Mitotic quiescence in hepatic cancer stem cells: An incognito mode

Kandasamy Ashokachakkaravarthy, Biju Pottakkat

Department of Surgical Gastroenterology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India

Abstract

Hepatocellular carcinoma represents one of the most aggressive cancers with high recurrence rates. The high recurrence is a major problem in the management of this disease. Cancer stem cells (CSCs) are often regarded as the basis of cancer recurrence. The anti-proliferative therapy kills the proliferating cells but induces mitotic quiescence in CSCs which remain as residual dormant CSCs. Later on, withdrawal of treatment reactivates the residual CSCs from dormancy to produce new cancer cells. The proliferation of these newly formed cancer cells initiates new tumor formation in the liver leading to tumor recurrence. HCC cells evade the immune surveillance via modulating the key immune cells by alpha fetoprotein (AFP) secreted from CSCs or hepatic progenitor cells. This AFP mediated immune evasion assists in establishing new tumors by cancer cells in the liver. In this review, we will summarise the CSC mechanisms of recurrence, mitotic quiescence, dormancy and reactivation of CSCs, metastasis and immune evasion of hepatocellular carcinoma.

Introduction

Hepatocellular carcinoma (HCC) accounts for 90% of the primary liver cancers and remains the sixth most common cancer globally. Among cancer related deaths, HCC represents a leading cause worldwide. Specific risk factors for HCC include hepatitis B virus (HBV), hepatitis C virus (HCV), alcoholism, hemochromatosis, aflatoxin, diabetes mellitus, smoking, obesity and non-alcoholic fatty liver disease (NAFLD). For early stage HCC, a partial hepatectomy is considered for curative resection in patients with preserved liver function. For intermediate stage HCC, transarterial embolization (TAE) (embolic particles without chemotherapeutic agents) or transarterial chemoembolization (TACE) (embolic particles with chemotherapeutic agents) offers advantage of tumor necrosis by selective cannulation of tumor feeding arteries with vaso-occlusive particles and/or chemotherapeutic agents. For advanced stage HCC, a systemic therapy of sorafenib, a multit kinase inhibitor remains the most common treatment. In patients who fail sorafenib therapy, cytotoxic chemotherapy, immunotherapy and antiviral therapy are being considered as second line treatment depending on the etiology and nature of disease. Recurrence after curative treatment remains a major obstacle in the management of HCC. Recurrences after HCC curative treatment are common even in early stage HCC where the 1 year, 3 year and 5 year recurrence rates were approximately 20%, 50% and 75% respectively. Overall survival after treatment remains poor in HCC due to increased recurrence rates. Accumulating evidence shows that cancer stem cells (CSCs) are responsible for HCC recurrence. In HCC, a distinct subpopulation of cells called CSCs or tumor initiating cells (TICs) shows properties of stemness such as self-renewal and proliferation. A growing body of evidence shows that critical biological processes of tumors such as invasion, metastasis and therapeutic resistance towards chemotherapy and radiotherapy are largely determined by CSC population in a tumor.

Correspondence: Biju Pottakkat, Department of Surgical Gastroenterology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, 605006, India. E-mail: bijupottakkat@gmail.com

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Existence of cancer stem cells and revival of stemness

CSC theory hypothesises that a distinct subset of cancer cells called CSCs give rise to its descendants, which are more differentiated and are commonly known as cancer cells. It also states that CSCs govern the growth and spread of tumors. The progenitor cells and cancer cells which are derived from CSCs have limited ability to undergo mitotic division and are predestined to stop proliferation at a time point. This form of cellular hierarchy exists in normal tissues as well, where the normal stem cells (NSCs) produce progenitor cells which gives rise to mature cells. This cellular hierarchy in cancer was first evidenced by Pierce and Wallace in 1971. They demonstrated that undifferentiated malignant cells give rise to the genesis of benign differentiated cells. Biologically, CSCs exist as a malignant equivalent of the NSCs and exhibit stemness to sustain cancer propagation, immune evasion and to interact with its environment for survival factors. Stemness is the ability to self-renew and give rise to differentiated cells by perpetuation of lineage in order to maintain the balance between quiescence, proliferation and regeneration.
evidences show that progenitor cells in many cancers acquire the ability of self-renewal which give rise to CSCs. This process of transformation of progenitor cells back to its predecessor stem cell state is called dedifferentiation.16-19

