

Review

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Advances in surface markers of liver cancer stem cell

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How to cite this article: Zhang JL, Gong LQ, Yan Q, Zhou NN, Lee VHF, Guan XY. Advances in surface markers of liver cancer stem cell. *Hepatoma Res* 2019;5:27. <http://dx.doi.org/10.20517/2394-5079.2019.13>

Received: 21 Feb 2019 **First Decision:** 10 Apr 2019 **Revised:** 16 May 2019 **Accepted:** 24 May 2019 **Published:** 23 Jul 2019

Science Editor: Jin-Lin Hou **Copy Editor:** Cai-Hong Wang **Production Editor:** Jing Yu

Abstract

Liver cancer stem cells (LCSCs), a small subpopulation that constitutes liver cancer heterogeneity, play a vital role in cancer initiation, invasion, recurrence, metastasis, and resistance to chemo-radiotherapy. It is believed that therapies targeting LCSCs can improve the efficacy of conventional chemotherapy and radiotherapy by completely eliminating tumors while preventing recurrence. Therefore, during last decades, numerous surface markers for LCSCs have been identified and characterized in many subtypes of liver cancer, especially in hepatocellular carcinoma (HCC). These well-recognized surface markers significantly promote the therapeutic efficacy that identifies, targets and destroys LCSCs. Meanwhile, there have been intensive studies that aim to investigate the molecular mechanism of how stemness contributes to liver cancer relapse, recurrence and resistance. However, liver cancer stemness seems to be regulated by a hierarchical organization and crosstalk of a wide variety of signaling pathways. Using individual or few LCSC surface markers may not be able to completely reveal the intrinsic stemness hierarchy. From an integrated perspective, understanding of recent advances in LCSC surface markers remains important and urgent. In this review, we concentrate on demonstrating the indispensable roles of LCSC surface markers in identification and characterization of multiple cancer stages including initiation, invasion, metastasis, resistance and highlighting the cutting-edge therapeutic strategies against cancer stem cells in HCC.

Keywords: Liver cancer, hepatocellular carcinoma, cancer stem cell, surface marker



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INTRODUCTION

Liver cancer is the seventh most frequently diagnosed cancer and the third death causing of cancer around the world, which has 841,080 newly diagnosed cases and caused 781,631 deaths in 2018^[1]. Hepatocellular carcinoma (HCC) comprises 75%-85% of the primary liver cancer cases and intrahepatic cholangiocarcinoma and other rare types comprise 15%-25%^[1]. Chronic hepatitis B virus infection, hepatitis C virus infection, steatohepatitis and cirrhosis are the most prevalent precursors to HCC. Despite of the recent advances in liver cancer therapies, the current treatment cannot effectively prevent tumor recurrence and metastasis due to the existence of (liver cancer stem cells) LCSCs. The concept of cancer stem cells (CSCs) is raised from clinical and experimental observations that there exists a subpopulation of cancer cells that possess stem cell-like characteristics including self-renewal and differentiation that eventually lead to cancer relapse and resistance. LCSCs have been reported in varied types of HCC and are deemed to be one of the major causes of HCC recurrence, metastasis, chemoresistance and radioresistance.

Conventional therapies against non-stem liver cancer cells such as chemotherapy and radiotherapy, have multiple limitations that result in cancer recurrence and metastasis due to acquired resistance. The survival LCSCs can re-initiate tumor development and invasion [Figure 1]. Hence, in order to develop feasible therapies that can prevent tumor recurrence and metastasis, it is important to specifically identify, target and eliminate LCSCs. Recent advances in LCSC surface markers and understanding of cellular features related to LCSC phenotypes greatly improve the efficacy of treatments that target LCSCs. Targeting the LCSCs with high expression of certain stemness surface markers, can manipulate the abilities of LCSCs in proliferation, growth, maintenance, differentiation, resistance and apoptosis via cellular signaling pathways so that tumor regeneration can be impeded.

In order to develop patient-specific therapies that target LCSCs, multiple stemness surface markers have been identified consisting CD133^[2], CD44^[3], CD90^[4], epithelial cell adhesion molecule (EpCAM)^[5], CD47^[6], CD34^[7], C-kit^[8], CD13^[9], CD24^[10], calcium channel $\alpha 2\delta 1$ isoform5^[11], oval cell marker OV6^[12], DLK1^[13], K19^[14], and Lgr5+^[15] [Table 1]. The integrated therapy using conventional anti-carcinogenic inhibitors such as sorafenib with LCSCs-targeting drugs, may provide an effective therapeutic strategy for complete elimination of liver cancer.

CD133

CD133, also referred to as PROM1, is a member of prominin family that has a structure of five transmembrane single-chain glycoprotein with a molecular weight of 115 ~ 120 kDa, including an extracellular N-terminus, two large extracellular loops, two small intracellular loops and an intracellular C-terminus^[16-19]. CD133 was originally identified as a surface marker of hematopoietic stem cells^[16]. In solids tumor, CD133 was firstly identified and further isolated in brain tumors^[20]. Later, the role of CD133 as a surface marker of CSC is been reported in a wide variety of tumor tissues such as lung cancer^[21], stomach carcinoma^[22], pancreatic cancer^[23], colon cancer^[24], and liver cancer that was identified by our team^[25-27].

