Surface markers of liver cancer stem cells and innovative targeted-therapy strategies for HCC (Review)

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Abstract. Liver cancer stem cells (LCSCs) have important roles in the occurrence, development, recurrence, therapy resistance and metastasis of hepatocellular carcinoma (HCC). Therefore, intensive studies are undergoing to identify the mechanisms by which LCSCs contribute to HCC invasion and metastasis, and to design more efficient treatments for this disease. With continuous efforts in LCSC research over the years, therapies targeting LCSCs are thought to have great potential for the clinical treatment and prognosis of liver cancer. Novel LCSC surface markers are continuously discovered and several have been used in targeted therapies to reduce HCC recurrence, metastasis, and drug resistance following tumor resection. The present review describes the surface markers characterizing LCSCs and the recent progress in therapies targeting these markers, including antibodies and polypeptides.

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1. Introduction

Hepatocellular carcinoma (HCC) is the seventh most common cancer worldwide. Traditional therapy strategies currently available for HCC include surgical procedures, radioactive particle implantation, radiofrequency ablation, hepatic artery chemoembolization (TACE), and chemotherapeutics. Recent studies have indicated that these therapy strategies are still not fully efficient and have multiple drawbacks, including post-treatment relapse, chemotherapy drug resistance and metastasis (1-5). Thus, discovering approaches to avoid recurrence and metastasis of liver cancer and to provide novel therapeutic strategies is of outmost importance in HCC. With the continuous progress in cancer stem cell (CSC) research, many specific studies overexpressed on the surface of CSCs have been discovered. These receptors are significantly associated with growth and proliferation of tumor cells. To date, scientists have isolated CSCs in various solid tumors, including liver, breast, lung and brain cancer. Liver cancer stem cells (LCSCs) represent a small fraction of cells in HCC cancer tissues that possess the abilities of self-renewal, multi-directional differentiation and indefinite proliferation, as well as high tumorigenic ability (6-10). As specific markers of CSCs, the CSC-specific overexpressed receptors may offer a new research direction as therapeutic targets for the diagnosis and treatment of tumors. Currently, potential clinical treatments targeting CSC include: Blocking signal transduction pathways in CSCs; inducing differentiation of CSCs; changing the microenvironment and inhibiting telomerase activity in CSCs; specific gene therapy targeting CSCs; specific compounds or drugs targeting CSCs; and ligands targeting CSCs. In conclusion, the CSC theory may provide an explanation as to the refractory nature of liver cancer and may provide useful insights for scientists to design novel therapies for HCC.

2. LCSCs and their origin

The liver has both exocrine and endocrine functions. It is estimated that the normal liver can completely self-renew within ~1 year (11), exhibiting a strong regeneration capacity, which is also an important feature of stem cells. While there is a large amount of endogenous stem cells in the liver, the duration of

their proliferative potential is short. These cells are commonly derived from undifferentiated liver oval cells, also known as hepatic precursor cells (HPC), and are located in the terminal bile canaliculi and beside the interlobular bile duct (12-14). Oval cells have both the ability to differentiate into hepatocytes and bile duct cells, which, in human HCC, display the properties of stem cells (15). Additionally, the majority of hepatic stem cell surface markers are the same as hepatic oval cell markers (OV)6, OV1, cytokeratin 7 (CK7) and CK19, α-fetoprotein (AFP), KIT proto-oncogene receptor tyrosine kinase (c-kit), and Thy-1 cell surface antigen (Thy-1). OV6 expression is a specific phenotype of oval cells that was originally identified in the livers of tumor-bearing rats, and is recognized as a surface marker of human liver progenitor cells (16). Yang *et al* (17) reported that overexpression of OV6 enhances the invasiveness and metastasis potential of HCC stem cells, and that increased numbers of $OV6^+$ CSCs in patients with liver cancer indicate worst clinicopathological features and poorer prognosis. In addition, Yang *et al* (17) demonstrated that the stromal cell derived factor (SDF)-1/C-X-C motif chemokine receptor (CXCR) 4 signaling pathway is significantly associated with the migration ability of $OV6^+$ HCC cells, suggesting that $OV6^+$ stem cells have an important role in HCC metastasis. By contrast, exogenous liver stem cells, which are derived from bone marrow or peripheral blood stem cells, are usually fewer in number, but exhibit a longer duration of proliferative potential (18).

Gene mutations, with the exception of mutations affecting self-renewal capacity, are important events occurring in the early stages of cancer. Previous studies have reported that CSCs originate from normal stem/progenitor cells and exhibit certain self-renewal ability (19). However, whether this hypothesis applies to HCC is unknown. Previous studies have demonstrated that there is indeed a small subset of cells in HCC that display the characteristics of CSCs. Side population (SP) cell sorting is widely used for the isolation and identification of CSCs from other types of tumors. The subsets of SP cells are identified by the ability of the ATP binding cassette transporter to export the DNA dye, Hoechst 33342. In the Huh7 and PLC/PRF/5 HCC cell lines, ~0.25-2.0% of the cells display an SP phenotype (20).

