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REVIEW

Hepatic cancer stem cells and drug resistance: Relevance in targeted therapies for hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of most common malignancies in the world. Systemic treatments for HCC, particularly for advanced stages, are limited by the drug resistance phenomenon which ultimately leads to therapy failure. Recent studies have indicated an association between drug resistance and the existence of the cancer stem cells (CSCs) as tumor initiating cells. The CSCs are resistant to conventional chemotherapies and might be related to the mechanisms of the ATP Binding Cassette (ABC) transporters and alterations in the CSCs signaling pathways. Therefore, to contribute to the development of new HCC treatments, further information on the characterization of CSCs, the modulation of the ABC transporters expression and function and the signaling pathway involved in the self renewal, initiation and maintenance of the cancer are required. The combination of transporters modulators/inhibitors with molecular targeted therapies may be a potent strategy to block the tumoral progression. This review summarizes the association of CSCs, drug resistance, ABC transporters activities and changes in signaling pathways as a guide for future molecular therapy for HCC.

Key words: Hepatocellular carcinoma; Liver; Cancer stem cells; Drug resistance; Hepatocellular carcinoma therapy

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INTRODUCTION

Primary liver cancer is the fifth most common neoplasm in the world and the third most common cause of cancer-related death[1]. Approximately more than 500 000 new cases are diagnosed per year[2]. Hepatocellular carcinoma (HCC) accounts for 85% to 90% of primary liver cancers[3]. Several major risk factors for HCC are known, the main ones are liver cirrhosis due to viral infections hepatitis B virus (HBV) or/and hepatitis C virus (HCV), excessive alcohol consumption, aflatoxin B and vinyl-chloride monomer[4], obesity-related disease and familial-related disorders such as primary hemochromatosis[5]. In Asia and Africa, as much as 70% of HCC is caused by the HBV infection, while in Europe and North America

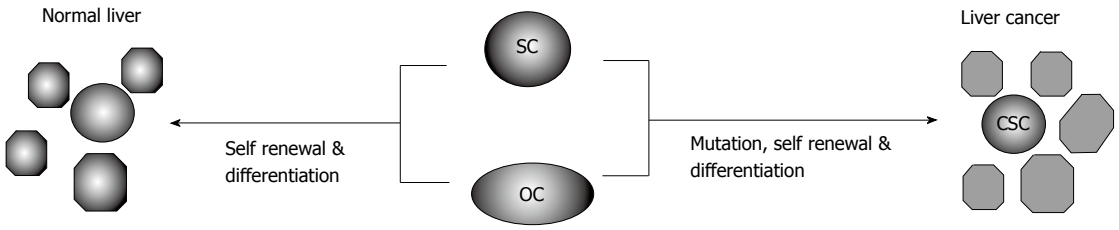

Figure 1 The liver cancer initiation hypothesis based on CSCs theory. The hepatic stem cells (SC) and oval progenitor (OC) cells have specific capacities to self renew and differentiate into multiple hepatic lineages. Mutations in SC and/or OC may modify the cells genetic property and switch the normal SC into CSCs leading to tumor initiation.

50%-70% is caused by HCV infection[2,6]. HCC without liver cirrhosis is also found although the annual HCC incidence is much lower than HCC with cirrhosis[7] indicating that chronic necro-inflammation is a key element of the occurrence of disease[8].

Until now, main treatments for HCC are surgical intervention (liver resection and liver transplantation) and local radiofrequency ablation. These approaches are curative only for localized small liver tumors, preferably in early stage (monofocal) when patients have a good life expectancy. In contrast, potential treatments for more advanced stages of HCC are more difficult. For HCC patients who cannot have any surgical intervention, survival has not significantly increased in the past 30 years[9].

Systemic treatment for more advanced stages of HCC is given as another option, although there are many limitations and the prognosis of unresectable HCC remains poor. Many chemotherapeutic agents have been tested but the response rate is still low, ranging between 10% and 15%[10]. Significant toxicity and decrease of the efficiency of the drugs also become limitations.

One of the most studied chemotherapeutic agents for cancer treatment for over 30 years is doxorubicin. A report from a phase Ⅲ trial in unresectable HCC patients compared the administration of doxorubicin as single-agent therapy and combination regimen therapy PIAF [cisplatin/interferon/doxorubicin (Adriamycin)/5-fluoruacil (5-FU)]. Although patients on PIAF showed a higher overall response rate (20.9%) than patients on doxorubicin alone (10.9%), the difference was not significant[11]. Since both single and combination therapies showed serious toxicity and an overall disappointing survival rate, the use of this systemic treatment should be carefully considered.

CANCER STEM CELLS

Stem cells are non-specialized cells which have potential capabilities to self-renew, differentiate into multiple cell types and proliferate extensively. They serve as the source of all cells types and have the capacity to divide without limit to replace damaged cells or generate new cells and tissues. These unique characteristics offer valuable advantages in regenerative medicine, tissue engineering and biotechnology applications[12]. Many studies demonstrate that stem/progenitor cells derived from several organs can replenish and express molecular characteristic and biological functions of adult cells. This benefit provides the basis for attempts for stem cell therapy in various diseases.