Dedifferentiation might be induced by various factors in biological systems. In 2006, two researchers from Japan named Takahashi and Yamanaka introduced a concept called induced pluripotency by using dedifferentiation as the principle behind this biological singularity. They demonstrated the transformation of adult cells into induced pluripotent stem cells (iPSCs) by introducing four molecular factors called Oct3/4, Sox2, c-Myc and Klf4 into the cells.20 The factors used to create iPSCs are called Yamanaka factors (Oct3/4, Sox2, c-Myc and Klf4), named after the researcher Yamanaka. The Yamanaka factors were critically involved in the regulation of developmental molecular signalling network which controls pluripotency and stemness.21-23 Recently researchers found that radiofrequency ablation (RFA) could induce dedifferentiation in human HCC tissues by heat shock response and also observed that rapid rise of temperature induced heat stress leads to phosphorylation of tyrosine and activation of protein kinase FA/Glycogen Synthase Kinase 3 (GSK) which are further involved in the neoplastic transformation and tumorigenesis.24,25

Identification of hepatic cancer stem cells

The concept of CSCs was proposed in the 1970s, but it gained the first experimental support in 1994, by an animal experiment where a particular subset of cells (CD34+/CD38-) caused xenograft tumors when inoculated to severe combined immunodeficient (SCID) mice but the other subset of cells was unable to create any tumors. This raised the notion that only cells having tumorigenic potential could develop new tumors. By the subsequent years of research, the first evidence for CSCs was documented in 1997 by John Dick, the pioneer in CSC biology who identified CSCs from acute myeloid leukemia (AML).26,27 Since then, the CSC research has been accelerated in pursuit of evidence for the existence and identification of CSCs in various cancers including HCC. The properties of CSCs, including tumorigenicity, pluripotency, functional characters and immunogenicity in hosts have been investigated since the identification of CSCs in various cancers.28 CD133 or prominin-1 was first identified as a marker of hematopoietic stem cell (HSC), which was then recognized as the first CSC marker of liver cancer in 2007 and the CSCs identified from liver were termed as hepatic cancer stem cells (HCSCs) or liver cancer stem cells (LCSCs).29,30 The discovery of HCSCs led to a drastic change in the etiological perspective of cancer pathophysiology. In the past few years of CSC research, it is reported that HCSCs possess few distinguishing markers such as surface markers (CD133, CD90, EpCAM, CD44, CD47, CD24, CD15, CD13), nuclear markers (Oct4, Sox2 and Nanog), enzymatic markers (ALDH1A1, PLSCR1) and cytoplasmic markers (CK19, OV6).31-33 A wide range of research on CSCs in HCC reported that HCSCs are responsible for initiation, progression, therapy resistance, the development of intrahepatic metastases, distant organ metastases, tumor relapse and recurrences in HCC.34-38

Cancer stem cells mechanism of recurrence

As stated earlier, HCC has a post-resection recurrence rate as high as 70% at 5 years which affects the overall survival of patients.4,39,40 The basis of this high recurrence rate was a mystery over the past few decades of research until researchers attempted to clarify the influence of surgical resection margins on post-operative recurrence. On the basis of a suspicion that cancer cells may remain in the peri-tumoral tissues of liver even after surgical removal of tumors, they wanted to investigate the effect of various surgical resection margins on recurrence free survival (RFS). As a result, they found that increasing the resection margin could actually decrease the recurrence and increase survival. But this could help only for patients having tumors of small size, single node and absence of macrovascular invasion.41,42 These results repeatedly raised a suspicion that surgical resection might not represent the exclusive factor which influencing the recurrence rates of HCC. Concurrently, various studies reported the presence of CSCs in HCC and the HCSC markers correlated with early recurrence of HCC. It is also found that HCSC-specific gene signature is associated with recurrence of HCC. Finally, experimental HCC also demonstrated the tumorigenic potential of HCSCs in SCID mice in which HCSCs produced new tumors suggesting that HCSCs play a critical role in initiating new tumors as well as recurrence of HCC.43-45 To explore the role of HCSCs in tumor recurrence, investigators created orthotopic HCC mouse models by inoculating human alpha-feto protein (hAFP) expressing HCC cells into immunodeficient mice. These mice were then treated with metronomic cyclophosphamide which caused complete tumor regression and increased the survival significantly but a fraction of circulating hAFP was found even after completion of cyclophosphamide treatment. This observation suggested that a proportion of residual tumor cells capable of producing hAFP may still remain in liver. Immunohistochemical analysis revealed that these residual cancer cells possess CD13 and hAFP but did not express proliferating cell nuclear antigen (PCNA) confirming that these are dormant CSCs with suspended proliferation. Finally, tumor was regrown in mice after withdrawal of treatment.46 This experiment demonstrates that residual dormant CSCs revive its proliferative and tumorigenic nature, initiating tumor recurrence after discontinuation of treatment.