LCSCs can self-replicate, differentiate, and present strong drug resistance. Liu *et al* (21) (Fig. 1) have hypothesized that CSCs are not derived from a specific source of cells in hepatitis-B (HBV)-associated HCC and may be derived either from hematopoietic stem cells (HSC) or from mesenchymal stem cells (MSC). The specific surface marker for HSCs is CD133, while the specific surface markers for MSCs are CD90 and CD44. Both HSCs and MSCs can differentiate into pluripotent stem cells (PSCs). PSCs can then differentiate into liver precursor cells/oval cells that express OV6 and epithelial cell adhesion molecule (EpCAM). PSCs and liver precursor cells can be induced into CSCs by the mechanism of 'maturation arrest', thus leading to the occurrence of liver cancer.

There are several theories regarding the origin of HCC cells. One theory proposes that they are derived from dedifferentiated mature liver cells. Gournay *et al* (22) have confirmed that dedifferentiation of mature liver cells occurs during the formation of HCC in mice, suggesting that proliferative liver cells may be one of the sources of LCSCs. Other scholars argue that HCC cells are derived from the abnormal differentiation of liver stem cells by 'blocked maturation'. For example, Sell *et al* (23) used chemical carcinogens and oncogenes to intervene in the differentiation of liver oval cells and to transform them into HCC pre-cancer cells. Dumble *et al* (24) subcutaneously inoculated oval cells into nude mice and reported the development of tumors similar to HCC. Results from the detection of surface markers demonstrated that the newly developed tumors were derived from differentiated oval cells, suggesting that oval cells may be involved in the occurrence of HCC (24). HCC tumors have also been demonstrated to include intermediate cells between HPC and mature hepatocytes. An increasing number of studies has demonstrated that LCSCs can originate from the 'blocked maturation' LSCs (25-27), because most HCCs consist of mixtures of mature cells and cells with a phenotype similar to HPCs. Immunophenotyping analysis of HCCs has further indicated that 28-50% of HCC cells express HPC surface markers, such as CK7 and CK19 (28). These tumors also include intermediate cells between HPC and mature liver cells. Furthermore, Yeh *et al* (29) reported that the expression levels of CD133 were negatively correlated with the expression levels of HBV surface antigen (HBsAg) in HBV-associated liver cancer tissue samples, indicating that LCSCs more likely originate from blocked liver stem cells, rather than differentiated liver cells post-infection. Therefore, various LCSC markers can be detected in HBV-associated clinical samples of HCC. There is also evidence suggesting that LCSCs may be derived from bone marrow stem cells (30) and SP cells (20,31).

Cancers exhibit immense tumor heterogeneity. If cancers originate from few CSCs and these stem cells offer various characteristics to the tissue, then the importance of cell abnormal differentiation ability needs to be redefined to better explain the heterogeneity of tumors. Dynamic analysis of the expression levels of LCSC markers can help clarify the changes of biological characteristics of LCSCs during hepatocarcinogenesis and explain the clinical significance of the changes in marker expression levels.

3. LCSCs and their characteristics in HCC

Drug resistance is associated with the recurrence and metastasis of cancer (32). CSCs resist chemotherapy-induced cell death through various mechanisms, including intrinsic and external mechanisms. The intrinsic mechanism consists of the self-renewal ability of CSCs, the enhancement of DNA damage repair pathways, the high expression of drug efflux-related proteins, the overactivation of growth pathways and other stem-related pathways. The external mechanism refers to the influence of tumor microenvironment factors on CSC resistance, including hypoxia stimulation, epithelial-mesenchymal transition (EMT) signals, and angiogenesis abnormalities (33). In HCC, SP cells or LCSCs expressing other molecular markers (including EpCAM, CD133, CD90, CD44 and CD13) exhibited resistance to radiotherapy and chemotherapy *in vitro* and *in vivo*. The mechanisms involved include increased expression of drug efflux-related proteins (31,34-36), activation of anti-apoptotic pathways (37-39), activation of stem cell-related pathways, and increased resistance and maintenance of a certain number of LCSCs (16,40). MicroRNAs (miRNAs)

