

Clinical and Biological Implications of Cancer Stem Cells in Hepatocellular Carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is a malignant tumor with poor prognosis, and is one of the leading causes of cancer-related deaths worldwide. Recently, the development of therapeutic drugs via novel mechanisms of action, involving molecular-targeted drugs and immune checkpoint inhibitors, has progressed in the field of HCC. However, the recurrence rate remains high, and further improvement of the prognosis of patients with HCC is urgently needed. Cancer stem cells (CSCs) are a promising target for further development of novel anti-cancer drugs because they are reportedly involved in tumor initiation, maintenance, recurrence, and resistance to conventional therapies. Although several studies have already been conducted, the functions and roles of CSCs in the development and progression of tumors remain to be elucidated. In this review article, we will clarify the fundamental knowledge of CSCs necessary for the understanding of CSCs and will outline so-far identified markers specific to liver CSCs and the pathological and therapeutic implications of CSCs in HCC.

Key words cancer stem cells; cell surface markers; hepatocellular carcinoma; lncRNA

Hepatocellular carcinoma (HCC), which is the most frequent primary liver cancer, is a poor-prognosis malignant tumor with a high recurrence rate. A study suggested that approximately 850,000 people have developed liver cancer in 2015, which is the sixth common cancer worldwide.¹ In the same year, approximately 810,000 people have died of liver cancer globally, which is the fourth leading cause of cancer-related deaths.¹ The etiologies accounting for HCC include hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol, and aflatoxin. In addition, non-alcoholic steatohepatitis (NASH) developed from a fatty liver without excessive alcohol consumption is becoming the major cause of HCC.² In Japan, the numbers of patients with HCC and HCC-related deaths are apparently decreasing because of the establishment of preventive interventions, including HBV vaccination, direct acting antivirals for HCV, and decontamination of aflatoxin.³ However, globally, the prevalence and death rate of HCC tend to increase,

and, interestingly, this increase was more profound in countries with high socio-demographic index, where the latest therapeutic options are believed to be more readily available to people.⁴ Although the research and development of drugs for NASH are currently underway, sufficient HCC prevention remains to be established.^{5, 6} Moreover, although several novel molecular-targeted drugs were recently approved for HCC treatment, these drugs have not attained a remarkable improvement in the prognosis of HCC patients.⁷ Thus, the identification of previously unrecognized molecular targets is keenly necessary to develop innovative drugs for HCC treatment.

We elaborated, in the present review article, the fundamental functions of cancer stem cells (CSCs) and summarized the molecular markers defining liver CSCs, all of which are suggested to have profound functions in HCC. Moreover, we explained the recent findings on the pathological and therapeutic significance of liver CSCs. This review emphasizes that CSCs will provide novel

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Abbreviations: 4Mu, 4-methylumbelliferone; ABC, ATP-binding cassette; AFP, α -fetoprotein; ALDH, aldehyde dehydrogenase; ANGPTL1, angiopoietin-like protein 1; CAF, cancer-associated fibroblast; CD44s, CD44 standard isoform; CD44v, CD44 variant isoform; CIK, cytokine-induced killer cell; COX2, cyclooxygenase 2; CSC, cancer stem cell; CTL, cytotoxic T lymphocytes; DC, dendritic cell; EMT, epithelial-mesenchymal transition; EPCAM, epithelial cell adhesion molecule; FASL, FAS ligand; FGF, fibroblast growth factor; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; HIF1 α , hypoxia-inducing factor 1 α ; HULC, highly upregulated in liver cancer; IL, interleukin; lncRNA, long non-coding RNA; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; MDSCs, myeloid-derived suppressor cells; MHC, major histocompatibility complex; MORC2, microorchidia family CW-type zinc finger 2; NASH, non-alcoholic steatohepatitis; NEAT1, nuclear paraspeckle assembly transcript 1; NF κ B, nuclear factor kappa B; NK, natural killer; PD-1, programmed cell death-1; PD-L1, programmed cell death-1 ligand-1; RIG-I, retinoic acid-inducible gene 1; PGE2, prostaglandin E2; SCF, stem cell factor; SP, side population; STAT3, signal transducer and activator of transcription 3; TAM, tumor-associated macrophage; TGF- β , transforming growth factor- β ; TP53, tumor protein p53; Treg, regulatory T

insights into the understanding of the development and progression of HCC and hold a promising potential as a therapeutic target for the diagnosis, treatment, and prevention of HCC.