On the other hand, if some mutations alter the genetic properties of the stem cells, they can become tumorigenic and may initiate cancer (Figure 1). These so-called cancer stem cells (CSCs) still possess the whole capacity as normal stem cells to proliferate and develop heterogeneous lineages of cancer cells that comprise the tumor[13]. CSCs are suggested to be one of the main players in the initial growth and maintenance of cancer. Evidences of CSCs were observed in the hematopoietic system[14-18] as well as in solid tumors breast[19], brain[20,21], prostate[22,23], gastric[24], lung[25], colon[26,27] and liver[28-32,41]. Many cancers are composed of heterogeneous lineages of stem cells, progenitor cells, less differentiated cells and differentiated cancer cells. Cancer is compiled by various types of cells, at different stages of differentiation and with different functions.

Current studies on several cancer types have revealed that CSCs are resistant to chemotherapy and radiotherapy. In chronic myeloid leukemia (CML), cells expressing CD34 remained viable in a quiescent state even in the presence of tyrosine kinase inhibitor STI571, a common drug for CML[33]. In HCC, purified cells expressing CD133 isolated from human cell line and xenograft mouse model survived chemotherapeutic agents doxorubicin and fluorouracil in a higher percentage compared to most tumor cells without CD133 phenotype[34]. Studies in glioblastoma demonstrated that CD133-positive cells were also resistant to radiotherapy. They were enriched after radiation and preferentially activated the DNA damage checkpoint and repaired radiation-induced DNA damage more efficiently than CD133-negative cells[35]. This mechanism is suggested to be a defense system of the cells.

Because CSCs are important in the initiation and maintenance of the cancer, their resistance to anticancer drugs is an obstacle for the total eradication of cancer. Conventional chemotherapies may recognize and kill most of bulk (differentiated) tumor cells but spare the CSCs. Therefore, to achieve a complete response in cancer therapy, it is crucial to target the CSCs first to eradicate the source of the cancer and then the more differentiated tumor cells.

CANCER STEM CELLS IN HCC

The common normal hepatic stem/progenitor cells can didates are proposed to be localized at the junction of the bile ducts and hepatic cords, known as canal of Hering[36]. The origin of stem cells in the liver has been a subject of discussion as to whether they are real resident hepatic stem cells or derived from bone marrow stem cells migrated to the liver. Different studies report that the progenitor cells population share phenotypic markers between common hematopoietic stem cells with the real hepatic markers.

A population of progenitor cells from adult liver identified as human liver stem cells (HLSCs) was reported. These cells expressed the mesenchymal stem cell markers CD29, CD73, CD44 and CD90 (Thy-1), but not hematopoietic stem cells markers CD34, CD117 (c-kit) and CD133 (Prominin-1). The HLSCs had multipotent capacities for hepatocytes, osteogenic, adipogenic and endothelial differentiation *in vitro*. *In vivo,* HLSCs contributed to mouse liver regeneration[37]. In contrast, the multipotent progenitor cells population originating from fetal liver expressed CD117 and CD34 markers. These cells had the capacity to differentiate to liver and mesenchymal cell lineages and replenish functional hepatocyes *in vivo*[38,39]. Interestingly, a study performed in a rat liver injury model characterized two distinct liver progenitor subpopulations of hematopoietic and hepatic origins. The hepatic (oval) progenitor cells population needed hepatic niche to proliferate *in vitro* while hematopoietic stem cells had a limited capacity to replicate and differentiate to hepatic lineage[40].

In HCC, surface marker CD133 is one of the most studied markers of the CSCs. CD133 was also identified as a CSCs marker in human leukemia, brain tumor, prostate cancer and laryngeal tumor. In the liver, $CD133^+$ cells possess higher proliferative output, greater capacity to form colonies and greater ability to induce tumor *in vivo* compared with $CD133^-$ cells[28,31,41]. Together with aldehyde dehydrogenase (ALDH), a hierarchical organization characterizing the tumorigenic hepatic CSCs population $CD133^+ALDH^+ > CD133^+ALDH^- > CD133^-ALDH^-$ was reported[42]. ALDH has been identified to be highly expressed in embryonic tissue as well as in adult stem cells[43]. Reactivated $CD133^+$ cells were frequently present in HCC and increased CD133 expression corresponded with higher stage tumors and indicated a poor prognosis for patients[44].

Other studies have proposed surface marker CD90 (Thy-1) since $CD90^+$ cells, but not $CD90^-$ cells, from HCC cell lines could induce tumor growth *in vivo*, and the number of injected cells paralleled with tumorigenicity. The detection of $CD45^-CD90^+$ cells in either tumor or blood was proposed to be a highly sensitive and specific circulating marker for the diagnosis of liver cancer[29,45].

A gene expression study showed molecular signature of hepatic progenitor cells including the presence of stem markers such as cytokeratin 19 (K19) and c-kit (CD117) in $EpCAM^+$ HCC while $EpCAM^-$ HCC displayed features of mature hepatocytes. This result proposed EpCAM as one of the stem cells markers. The $EpCAM^+$ cells also showed Wnt/β-catenin signaling activation[46]. The cells with EpCAM and alpha fetoprotein (AFP) co-expression had the capabilities to self-renew, differentiate and initiate highly invasive HCC in mice[47]. In human hepatic adenoma and focal nodular hyperplasia, distinct cytokeratin 7 (K7) and K19, together with neuronal cell adhesion molecule expression, suggested different subsets of hepatic progenitor cells[48].