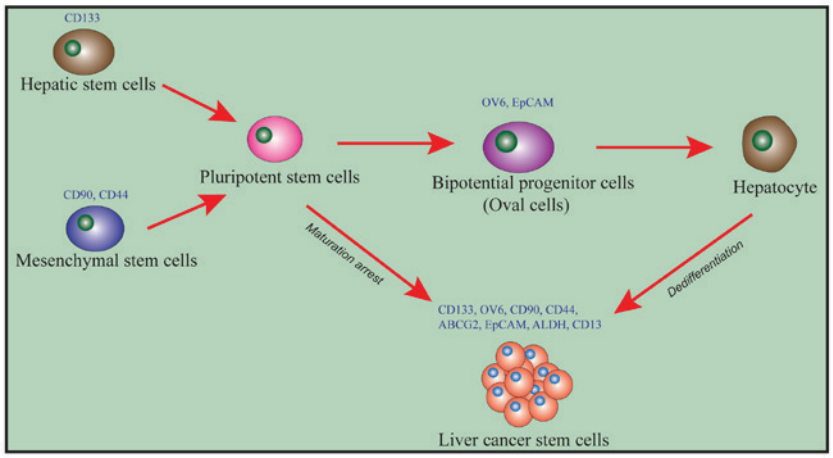

Figure 1. Possible cellular origins and markers of LCSCs. HCC may arise from cells at various stages of differentiation in the hepatic stem cell lineage: Mature liver cells; liver progenitor cells or oval cells as bipotential stem cells; and bone marrow stem cells, including hematopoietic and mesenchymal stem cells as multipotent liver stem cells. HCC could originate from stem cells either due to 'maturation arrest' or to 'dedifferentiation of mature cells'. LCSCs, liver cancer stem cells; HCC, hepatocellular carcinoma; CD133, prominin-1; OV, oval cell marker antibody; EpCAM, epithelial cell adhesion molecule; ABCG2, ATP binding cassette subfamily G member; ALDH, aldehyde dehydrogenase.

participate in the maintenance of the CSC phenotype by regulating the expression of oncogenes and stem cell-related genes (41,42). The half-life of mature tumor cells in the circulation is very short and most of them die from natural apoptosis, with a relatively small effect on tumor invasion and metastasis. A previous study revealed that the viability, distant metastasis, and homing ability of LCSCs in the circulatory system were significantly higher than that of other tumor cells (43). This may be explained by the EMT status of LCSCs, which enables them to serve a leading role in the metastasis and invasion of HCC and to become the source of HCC recurrence (43). Theoretically, tumor recurrence may be effectively prevented if a method to eliminate CSCs could be developed, making CSCs a desirable diagnosis and treatment target for resistant tumors, including HCC (44). This would be especially true for cases with poor therapeutic effect by traditional methods. LCSC-targeted therapy is thus hypothesized to achieve excellent antitumor effects and to reduce the side effects of chemotherapy, providing novel more efficient strategies for the treatment of cancer.

4. Currently known LCSC surface markers

With the identification of specific surface markers, LCSCs can be successfully separated and enriched through screening for these markers by fluorescence-activated and magnetic-activated cell sorting methods (45). If LCSC-specific molecular markers are targeted and blocked, the number of LCSCs may be reduced, potentially resulting to inhibited tumor growth and recurrence.

To date, the commonly-reported LCSC surface markers are EpCAM (also known as CD326), CD133, CD90 (also known as Thy-1), CD44, and CD13 (40,46-49). In addition, other surface markers have also been demonstrated to be involved, including OV6, K19, c-kit (also known as CD117), ATP binding cassette subfamily G member 2 (ABCG2), and aldehyde dehydrogenase (ALDH).

Most of these markers normally exist on the surface of HSCs. Cells expressing these markers have similar stem cell properties, therefore these markers are hypothesized to be used as molecular therapeutic targets to eliminate LCSCs and to overcome cancer recurrence. Although certain other surface markers have been reported on cancer stem cells, they are not specific to LCSCs (50).

5. LCSC surface markers and targeted therapies

Prominin-1 (CD133). CD133 is one of the most studied stem cell surface markers in recent years. In solid tumors, CD133 was first discovered and isolated in brain tumors; Singh *et al* (51) successfully isolated $CD133^+$ tumor cells from glioblastoma and demonstrated that $CD133^+$ glioblastoma tumor cells can form neurosphere-like clones, with a strong self-renewal capacity, differentiation potential and tumorigenicity *in vivo*. The tumor cells and subtypes of tumors formed in mice were the same as those obtained by orthotopic grafts, but could also be passaged consecutively; therefore, $CD133^+$ cells were identified as tumor stem cells. Furthermore, $CD133^+$ cells have an important role in multiple other solid tumors, including gastric (52), liver (53-55) and colon cancer (56,57). CD133 is a transmembrane glycoprotein with a unique structure of five transmembrane domains and two large extracellular glycosylation chains, expressed in both hematopoietic and neural stem cells (58). In several studies of HCC, the HSC surface marker CD133 was used to isolate LCSCs (47,54). CD133 is expressed on the surface of stem cells in many solid tumors, including liver, colon, brain, lung and prostate cancer, and in B16 melanoma (59). In human HCC cell lines, ~0-65% of cells are $CD133^+$ cells. CD133 is considered one of the main LCSC markers, with self-renewal, multi-lineage differentiation and chemoresistance abilities (37). Methionine adenosyltransferase (MAT) is the only enzyme that can catalyze the biosynthesis of S-adenosylmethionine (SAMe), which is the principal biological methyl donor in cells. SAMe can regulate hepatocyte growth and apoptosis. Exogenous SAMe inhibits the growth of hepatoma cells and prevents HCC development. Similar results were observed in a MAT deficiency-induced HCC mouse model (60). Xenografts of $CD133^+$ cells in nude mice formed