In 2006, Suetsugu *et al.*^[28] reported that CD133+ liver cancer cells, sorted from the Huh7 cell line, exhibited a more potent capability of proliferation and metastasis compared to the CD133- counterparts. Our previous study indicated that CD133+ cells also processed a stronger colony-forming characteristic, greater tumorigenicity and potential to differentiate into angiomyogenic-like lineages^[2]. We also further characterized the liver CD133+ CSCs, revealing that CD133+ cells were endowed with high *in vivo* tumorigenicity and the capability to form spheroids with an upregulated expression of stemness-associated genes *in vitro*^[29-31]. Liu *et al.*^[32] reported that CD133 was crucial to monitor the migratory capability of LCSCs, tumor-initiating properties, and the epithelial-mesenchymal transition (EMT) process. Tang *et al.*^[33] demonstrated that CD133+ liver tumor-initiating cells (TICs) had angiogenesis ability. In addition, Li *et al.*^[34] and other researchers^[35] also found that CD133+ HCC cells could exploit autophagy to maintain their survival. Liver CD133+ CSCs are

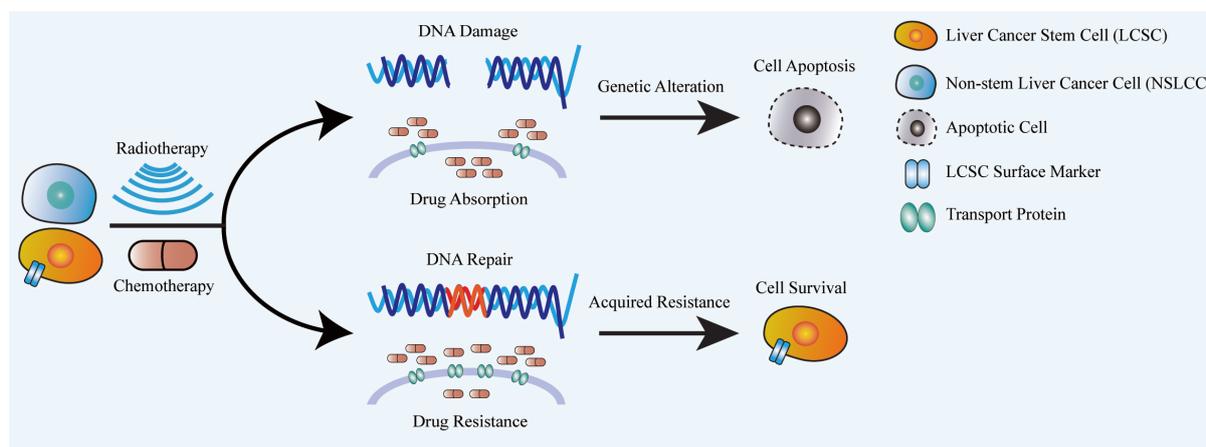


Figure 1. Acquired chemo- and radioresistance in liver cancer stem cells. Traditional chemo-/radiotherapy can induce genetic alteration in non-stem liver cancer cells (NSLCCs) via DNA damage and cytotoxic agent intake, in order to activate cellular apoptosis. However, upon treated with traditional chemo-/radiotherapeutic agents, liver cancer stem cells (LCSCs), can acquire chemo-/radioresistance including an increased level of drug intake and an enhanced DNA repairing mechanism, which eventually lead to a higher survival rate of LCSC subpopulation.

Table 1. Summary of liver cancer stem cell biomarkers and related pathways

Surface markers of LCSCs	Related pathways
CD133	AKT/PKB ^[30] , EGFR-AKT ^[38] , IL-8/CXCL1 ^[33] , Aldehyde dehydrogenases ^[25] , JNK ^[39] , mTOR ^[40] , TGF- β ^[41,42] , Aurora kinase/RalA ^[43] , Notch1 ^[44] , PTEN ^[45] , NF- κ B ^[45] , ZFP42/REX1 ^[46] , miR-150/c-Myb ^[47] , miR-142-3p ^[48] , miR-152/KIT ^[49] , miR-130b/TP53INP1 ^[29] , miR-1246/Wnt/ β -catenin ^[50] , LncSox4/Stat3 ^[58]
CD44	AKT ^[81] , YAP1/TEAD ^[82] , anti-miR-27a/QD-HA-PEI ^[84] , TGF β /ALK5 ^[85] , mTOR ^[86] , FoxM1/ROS ^[83]
CD90	SHH/Gli and IL6/JAK2/STAT3 ^[91] , ABCG2 and Oct5 ^[93] , miR-125a/b ^[96] , has 0067531 ^[97]
EpCAM	Wnt/ β -catenin ^[5,104] , CHD4 ^[105] , OSM ^[106] , ATRA ^[107] , EZH2 ^[108] , miR-155 ^[109] , miR-181 ^[111] , miR-216a/217/PTEN/SMAD7 ^[110]
CD47	CTSS/PAR2 ^[6,120] , NF- κ B ^[122] , SIRP α ^[119]
CD34	OCT4, SOX2, NAONG, Klf4, c-Myc, and Lin28 ^[7]
C-kit	TGF- β /SMAD2 and c-KIT/JAK1/STAT3 ^[132]
CD13	TGF- β -/EMT ^[139]
CD24	STAT3/NANOG ^[10,144] , Twist2 ^[144]
α 2 δ 1	OCT4, SOX2, NANOG, and BMI1 ^[111] , miR-31/ISL1 ^[148]
OV6	Wnt/ β -catenin ^[12]
DLK1	Nanog, SMO, SOX2, Oct3/4 ^[153]
K19	EMT and TGF β /Smad ^[14,155] , PDGFR α -laminin ^[156] , MET-ERK1/2-AP1 and SP1 ^[157]
LGR5	HGF/ Rspo1 ^[173] , LSD1/Prickle1/APC/ β -catenin ^[175]

shown to be more resistant to radiotherapy^[36] and chemotherapy^[37]. Our previous study found that CD133+ cancer stem cells conferred chemoresistance caused by abnormal activation of the Akt/PKB pathway^[30]. Other Aberrant signaling pathways related to CD133+ LCSCs have also been reported and characterized including EGFR-AKT^[38], IL-8/CXCL1^[33], aldehyde dehydrogenases^[25], JNK^[39], mTOR^[40], TGF- β ^[41,42], aurora kinase/RalA pathway^[43], Notch1 signaling pathway^[44], PTEN signaling pathway^[45], NF- κ B signaling pathway^[45]. Recently, our team identified ZFP42/REX1 as a key regulator of cancer stemness in CD133+ LCSCs by genome-wide DNA methylation analysis^[46]. A panel of miRNAs that include miR-150, miR-142-3p, miR-152, miR-130b and miR-1246 have also been found to regulate proliferation, tumorigenicity, invasion, migration and angiogenesis in CD133+ HCC cells^[29,47-50].

