·Review·

Progress and prospects in cancer stem cell research for hepatocellular carcinoma

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[Abstract] The cancer stem cell (CSC) theory stipulates that it is a small population of cells called CSCs that initiates tumor formation and maintains its growth. CSCs are scarce within the bulk of the tumor mass, and possess stem cell-like properties such as self-renewal, differentiation and resistance to therapies, and so on. In the past few years, by using side population technique and approaches based on surface markers, including CD133, CD90, OV6 and EpCAM, researchers have identified and isolated a subpopulation of liver cancer cells with enhanced colony-forming and tumorigenic ability, which is strong evidence for the existence of liver CSCs. In this review, we summarized the progress of research on liver CSCs, discussed the significance of liver CSCs in the diagnosis and treatment of hepatocellular carcinomas, and put forward the future research directions as well as the challenges and opportunities.

Key words: hepatocellular carcinoma, cancer stem cells, stem cells

For the past a few years, with the introduction of the concept of stem cell into oncology as well as the isolation and identification of diverse cancer stem cells, the theory of cancer stem cells (CSCs) is formed gradually. [1, 2] Cancer cells are consisted of cells with different degrees of differentiation like normal tissues, among which a small population of stem cell like cells, e.g. the so-called "CSCs", have the potential of self-renewal, infinite proliferation and multi-directional differentiation. They are the initiator cells for tumor formation and help maintain continuous tumor growth. They are also called tumor-initiating cells (TICs), playing a pivotal role in the genesis, development, metastasis and recurrence of tumors. Most of other tumor cells only have relative proliferation ability and would ultimately die after limited proliferation and differentiation. The theory of CSCs not only provides a new idea for the study on genesis, development and metastasis of tumors, but only brings new hope for the clinical diagnosis and treatment of tumor. It is a completely new theory with epoch-making significance in the field of oncology. There have been long-term studies on the relationship between hepatocellular carcinoma (HCC) and stem cells. With the theory of CSCs, the study on liver cancer stem cells has become a hotspot, which has attracted the interest of

many investigators and achieved some noteworthy results.

Theory of CSCs

Proposing of the theory of CSCs. As early as 150 years ago, pathologists discovered some similarities between cancer and embryonic tissues, and thus hypothesized that cancer cells originates from embryo-like tissue. In 1960s, serial dilutions of mouse leukemic cells were implanted into mice of the same strain and only 1%-4% of the implanted tumor cells were found to be able to form intrasplenic clones.[3] Human acute myeloid leukaemia (AML) stem cells with the $CD34^{+}CD38^{-}$ phenotype, which were reported in 1994, was the first discovery of human malignant CSCs[4] and it was soon validated by other report[5]. In 2001, after reviewing studies on haemopoietic stem cells and tumors of the hematological system, Reya et al.[1] proposed the theory of CSCs, and presumed that CSCs are present not only in tumors of the hematological system, but also in numerous solid tumors.

Evidence on the presence of solid tumor stem cells. Currently, there have been extensive evidences supporting the presence of CSCs in solid tumors.[6] CSCs were first isolated from solid tumor in 2003. A1-Hajj et al[7] sorted $CD44^{+}CD24^{-/low}$ cells from breast cancer cells using flow cytometry. Only 200 cells with such a phenotype formed transplanted tumors in mice with non-obese diabetic/severe combined immunodeficiency (NOD/SCID) and could be serially passaged in vivo. The transplantated tumors formed at each time contained both $CD44^{+}CD24^{-/low}$ cells and tumor cells with other phenotypes, which were non-tumorigenic in the primary tumor. Almost at the same time, Singh et al.[8, 9] sorted $CD133^{+}$ tumor cells from a series of brain tumor tissues, which had similar growth features in vitro as normal nerve stem cells, were able to self-renew and proliferate, and differentiate into tumor cells with the similar phenotype with the primary tumor after induction in vitro. However, only 100 $CD133^{+}$ cells were required to form tumor in NOD/SCID mice and the phenotype of the tumor was as the same as the primary tumor. Soon after, CSCs were found successively in solid tumors including lung cancer,[10] prostatic cancer,[11] pancreatic cancer,[12] colon cancer,[13] and endometrial cancer,[14] strongly supporting the theory of CSCs.