tumors, while $CD133^-$ cells did not (61); Yang *et al* (48) also reported that $CD133^+$ liver cancer cells exhibited higher tumorigenic and proliferative abilities, properties that are similar to the features of HPCs (47). CD133 knockout may reduce the tumorigenicity and change the cell cycle distribution in these cells. Additionally, HCC patients with high CD133 expression in their tumors have poor prognosis and increased recurrence, indicating that CD133 expression may be associated with the prognosis of liver cancer (47,54,62-64). A previous study has demonstrated that the migration of $CD133^+$ and $CD133^-$ cells was not significantly different, and that the CD133 expression pattern was inconsistent with the clinical manifestations (65). A recent study reported that $CD133^+$ LCSCs were resistant to interferon-induced autophagy (66). Therefore, the identification of targeted molecular markers is of great significance.

Monoclonal antibodies are commonly used as ligands in CD133-targeted therapy. These antibodies can carry various drugs or toxins to the target in order to enhance the immune response of the human body towards the disease. Such methods have several advantages that are absent in traditional anticancer drugs, namely, relatively high target specificity, low molecular weight, less side effects, and better patient compliance. Currently reported antibodies against CD133 are AC133, 293C3 and AC141, among which AC141 and 293C3 are antibodies targeting CD133/2. CD133/2 is a variant of the CD133 antigen, which is predominantly expressed in the fetal liver and kidney, but not in the adult pancreas and placenta tissues. Prasad *et al* (67) prepared a compound antibody from CD133 and CD3 antibodies. This compound could specifically identify glioma stem cells and recruit T cells to kill these stem cells, demonstrating an excellent targeted therapeutic effect. Smith *et al* (68) combined a mouse anti-human CD133 antibody with the anti-microtubule cytotoxic drug monomethyl auristatin E, and confirmed that this complex inhibited the growth of $CD133^+$ LCSC-like cells *in vivo* and *in vitro*. Lang *et al* (69) prepared a ^{131}I-CD133 monoclonal antibody (mAb) with high stability and specificity *in vitro*. Additionally, *in vivo* experiments demonstrated that the ^{131}I-CD133 mAb had a high selectivity, and the binding rates with $CD133^+$ colon adenocarcinoma CSCs and $CD133^-$ cells were 70.01±6.02 and 2.73±0.26%, respectively (69). Imaging of the transplanted tumor mice demonstrated that most of the ^{131}I-CD133 mAb was deposited in the $CD133^+$-transplanted tumor sites in the mice, while this was not observed in CD133 mice. The ^{131}I-CD133 mAb may be therefore applied with high selectivity and high stability in the clinical diagnosis of LCSCs, as well as for immune imaging and radiation therapy of LCSCs, and clinical trials are currently ongoing.

In the field of CD133-targeted polypeptides, Sun *et al* (70) successfully identified a short peptide LQNAPRS (LS-7) which can highly bind to mouse CD133, by using phage display technology. Wang *et al* (71) prepared a DSPEPEG micelle system loaded with a 7-amino-acid peptide (TR short peptide), which was used to investigate its targeting effect on brain stem cells. Compared with the unmodified micelles, the uptake rate of the TR-modified micelles was significantly increased in brain glial stem cells. TR peptide-modified micelles exhibited specific binding of the TR peptide to the CD1333 receptor, and improved anticancer effects by targeting $CD133^+$ glioma stem cells. In conclusion, despite these studies demonstrating that CD133 can be used for the isolation and identification of LCSCs *in vitro* as well as for targeted therapy, the application of a single surface marker remains limited.

CD13. CD13 is the earliest identified marker of normal and malignant myeloid cells and has been used for many years to characterize and classify leukemia or lymphoma cells (72). CD13, also known as aminopeptidase N (73), is a zinc-binding protein. In addition, $CD13^+$ cells exhibit features similar to that of stem cells, such as increased cell proliferation and tumor cell formation, and increased resistance to chemotherapy. $CD13^+$ cells are resistant to adriamycin and fluorouracil (5-FU) treatment, and expression of CD13 is enhanced by chemotherapy. This is associated with the high resistance of $CD13^+$ cells; in the presence of chemotherapy drugs, $CD13^-$ cells exhibit an increased response to oxygen clusters, leading to DNA breakage and cell death. In $CD13^+$ cells, the expression of the glutamate-cysteine ligase (GCLM) gene is significantly increased compared with other cells. GCLM catalyzes intracellular antioxidant glutathione synthesis, against reactive oxygen species induced by chemotherapy/radiotherapy, thereby protecting DNA from DNA damage, preventing apoptosis, and resulting in drug resistance (74). The classical cytotoxic antitumor drugs, doxorubicin and 5-FU, kill $CD90^+$ hepatoma cells, but the proportion of the surviving $CD13^+$ cells increases. The percentage of $CD13^+/CD90^-$ cell subpopulation in clinical tissues from patients with HCC who relapsed following arterial chemoembolization is significantly higher compared with untreated HCC tissues. Following administration of a low dose gradient of cyclophosphamide, the remaining tumor cells exhibit a $hAFP^+/CD13^+/PCNA^-$ phenotype, with the $CD13^+$ cells increased, indicating that $CD13^+$ cells are resistant to this chemotherapy. However, combined treatment with Tegafur, a prodrug of 5-fluorouracil (5-FU), and cyclophosphamide, using a low-dose rhythmic administration, significantly reduced the number of tumor cells (75,76). Studies have demonstrated that the expression of CD13 may enhance the semi-static activity of CSCs. Haraguchi *et al* (40) observed that $CD13^+$ cells are predominantly in the G_1/G_0 phase of the cell cycle, suggesting that CD13 may be a marker of the dormant or semi-stationary status of LCSCs.