Tumorigenesis consists of a multisteps process from normal to cancerous cells. Wide variations in the HCC prognosis among individuals imply that HCC may have different phenotypes. A genomic study using oligonucleotide microarrays revealed 2 subtypes of HCC with different prognosis. Individuals in subtype sharing a gene expression pattern with fetal hepatoblast had a poor prognosis compared to another subtype which shared pattern with adult hepatocytes. This hepatoblast subtype may arise from bipotent progenitor cells. These cells highly expressed the K7 and K19 markers for early hepatoblast and mature hepatic progenitor cells[49,50]. K7 and K19 were also associated as a predictor of postoperative recurrence due to increased invasiveness[51,52].

However, the heterogeneity and hierarchy of liver cancer remain elusive and the characteristic of the hepatic CSCs is still unclear. Although several biological markers have been proposed to identify the hepatic CSCs, supporting data are contradictory and no agreement has been reached. In addition, the markers between normal stem cells and CSCs markers might overlap. The CSCs characterizations which distinguish them from normal stem cells will be significantly important.

ABC TRANSPORTERS AND DRUG RESISTANCE

The ATP Binding Cassette (ABC) transporters are one of the largest families of membrane transport proteins. These proteins utilize a pair ATP (Adenosine-5'-triphosphate) molecule to export specific compounds or to flip them from inner to outer leafs of the membranes[53]. Thus, they are responsible for translocations of various substrates such as metal ions, sugars, peptides, proteins, amino acids and a large number of hydrophobic compounds and metabolites across the membrane barrier[54]. In humans, there are 49 members of ABC transporters gene which are classified into seven subfamilies based on the sequence homology and ATP-binding proteins[55], as described in Table 1.

The ABC genes are composed either as full transporters containing two transmembrane domains (TM) and two nucleotide binding folds (NBF) domains or as half transporters containing one of each TM and NBF[56]. While NBFs are responsible for the binding and hydrolysis of ATP creating the motional force, TM builds the translocation pathway for compound translocation[57].

The main role of the ABC transporters is to protect

Table 1 Human ABC transporter genes and proteins conferred drug resistance

Official symbol	Alternative name	Members	Proteins associated with drug resistance	Resistant drugs
ABCA		13 (ABCA1 to ABCA13)	ABCA2	Mitoxantrone
ABCB	MDR	11 (ABCB1 to ABCB11)	ABCB1 (MDR1/PGP)	Doxorubicin, colchicine, etoposide, paclitaxel, cisplatin, methotrexate, daunorubicin, camptothecin
			ABCB11 (BSEP)	5-fluorouracil, paclitaxel
ABCC	MRP	13 (ABCC1 to ABCC13)	ABCC1 (MRP1)	Doxorubicin, daunorubicin, methotrexate, colchicine
			ABCC2 (MRP2)	Doxorubicin, cisplatin, etoposide
			ABCC3 (MRP3)	Methotrexate, etoposide
			ABCC6 (MRP6)	Etoposide
			ABCC10 (MRP7)	Vinorelbine, paclitaxel, docetaxel
			ABCC11 (MRP8)	5-fluorouracil, tamoxifen, paclitaxel
ABCD	ALD	4 (ABCD1 to ABCD4)		
ABCE	OABP	1 (ABCE1)		
ABCF	GCN20	3 (ABCF1 to ABCF3)		
ABCG	White	5 (ABCG1, ABCG2, ABCG4, ABCG5, ABCG8)	ABCG2 (BCRP)	Mitoxantrone, topotecan, doxorubicin, daunorubicin, cisplatin, etoposide

the cells from accumulation of toxic compounds since these proteins have the capacity to export drugs and decrease the cell sensitivity to drugs. This explains the close association between ABC transporters proteins and drug resistance (Table 1). Extensive reviews on the molecular basis of the multidrug transport by ABC transporters are available[57].

Many normal stem cells and cancer cells express high level of specific ABC transporters[58]. Some ABC transporters such as multidrug resistance 1 (ABCB1/MDR1/PGY1), multidrug resistance-associated protein-1 (ABCC1/MRP1), multidrug resistance-associated protein-3 (ABCC3/MRP3) and breast cancer resistance protein (ABCG2/BCRP/MXR) were found in hepatic progenitor cells and hepatocytes in severe liver diseases[59,60]. ABCB1 was expressed primarily in the liver and blood brain barrier and supposed to be involved in cell protection[54]. ABCB1 over expression in drug resistant cells has been studied for more than 20 years[61]. This protein has broad substrates specificity and mediates resistance to a wide variety of drugs such as doxorubicin, colchicines, vinblastine and many more. In HCC, the expression of ABCB1 is variable, being either high or low expressed, or even not expressed. A study showed that ABCB1 over-expression was associated with HCC aggressiveness and reduction of survival, and ABCB1 was proposed as a prognostic marker after surgical resection in patients[62]. In contrast, another study showed that ABCB1 was less expressed in HCC than non tumorous tissue and not related with a more aggressive tumor phenotype and survival[63]. Since ABCB1 expression is closely associated with histological cellular differentiation[64], this could be the reason of the divergent expression among individuals with different HCC phenotypes. Another explanation might be the presence of the polymorphisms of ABCB1. The association between HCC recurrence-free and 2677A carrier (carrying at least one variant A allele) was significant compared to other polymorphisms on ABCB1 nucleotide sequences[65].