In summary, aforementioned studies demonstrate that the maintenance of CD133+ LCSCs is modulated by an intricate network of signaling pathways. Cells with varied morphological structures primarily constitute HCC and express distinct hepatic lineage genes. Thereby, there might also be functionally different cancer cell subpopulations that express distinct stemness-associated markers. Wilson *et al.*^[51] have shown that the

most widely used CSC markers including CD133, CD44, CK19, CD90, EpCAM, and ALDH are not specific to LCSCs. CD133+/ALDH+ cells showed to possess stronger tumorigenicity than their CD133-/ALDH- or CD133-/ALDH+ counterparts^[25]. We also found and confirmed^[30] that CD133+/ALDH+ cells possess stronger tumorigenicity than their CD133-/ALDH- and CD133-/ALDH+ counterparts both *in vivo* and *in vitro*. Furthermore, we established a hierarchical organization in HCC to demonstrate HCC tumorigenicity from the highest to the lowest: CD133+/ALDH+ > CD133+/ALDH- > CD133-/ALDH-. Zhao *et al.*^[11] reported that some subpopulations of liver cancer cells, including CD133+/1B50-1+, CD13+/1B50-1+ and EpCAM+/1B50-1+ cells, exhibited high tumorigenicity. CD133+/EpCAM+ cells displayed the highest tumor-initiating activity, compared to CD133+/EpCAM- and CD133-/EpCAM+ cells^[52]. Elevated CD133 expression is associated with tumor differentiation grades, disease stages and alpha-fetoprotein (AFP) levels. Furthermore, a higher CD133 expression level indicates higher recurrence rates as well as poorer overall survival^[36,53-57]. Recently, Chen *et al.*^[58] reported that a long noncoding RNA termed LncSox4, is upregulated in CD133 and EPCAM high-expressed HCC tissues, modulating the self-renewal of liver tumor-initiating cells via Stat3-mediated Sox4 expression.

When CD133 as a target was concerned, Sasaki *et al.*^[54] developed a DC-based vaccine inhibited the tumorigenicity of CD133+ HCC cells subcutaneously injected into nude mice. Our previous study demonstrated that AKT1 inhibitor can significantly reduce the expression of the survival proteins that was primarily expressed endogenously in CD133+ HCC cells^[30]. Smith *et al.*^[59] developed an anti-CD133 antibody-drug conjugate that could inhibit growth of CD133+ HCC cells. Lang *et al.*^[60] prepared a 131I-CD133 monoclonal antibody (mAb) with specific selectivity that could lead to clinical significance in liver cancer treatment. Huang *et al.*^[61] developed a bispecific antibody (BsAb) of anti-CD3/anti-CD133 and coagulate it to the cytokine-induced killer (CIK) cells to effectively target and kill CD133+ cells.

CD44

CD44, firstly was recognized as a lymphocyte homing receptor^[62], can be broadly detected in multiple tissues including embryonic^[63], hematopoietic^[64], mesenchymal^[65], and cancer stem cells^[66-69]. In humans, CD44 gene comprises 20 exons and 19 introns and undergoes complicated alternative splicing to generate CD44 standard form (CD44s)^[70-72] and CD44 variant splice isoforms^[73]. CD44 is involved in the interaction between cells and extracellular matrix^[74].

Williams *et al.*^[75] emphasized on the behavior of CD44-regulating stem cell, including cell differentiation and self-renewal and cell-matrix interactions during tumor progression and migration. Isolated CD44s+ cells can effectively form colonies and possess hepatic markers^[76]. In HCC, CD44s expression is involved to modulation of the mesenchymal phenotype mediated by TGF-beta and its expression level is an unfavorable prognosis factor^[77]. Proliferation of CD44+ cells and its tumorigenesis can be stimulated by IL6 produced by tumor-associated macrophages (TAMs)^[78]. CD44 expression is known to be related to invasive and metastatic behavior of liver cancer^[79]. For instance, FAM83D promotes HCC recurrence by increasing CD44 expression and modulating CD44+ CSCs malignancy^[80]. Coexpression of CD44 with other markers such as CD133 and CD90 help well identify LCSC phenotypes. CD133+/CD44+ subpopulation is associated with the metastatic capability in the xenotransplantation assay in nude mice^[36]. CD133+/CD44+ HCC cells exhibits elevated expression of many CSC-related genes and are more chemotherapy-resistant owing to the increased expression of transporters that belong to ATP-binding cassette superfamily^[79]. Most of CD90+ cells coexpress CD44 and these CD90+/CD44+ cells exhibit an aggressive behavior than the CD90+/CD44- counterpart and easily develop metastases in the nude mice lung^[4]. Yang *et al.*^[4] found that administration of anti-CD44 antibody was able to induce apoptosis of the CD90+ and CD90- cells in a dose-dependent manner, and prevented CD90+/CD44+ CSC-derived tumor both locally generated and distantly metastasized^[4].

The mechanism of the conversion from terminally differentiated cells that expose to oncogenic factors into CSCs remains largely uninvestigated. Dhar *et al.*^[81] explained this phenomenon that CD44 could activate AKT to induce Mdm2 phosphorylation nuclear translocation, which terminated the p53 DNA-damage surveillance. This process enables DNA- sequestered hepatocytes to avoid p53-induced apoptosis and to respond to proliferation-related signals that promotes daughter cells transfer to HCC progenitors. CD44s, regulated by the YAP1/TEAD axis, can positively modulate the YAP1 expression along with its target genes through the PI3K/Akt pathway in HCC. This processes composed a feedback loop consisting of CD44s and YAP1, promoting HCC tumorigenesis by regulating cell proliferation and invasion during^[82]. Kopanja *et al.*^[83] find that FoxM1 expression level is associated with CD44 expression, suggesting that FoxM1 is required for the expression of CD44 in HCC cells. In liver cancer, anti-miR-27a/QD-HA-PEI exhibit effective anti-cancer effects *in vitro* and *in vivo* via down-regulation of FOXO1 and PPAR- γ ^[84]. Galunisertib (LY2157299), a selective ATP-mimetic TGF- β inhibitor, can effectively reduce tumor cell vitality via alleviating expression of CD44 and THY1^[85]. INK128, an ATP-competitive mTOR inhibitor, can suppress CD44+ and sorafenib insensitive HCC *in vitro* and *in vivo*^[86].