Tumor dormancy, mitotic quiescence and reversibility of G0

The concept of tumor dormancy was first introduced by Hadfield in 1954, who described dormancy as a state of temporary mitotic and growth arrest. Dormant cancer cells are the malignant cells having potential to proliferate but show no sign of multiplication.47 It has been defined that cancer cell dormancy is a state of quiescence exhibited by cancer cells with no constant growth as well as absence of apoptotic and proliferative markers.48 But these attributes of quiescence are also exhibited by few other adult cells such as skeletal muscle cells, adipocytes, cardiomyocytes and neurons which exits the cell cycle and enters G0 phase to irreversibly suspend proliferation. Although, these cells may undergo physiological senescence by stimulation of growth promoting pathways which enforces hypertrophy and hyperactivity but they fail to reenter the cell cycle and replicate.49 These cells were considered to be in a state of senescence where the proliferative potential is lost. Senescence is a physiological state of cell cycle arrest but not growth arrest.50 In contrast to this, Dormancy is a reversible quiescent state in which the cells undergo both cell cycle arrest and growth arrest where there is no evidence of hyperplasia or hyper trophy.

In the early postulates of cell cycle, it was first generalized that G0 is an irreversible, inactive and non-cycling state by which cells undergo senescence. Later it was rediscovered by an experiment...
which demonstrated that a temporary serum starvation could enforce the cells to undergo a reversible G0 phase called Quiescence. Since then, senescence was distinguished from quiescence which is a non-dividing state of the cell, achieved by reversible arrest of cell cycle, growth and proliferation. The reversibility of G0 and mitotic arrest marks the quiescence more advantageous than senescence for a cell. In consequence, the dormant cancer cells exist in a state of mitotic quiescence where there is no sign of proliferation or growth. Among various distinguishing properties of CSCs, quiescence exists potentially as a regulatory trait for the maintenance of stemness which is one of the regulatory traits of NSCs.

Hibernating HCSCs

Among CSCs, a subset of cells remains dormant and exist in a quiescent state. These are called hibernating CSCs. Dormant tumors contain a few quiescent CSCs that can be identified by markers such as CD13 and lack of expression of PCNA. CD13 is also known as aminopeptidase N, which is Zn(2+) dependent enzyme. It is a membrane bound ectopeptidase which catalyses the degradation of N-terminal neutral amino acid containing proteins and peptides. Recently, the hibernating HCSCs such as quiescent and dormant states of HCSCs were documented by a human study which analyzed the existence of quiescent HCSCs in human HCC tissue samples by analyzing the expression of CD13. The authors found a high expression of CD13 in HCC tissues, significantly correlating with overall poor survival. Similar results were reported by other studies on pancreatic and non-small cell lung cancers in which the high expression and serum levels of CD13 are strongly associated with poor survival.

