. A recent study suggested that the CSC phenotype could be precisely defined by co-expression of CD133 and CD44 cell surface markers. $CD133^+/CD44^+$ HCC cells showed stem cell properties, including extensive proliferation, self-renewal and differentiation into the bulk of cancer cells. Additionally, recent studies

also revealed that blocking CD44 signaling using an anti-CD44 antibody might be a potential strategy to eradicate liver CSCs and consequently cure those patients[40]. A previous study demonstrated that $CD90^+CD44^+$ HCC cells possess a high capacity for tumorigenicity. Researchers who have characterized this subpopulation of cells have also examined the potential benefits of targeting CD44 *via* a neutralizing antibody approach. The systemic administration of anti-human CD44 antibodies in immunodeficient mice, formed by the intrahepatic inoculation of CD90+ liver CSCs, suppressed tumor nodule formation of liver tissue and metastatic lesions in lung tissue. Furthermore, the administration of CD44 antibodies was also shown to induce apoptosis in both CD90+ and CD90- cells *in vitro*[39]. In research on $CD13^+$ liver CSCs, Haraguchi *et al*[52] have also indicated that the combination of a CD13 inhibitor and 5-FU dramatically reduced tumor volume compared with that of either agent alone. 5-FU inhibited proliferating $CD13^+$ semi-quiescent CSCs, and the self-renewing and tumor-initiating abilities of liver CSCs were suppressed by CD13 inhibition. These studies demonstrated a novel treatment strategy of liver cancer by combining a CD13 inhibitor with reactive oxygen species (ROS) -inducing chemo/radiation therapy. Currently, several EpCAM-targeting antibodies are in clinical development, which include Catumaxomab and Adecatumumab. Clinical trials have been conducted in various cancers, including breast, prostate and colon cancers[84,85]. In liver cells, RNAi targeting of EpCAM significantly decreased the CSC pool and reduced both tumorigenicity and invasive capacity of CSCs[52,57]. Because EpCAM expression is a downstream target of Wnt/β-catenin, these results may have implications for the development of novel target therapies.

In addition to antibody-targeted therapy, a recent discovery by Lee *et al*[86] showed that lupeol, a phytochemical present in fruits and vegetables, could target $CD133^+$ liver CSCs by inhibiting their self-renewal and tumorigenic capacity. In addition, lupeol was able to sensitize HCC cells to chemotherapeutic agents (doxorubicin and cisplatin) *via* the PTEN-AKT-ABCG2 signaling pathway. The combination of lupeol, doxorubicin and cisplatin was found to exert a synergistic effect on tumor suppression, allowing the use of a lower dosage of conventional chemotherapeutic drugs, which may substantially reduce the cytotoxic side effects.

Other approaches have also been applied to target liver CSCs utilizing mechanisms that are not dependent on CSC-specific markers. Research studies targeting stem cell-related signaling pathways have shown some efficiency, and these therapeutic studies have been reviewed elsewhere[87,88].

CONCLUSION

During the past few years there has been a great quantity of work researching markers that identify liver CSCs, and these discoveries have contributed to one of the most important developments in cancer treatment. Nevertheless, some important issues still need to be resolved. For example, some of the pivotal markers that are significant to CSCs are also shared by normal stem cells; thus, drugs targeting these markers could have a negative effect on normal stem cells. To specifically target CSCs without unnecessarily affecting normal stem cells, molecular differences between them need to be delineated. In addition, in the coming years, one of the major challenges will be to determine how these different liver CSC markers relate to one another. There is growing concern that a single marker cannot isolate a LCSC population. Increasing evidence has demonstrated that combinations of multiple markers can specifically label CSC populations.

In summary, in the future, more effective liver CSC markers are required to identify and design more specific anti-CSC marker therapies. The apparent advantages of specifically targeting CSCs in improving the potency of existing therapies are revealed in current knowledge, leading to long-term clinical benefits by providing an important framework for developing a novel therapeutic regimen.

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