Downregulation of CD13, by use of a CD13 neutralizing antibody or inhibitors, can induce apoptosis in the HCC cell lines Huh7 and PLC/PRF/5. When $CD13^+$ hepatocytes were treated with 5-FU, which is directly targeted at CD13 molecules, the number of cells with tumorigenic and self-renewal abilities was significantly reduced (77). The coexpression of CD13 and CD90 has an important role in the occurrence of liver cancer. The combined application of CD13 and CD90 inhibitors significantly reduces tumor volume, compared with the application of each individual inhibitor alone. Reduction or inhibition of CD13 molecules on the surface of HCC cells by interfering techniques also affect, to a certain extent, the self-renewal and tumorigenic ability of LCSCs (40).

CD90 or Thy-1. In 1964, CD90 was first identified in the CH3 AKR strain mice in an effort to develop an antileukemia xenoantibody, and was named as θ antigen (78). In 1969, due to the fact that a study had demonstrated that the precursors of T cells

are mature in the thymus (79), the important surface marker of T cells, θ antigen, was renamed as Thy-1. In the 1980s, CD90 was isolated from the human T-cell leukemia cell line, MOLT-3, indicating the presence of CD90 in humans (80). CD90 overexpression was demonstrated to be associated with age in patients with HCC and HBV infection, tissue staging and high CD90 expression were associated with poor prognosis (81). The $CD90^+$ cell population was selected from a HCC cell line, as well as tissues and blood from patients with HCC, and it was demonstrated that they presented increased tumorigenic abilities and indefinite proliferation compared with the $CD90^-$ cell population, suggesting that the $CD90^+$ cells might be a 'hepatocellular stem cell' population (48,82). CD90 is a surface marker expressed in human HCC cell lines and mesenchymal stem cells with a positive rate of ~0-2.5%, and often serves as a surface marker for various stem cells. Yang *et al* (48) noted that HCC tumor samples and the majority of blood samples contain highly tumorigenic $CD90^+/CD45^-$ cells, while samples from normal individuals or patients with chronic hepatitis do not. Similarly, aspects of the aforementioned study, which focused on the expression of CD90 in HCC cell lines, revealed that only $CD90^+$ cells exhibited tumorigenic ability. If the surface marker glycoprotein CD44 was also expressed in the $CD90^+$ cells, the invasive phenotype was even stronger, with increased metastatic and self-renewal capacities. When CD44 was blocked by an inhibiting antibody, the tumor formation and metastasis abilities of $CD90^+$ cells were decreased and apoptosis was induced. In the same study, it was also noted that $CD45^-/CD90^+$ cells were present in all tissue samples and ~90% of blood samples from liver cancer patients, and exhibited a more aggressive phenotype in immunodeficient mice; while only a small population of $CD90^+$ cells existed among 6 different liver cancer cell lines, and exhibited a low aggressive phenotype in immunodeficient mice. Transplant experiments in nude mice demonstrated that $CD90^+$ HCC cells had a tumorigenic ability that was not present in the $CD90^-$ cells. A further study has indicated that $CD45^-/CD90^+$ cells also express other stem cell markers, including CD133, epithelial specific antigen (ESA), CXCR4, CD24, kinase insert domain receptor and CD44 (48). CD90, possibly one of the surface markers of LCSCs, has been used in the identification of LCSCs in recent years. $CD45^-/CD90^+$ cells may become a new target for diagnosis and treatment of liver cancer. CD90 has been shown to upregulate the expression of the molecular marker CD133, and this abnormal expression can promote tumor progression. The CD90/integrin/mechanistic target of rapamycin kinase (mTOR)/AMP-activated protein kinase (AMPK)/CD133 signaling pathway serves an important role in tumor formation, and inhibition of this pathway by the energy-limited simulant, OSU-CG5, reduced the proportion of $CD90^+$ cells in fresh HCC specimens and inhibited tumor growth (83).