As said earlier, serum starvation lets the cell to enter into quiescent state. This observation is further supported by an experiment demonstrating the effect of ischemia on HCC. Gade et al., demonstrated that over 79% of HCC cells survived under severe ischemia via undergoing a quiescent state by means of residing in G0/G1 phase of cell cycle after severe ischemic stress. These cells died after treatment with autophagy inhibitor, which confirmed that ischemia causes quiescence and autophagy dependence in HCC. To evaluate the antiproliferative effect, Carr et al., examined the effect of regorafenib (fluoro-sorafenib) on HCC cells. They found that more than 20% of detected DTCs were CSCs and the results of an animal experiment on DTCs and CSCs in which it was demonstrated that a temporary serum starvation could enforce the cells to undergo a reversible G0 phase called Quiescence. Since then, senescence was distinguished from quiescence which is a non-dividing state of the cell, achieved by reversible arrest of cell cycle, growth and proliferation. The reversibility of G0 and mitotic arrest marks the quiescence more advantageous than senescence for a cell. In consequence, the dormant cancer cells exist in a state of mitotic quiescence where there is no sign of proliferation or growth. Among various distinguishing properties of CSCs, quiescence exists potentially as a regulatory trait for the maintenance of stemness which is one of the regulatory traits of NSCs.

Homing DTCs acquire dormancy and stemness

To detect the source of recurrence, an animal study was conducted in which HCC was induced in male rats by diethylnitrosamine (DEN). These male rat HCC livers were then replaced by the livers of healthy female rats by orthotopic liver transplantation. Subsequently, after recurrence, the transplanted female rat livers were analyzed for Y chromosome using in situ hybridization technique. By detection of Y chromosome from DTCs, they found that DTCs were present in 65% of HCC patients and these patients had high levels of preoperative AFP. Furthermore, DTC positive HCC patients were positive for portal vein invasion and had distant metastases. The distant metastasis-free survival was more in DTC negative HCC but there was no significant difference in liver specific recurrence free survival and overall survival between DTC negative and DTC positive HCC, which indicates that DTC positive patients either have intrahepatic metastases or distant organ metastases leading to recurrence and poor survival.

Acquisition of dormancy in residual cancer cells

The mechanisms of DTC acquisition of dormancy and stemness have been proposed previously. DTCs were reported to acquire dormancy after intra-organ metastasis or distant organ metastasis. In addition, they initiate intrinsic dormant programs and constrains self-renewal to sustain their survival. It is reported that growth arrest-specific-6 (GAS6) regulates the transi-
tion between residual DTCs into CSCs. Emerging evidence shows that microenvironment niche-derive GAS6 regulates a crucial role in the process of homing, survival and conversion of DTCs to CSCs. AXL, the receptor of GAS6 was reported to be implicated in HCC progression and metastasis. A study found that high expression of AXL in human HCC tissues correlate with high recurrence rates in HCC and the GAS6/AXL axis plays significant role in the tumor recurrence. It is also shown that GAS6/AXL axis not only involved in cancer progression and metastasis, but is also associated with recurrence and treatment resistance.

Emerging evidences support the notion that homing DTCs deploy dormancy as a survival mechanism during treatment by inhibiting proliferation and self-renewal. Most clinical evidence show that DTCs are in either non-proliferative or slow cycling state which indicate that mitotic arrest is an essential biological event of DTCs which supports in the acquisition of dormancy. These findings collectively suggest that DTCs metastasize into different regions by homing and transform into residual CSCs and go into a dormant state which later might be reactivated to initiate tumor formation and recurrence. There are other mechanisms by which residual cancer cells acquire dormancy such as drug induced dormancy. Studies have shown that MYC oncogene inactivation induces dormancy in cancer cells. The consensus of MYC oncogene reveals that tumor cells remain dormant as long as MYC remains inactivated. It is a well-known fact that sorafenib inhibits proliferation by inhibition of MEK/ERK pathway, but the consequence of MEK/ERK on MYC oncogene remained unknown for a long period. Recent studies revealed that sorafenib as well as foretinib inactivate MYC via inhibition of the MEK/ERK pathway. To verify that MYC inactivation could help in effective treatment of liver cancer, MYC transgenic mice with liver cancer were treated with doxycycline. Mice treated with doxycycline did not show any expression of MYC oncogene after treatment and became free from disease. Inactivation of MYC blocked the proliferative ability of cancer cells. Previous studies show that sorafenib induces tumor regression in HCC patients but not complete eradication of residual cancer cells and a significant number of HCCs are refractory to sorafenib treatment. This suggests that HCC cells acquire drug induced dormancy which may be a consequence of anti-proliferative treatment. These observations provide a possible explanation for the link between anti-proliferative therapy and drug induced dormancy. Drugs that inhibit proliferation via MEK/ERK inhibition cause MYC inactivation which induce dormancy in residual cancer cells which survived the drug effect.