CD90

In 1964, CD90 was initially named as θ antigen because it had identified in a process to develop an antileukemia xeno-antibody in CH3 AKR strain mice^[87]. Later in 1969, θ antigen was renamed as Thy-1 since the thymus was found to the location where precursors of T cells got mature^[88]. In the 1980s, Ades *et al.*^[89] isolated CD90 from MOLT-3, a human T-cell leukemia cell line, demonstrated the presence of CD90 in human. CD90 is a 25-37 kDa glycosylphosphatidylinositol-anchored glycoprotein, and a crucial modulator of multiple cellular events, including immunologic function of promoting T cell activation and nonimmunologic functions such as nerve regeneration, tumorigenesis, metastasis, inflammation, and fibrosis^[90]. The CD90+ LCSCs isolated from liver cancer tissue specimens shows a strong tumorigenic potential after being implanted into nude mice^[4]. Zhang *et al.*^[91] illustrated that by activating the IL6/JAK2 pathway, SHH/Gli could regulated the stem-cell like characteristics of CD90+ LCSC. Cytotoxic drugs 5-FU or epirubicin treatment result in the generation of CD90+ and CD105+ cells *in vitro* in Huh1 and Huh7 cells, which primarily have no CD90+ nor CD105+ cells^[92]. It was shown by Jia *et al.*^[93] that being as a closely related cause to chemoresistance, the overexpression of ABCG2 and Oct5 was frequently enriched in CD90+/CD133+ LCSCs. Subcutaneous transplantation of CD90+/CXCR4+ HCC cells to NOD/SCID mice are easily detected in the peripheral blood and able to develop distal metastatic tumors^[94]. The expression of CD90+ does not overlap with the expression of EpCAM+. Gene expression analysis shows that EpCAM+ cells display epithelial characteristics, while CD90+ cells exhibit a vascular endothelial type of gene profile^[95]. Exosomes containing miR-125a and miR-125b derived from TAMs mediate stem cell properties in HCC by targeting CD90^[96]. Zhang *et al.*^[97] demonstrated that has 0067531 affected the biological functions of CD90+ HCC cells by regulating P13K-AKT signaling pathway. Moreover, CD90 overexpression is shown to be associated with unfavorable prognosis^[98]. Overall, the results of present studies have suggested that CD90 is a potential biomarker for HCC diagnosis and targeting therapy.

EpCAM

EpCAM is the first human tumour-associated antigen identified with monoclonal antibodies (mAb)^[99], and also the first monoclonal antibody manufactured against for human cancer is murine mAb 17-1A targeting EpCAM^[100,101]. According to an early elaborate review about EpCAM in cancer^[102], it is a type I membrane protein of 314 amino acids, containing two epidermal growth factor-like domains at the extracellular domain and 26 amino acids at intracellular domain. EpCAM is a cell surface marker expressed in almost all the epithelial tumors^[103]. The EpCAM+ HCC cells possess CSC-like characteristics including an enhanced self-renewal ability and differentiation potential, and are able to initiate the development of highly tumorigenic cancer in NOD/SCID mice. EpCAM is a target gene in Wnt- β -catenin signaling pathway^[5,104]. Chemoresistance as well as stemness of EpCAM+ LCSCs are modulated by abnormal expression of CHD4^[105],

OSM^[106], ATRA^[107], EZH2^[108]. A group of microRNAs including miR-181, miR-155, miR-181, miR-216a/217 have been found involved in regulating stemness of EpCAM+ HCC cells^[109-111]. Patients with EpCAM+/AFP+ HCC have higher frequency of portal vein invasion and significantly shorter survival than EpCAM-/AFP- HCC patients^[112]. Chen *et al.*^[113] proposed a novel EpCAM-antibody-labeled polymer in nano-vesicles for cancer stem cells-targeted drug and siRNA and displayed higher tumor selectivity and killing efficacy. A recent study revealed that metformin decreased both the EpCAM+ HCC cells abundance and self-renewal capability^[114]. Babaei *et al.*^[115] reported that EpCAM targeted nanoparticles of PEG-Au@Si-5-FU exhibited higher cytotoxicity than nontargeted PEG-Au@Si-5-FU in 2D and 3D HepG2 cell cultures. Many EpCAM antibodies are currently available to treat patients with EpCAM+ malignant ascites in preclinical and clinical trials including edrecolomab, adecatumumab, MT110 and catumaxomab^[116].