CD44. CD44 is a glycoprotein encoded by a single gene, and hyaluronic acid is its main receptor. As an important class of adhesion molecules, CD44 is widely distributed on the cell surface of various cell types, including lymphocytes, monocytes and endothelial cells (84), and it is involved in intercellular cell adhesion and cell migration. CD44 may be associated with tumor cell invasion and metastasis of liver cancer (36). Under normal circumstances, CD44 is in a relatively quiescent state on the cell surface. However, CD44 is overexpressed in tumor cells and mainly involved in heterotypic adhesion (the adhesion of tumor cells to the host cells and the host matrix), thereby promoting tumor cell invasion and metastasis. The relationship between CD44 and tumor infiltration and metastasis has been investigated (85). CD44 is a stem cell marker of pancreatic, gastric, and colorectal cancer. Subsequent studies indicated that it is also one of the important markers of LCSCs, and its coexpression with other markers can better identify LCSC phenotypes. Mima *et al* (49) observed in a nude mouse model that the tumor formation rate of $CD44^+$ cells was faster compared with $CD44^-$ cells, and that only $CD90^+/CD44^+$ cells appeared in the lung metastasis sites. Compared with $CD133^+/CD44^-$ cells, $CD133^+/CD44^+$ HCC cells were more prone to tumor formation and drug resistance, and expressed more stem-associated genes (36). $CD133^+/CD90^+$ cells were more aggressive than $CD44^+$ cells alone.

Blocking CD44 activity by use of a CD44-targeting antibody can induce the apoptosis of $CD90^+$ cells *in vitro* and inhibit tumor formation of $CD90^+$ cells in immunodeficient mice *in vivo* (86). IM7 is a murine monoclonal antibody specifically targeting CD44, which has a confirmed inhibitory effect on tumor growth. Zhang *et al* (87) reported that RG7356, a humanized antibody against CD44, could induce apoptosis of chronic lymphocytic leukemia cells.

To date, given the characteristics of CD44, studies on short peptides targeting CD44 are gradually increasing. Cho *et al* (88) prepared a novel short peptide complex PDPP targeting CD44 by combining the short peptide with D-polylysine. The binding capacity of PDPP and CD44 was 4-10 times stronger than that of the CD44 antibody, suggesting that PDPP may serve as a probe for the diagnosis and treatment of cancer stem cells. Park *et al* (89) successfully identified a short peptide, P7(FNLPLPSRPLLR), which can specifically bind to CD44 expressed on the surface of breast cancer CSCs. Similar to the CD44 antibody, the binding rate of P7 on MCF7 cells was high. Therefore, the authors suggest that the short peptide P7 could be used for the treatment of CSCs as a substitute for antibodies.

EpCAM. EpCAM is a transmembrane glycoprotein with a relative molecular mass of 40,000 Da and currently used in research for various tumor types (90-93). EpCAM is expressed during the early liver development process, but not in normal mature liver cells. EpCAM is expressed in human epithelial tissue and tumors, as well as in precursor cells and stem cells. It is also present in liver stem cells and hepatoblasts. Nevertheless, the high expression of EpCAM is significantly associated with activation of cell proliferation (94). EpCAM is also expressed on the surface of LCSCs and pancreatic CSCs (95,96). Recent studies have demonstrated that the tumor formation and invasion abilities of $EpCAM^+$ HCC cells were significantly higher compared with $EpCAM^-$ HCC cells (97). Liver stem cell surface markers were expressed in $EpCAM^+$ cells, while the expression of mature hepatocyte markers was significantly increased in $EpCAM^-$ cells (91). Yamashita *et al* (98), EpCAM expression was utilized to classify patients with HCC, and the differential expression of AFP and EpCAM was verified in tumor samples from two HCC cell lines. $EpCAM^+$ cells

exhibited CSC-like characteristics, with high tumor formation ability *in vivo* and *in vitro*. Compared with $EpCAM^-$ cells, the increased CSC characteristics of $EpCAM^+$ cells in primary liver cancer samples were further confirmed. The aforementioned study indicated that activation of the Wnt/β-catenin signaling pathway may increase the proportion of $EpCAM^+$ cells and block the reduction of EpCAM-induced tumorigenic ability of these cells. Results of CD90 and EpCAM expression obtained from HCC tumor cell lines were confirmed in human HCC samples. The aforementioned studies offer direct evidence for the existence of LCSCs in human HCC.