The role of ABCC1 is to serve as primary transporters for compounds conjugated to glutathione, glucuronate and sulfate conjugated and cytotoxic drugs, indicating its importance in defending cells from oxidative stress. The transporters ABCC2 and ABCC3 had overlapping substrate specificities with ABCC1 but different distribution in the tissue[66]. Rat liver progenitor cells expressed high levels of active ABCC1 and ABCC3[67]. ABCC1 expression was higher in HCC with poor survival and hepatoblast subtype of HCC and correlated with K19 expression[50,68]. Together with ABCC3 and ABCG2, they co-localized with K7/K19, markers for hepatic progenitor cells in the tumor[68].

ABCG2 was first identified in human breast carcinoma cells. This protein is expressed in many normal tissues such as placenta, brain, prostate, small intestine, testis and liver. The spectrum of anticancer drugs transported by ABCG2 included mitoxantrone, camptothecin-derived and indolocarbazole topoisomerase I inhibitors, methotrexate and flavopiridol[69]. ABCG2 was one of the chemosensitivity determinants of irinotecan hydrochloride (CPT-11), an effective anticancer drug[70]. The study of ABCG2 expression in a variety of solid tumors demonstrated its presence in 40% of tumors with different degrees of positivity[71]. The ABCG2 expression is assumed to be correlated with stem cells and CSCs. ABCG2 expression in the progenitor cells/reactive ductules could contribute to the resistance to cytotoxic agents and xenotoxins[60].

SIDE POPULATIONS OF STEM CELLS

The isolation of side population (SP) rich on stem cells was first developed to purify the hematopoietic stem cells from the murine bone marrow cells following the Hoechst 33342 efflux activity by FACS[72]. This population was composed of primitive and progenitor hematopoietic cells subpopulations, one of which expressed stem cell markers Sca^{+} and $CD34^{-}$ as the most primitive[73]. SP cells were visualized as "dull cells" with low or negative fluorescence in dot plot due to the unique feature of SP cells capability

Table 2 Several potential inhibitors and targeted agents of growth factor receptors, signaling pathways and ABC transporters in liver cancer

Targets		Agents	Ref.
ABC transporters	ABCB1/ MDR1	Verapamil, cyclosporine, GF120918, PSC833, GG918, biricodar	[146-149]
	ABCC1/ MRP1	Cyclosporine, biricodar	[146,149]
	ABCG2/ BCRP	FTC, Kol43, GF120918, novobiocin, naringenin	[69,70,147,150,151]
Growth factor receptors and signaling pathways	VEGF receptors	Sorafenib, sunitinib, bevacizumab	[139,142-145]
	EGF receptors	Erlotinib, gefitinib, cetuximab	[120-126]
	IGF receptors	IMC-A12	[132-134]
	Hedgehog pathway	Cyclopamine, anti-Rab23, SHH neutralizing antibodies	[107,108]
	Wnt/ β-catenin	Anti-Wnt antibody, AKT1 inhibitor	[34]
	Notch	TW-37	[136]

VEGF: Vascular endothelial growth factor; IGF: Insulin growth factor; EGF: Epidermal growth factor; ABC: ATP binding cassette.

to pump out the dye out of the cells. The activity of SP cells in exporting many types of substrates including dyes and drugs is assumed to have close association with the drug resistance.

The purified SP cells had been obtained from many solid tumors, including isolation of stem/progenitor cells from cancer originating from prostate[74], pancreas[75], stomach[76] and liver[30]. These studies confirmed the existence of a distinct hierarchy in malignancies and many SP cells obtained from different cancers demonstrated tumor initiating potentials. For example, SP from pancreatic cancer cell line had high capacity of the epithelial to mesenchymal transition (EMT), invasion and metastasis[75].

In humans, SP cells derived from an adult normal liver had the capacity to generate hepatocyte-like cells *in vitro*, irrespective to their CD45 marker status[77]. In the murine model, SP cells isolated from liver had potential to generate various liver cells such as mature hepatocytes and bile duct epithelium. As much as 75% of these SP cells expressed $CD45^+$ but the highest efflux activity was found in $CD45^-$ cells. Moreover, both $CD45^+$ and $CD45^-$ SP cells expressed CD34, CD117, Sca-1 and CD90[78].

A study using murine models demonstrated that bone marrow cells from MDR1A/$1B^{-/-}$ mice contained a normal number of SP cells, indicating that MDR1A/1B was not required for SP phenotype. By contrast, a significant reduction of SP cells in bone marrow and skeletal muscle was observed in $BCRP1^{-/-}$ mice, suggesting BCRP1 as molecular phenotype of SP[79,80].