CD47

CD47 is firstly discovered in 1992 as a surface protein that is frequently expressed in ovarian carcinoma^[117]. Later studies have exhibited that CD47 is a highly expressed transmembrane protein with various functions^[118,119]. Lee *et al.*^[6] identified that CD47 was preferentially expressed in liver TICs, which result in cancer development, self-renewal, metastasis and chemoresistance and significantly influence the clinical prognosis of patients. CD47+ HCC cells preferentially secrete cathepsin S (CTSS), which manipulates liver TICs through the CTSS/protease-activated receptor 2 (PAR2) loop. Suppression of CD47 by morpholino decreases HCC viability and exerts a chemo-sensitization effect through blockade of CTSS/PAR2 signaling pathway^[6,120]. Increased CD47 expression level has been considered as a negative prognostic factor for a wide variety of cancer^[118,121]. Lee *et al.*^[6] unraveled that CD47 expression was enriched on CD133+/CD24+ TICs isolated from a HCC cell line and was increased by serial passage in the presence of doxorubicin and cisplatin, and high CD47 expression conferred chemoresistance and increased the stemness characteristics of TICs. CD47 blockade or down-regulation suppresses HCC development and elevated sensitivity to chemotherapeutic drugs such as sorafenib^[6,122-124], while NF- κ B-mediated CD47 up-regulation enhances sorafenib resistance^[122]. Importantly, not only being considered as a LCSC surface marker, expression of CD47 is also involved in innate immune response^[123]. CD47 is a ligand for signal regulatory protein- α (SIRP α), which expressed on macrophages and dendritic cells^[125]. After binding CD47, SIRP α activates a signaling cascade that leads to the inhibition of phagocytosis^[119]. Macrophage phagocytosis of HCC cells is enhanced after treatment with CD47 antibodies (CD47mAbs) that impede CD47 binding to SIRP α ^[118,126]. Treatment to mice with tumor burden with antibodies that blockade CD47 signaling can produce intensive tumor regression when used solely or integrated with existing therapeutic strategies^[118,121,127,128] and humanized CD47 antibodies have recently entered human clinical trials (NCT02678338, NCT03717103, NCT03763149, NCT02216409, NCT02367196).

CD34

Park *et al.*^[7] identified CD34+ as a newfound LCSC surface marker. SOX2 is one of the vital factors maintaining CD34+ LCSC stemness before colonization, and OCT4, SOX2, NAON, Klf4, c-Myc, and Lin28 are supposed to be associated with stemness maintenance of CD34+ LCSC on feeder cells^[7]. Park *et al.*^[129] found that CD34+ LCSCs possessed stemness characteristics and three types of liver carcinomas were directly produced from CD34+ PLC/PRF/5 hepatoma cells (PLC): hepatocellular carcinoma (HCC), cholangiocarcinoma (CC), as well as combined hepatocellular cholangiocarcinoma (CHCs). CD34+ PLCs that express OV6 and their progeny OV6+ cells primarily produce CHC and CC, suggesting that the OV6+ antigen is correlated with human CHC and CC^[129]. Crosby *et al.*^[130] addressed that c-kit+ or CD34+ liver cancer cells had the potential to transfer to biliary epithelial cell lineage and might represent biliary epithelial stem cells. Zeng *et al.*^[131] demonstrated that CD34+ LCSCs and xenografts generated by CD34+ LCSCs exhibited a blended phenotypes, coexpressed stemness and myelomonocytic cell markers. CD34+ LCSCs are often coexpressed with CD45, suggests that the origin appears to be from a hematopoietic precursor, which illuminate a comprehensive understanding of the molecular mechanism of how LCSCs are originated and developed^[131].

C-kit

C-kit, also named as stem cell factor receptor, is a receptor protein of transmembrane type III with intrinsic tyrosine kinase activities to generate human embryonic stem cells. Besides having been used to identify human hematopoietic progenitor cells or hepatic stem cells, c-kit also is capable to sustain the stem cells in an undifferentiated state. The presence of c-kit on HCC cell lines suggests that stem cell factor (SCF) have been considered to play an indispensable role in the manipulation of the proliferative capability of liver cancer cells^[8]. Fujio *et al.*^[132] demonstrated that C-kit could be an important factor in the receptor systems, a growth factor related to the biological functions of liver stem cells and the development of bile ducts. It has been reported^[133] that TGF- β /SMAD2 signaling pathway mediates the expression of the c-KIT receptor ligand in a transcriptional level by activating c-KIT/JAK1/STAT3 signaling pathway. SCF activates TGF- β 1 ligand expression through STAT3, thereby result in a positive feedback loop between TGF- β /SMAD and SCF/c-KIT signaling pathway. The signaling network attenuates TGF- β -mediated cell cycle arrest and activates tumor cell to into proliferation, epithelial-to-mesenchymal-transition, migration, and invasion^[133]. Blockade of C-kit in late cirrhosis might restore TGF- β inhibitory effect on normal liver stem cells and prevent initiation and progression of HCC^[134]. The expression of C-kit is significantly higher in liver cancer patients with advanced clinical stage and is an independent poor prognostic factor of DFS in HCC patients^[135].

CD13

CD13, also referred as aminopeptidase N, is a membranous glycoprotein that has been used to identify leukemia or lymphoma cells^[136]. CD13 plays vital roles in cancer progression including cell proliferation, invasion, and angiogenesis^[137-139]. Nagano *et al.*^[140] demonstrated that CD13 is a surface marker of CSCs in human liver cancer and may have promising therapeutic potentials. It was found by Haraguchi *et al.*^[9] that CD13 attenuated ROS-induced DNA injury after chemo/radiation treatment and protected cells from apoptosis. They also found that ubenimex, a CD13 inhibitor, alleviated oncogenic and self-renewal ability of CSCs and suppressed CD13+ tumor growth with co-treatment of 5-FU. Kim *et al.*^[141] reported that upregulated CD13 expression was associated with TGF- β -induced EMT-like process, which prevents further increasing of ROS level as well as the induction of apoptosis, supporting the survival of CD13+ CSCs in liver cancer cells. It was also shown by Yamashita *et al.*^[142] that ubenimex synergistically enhanced the antitumor effects of a chemotherapy regimen composed of 5-FU, CDDP and DXR on HCC cells, and the functions of ubenimex were associated with enhanced intracellular ROS levels.