TUMOR-INITIATING PROPERTY OF CSCS

CSCs are defined as cancer cells that are capable of self-renewal and differentiation into non-CSCs and were first determined as tumor-initiating cells.^{8,9} That is, only a small subpopulation of cancer cells has been demonstrated to be able to initiate a tumor tissue in an immunocompromised mouse.^{8,9} The stemness has been postulated to have conferred to cancer cells via (1) the transformation of tissue stem or progenitor cells with maintaining the self-renewal ability or (2) the reprogramming of cancer cells transformed from differentiated cells.¹⁰ The underlying molecular mechanisms remain unclear, although the above scenarios may occur depending on the context of tumor development.

In order to explain the process of cancer cell expansion, two major models have been proposed, namely, the stochastic (or clonal evolution) model and the hierarchy (or CSC) model.¹¹ The former proposes that cancer cells with CSC-like properties are randomly produced via the gain and loss of a wide variety of cellular traits. Thus, all cancer cells could have the potential to be CSCs, and the proportion of CSCs would be varied. However, an autologous transplantation of leukemia cells demonstrated that the frequency of tumor-initiating cells was constant at approximately 1:100.¹² A similar result was also observed by an *in vitro* colony formation assay.¹³ Hence, these observations better fit the hierarchy model that has been originally established in stem cell biology. In this model, it is proposed that a subpopulation of cells undergoing self-renewal and differentiation would exist at a certain proportion in a tumor tissue. However, following the long-term culture of isolated CSC marker-negative cancer cells, it was demonstrated that CSC marker-positive cells appeared at a proportion similar to that observed before the isolation.¹⁴ This is not the case for normal stem cells that, once differentiated, cannot revert to original cells without specific conditions and better suits the stochastic model rather than the hierarchy model. Since these observations are difficult to be elaborated by either one model, the heterogeneity of tumor tissues has been attempted to be expounded by constructing a hybrid model of both.¹⁵ The precise molecular mechanisms of tumor initiation by CSCs will provide novel insights for the understanding of the process of tumor development and will thus be potential therapeutic targets for the prevention and treatment of tumors.

CSC MARKERS IN HCC

Side-population cells

As described above, CSCs have been determined as a subpopulation of cancer cells that can initiate tumor tissues in immunocompromised mice.¹⁰ However, CSC and non-CSC should be separated from a mixed cellular population in order to understand their detailed functions. As such, side-population (SP) cells have been taken advantage of since before CSC markers have been sufficiently elucidated.¹⁶ CSCs and normal stem cells are known to strongly express ATP-binding cassette (ABC) transporters that are involved in drug efflux and resistance to chemotherapy. Following staining with Hoechst 33342, which is a substrate for ABC transporters, dim cells, that is, SP cells, were isolated by a flow cytometer. This method was originally adopted to isolate hematopoietic stem cells and also oval cells, which are considered as hepatic progenitor cells.^{17,18} SP cells derived from HCC cell lines have been demonstrated to be enriched with cancer cells with stemness and a high tumor-initiating potential. Chiba *et al.*, for instance, found that two of four HCC cell lines contain SP cells with a high expression level of ABCB1 but at a quite low frequency of less than one percent of total cells and that these SP cells exhibited both hepatocytic and cholangiocytic features as observed in hepatic progenitor cells.¹⁶ Furthermore, SP cells from HCC cell lines showed more malignant characteristics than did non-SP cells and efficiently initiated tumor tissues in immunocompromised mice.¹⁶ Further investigation by the same research group also revealed that the polycomb protein, BMI1, was upregulated in liver SP cells, compared with that in non-SP cells and played a critical role in self-renewal and tumor-initiating property of SP cells.¹⁹ Another group also demonstrated the presence of SP cells with a high metastatic potential in HCC cell lines.²⁰ It was suggested through a comparative proteomics approach that the AKT and nuclear factor kappa B (NFκB) pathways are involved in the regulation of liver SP cells.²⁰

Further studies have thus far elucidated that CSCs and SP cells express several specific cell surface proteins that are not expressed in non-CSCs and non-SP cells and that there exist cells that express different CSC markers even in the same tumor tissue. Intriguingly, the functions of CSCs are slightly different from each other depending on the cell surface markers, although they share common properties, such as the tumor-initiating property. In HCC, as described below, several CSC markers, including CD133,²¹ CD44,^{22,23} CD24,²⁴ epithelial cell adhesion molecule (EPCAM),²⁵ CD90,^{26,27} CD13,²⁸ OV6,²⁹ and aldehyde dehydrogenase (ALDH),

have been reported (Fig. 1).³⁰ In addition, a huge number of long non-coding RNAs (lncRNAs) are expressed in cells and involved in a variety of cellular functions, including carcinogenesis. Recently, we demonstrated that nuclear paraspeckle assembly transcript 1 (NEAT1), a lncRNA, is involved in the enhancement and maintenance of CSC-like properties and is required for CD44 expression in HCC.³¹