In HCC, the SP population has also been reported. SP cells, sorted from HCC cell lines HCCLM3, MHCC97-H, MHCC97-L and Hep3B harboured CSCs-like, might be related to the metastatic potentials and therapeutic-resistance[81]. The SP from cell lines PLC/PRF/5 (0.80%) and HuH7 (0.25%) showed high proliferations, anti-apoptotic properties and capabilities to initiate tumor formation in non-obese diabetes/severe combined immunodeficiency (NOD/SCID) mice[30]. Further studies on ABCG2 expression in these cell lines showed that the sorted $ABCG2^+$ cells generated both $ABCG2^+$ and $ABCG2^-$ cells while $ABCG2^-$ cells only gave $ABCG2^-$ cells. Additionally, GATA6, an essential factor of earliest phase of hepatic development, was intensely expressed in $ABCG2^+$ cells and C/EBPβ, a factor for late phase of liver development, was expressed more in ABCG2- cells[82]. Using a 2-acetylaminofluorene partial hepatectomy (AAF/PH) rat model, it has been demonstrated that hepatic oval cells of non-parenchymal cells had the side population phenotype defined by expression of ABCG2/BCRP1[83].

A study by Hu *et al*[84] showed that ABCG2 expression significantly influenced the levels of drug efflux from HCC cell lines. The SP cells were importantly involved in the drug efflux-related chemotherapy resistance and the SP analysis was found to be an efficient method to evaluate the functional activity of ABCG2.

However, since ABCG2 is also expressed in other normal tissues[69], ABCG2 is perhaps not the best single marker to identify stem cells. The molecular phenotype markers of stem cells from various tissues obtained by SP technique were diverse. In human bone marrow, ABCG2 co-expression with CD34 and CD133 was found to be very low or undetectable and the use of single marker ABCG2 only harbored little colony forming potential[85]. In human liver, SP cells with $CD45^+$ phenotype could generate hepatocytes similar to their $CD45^-$ counterpart[77].

Furthermore, the characterization and definition of SP stem cells has several limitations. The SP population is usually very low and further characterization will be inadequate. Hoechst dye is toxic to the cells and the efflux is a biological process that may affect the results. High variations found in different studies might be caused by different ways of tissue dissociation, cells counting, dye concentration, staining condition and stringency in selection of SP cells. These parameters dramatically affected the viability, homogeneity and the apparent yield of SP cells[86].

ABC TRANSPORTER INHIBITORS

As mentioned previously, one of the most important appearances of the CSCs is their resistance to standard chemotherapies. The combination of chemotherapy drugs and specific inhibitors targeting ABC transporters could be a potential strategy to kill both tumor cells and the CSCs[87]. This approach focuses on killing the CSCs as the main source of the tumor by sensitizing the cells to drugs and inhibiting the drugs efflux from the cells. Total eradication of the tumor will prevent the reoccurrence of cancer (Figure 2).

Experimental and clinical studies focused on increasing the sensitivity of cancer cells to anticancer drugs are ongoing. Several compounds have been introduced to