According to a previous study which investigated the effect of metronomic therapy on tumor recurrence in orthotopic HCC mouse model, the residual dormant CSCs formed new tumor outgrowth after withdrawal of treatment. Initially they implanted hAFP producing human HCC cells (TIL) into immunodeficient mice to form new HCC tumors. Serum hAFP showed significant correlation with tumor size. Cyclophosphamide administration induced regression of tumor mass completely but a residual hAFP were detected in serum, suggesting that there could be some residual hAFP producing cancer cells remaining in liver. This was verified using immunohistochemical analysis which showed that those cells were positive for hAFP and CD13 but negative for PCNA. Surprisingly, tumors were regrown after discontinuation of treatment following complete tumor regression. This observation confirms that hAFP+/CD13+/PCNA- cells residing after cyclophosphamide treatment are dormant CSCs. Moreover, these findings suggest that drug induced tumor regression causes dormancy in residual CSCs but do not affect the tumorigenic potential of these cells which is the basis of new tumor formation after treatment withdrawal.

**Revoking the dormancy to normalcy and resurrection of CSCs in HCC**

CSCs remain dormant until the microenvironment becomes favorable for proliferation, and it can be reactivated to active state by any growth stimulus leading to new tumor formation commonly known as tumor recurrence. These dormant cells are the residual cancer cells which failed to respond to conventional anti-proliferative and antiangiogenic therapies. Recently it was found that, quiescent CSCs undergoes epithelial to mesenchymal transition (EMT) under the influence of interleukin-17 (IL-17) which transforms the quiescent CSCs into invasive phenotypes leading to acquisition of metastatic characters in gastric cancer. But the central player in bringing back the dormant CSCs to normalcy is MYC oncogene. Dormancy and normalcy of CSCs primarily depend on inactivation and activation of MYC respectively which switch the cells between these two states. To analyze the effect of doxycycline on MYC inactivation and activation, two groups of MYC transgenic mice with liver cancer were treated with doxycycline. Complete tumor regression and MYC inactivation were observed during doxycycline treatment. Then, one group was discontinued for doxycycline treatment and another group continued the treatment. Doxycycline and MYC inhibition became free of disease but doxycycline discontinuation induced reactivation and high expression of MYC in the other group which led to high expression of AFP and CSC markers. This study concluded that MYC inactivation by doxycycline induces dormancy in HCCSCs and reactivation of MYC upon cessation of doxycycline treatment induced tumorigenic properties in these residual dormant CSCs.

The fact that MYC plays significant roles in organ, as well as embryonic development, has been well established and cannot be ignored and the involvement of MYC in the tumor biology was found to be significant. Accordingly, it is undeniable that programs found in stem cells and MYC regulated cellular programs in cancer cells are common. As defined earlier, quiescent cancer cells remain in dormant state with their preserved proliferative potential. Proliferative stimulus such as growth factors and cessation of anti-proliferative therapy play crucial roles in provoking the residual dormant CSCs to normalcy. Recovery from dormancy restores the inherent proliferative programs of CSCs forming new tumors which in turn cause recurrence.