It has been demonstrated that the knockdown or knockout of these CSC markers in cancer cells result in the impairment of CSC-like properties, which suggests that these molecules and their downstream signaling pathways play crucial roles in the regulation of CSC-like properties. Accordingly, these CSC-specific molecules not only attract interest in terms of molecular cancer biology but also are expected as novel therapeutic targets for HCC treatment. This section will briefly describe the pathological implications of each CSC marker in HCC.

CD133

CD133 encoded by the *PROM1* gene is expressed as a membrane glycoprotein mainly in neurons and bone marrow progenitor cells and is suggested to have functions required for the maintenance of undifferentiated status.^{32, 33} It was demonstrated that CD133-positive HCC cells exhibited strong expression of a liver progenitor marker, α -fetoprotein (AFP), and weak expression of mature hepatocyte markers, glutamine synthetase, and cytochrome P450 family 3 subfamily A member 4.²¹ These findings suggest that CD133 also plays a regulatory role in the maintenance of the undifferentiated status of CSCs in HCC. In addition, it was shown that CD133 induced the activation of the mitogen-activated protein kinase cascade through interleukin-8 (IL-8) and neurotrophine.³⁴ Moreover, CD133 expression in HCC was correlated with the activation of AKT³⁵ and NF κ B,³⁶ both of which are crucial regulators of liver CSCs and SP cells.²⁰ Conversely, the suppression of