CD24

CD24 is a glycosylated and mucin-like cell surface glycoprotein with relatively high expression in stem/progenitor cells and related to formation and development of CSCs isolated from breast, colon, ovary, pancreas^[143,144]. Huang *et al.*^[145] firstly cloned the full-length CD24 cDNA sequence from human HCC cells and identified that CD24 mRNA overexpression was associated with p53 mutation and tumor differentiation. Lee *et al.*^[10] reported CD24 as a surface marker of LCSCs. They^[10] also documented that CD24 was upregulated in chemoresistant tumors after cisplatin treatment in immunodeficient mice model. Significantly, CD24 expression largely overlaps with expression of CD133 and EpCAM in HCC^[10]. CD24+ HCC cells have a great impact on clinical prognosis of patients, and play a vital role in self-renewal, differentiation, maintenance, and metastasis of tumors^[10]. Self-renewal and tumor initiating behavior of CD24+ LCSCs is regulated by STAT3-mediated NANOG regulation^[10]. It was demonstrated by Liu *et al.*^[146] that the pathway of Twist2-CD24-STAT3-NANOG was crucial to the regulation of self-renewal of CD24+ LCSCs.

$\alpha 2\delta 1$

In 2010, García *et al.*^[147] reported that when the expression of calcium channel $\alpha 2\delta 1$ subunit was inhibited, migration, adhesion as well as spreading of myoblasts were impaired, whereas the L-type calcium maintained unaffected, suggesting a newfound function of the $\alpha 2/\delta 1$ subunit in extracellular signaling. Later studies

have confirmed that calcium channel $\alpha 2/\delta 1$ subunit is a potential marker for CSCs in laryngeal squamous cell carcinoma^[148] and in non-small cell lung cancer^[149]. Zhao *et al.*^[11] identified $\alpha 2/\delta 1$ subunit as a LCSCs marker and developed its monoclonal antibody named 1B50-1, which had positive therapeutic effects on HCC xenograft by eradicating LCSCs. $\alpha 2/\delta 1$ + liver cancer cells have stemness characteristics, including the expression of stemness-related genes such as SOX2, OCT4, BMI1, and NANOG, the capability of self-renewal, invasiveness, and to produce both $\alpha 2/\delta 1$ + and $\alpha 2/\delta 1$ - cells^[11]. Recently, Zhang *et al.*^[150] discovered that miR-31 could negatively manipulate the self-renewal capability of $\alpha 2/\delta 1$ + LCSCs via sequestering ISL1, implying a potential therapeutic strategy for directly targeting liver TICs.

OV6

1998, Roskams *et al.*^[151] identified that reactive ductules and intermediate hepatocyte-like cells originated partially from differentiation and activation of progenitor cells. It has been put forward that OV6 in human liver can help identify cells owing a progenitor stem cell-like characteristics, which has the ability to differentiate into OV6+ ductular cells or lobular hepatocytes. OV6+ is a specific phenotype of oval cells that has been originally identified in the livers of tumor transplanting rats, and is identified as a surface marker of human liver progenitor cells in 2008^[152]. In 2012, Yang *et al.*^[12] further demonstrated that OV6+ HCC cells not only possessed a stronger capacity to form spheroids, but also showed stronger tumorigenic and metastatic characteristics. These results suggest that OV6+ HCC cells are highly capable of self-renewal and forming tumors. Wnt/ β -catenin signaling plays an indispensable role in the induction and expansion of OV6+ subpopulation within tumor tissues. Thereby, OV6 is considered as an effective LCSCs surface marker. Additionally, Yang *et al.*^[12] also demonstrated that overexpression of OV6 enhanced the invasive and metastatic characteristics of HCC CSCs so that the number of OV6+ CSCs increased in patients diagnosed with liver cancer indicated poorer clinical outcomes and prognosis.

DLK1

DLK1 has shown to be expressed in fetal liver, but scarcely expressed in neonatal and adult liver in mice and rats^[153]. Huang *et al.*^[154] demonstrated that proliferation of SMMC-7721 cells was significantly enhanced by exogenous DLK1, whereas colony-forming ability, cell growth, and tumorigenicity of Huh-7, Hep3B, and HepG2 cells were significantly impeded by the suppression of endogenous DLK 1 via RNA interference. It was identified by Li *et al.*^[13] that the enhancing effect of DLK1 in tumorigenicity and cancer stemness could potentially be used as a molecule target for therapies against LCSCs. DLK1+ cells have been discovered in all 17 HCC cell lines and showed a more potent capability of clonogenicity *in vitro* and tumorigenicity in animal models. In addition, some stemness markers have been identified upregulated in DLK1+ Huh-7 and Hep3B cells including NANOG, SMO, SOX2, Oct3/4, CD133, CD90, and EpCAM. The isolated DLK1+ HCC cells are possess strong therapeutic resistance to conventional cytotoxic agents such as doxorubicin, cisplatin, epirubicin, and 5-FU^[155].

K19

Cytokeratin 19(K19) is a newfound CSC surface marker associated with EMT and TGF β /Smad signaling pathway^[14]. K19 disappears from liver cells but remains in bile duct cells at the 10th differentiation week, which is an important step in the organogenesis of liver^[156]. It has been reported that using 18F-FDGPET and CYFRA 21-1 can identify K19+ LCSCs in HCC. In patients with HCC, K19 expression is significantly correlated with GLUT1 expression and FDG accumulation, and K19 regulated 18F-FDG uptake via TGF β /Smad signaling pathway^[157]. Besides the TGF β /Smad signaling pathway, many other signaling pathways have been documented as well. The PDGFR α -laminin B1-K19 cascade drives tumor development at the invasive front of HCC^[158]. Rhee *et al.*^[159] reported that expression of K19 in HCC is modulated by fibroblast-derived HGF via a MET-ERK1/2-AP1 and SP1 axis. K19+ cells have high proliferation potential and doxorubicin, 5-fluorouracil and sorafenib resistance^[14,160]. K19 expression exhibits strong correlation with increased

tumorigenicity, decreased tumor differentiation potential, metastasis and invasion and poor prognosis in HCC^[160-163], with profiling study shows that K19+ HCCs highly express invasion or metastasis-related genes (TACSTD2, VASP, LAMC2, LAMB1, PDGFRA), biliary/HPC markers (NOTCH2, GSTP1, CD133, JAG1) and members of the miRNA-200 family (miR-200c, miR-141)^[160]. A recent study showed that K19+ cells were not involved in the early clonal expansion of rat hepatocarcinogenesis, and K19 expression arose in preneoplastic hepatocyte lesions undergoing malignant transformation. In addition, they also indicated that K19 positivity in HCCs did not necessarily reflect the cell of origin of the tumor, but rather the plasticity of preneoplastic cells during the tumorigenic process^[164].