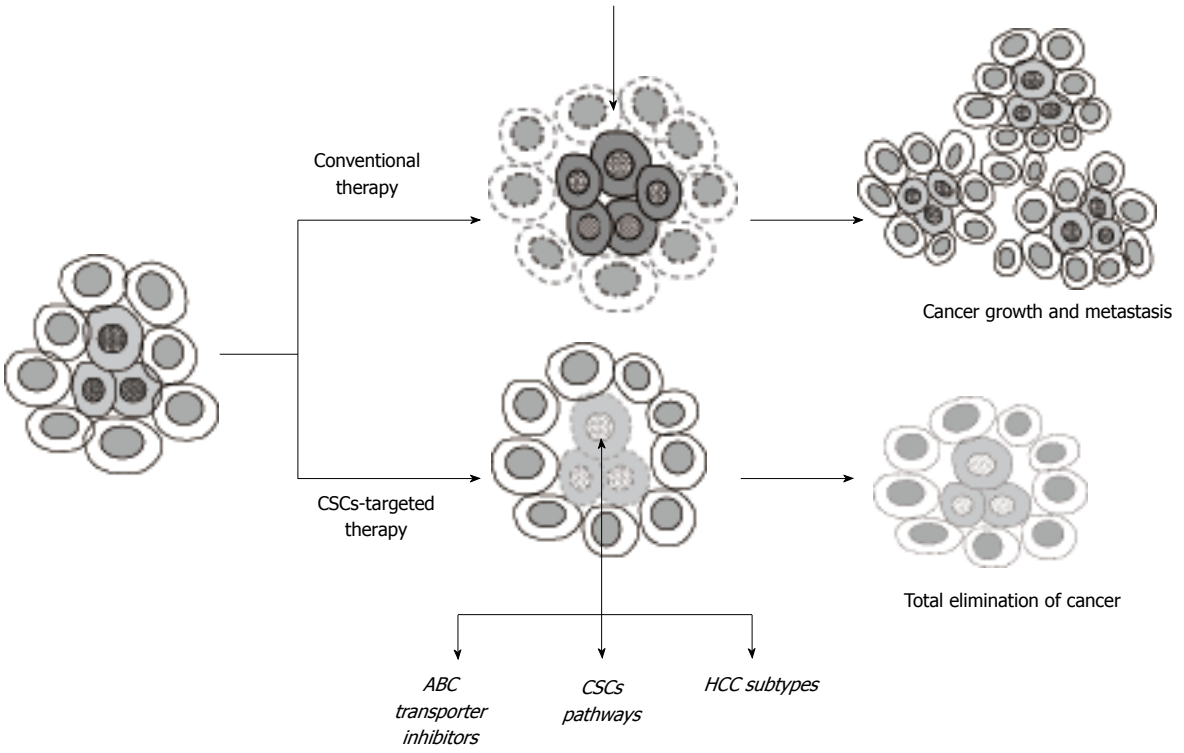

Figure 2 The CSCs-targeted therapy strategy. Conventional therapies affecting mainly the differentiated cells might not be sufficient to eradicate total tumor. In the CSCs-targeted therapy, chemotherapeutic agents is specially designed to target the CSCs. This strategy may primary block the main source and consistently inhibit the growth of tumor. Some factors such as CSCs pathways, tumor subtypes and drugs transporters inhibitions should be considered to increase the efficiency and safety of the treatment.

block ABCB1, ABCC1 and ABCG2 in HCC, including the use of modulators and monoclonal antibodies, as listed in Table 2. Combination therapies between modulators and antibody against transporter proteins have also been explored. For example, the synergistic effect of bromocriptine and tumor necrosis factor-alpha reversed the cancer growth in nude mouse ABCB1 model of liver neoplasm[88]. Drug delivery system is also becoming a subject of interest. The use of liposome-encapsulated drugs administered through the hepatic artery[89] and adenoviral delivery of ABC transporters nucleic acid constructs are proposed to be an efficient and safe system[90,91].

Nevertheless, serious cell toxicity of the inhibitors requires careful consideration due to drug sensitization and accumulation[92]. Combination regimens therapy containing optimum concentration of anticancer drugs and inhibitors with a better targeting system will be useful for the success of the therapy.

SIGNALING PATHWAYS

Another way to approach potential HCC treatment with minimal systemic toxicity is to target the essential CSCs pathways. Several signals pathways coordinate together in the development and differentiation of stem cells in a complex network which has not been fully described. New therapeutic strategies targeting signaling pathways which are involved in the self renewal of CSCs and block differentiated cancer cells have been suggested[93]. These therapies aim to modulate important steps of the networks such as growth factors, growth factor receptors or kinase involved in the cell cycle, cellular survival, angiogenesis, and metastasis.

The transforming growth factor beta (TGF-β) family proteins are responsible for controlling cell proliferation, differentiation and other functions. These proteins are involved in regulating the biology of embryonic stem cells and tumor suppression and help the selection of cell fate and the progression of differentiation[94]. However, a recent study showed that TGF-β signaling network is a dynamic process in which different signals function in parallel to induce different early genes[95]. Moreover, the role of TGF-β in early events of differentiation depended on the cell types. The bone morphogenetic protein (BMP), a member of TGF-β family, induced differentiation of mesenchymal cells into chondroblast or osteoblast phenotypes *in vitro*. The activin/TGF-β provided competence for chondroblast differentiation at early stages, while TGF-β inhibited osteoblast maturation at late stages in the differentiation pathway[96]. In fetal murine hepatoblasts, the TGF-β signaling pathway members were significantly up-regulated during ductular differentiation *in vitro* but not during hepatocyte differentiation[97]. The inhibition of activin/TGF-β signaling by the Onecut transcription factors HNF-6 and OC-2 allowed normal hepatocyte differentiation[98].

Cell fate decisions in the liver were suggested to be also

related with the role of microRNAs, in which miR-23b clusters miRNAs repressed bile duct gene expression by down-regulating Smads[99]. Interestingly, TGF-β treatment induced dedifferentiation of fetal rat hepatocytes to liver stem cell-like phenotype, suggesting that TGF-β might play an essential role in the transdifferentiation process[100].

TGF-β family members may also have implications in the maintenance of somatic stem cells and cancer stem cells. During carcinogenesis, the TGF-β signaling play important roles in inducing EMT by up-regulating the expression of Snail transcription factor family members[101]. A recent study demonstrated that TGF-β pathway was deregulated in human HCC. Both normal tissues and HCC specimens contained progenitor/stem cells which express signal transducer and activator of transcription 3 (STAT3), ornithine carbamoyl-transferase 4 (OCT4) and NANOG. The signaling proteins TGFBR2 (TGF-β receptor 2) and embryonic liver fodrin (ELF) which were prominently found in the normal tissues, were absent in HCC tissues suggesting that the change in TGF-β pathway may induce HCC trough interruption of differentiation by hepatic progenitor/stem cells. STAT3/OCT4 stem cells with disrupted TGF-β signaling were likely cancer progenitor cells and modulation on stem cell renewal factor may reduce tumor construction[102].

A functional link between IL-6, a major stem cell signaling pathway and TGF-β pathway has been revealed. Gene expression analysis of HCC in $ELF^{+/-}$ mice showed that HCC could arise from an IL-6-driven transformed stem cell with inactivated TGF-β signaling[102,103]. Additionally, the absence of inter-α-trypsin inhibitor-4 (ITIH4), an IL-6 target and a biomarker of foregut cancer, appeared to decrease the expression of IL-6/STAT3. The tumor size of $ELF^{+/-}$/$ITIH4^{-/-}$ mouse was smaller than $ELF^{-/-}$ mouse[102].