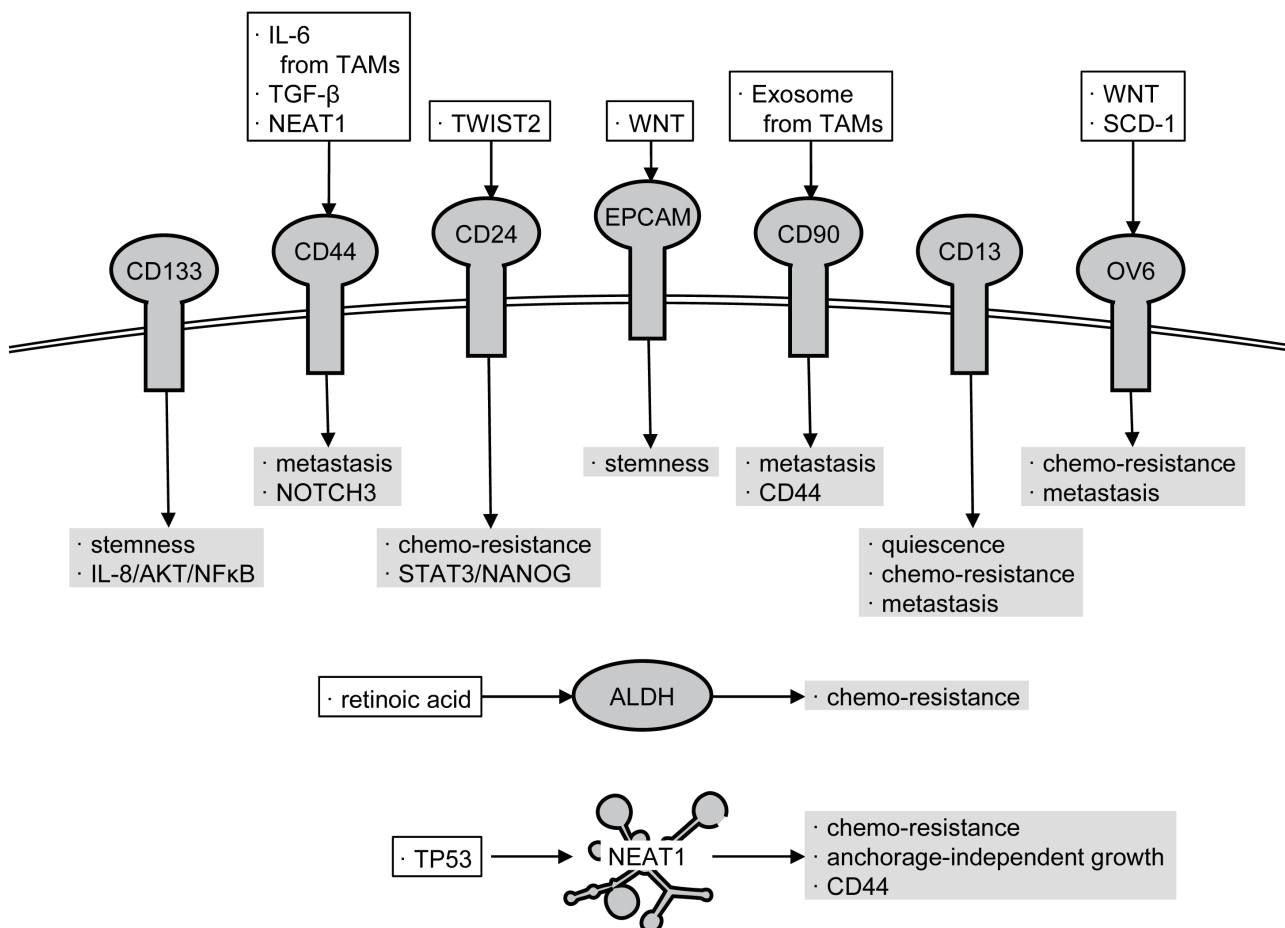


Fig. 1. Factors defining liver CSCs. To maintain their properties in HCC, CSCs express the factors including specific cell surface proteins as well as intracellular proteins and lncRNAs. The proposed regulators of the factors are shown in open squares. Their target signaling pathways and phenotypes are shown in gray squares. Details are discussed in the main text.

CD133 attenuated CSC-like properties and tumorigenic and metastatic potential, which suggests that CD133 would be a promising target for HCC treatment in a study in which mice are used.^{35,36}

CD44

CD44 is expressed as a variant isoform (CD44v) incorporating variant exons, as well as standard isoform (CD44s).^{22,23} Reportedly, CD44v is expressed in CSCs derived from a wide variety of tumor tissues and potentiates cellular antioxidant capacity by enhancing the synthesis of glutathione.^{37,38} However, CSCs in HCC mainly express CD44s. This fact prompted us to examine the function of CD44s in HCC by constructing CD44-knocked out HCC cells.²³ In our previous report, CD44s has been demonstrated to be involved in the induction of antioxidant enzyme genes expression and the maintenance of CSC-like properties in an HCC cell line, possibly via NOTCH3.²³ Meanwhile, another research group reported that CD44 expression in HCC was induced by IL-6 secreted by tumor-associated macrophages (TAMs), which results in the enhancement of CSC-like properties.³⁹ In addition, CD44s was suggested to enhance the metastatic potential of HCC cells rather than the maintenance of stemness, under the control of transforming growth factor- β (TGF- β).²²

CD24

Lee *et al.* analyzed the gene expression profile of tumor tissues that survived after treatment with cisplatin in mice transplanted with HCC cells and found that CD24 was significantly upregulated in the survived cancer cells.²⁴ CD24 is known as a tumor-related gene highly expressed in various tumor tissues and is suggested to regulate CSC-like properties by inducing NANOG expression via signal transducer and activator of transcription 3 (STAT3) in HCC.²⁴ Moreover, it was proven that TWIST2, a transcription factor involved in epithelial–mesenchymal transition (EMT), enhances CSC-like properties by inducing CD24,⁴⁰ suggesting the TSWIST2-CD24-STAT3-NANOG pathway as a novel regulatory mechanism for CSC regulation in HCC.

EPCAM

In a healthy liver, EPCAM is expressed in bile duct epithelial cells. Liver progenitor cells also express EPCAM, but its expression is diminished as hepatocyte differentiation progresses and thus is absent in mature hepatocytes. CSCs in HCC also exhibit the high expression levels of EPCAM under the control of the WNT signaling pathway.⁴¹ The expression of EPCAM and AFP has been demonstrated to be possibly used

to discriminate between malignant HCC and bile duct epithelial cells.^{25,42} In HCC cells double-positive for EPCAM and AFP, the expressions of CD133, liver progenitor markers, and WNT target genes were concomitantly upregulated, whereas those of mature hepatocyte markers were downregulated.⁴² Moreover, the knockdown of EPCAM in HCC cells impaired CSC-like properties.⁴² In addition to its regulatory function of stemness, the co-culture of HCC cells with major histocompatibility complex (MHC)-independent $\gamma\delta$ T cells in the presence of a bi-specific antibody recognizing EPCAM and CD3 resulted in the increased cell lysis of HCC cells,⁴³ which suggests that EPCAM can be applied as a target for HCC immunotherapy. Nevertheless, to distinguish between HCC and bile duct epithelial cells, further studies are required for the development and clinical application of EPCAM-targeting drugs.