Targeted therapy towards the LCSC molecular marker EpCAM can effectively eliminate the expression of EpCAM in LCSCs (99,100). EpCAM antibodies currently available in preclinical or clinical studies include edrecolomab, adecatumumab, MT110 and catumaxomab, and they have been approved in the EU for patients with $EpCAM^+$ malignant ascites. Chen *et al* (7) used an EpCAM antibody (EpCAM-Ab) as the target to modify micelles loaded with anticancer drugs or genes, and to develop a gene delivery system targeting CSCs. This delivery system exhibited characteristics of pH-responsive drug release, with the amount of drug released at pH 5.0 being 40% higher than that at pH 7.4. *In vitro* experiments showed that the inhibitory effect of EpCAMAb-modified adriamycin-loaded micelles on LCSCs was significantly enhanced, with an IC_{50} of 0.051 mg/l, while the IC_{50} of the EpCAMAb-unmodified adriamycin-loaded micelles was 0.24 mg/l, which was 5 times that of the former. This targeted drug delivery system offers a significant therapeutic effect, indicating the feasibility of this antibody-mediated active CSC targeted therapy, as well as its potential value for the clinical treatment of cancer. Because RNA interference (RNAi) of EpCAM has been confirmed to significantly reduce the number of stem cells and their tumorigenic and invasive abilities (91,95), Bae *et al* (101) reported that, following EpCAM gene silencing by RNAi, HCC tumor grade, proliferation, invasiveness and AFP levels were significantly decreased.

6. Other LCSC targets and their applications in tumor therapy

Delta-like 1 non-canonical Notch ligand 1 (DLK1) is a progenitor marker in fetal liver and serves an important role in the carcinogenesis of HCC. Tanimizu *et al* (102) in order to isolate and characterize hepatobrobocytes, used the signal sequence trap method to search for cell surface antigens expressed in mouse fetal hepatocytes. They demonstrated that DLK1 (also known as Pref-1) was highly expressed in fetal liver and reported that most of the colony-forming $DLK1^+$ cells could differentiate into hepatocytes and bile duct epithelial cells. In addition, 7% of the colony-forming $DLK1^+$ cells exhibited a high degree of proliferation, and were able to form a large colony containing >100 cells following 5 days in culture. When they transplanted donor $DLK1^+$ cells into recipient spleens, they found donor-derived hepatocytes in the recipient liver, indicating that $DLK1^+$ cells were able to differentiate into hepatocytes *in vivo*. These results clearly suggest that DLK1 is a liver hepatocyte marker (102). The biological behavioral differences between $DLK1^+$ and $DLK1^-$ cells were assessed by growth curve, colony formation assay, spherical colony formation, chemical resistance and *in vivo* tumorigenicity. Knockdown of DLK1 reduced the malignancy of HCC cells and may kill LCSCs directly (103), suggesting that DLK1 may be a potential therapeutic target for LCSCs.

Assis *et al* (104) reported that $CD24^+$ HCC cells were highly important in the maintenance, self-renewal, differentiation and metastasis of chemotherapy-tolerant HCC cell xenografts, significantly affected the clinical prognosis, and tumor recurrence following chemotherapy. The authors used experiments based on lentivirus knockdowns and demonstrated that CD24 is a functional LCSC marker that is regulated by signal transducer and activator of transcription 3-mediated NANOG homeobox regulation to generate CSCs. These results suggested that the CD24 cascade in LCSCs may provide an attractive therapeutic target for HCC.

De Francesco *et al* (105) isolated the population of cells with CSC properties and labeled the calcium channel α2δ1 in primary hepatocellular carcinoma and their surgical margins using the monoclonal antibody 1B50-1. It was demonstrated that α2δ1 serves an important role in regulating the calcium oscillation amplitude, which is important in maintaining the properties of CSCs. 1B50-1 can bind α2δ1 in CSCs and may have potential as a drug against HCC by targeting α2δ1.

Recent studies have demonstrated that ICAM-1 is expressed on a variety of stem cells, including bone marrow mesenchymal stem cells, adipose-derived stem cells, periodontal ligament stem cells and placental mesenchymal stem cells (103-106). Based on the above findings, intercellular adhesion molecule-1 (ICAM-1) is also considered to be one of the surface markers of LCSCs. Liu *et al* (107) measured the sphere formation and tumor formation abilities of $ICAM\text{-}1^+$ cells *in vivo* and *in vitro*, respectively. They also used a specific targeting system that inhibited ICAM-1 expression in HBV transgenic mice (M-TgHBV) to study whether inhibition of ICAM-1 expression reduced the incidence and metastasis of tumors *in vivo*. ICAM-1 was demonstrated to be significantly expressed in HCC tumor cell lines, tumor tissue from patients or transgenic mice, and in circulating tumor cells from patients. Compared with $ICAM\text{-}1^-$ tumor cells, $ICAM\text{-}1^+$ tumor cells exhibited greater tumorigenic ability and increased expression of stem cell-related genes. Specific inhibition of ICAM-1 reduced tumor formation and metastasis in M-TgHBV mice. Increased numbers of $CD45^-/ICAM\text{-}1^+$ cells in blood samples from patients with HCC was an indicator of poor prognosis. Finally, this review also reported that ICAM-1 expression was regulated by the stem cell transcription factor Nanog.