LGR5

In 2007, LGR5, a G protein-coupled receptor with a seven transmembrane domains^[165], was firstly identified as a surface marker of intestinal stem cells^[166]. Later, it has been applied to identify homeostatic stem cells in various organs such as ovaries, hair follicles, mammary gland, and stomach^[166-169]. LGR5 has been reported to involved in regeneration of damaged tissues in the small intestine and colon, liver, pancreas, and stomach^[15,170-172] and in CSCs that regulates tumor proliferation^[173,174]. Carbon tetrachloride treatment enhances both fibrosis and LGR5+ liver stem cell growth, whereas LGR5 downregulation aggravates fibrosis. HGF together with Rsp01 increases the number of LGR5+ liver stem cells and enhances hepatic function by inhibiting fibrosis^[175]. Both Carbon tetrachloride-induced acute damage and oval cell response to damage can induce LGR5+ stem cells/progenitors actively engaged in hepatic reconstitution via *de novo* generation of hepatocytes^[15]. Effendi *et al.*^[176] addressed that LGR5 upregulated HCC cells showed more potent colony-forming capability and possessed higher therapeutic resistant to a cytotoxic drug and weakened migration ability than the controls. Further, LGR5 overexpressed HCC cells produces nodule-type metastases in the livers of immunodeficient mice, whereas vector-transfected HCC cells generates more invasive tumors^[176]. Lei *et al.*^[177] unraveled that the LSD1/Prickle1/APC/ β -catenin signaling axis is engaged in regulating the stem characteristics and chemoresistance of hepatic LGR5+ LCSCs.

TREATMENT TARGETING LCSCs

Using surface markers to identify and isolate LCSCs remains an initial and important step of CSC-targeting therapy. Immunotherapy uses specific antibodies to target LCSC surface makers can be integrated with conventional chemotherapy, radiotherapy and surgery to promote therapeutic effects. The most frequently-used LCSC-associated surface markers along with clinical strategies that target them are demonstrated as follows.

One of the current therapeutic approaches to target directly LCSCs is nanomedicine-based therapy, in which medication delivery and intake are effectively controlled in nanoscale^[178]. Epirubicin-adsorbed nanodiamonds displayed high efficacy in inducing the elimination of chemoresistant LCSCs^[179]. Poly lactic-co-glycolic acid-encapsulated disulfiram strongly inhibits LCSCs and has a synergistic cytotoxicity with 5-FU or sorafenib^[180]. Gao *et al.*^[181] developed a GPC3-targeted CAR and found that it obviously suppressed HCC growth. Overexpression of ANXA3 increased the number of CD133+ cells and positively associated with tumorigenicity of CD133+ cells. The underlying mechanism of ANXA3-mediated maintenance of LCSCs stemness involved the HIF1A/Notch pathway. ANXA3 upregulated dendritic cells could induce more active T cells, which could preferentially kill CD133+ LCSCs^[182]. Xu *et al.*^[183] addressed that Hep-12 cells owing stemness properties, are susceptible to autologous-activated tumor-infiltrating lymphocytes-mediated recognition and cytotoxicity. What's more remarkable, the authors put forward that it may be the first evidence to demonstrate the hypothesis that immunotherapy can be used to target recurrent HCC cells with stem cell-like properties. Bone morphogenetic protein-9 is a potent growth inhibitor of hepatocellular carcinoma and reduces the liver cancer stem cells population by suppressing the expression of five prominent LCSC markers, including CD44, CD90, AFP, GPC3 and ANPEP^[184]. In current clinical practice (according to

NCCN guidelines for hepatobiliary cancers, version 4.2018), several oral targeted drugs have been approved, including sorafenib, lenvatinib, regorafenib, showed a median overall survival of 10.7 to 13.6 months^[185-187]. Immunotherapy has also been considered as one of the promising treatments and is being actively studied and optimized in liver cancer progression and metastasis^[188]. The most *ex vivo* investigated and clinically relevant check-point proteins are CTLA-4, PD-1, and PD-L1. Nivolumab and pembrolizumab, both as PD-1 antibodies with similar efficacy, are now approved to treat liver cancer clinically. Nivolumab showed an objective response of 20%, contained 1% complete response and 18% partial response, and stable disease is 45%^[189], when pembrolizumab is concerned, the objective response is 17%, and the complete responses, partial response, and stable disease were 1%, 16% and 44% respectively^[190]. The development of new drugs enable the improvement of object responses and survival of advanced liver cancer, what's deserved notification is that drugs such as sorafenib, lenvatinib, regorafenib, nivolumab and pembrolizumab now available in clinic can obtained about 1% clinical complete response in small number of patients, revealing the pathways these drugs targeted may have the potential to diminished almost the whole tumor including the LCSC, and further exploration of the underlying mechanism of cancer development and progression is promising.

Alternative therapies including induction of LCSC differentiation and apoptosis are also promising. Conventional chemotherapy and radiotherapy have been proven to successfully eradicate terminally differentiated cancer cells but fail to influence CSCs^[191,192]. Therapies that induce LCSC differentiation can be combined with those conventional therapies to efficiently diminish LCSC subpopulation and impede cancer development since the differentiation process obtains higher priority than cancer self-renewal process. There are intensive studies developing and optimizing the differentiation-inducing agents including retinoic acid, histone deacetylase inhibitors, tyrosine-kinase, Hippo/YAP signaling pathway inhibitors^[192-195]. Apoptosis is also a vital cellular mechanism that regulates cell death through a complicated signaling network. LCSCs can escape from apoptosis process, therefore they possess unlimited and uncontrollable self-renewal ability to initiate cancer development and invasion. Induction of apoptotic mechanism in LCSCs by using microRNA hold great promise in cancer treatment so that many studies have been focused on developing therapies to activate apoptotic pathways in LCSCs^[196].