CD90

Using six human HCC cell lines and an immortalized hepatocyte line, Yang *et al.* investigated the relationship between several liver progenitor markers and tumor-initiating potential.^{26,27} Consequently, they identified CD90 as an HCC-specific protein whose expression was correlated with tumor-initiating potential.^{26,27} CD90 expression in HCC has been suggested to be regulated by exosomes secreted from TAMs.⁴⁴ Intriguingly, most CD90-positive cells are also positive for CD44 whose expression is also regulated by TAMs.³⁹ Consistently, the metastatic potential of CD90-positive cells has been shown to be suppressed by an anti-CD44 antibody.²⁶ Moreover, it has been observed that, whereas EPCAM and CD133 are co-expressed with immature hepatocyte markers, CD44 and CD90 are co-expressed with mesenchymal markers that are indicative of high metastatic potential.⁴⁵ These results suggest that CD90 mainly regulates the metastatic potential of HCC cells via CD44.

CD13

CD13, also known as aminopeptidase N, was identified via gene expression profiling, as a cell surface protein that is highly expressed in SP cells of HCC.²⁸ Notably, unlike other CSC markers, cells highly expressing CD13 are mostly in the G0/G1 phase,²⁸ and thus, CD13 is considered as a marker of quiescent CSCs. Cancer cells in a quiescent state are generally known to be relatively resistant against various anti-cancer drugs because most of those drugs target cellular machineries required for cell proliferation. However, ubenimex, a protease inhibitor, which also inhibits the aminopeptidase activity of CD13, has been shown to have potentiated the

sensitivity of HCC cell lines to 5-fluorouracil.²⁸ It has also been demonstrated that CD13 was co-expressed with N-cadherin, a mesenchymal marker, and moderately alleviated oxidative stress during the induction of mesenchymal phenotype in HCC cells.⁴⁶ Thus, it is suggested that CD13 contributes to the efficient induction of EMT by protecting HCC cells from the oxidative stress associated with EMT and thereby promotes the acquisition of metastatic potential. However, how this aminopeptidase activity regulates CSC-like properties in HCC remains to be elucidated.

OV6

OV6 is a monoclonal antibody reacting with oval cells that appear in the canals of Hering after severe liver damage of rats. This antibody was produced by using neoplastic nodules in the rat liver as an antigen.⁴⁷ The target protein has not been fully specified, although it was shown that a cytoskeletal protein with a molecular weight of 56 kDa was reacted with OV6.⁴⁷ The oval cells are considered as hepatic progenitor cells found in the rat livers, as described above. However, it is known that oval-like cells that are reacted with OV6 also appear in damaged liver and HCC of humans. Consistently, it was demonstrated that OV6-positive cells isolated from HCC also expressed other CSC markers and had high tumor-initiating potential.²⁹ WNT/ β -catenin signaling increased the number of OV6-positive cells and contributed to cisplatin resistance in HCC.²⁹ Moreover, the frequency of OV6-positive cells was significantly correlated with poor prognosis of patients with HCC.⁴⁸ Intriguingly, OV6-positive cells were mainly found in the invasion front of HCC tumor tissues, and an *in vitro* assay revealed that these OV6-positive cells have a high-migrating and invasive potential.⁴⁸ Additionally, the same group also demonstrated that stromal cell-derived factor 1 induced the expression of OV6 antigen through C-X-C motif chemokine receptor 4 and thereby promoted metastasis of HCC cells.⁴⁸

ALDH

A proteome analysis of HCC cell lines identified ALDH as a protein highly expressed in CD133-positive cells.³⁰ It has been shown that ALDH and CD133 double-positive cells had higher CSC-like properties than those of ALDH-negative and CD133-positive cells.³⁰ Additionally, ALDH has been suggested to enhance the survival of cancer cells via the detoxification of endogenous and exogenous aldehydes, and the scavenging of reactive oxygen species.⁴⁹ Conversely, ALDH is also known to be a critical enzyme for the synthesis of retinoic acid, which has an antitumor activity, in general.