Barclay and Brown (108) reported that CD47, a widely expressed integrin-related protein, was upregulated in LCSCs. Since CD47 acts as a ligand for signal-regulatory protein α (SIRPα), which is mainly expressed on phagocytic cells (including macrophages and dendritic cells), the activation of CD47 receptors can initiate a signal transduction cascade and inhibit macrophage cell phagocytosis (108-112). Majeti *et al* (113) demonstrated that CD47 expression in leukemia stem cells was increased compared with normal controls, and high expression of CD47 was associated with poor overall survival in three independent adult AML patients. In addition, the monoclonal antibody CD47 can cause leukemia stem cells to be engulfed by macrophages. Anti-CD47 antibody was used to target AML LSC in human

AML LSC-transplanted mice, and the results demonstrated that high expression of CD47 is an independent factor indicating poor prognosis, and may be used as a target for the treatment of AML. Chao *et al* (114) demonstrated that calreticulin is a major pre-phagocytic signal in several human cancers. It provides an explanation for the role of anti-CD47 antibody in selectively targeting tumor cells and highlights the balance between phagocytosis and anti-phagocytosis in hepatocellular carcinoma immune escape. Willingham *et al* (115) blocked CD47 by use of targeting monoclonal antibodies and demonstrated that inhibition of macrophage phagocytosis by CD47 was relieved in *in vitro* experiments. Then, they established a tumor model by transplanting human tumor cells into immunodeficient mice, and treated them with the anti-CD47 antibody. The results indicated that increased treatment duration extended survival in mice. Treatment of larger tumors with the anti-CD47 antibody inhibited tumor growth and metastasis. Anti-CD47 antibodies have potential effects on the treatment of smaller tumors, as well. The results demonstrated that all human tumor cells require the expression of CD47 to inhibit the innate immune monitoring and clearance of phagocytic cells, while CD47 is a widely expressed marker in all cancers that help tumor cells escape from phagocytosis and clearance. Thus, CD47 may be an effective target for the treatment of cancer. Lee *et al* (116) revealed that transplantation of freshly isolated $CD47^+$ cells in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice exhibit strong tumorigenicity, self-renewal and distant metastasis. CD47 mRNA is preferentially expressed in $CD133^+/CD24^+$ LCSCs. In addition, the increased expression level of CD47 mRNA in HCC clinical samples is positively correlated with patient survival. Knockout of CD47 using lentivirus-based short hairpin RNA (shRNA) inhibited the characteristics of stem cells, suggesting that CD47 has a key role in regulating the stem cell characteristics of HCC. In addition, cathepsin S (CTSS) is a downstream effector of CD47, which is preferentially secreted by $CD47^+$ HCC cells and could regulate the function of hepatic CSCs by activating protease-activated receptor 2 (PAR2) through autocrine pathways. Clinically, the serum level of CTSS was significantly associated with advanced tumor behavior in human HCC. HCC cell lines and patient-derived xenograft models were established and the CTSS/PAR2 signaling pathway was blocked by the morpholino approach to achieve chemosensitization effects. This review elucidates the signal transduction function of CD47 and its role in the pathogenesis of cancer through the CTSS/PAR2 pathway, suggesting a novel target in HCC treatment.

Lei *et al* (117) used the lysine-specific demethylase 1A/prickle planar cell polarity protein 1/adenomatous polyposis coli/β-catenin signal axis as a novel molecular circuit to regulate the hepatocyte properties and chemoresistance of $Lgr5^+$ LCSCs in liver, and the results confirmed that this signal axis may be used as a target for chemotherapy sensitization of liver cancer.

7. Conclusions and perspectives

Given the lack of sensitivity of liver cancer to radiochemotherapy, current treatments of liver cancer include surgical procedures, interventional therapy (including TACE, microwave ablation, and particle implantation therapy), and biological therapy (including immunotherapy and gene therapy). However, even surgery-based comprehensive treatment cannot prevent HCC recurrence and metastasis. Exploration of possible targeted therapies towards LCSCs may offer the only way to break through the bottleneck of HCC treatment. Current targeted therapy strategies for HCC include the inhibition of LCSC proliferation and induction of apoptosis; induction of LCSC differentiation to improve sensitivity to radiochemotherapy; and destruction of the LCSC microenvironment. Furthermore, direct targeting of LCSC surface markers, including CD133, CD90 and EpCAM, may represent another research direction. Although the molecular targeted drug, sorafenib, alone or in combination may inhibit the progress of HCC, drug resistance occurs fast and the numbers of $CD90^+$ cells are not reduced. Therefore, radical treatment of HCC should begin by eliminating the stem cells. Although various LCSC surface markers have been identified, LCSCs of high purity cannot be independently isolated using only one molecular marker, and some regulation mechanisms of stem cells remain unclear. Findings on LCSCs have been mainly obtained from *in vitro* experiments. Without examining the role of LCSCs within their microenvironment in the body, it is unclear whether the direct application of these results to the human body will work effectively. Further studies are therefore urgently required in order to develop efficient novel treatments for liver cancer.

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