These inducing therapies would be feasible and efficient if LCSCs could be specifically identified according to the expression profile of varied LCSC surface markers. However, there is no LCSC surface marker has been identified and proven to have the ability to represent the entire subpopulation of LCSCs, thereby the inducing therapies remain challenging so far. It leads us to consider that whether or not the potential combination of varied LCSC surface markers can improve the specificity in identification of LCSCs.

SUMMARY

The above-mentioned discussion offers a promising insight of how LCSCs can be employed in clinical diagnosis and treatment for liver cancer development, progression, metastasis and resistance [Figure 2]. During the last decades, the compelling knowledge about CSCs has enabled rapid advances of drugs targeting CSCs and gradually emerged as an indispensable class of therapies. Numerous agents with the capabilities to inhibit CSC-associated signaling pathways, including Notch pathway, Hedgehog pathway and WNT pathway, have been approved for clinical use. The recent development of culture condition allows CSCs to undergo long-term proliferation in spheroids and organoids, thus offer researchers with an innovative platform for identifying new CSC markers with high specificity and efficacy. Moreover, because organoids are directly derived from primary tumor tissues, the organoid technique provides a unique perspective to researchers so that we can comprehensively investigate the heterogeneous functions of CSCs in recurrence, metastasis, chemoresistance and radioresistance.

From a broader perspective, there is no doubt that drugs targeting CSC should be considered as a promising clinical strategy for therapeutic intervention, although the rate of treatment failure that aims to effectively

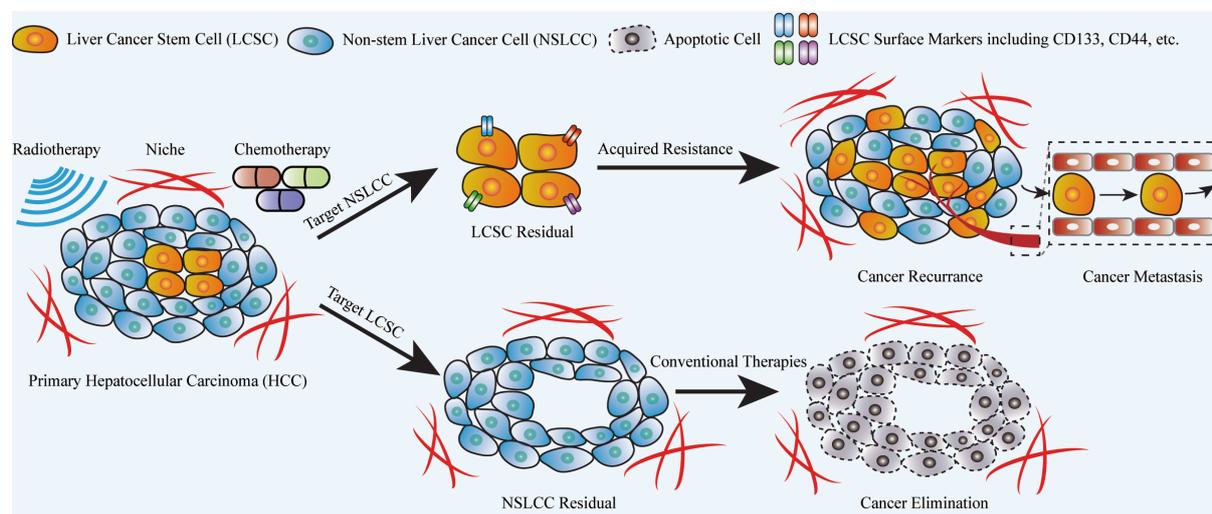


Figure 2. Clinical implication of conventional cancer therapy and LCSC-targeting therapy. Conventional chemotherapy and radiotherapy are frequently used to treat liver cancer, effectively targeting the non-stem liver cancer cells (NSLCCs) but not liver cancer stem cells (LCSCs). The LCSC residual can be re-activated to enrich the LCSC subpopulation and eventually trigger cancer recurrence and metastasis with a more aggressive phenotype. With the help of varied LCSC surface markers that can specifically and effectively identify LCSC heterogeneity, LCSC-targeting therapy is believed to be capable of eradicating the CSC subpopulation in liver cancer. Integration of LCSC-targeting therapy and conventional chemo-/radiotherapy might lead to complete cancer elimination without further development and invasion. LCSC-targeting therapy has generated many promising results in pre-clinical trials and there are intensive efforts from researchers and clinicians for further research.

eliminate CSCs remains relatively high so far. It is worth considering that such treatment failure might be resulted from inefficiency of drug delivery instead of inefficiency of the drugs. Promoting the efficacy of drug delivery and developing alternative approaches to target CSCs represent one of the most foremost fields to be explored. In addition, early diagnosis of cancer by using CSC markers remains as an important ramification that can prevent development, metastasis and resistance. Hence, there is an emergency for greater concentration on identification of CSC markers that can specifically and effectively represent tumor grades and disease stages. Integrating the development of early diagnosis techniques with a comprehensive understanding of CSC surface markers that drive a benign stage to a malignant stage can enable patient-specific and efficient early intervention and offer a balanced approach to regulating cancer development and invasion.

DECLARATIONS

Authors' contributions

Designed the structure and outline of the manuscript, instructed writing the intellectual part, and approved final publication: Guan XY

Reviewed the literatures and wrote the draft: Zhang JL

Revised the manuscript and drew the pictures: Gong LQ

Review and minorly revised the manuscript: Yan Q, Zhou NN, Lee VHF

Availability of data and materials

Not applicable.

Financial support and sponsorship

This study was supported by a grant of the Basic Research (Discipline Layout) of Shenzhen Science and Technology Project (No. JCYJ20180508153249223).

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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