**The origin of AFP: hepatic progenitors versus HCSCs**

AFP synthesis and secretion is one of the important features of HCC over other cancers, though few reported that some HCC patients had low AFP levels in serum. However, high AFP in HCC was significantly associated with post-operative complications and recurrence. Previous reports showed that 5-year survival of patients who underwent liver transplantation for HCC were 52.7% and 80.3% for patients with AFP levels >1000 ng/mL and ≤1000 ng/mL respectively. An elevated AFP level predicts poor prognosis of HCC after liver resection and transplantation. A serum AFP level more than 200 ng/mL has 3.32 fold more risk for recurrence than in patients with AFP levels less than 200 ng/mL. AFP levels were often correlated with the size of the tumors. A majority of small HCC less than 2 cm do not raise AFP levels. Studies also found a significant correlation between CSC marker CD133 and AFP in HCC as well as HCV infection. On the other hand, the levels of AFP were also found to be increased in chronic liver disease with hepatocellular damage associated with HCV infection. These observations demonstrate that AFP is not only involved in
HCC genesis, but primarily associated with regenerating hepatocytes. AFP is an oncofetal protein secreted by fetal liver and yolk sac. AFP synthesis declines after fetal development and birth. AFP is secreted in liver cancers due to the presence of undifferentiated hepatocytes.96,97 The well-known property of liver tissue to regenerate after partial hepatectomy is due to its ability to restore the loss of hepatocytes by rapid proliferation. This led to the notion that liver consists of undetermined stem cells which are responsible for rapid proliferation.97 Hepatocyte proliferation starts immediately within 48 hours of hepatectomy with simultaneous rise in AFP for about five days, which is often detected in liver cells undergoing mitosis during this regeneration process.98,99 Moreover, AFP is considered as a liver progenitor cell marker in fetal liver and it is also evident that proliferating liver cells re-express stem cell markers.97 Thus AFP secreting cells are undifferentiated hepatocytes which tend to exhibit a progenitor state mimicking the functional characters of embryonic stem cells as a consequence of dedifferentiation of mature hepatocytes. Previous studies reported that HCCs express markers of dedifferentiation including CD133 and AFP which correspond to poor prognosis with clinical implications. These are not specific HCC markers since they are expressed by hepatic progenitor cells as well as HCSCs.100 The proportionate increase in AFP corresponds to the regenerating hepatocytes either in an undifferentiated state or merely a consequence of ongoing dedifferentiation of hepatocytes back to its embryonic stem cell state.97,101 It is conceivable that initial liver response to damage by rapid proliferation leads to reappearance of undifferentiated hepatocytes which are the sources of AFP. On the other hand, the acquisition of stemness in differentiated hepatocytes plays a crucial role in the genesis of HCSCs, which in turn become the source of AFP.97,100

**AFP and immune deceptions**

The function of AFP is largely unknown, although it is hypothesized that it plays a transport role in serum, similar to albumin.102 Recent studies came up with evidences supporting the novel immunomodulatory functions of AFP which influence the proliferation and growth of tumor cells in HCC.103 Previously it was assumed that fetal AFP circulated in maternal circulation protects the fetus from rejection from the maternal immune system during embryo development.104,105 To support this notion, various studies emerged with evidences culminating that AFP acts as a detrimental molecule for immune cells. Tumor derived AFP is shown to induce apoptotic cell death in antigen presenting cells (APC) which leads to ineffective immune surveillance. In addition to impairment of APCs, it was also shown to suppress the proliferation of natural killer (NK) cells and T cells.106 Further studies showed that, tumor derived AFP is involved in impairment of dendritic cells (DCs) function thereby inhibits antigen presentation process to immune system. It was also shown to inhibit the differentiation of DCs.102 In vitro studies demonstrated that incubation of human peripheral blood DCs with AFP resulted in apoptosis via expression of p38-MAPK and caspase3 in DCs which further resulted in immune evasion of tumor cells.107 HCC immune surveillance system is mainly constituted by DCs, NK cells and T cells. It is confirmed that immune escape of HCC cells is merely a consequence of the effect on these three cells by AFP.108,109 Thus, AFP plays critical roles in decreasing immunity to overcome the immune responses mounted against the HCC cells. Similar to fetal AFP mechanism, hepatic progenitor cell or CSC derived AFP, tunes the immune system in favor of HCC development by modulating the biology of key immune cells such as DCs, NK cells and T cells.

**Conclusions**

CSC theory has gained significant attention since the discovery of induced pluripotency and CSCs. The HCSCs found in liver cancer possess stem cell features which makes them relatively imperishable. The high recurrence rates after treatment phase characterizes the HCC as an aggressive cancer type. These unsatisfactory outcomes of anticancer and antiproliferative therapies intensified the scrutiny on the field of HCC recurrence and HCSCs. Recent researches had evidenced that, stem cell nature protects and transforms the HCSCs to a quiescent state, making it dormant during therapeutic interventions. These mitotically quiescent cells remain in a biologically silent state of existence which makes them durable. Dormant HCSCs surviving the cancer therapies revokes back to its normal cellular state that brings up active proliferation and self-renewal which leads to cancer recurrence. Hence, we propose that, the functionally mutated state of mitotic quiescence is the basis of therapeatic resistance, tumor dormancy and cancer recurrence after therapy.

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