How ALDH activity in CSCs affects retinoid signaling remains to be elucidated. Notably, a CSC-targeted therapy using retinoic acid was proposed because the retinoic acid lowered the ALDH activity in lung cancer cell lines in a negative feedback manner.^{50, 51}

LncRNA

The lncRNA, NEAT1, is expressed as short variant 1 (3.8 kb; NEAT1v1) and long variant 2 (22.7 kb; NEAT1v2). Although the expression of NEAT1 was induced by tumor protein p53 (TP53), it was demonstrated that NEAT1 promoted carcinogenesis by activating DNA repair and cell proliferation signals.^{52, 53} We recently elucidated that the knockout of the *NEAT1* gene resulted in decreased CSC-like properties of HCC cell lines, which were concomitant with the abolishment of CD44 expression.³¹ Rescue experiments supported the notion that CD44 expression in HCC was highly dependent on NEAT1 expression. As described above, since CD44 regulates CSCs, it was postulated that NEAT1 might maintain CSC-like properties via CD44. However, we found that NEAT1 overexpression restored the CSC-like properties even in CD44-deficient HCC cells.³¹ These findings suggested that NEAT1 maintained the CSC-like properties of HCC cell lines in both CD44-independent and CD44-dependent manners.

In addition to NEAT1, several lncRNAs have been reported to be involved in the maintenance of CSC-like properties, including highly upregulated in liver cancer (HULC),⁵⁴ metastasis-associated lung adenocarcinoma transcript 1 (MALAT1),⁵⁵ and LINC00324.⁵⁶ HULC is a target gene of a lncRNA, cancer upregulated drug resistant (CUDR), which induced hepatocytic differentiation of embryonic stem cells and promoted the malignant growth of the hepatocyte-like cells.⁵⁷ In liver CSCs, HULC enhanced autophagy by inducing the expression of sirtuin 1 and thereby promoted the cell cycle in a cyclin D1-dependent manner.⁵⁴ MALAT1 has been shown to be upregulated in HCC.⁵⁵ The knockdown of MALAT1 led to a decrease in CD133 and CD90 double-positive CSC populations in association with the suppression of WNT/ β -catenin signaling, although its detailed mechanism is unclear.⁵⁵ LINC00324 was also identified as a lncRNA highly expressed in HCC, and, interestingly, its expression was significantly associated with FAS ligand (FASL).⁵⁶ Mechanistically, LINC00324 has been shown to have an interaction with PU box binding protein, which is a transcription factor that directly regulates FASL expression.⁵⁶ Moreover, the knockdown of LINC00324 or FASL decreased the expression of stemness-related genes.⁵⁶ However, it is known that FASL induces apoptosis in cells that express

FAS. How LINC00324 regulates CSC-like properties via FASL remains unclear. These lncRNAs provide more insights into the regulatory mechanisms of CSC by identifying their target genes, although, unlike surface protein markers, they cannot be used for the isolation of CSCs.

MALIGNANT FUNCTIONS OF CSCS IN HCC

As described above, CSCs exhibit not only tumor initiation but also other malignant features, including resistance to conventional therapies, EMT phenotype, and immunomodulatory property. Sorafenib is a standard molecular-targeted drug used for the treatment of advanced HCC. Liver CSCs are frequently endowed with sorafenib resistance through several pathways. The downregulation of angiopoietin-like protein 1 (ANGPTL1) in HCC has been shown to be associated with sorafenib resistance.⁵⁸ Conversely, the overexpression of ANGPTL1 suppressed sorafenib resistance, and CSC-like properties, in association with decreased EMT phenotype through the downregulation of an EMT factor SLUG.⁵⁸ Metformin, a type 2 diabetes drug, inhibited the EMT process and reduced CSC-like properties and sorafenib resistance in an HCC cell line.⁵⁹ Conversely, microorchidia family CW-type zinc finger 2 (MORC2) also enhanced CSC-like properties and sorafenib resistance.⁶⁰ Mechanistically, MORC2 induced DNA methylation of *neurofibromatosis 2* and *kidney and brain protein* genes by DNA methyltransferase 3A, leading to the suppression of the anti-tumorigenic Hippo signaling pathway.