The Hedgehog (HH) signaling pathway is one of the key controllers in cell development. HH pathway is most active during embryogenesis and may be involved in the regulation of adult stem cells, mainly in maintenance and self renewal[104]. The disregulation in HH pathway has been proposed to be a component in stem cell activation in cancers and therefore represents an attractive agent for cancer therapy[105]. Cyclopamine, a steroid-like compound against smoothened (SMO) in the HH pathway, was found to significantly down regulate the SHH, SUFU, PTCH, GLI2 and GLI3 on prostate cancer cells DU-145[106]. In HCC, HH pathway was over expressed in cancer tissues compared with non-cancer tissues and linked with histological differentiation and portal venous invasion. Cyclopamine was reported to block HH signaling pathway also in HCC[107,108] by inducing the reduction of DNA synthesis and inhibiting cell growth, thus causing a significant reduction in HCC invasiveness and motility of HCC cells[107]. The new inhibitor HhAntag691 (GDC-0449) has entered clinical trials for a variety of solid tumors. This molecule inhibits both HH signaling and ABC transporters ABCG2 and ABCB1[109,110]. The SHH neutralizing antibodies were reported to decrease the expression of HH target genes, inhibit cell growth and result in apoptosis[108].

The Wnt signaling pathway consists of a large network of proteins involved in embryogenesis and cancer. The Wnt proteins were assumed to act as stem cells growth factor and maintain the proliferation of stem cells[111]. The Wnt signaling may crosstalk with TGF-β signaling and regulate the mesenchymal stem cells proliferation[101].

Active Wnt/β-catenin signaling pathway was shown to occur preferentially in liver progenitor cells and to be closely related with drug resistance[112]. An activation of Akt signaling and impaired expression of phosphatase and tensin homolog (PTEN) has been reported in about 40% of human HCC[113]. AKT1 inhibitor treatment to $CD133^{+}$ HCC cells significantly reduced the expression of survival protein[34]. A study from the SP population demonstrated that the Akt signaling inhibition attenuated the drug efflux and increased drug efficacy[84].

PTEN is one of the most frequently mutated tumor suppressors in cancer[114]. Decreased PTEN expression was correlated with HCC progression, high AFP levels, p53 over expression and poor prognosis[113]. Chemoresistance to interferon-alpha/5-FU combination therapy for HCC was induced by Wnt/β-catenin signaling pathway[115]. The PTEN-Akt pathway activated stem cells by helping control nuclear localization of the Wnt/β-catenin[116]. Data obtained from gene expression analysis showed that the activation of Wnt/β-catenin pathway led to enrichment of the proposed progenitor cells $EpCAM^{+}$ population. The RNA interference-based blockage of EpCAM, Wnt/β-catenin target attenuated the activities of the cells[47,117].

Epidermal growth factor (EGF) is a single strand polypeptide involved in regulation of a wide variety of physiological and pathological processes including embryogenesis, growth, tissue repair, regeneration and neoplasia. EGF works by binding with the EGF receptor (EGFR). Microarray analysis on liver-specific non-mutated β-catenin over expressing transgenic mice demonstrated increase levels of activated EGFR and Stat3. The EGFR inhibition decreased liver size and seemed to be a direct target of the pathway[118]. EGFR was found to be over expressed in HCC and associated with tumor aggressiveness and poor prognosis[119].

Several agents targeting EGFR are currently under development. In phase Ⅱ clinical trials, erlotinib, an oral receptor tyrosine kinase inhibitor specific for the EGFR/HER1, has been evaluated and is reported to give progression-free survival in advanced HCC with median overall survival 10 to 13 mo[120,121]. In experimental models with mouse and human HCC cells, gefitinib, another EGFR inhibitor for lung cancer treatment, inhibited the growth of HCC and its combination with cisplatin enhanced inhibitory effect[122,123]. In the cirrhotic rat model, the number of HCC nodules was reduced after gefitinib administration and EGFR was activated lower in the diseased and tumoral tissues[124]. Cetuximab, a chimeric monoclonal antibody against EGFR, is also under inve-

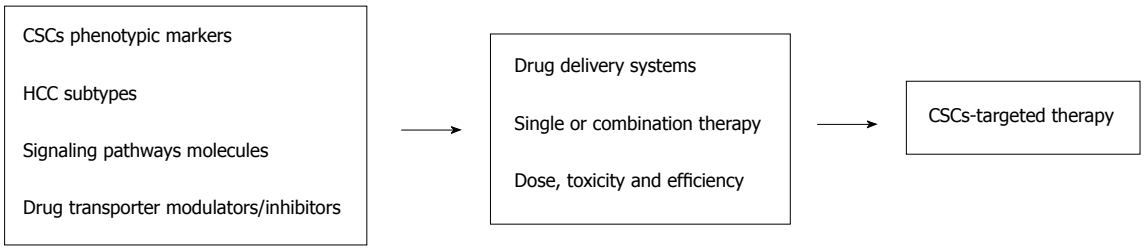

Figure 3 A collaborative approach to target the CSCs. The hepatic CSCs identifications and their functional significances, including multidrugs resistance behavior and aberrant signaling pathways should be clearly identified. Together with CSCs markers, clinical aspects such as drug delivery system, single or combination therapy, drug dose and toxicity will support the potential of therapy. Both biological and clinical considerations will be potent means to improve the safety and efficiency of CSCs-targeted therapy.

stigation. A phase Ⅱ clinical study of cetuximab in advanced HCC demonstrated that although cetuximab was tolerable in terms of toxicity, it had no antitumor activity[125]. However, even though cetuximab seemed to have no effect as single agent, combination of cetuximab and gemcitabine plus oxaliplatin in advanced stages HCC patients appeared to be active and to have a manageable toxicity[126].