The induction of EMT is suggested to be an important process to acquire CSC-like properties. The EMT factor TWIST2, which is overexpressed in HCC, induced liver CSCs by directly augmenting CD24 transcription as well as increased migration and invasion abilities.⁴⁰ SLUG is also a well-known EMT factor and plays an essential role in the induction of EMT by hypoxia-inducing factor 1 α (HIF1 α).⁶¹ SLUG induced by HIF1 α further enhanced the CSC-like properties in HCC through NOTCH1 signaling.⁶¹ The cancer stemness of leukemia cells has been demonstrated to be suppressed by the retinoic acid-inducible gene I (RIG-I).⁶² The knockdown of RIG-I in HCC induced CSCs and upregulated TGF- β expression in the HCC cells.⁶³ TGF- β is a multi-functional cytokine that induces not only EMT phenotype but also CSC-like properties in HCC.⁶⁴ Consistently, TGF- β secreted from the cells knocking down RIG-I expression directly suppressed the maturation of dendritic cells (DCs), which leads to the evasion of tumor immunity.⁶³

In tumor tissues, vascular endothelial cells and

immune cells also exist. Drugs that target these non-tumor cells have already been developed, for instance, vascular endothelial cell growth factor receptor inhibitors and programmed cell death-1 (PD-1)/PD-1 ligand-1 (PD-L1) inhibitors.⁷ In addition, non-tumor cells with tumor-specific functions, including cancer-associated fibroblasts (CAFs),⁶⁵ tissue-associated myeloid cells,⁶⁶ and pericytes,⁶⁷ have been recently found in tumor tissues, and it is revealed that these cells promote the growth and metastasis by interacting with tumor cells.⁶⁸ Moreover, these non-tumor cells resident in tumor tissues also provide a niche for the induction and maintenance of CSCs. For instance, TAMs induced CSCs in HCC by secreting TGF- β ⁶⁹ or IL-6.³⁹ These factors also induced EMT, drug resistance, and further recruited myeloid-derived suppressor cells (MDSCs) that suppress tumor immunity.^{39, 69, 70} The stem cell factor (SCF)/c-KIT axis plays an essential role in maintaining CSCs in ovarian and lung cancers and leukemia.⁷¹⁻⁷³ It was demonstrated that SCF produced by cancer cells, such as in HCC as well as breast, lung, ovarian, and gastrointestinal cancers, induced the tumor infiltration and activation of mast cells.⁷⁴ The tumor-infiltrated mast cells suppressed tumor immunity by recruiting regulatory T (Treg) cells and releasing adenosine.⁷⁴ The mast cells also produced C-C motif chemokine ligand 2, which further recruited MDSCs to HCC tissues, and promoted IL-17 production by MDSCs.^{74, 75} IL-17 sequentially activated the immunosuppressive function of Treg cells.⁷⁵ These results suggest that SCF produced by HCC cells creates an immune-suppressive tumor microenvironment, possibly in association with the induction and maintenance of liver CSCs. CD206, a mannose receptor, is expressed in immature DCs and immunosuppressive M2-macrophages and is suggested to be involved in immunosuppression.^{76, 77} Moreover, CD206 expression was found in HCC and was correlated with tumor size, metastasis, and poor prognosis.⁷⁸ CSCs in HCC cells exhibited a higher expression of CD206 than did non-CSCs, and the knockdown of CD206 suppressed their metastatic potential.⁷⁸ This result suggests that CD206 regulates multiple aspects of liver CSCs.

CSC AS A THERAPEUTIC TARGET FOR HCC

Cyclooxygenase 2 (COX2) and its products, prostaglandins, are also essential regulatory factors for CSCs. It was demonstrated that the expression level of COX2 was correlated with the presence of CSCs in not only HCC but also in other types of cancers.⁷⁹⁻⁸¹ Celecoxib, a COX2 inhibitor, decreased CD44⁺/CD133⁺ and SP cells in HCC cells.⁸² Mechanistically, celecoxib inhibited the production of prostaglandin E2 (PGE2) and suppressed

the PGE2-induced activation of AKT signaling by the upregulation of PTEN.^{79, 82} Moreover, celecoxib sensitized liver CSCs to epirubicin by downregulating multi-drug resistance protein 1 (ABCB1) besides the downregulation of CD44 and CD133.⁸³ Celecoxib, combined with epirubicin, increased tumor-infiltrating CD8⁺ T cells, decreased Treg cells, and downregulated PD-L1 expression in HCC tissues.⁸³

Extracellular matrix provides stem cells with a niche, which maintains their stemness, and supports stable self-renewal and asymmetric cell division. Hyaluronan has been demonstrated to be an essential extracellular matrix for breast and liver CSCs.^{84, 85} Breast CSCs interacted with TAMs via hyaluronan produced by CSCs and thereby promoted the production of platelet-derived growth factor-BB by TAMs to activate CAFs.⁸⁴ The activated CAFs, sequentially, secreted fibroblast growth factor (FGF) 7 and FGF9, which enhanced CSC-like properties in breast cancer.⁸⁴ This vicious cycle was blocked by 4-methylumbelliferone (4Mu), an inhibitor of hyaluronan synthase 2, which is upregulated in breast CSCs.⁸⁴ 4Mu also suppressed HCC growth, concomitant with the downregulation of CSC markers, including CD133, CD90, EPCAM, CD44, and CD13 as well as CD47, which acts as a “don't eat me” signal to protect cancer cells from phagocytosis by macrophages.⁸⁵ Consistently, 4Mu combined with adenovirus expressing IL-12 significantly potentiated phagocytosis by macrophages and antitumor CD8⁺ cytotoxic T cell response.⁸⁵

Immunotherapy that targets liver CSCs is currently investigated. Kiatomab is a specific monoclonal antibody against KIAA1114, which is a recently identified cell surface marker of liver CSCs with unknown function.⁸⁶ The inhibitory effect of kiatomab on HCC tumor growth and metastasis was demonstrated in a murine syngeneic tumor transplantation model and was suggested to involve antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity.⁸⁷

DCs are potent immune adjuvant by presenting antigens to naïve T cells and releasing cytokines. DCs fused with CD90⁺ CSCs of HepG2 hepatoma cell line showed the increased expression of co-stimulatory molecules, such as CD80, CD83, and CD86, and MHC class I and II molecules (human leukocyte antigen-A, -B, -C, and -DR).⁸⁸ Moreover, the fusion with CSCs induced more potent activation of DCs, including higher expression of inflammatory cytokines, than that with bulk HepG2 cells.⁸⁸ Interestingly, DCs fused with CSCs activated cytotoxic T lymphocytes (CTL) against both HepG2 CSCs and bulk cells.⁸⁸ However, DCs fused with bulk cells induced less activation of CTL against

bulk cells than did CSCs-fused DC and failed to induce CSCs-specific CTL response.⁸⁸

Cytokine-induced killer cells (CIKs) are a population of cytotoxic effector cells generated by treating peripheral blood mononuclear cells with specific cytokines, such as IL-2 and interferon- γ , and contain CD3⁻/CD56⁺ natural killer (NK) cells, CD3⁺/CD56⁻ T cells, and CD3⁺/CD56⁺ CIKs.⁸⁹ These “bona fide” CIKs are also positive for NK-activating receptor NKG2D and exhibit NK-like MHC-unrestricted cytotoxicity against a wide variety of malignant cells possibly through NKG2D but not toward normal cells.⁸⁹ Since antigen-pulsed DCs directly or indirectly activate the killer activity of CIKs, it was demonstrated that autologous CIKs co-cultured with autologous DCs from patients with HCC efficiently suppressed liver CSCs-derived tumor growth *in vivo*.⁹⁰ Notably, when DCs were pulsed with liver CSCs, the suppression effect was more prominent than when DCs were pulsed with bulk HCC cells.⁹⁰ These results indicate that liver CSCs are promising therapeutic antigens for the establishment of immunotherapy for HCC.

CONCLUSIONS

A large part of the regulatory mechanisms of CSCs remains unclear although enormous attempts have been made to understand CSCs, and the existence of CSC itself remains debated. The CSC markers and functions depicted in the present review are just a part of those identified so far. Some of these CSC markers solely regulate CSC-like properties whereas others cooperate with each other to promote CSC-like properties. By contrast, some CSC markers are mutually exclusively expressed in cancer cells even those of the same HCC tissue. To develop innovative medicines to combat HCC, the complex functions of these CSC-related molecules in the maintenance of CSC-like properties should be urgently elucidated.

Clinical needs for HCC treatment are being met owing to the recent approval of several molecular-targeted drugs.⁷ Moreover, with the advancement of surgical treatments, the prognosis of HCC patients is slowly but steadily being improved. However, in line with the prolonged prognosis by those recent advances, recurrence risk appears to be slightly increased. Because CSCs are involved in recurrence as well as *de novo* tumor occurrence, CSC-targeted therapy will also be a good option for the prevention of cancer recurrence in the future. We believe that deepening our understanding of CSCs will provide a great advance toward achieving the complete eradication of HCC.

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