Transforming growth factor-α (TGF-α), one of the most important ligands for EGFR, was commonly over-expressed in HCC[127]. In HCC, serum TGF-α levels were found to be closely related to severity of liver dysfunction, and hepatic expression of TGF-α and EGFR correlated with proliferation of normal and neoplastic hepatocytes[128]. A recent study reported that TGF-α interacted with MYC oncogene. The expression of MYC and TGF-α in liver progenitor cells resulted in enhanced cell proliferation in culture and the generation of poorly differentiated tumors after inoculation into nude mice. However, further study using the apoptosis-deficient mutants T58A and S71F showed that T58A allele had an increased ability to interact with TGF-α in promoting cell proliferation and tumorigenesis, while the interactions between S71F allele and the TGF-α had opposite effects[129].

Current study is also focused on the insulin growth factor (IGF) which is involved in the hepatogenesis. Alteration of IGF and its receptor was associated with tumor stages, reduced survival, development of metastasis and dedifferentiation[130]. A study on human hepatoma cell lines showed that the IGF2/IGF1-R activation triggered proliferative and survival signals through EGFR-dependent and -independent mechanisms. The IGF2/IGF-1R survival pathway may contribute to gefitinib resistance in these cells[131]. Currently, monoclonal antibody inhibits IGF1-R is under clinical phase Ⅱ study for HCC[132-134].

The Notch signaling pathway is involved in the development of many organs and has important role in keeping the balance of cell proliferation, differentiation and apoptosis. The activation of Notch signaling in mouse hepatoblast resulted in inhibition of hepatic differentiation and induction of several cholangiocytic characteristics, suggesting that Notch signaling plays a key role in the differentiation of hepatoblast[135].

Alteration disturbed Notch might induce tumorigenesis and changes in the expression of Notch receptors were found in many malignant tumors including HCC. In pancreatic cancer, antitumor drug TW-37, a small-molecule inhibitor of Bcl-2 family proteins, inhibited cell growth and induced apoptosis. It has been suggested that the activity of TW-37 was mediated through a novel pathway involving inactivation of Notch-1 and Jagged-1[136]. In adult human liver, the expression and localization of Notch receptors has been observed to be altered during liver damage[137]. The over-expression of Notch1 using cDNA encoding its constitutively active form was able to inhibit the growth of HCC cells *in vitro* and *in vivo*[138].

The angiogenesis pathway also has become an effective target of current pharmacologic strategies[139]. The vascular endothelial growth factor (VEGF) expression was closely related with vascularity of HCC compared with a non-cancer specimen[140] and associated with the invasion and metastasis[141]. Several VEGF signaling inhibitors might be promising therapeutic agents for HCC. Sorafenib, a multi-tyrosine kinase inhibitor including VEGFR-2 and VEGFR-3 targeting, was demonstrated to prolong median survival and time to progression by nearly 3 months in patients with advanced HCC in a large phase Ⅲ trial[142]. Another inhibitor on a phase Ⅱ clinical trial, sunitinib, has demonstrated tolerability and efficacy in patients with advanced HCC[143]. Bevacizumab, a recombinant monoclonal antibody against VEGF has been used as single or combination therapy agent[144,145]. Combination of bevacizumab and erlotinib in advanced HCC patients showed significant anti-tumor activity[145]. Still, further evaluation is needed to avoid the negative side effects of the agents.

CONCLUSION

The existence of CSCs in HCC has been supported by a growing body of evidence from basic and clinical research. However, until now the CSCs characteristics in HCC are still unsettled and CSCs signaling pathways network is not fully described. More information of CSCs uniqueness and activation would be one of the main keys in understanding the initiation and development of cancer. Furthermore, to achieve a better strategy for a

total elimination of HCC, several biological and clinical aspects should be considered for an effective CSCs-targeted therapy (Figure 3). First, characterization and identification of the CSCs phenotypes which distinguish them from normal stem cells will be important. Specific CSCs-targeted therapies which recognize only CSCs and not normal stem cells will greatly increase the efficiency while avoiding the 'wrong' target. Therefore, further investigations on signaling pathways involved in CSCs-induced tumor will be a potent means in finding the best target of therapies. Second, understanding of the biological properties of CSCs that makes them resistant to treatments will help to decrease drug resistance and increase drug sensitivity. Application of ABC transporters inhibitors and combination therapies of drugs and inhibitors may enhance treatment efficacy and at the same time decrease drug toxicity. Third, drug design and administration to obtain a correct delivery target in the diseased tissue will greatly improve toxic effect where needed and remove toxicity where this is harmful. And fourth, the analysis of HCC prognostic subtypes might form a basis to decide a better personalized approach to the patients. Combining all data together, more studies on HCC and hepatic CSCs are needed to have a better view of the mechanism underlying HCC and to find potent novel molecular therapies in the future.

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