Evidence on the presence of liver CSCs. The presence of liver cancer stem cells (LCSCs) has been indicated in some studies.[15-18] Like many other kinds of tumors, liver cancer is also generated and driven by subpopulation of hepatoma carcinoma cells that have features of stem cells, including self-renewal and differentiation potential.

Oval cells are a kind of hepatic stem/precursor cells that can differentiate into hepatocytes and bile duct cells. CC-62 cell line, which was established using mixed type HCC-cholangiocellular carcinoma tissues and expresses markers of oval cells, such as albumin, CK-7, CK-19, OV-6, can form tumors in nude mice,[19] It is the first attempt to identify liver cancer stem cells. Side population (SP) cells that sorted by flow cytometry are a population of cells that can pump Hoechst 33342 dye and thus are slightly stained due to the expression of membrane transport protein ABCG2 (adenosine triphosphate-binding cassette, sub-family G, member 2). Recently, it has been shown that SP cells of tumor cell lines and from primary tumor tissues have CSC like features. Chiba et al.[20] analyzed four kinds of human liver cancer cell lines and isolated SP cells from Huh7 and PLC/PRF/5. On the other hand, no SP cell was found in HepG2 or Huh6. It was found in immunohistochemical examination that most SP cells can express marker proteins of hepatocyes and bile duct cells at the same time. In vitro experiment showed that theses SP cells had potent clone formation ability and anti-apoptotic ability. In vivo experiment showed that only 1×10^{3} SP cells can form transplanted tumors in NOD/SCID mice, while 1×10^{6} non-SP cells can not form tumors. Analysis on the transplanted tumors found that SP cells can produce SP and non-SP cells, and only SP cells can have tumor formation ability. Gene chip

analysis found that several stem cell genes in SP cells were up-regulated compared with non-SP cells, which is very important to maintain the function and phenotype of stem cells. Based on the above results, the investigators believe that SP cells are human liver cancer stem cells. Shi et al.[21] also isolated SP cells from four kinds of liver cancer cells (HCCLM3, MHCC97-H, MHCC97-L, and Hep3B) and found that the percentage of SP cells in various liver cancer cells was positively related with the metastatic ability of the cell line itself. These SP cells all had self-renewal ability, high clone formation potential and significant anti-chemotherapy property in vitro. As a few as 2×10^3 SP cells can form tumors in NOD/SCID mice.

Although the SP method has been widely used in the identification of tumor stem cells, it has its own limitations for the following reasons: Hoechst 33342 has cytotoxicity and SP cells are protected because of its membrane transportation ability, while non-SP cells are not protected and thus can be damaged by cytotoxicity, leading to a failure in tumor formation. Therefore, the difference in tumor formation ability between SP cells and non-SP cells may be a false front that results from the cytotoxicity of Hoechst 33342, rather than the real difference in "stem cell property.[22] On the contrary, ABCG2 on the surface of SP cells can be considered as a marker of liver CSCs instead of SP cells.

CD133 is an accepted marker of hematopoietic stem cells and nerve stem cells and considered as the marker of brain and prostate CSCs.[6] Recently, liver CSCs with $CD133^+$ phenotype have been isolated and identified from human liver cancer cell lines by many study groups.[23-25] Ma et al.[25] found that $CD133^+$ liver caner cells had more potent clone formation and proliferation ability, compared with $CD133^-$ liver cancer cells. It was found in immunohistochemical examination that $CD133^+$ cells were present in human liver cancer specimens, paired non-tumor liver tissues and cirrhotic liver tissues in a small amount, while not in normal liver tissues. This result was consistent with previous reports that the percentage of tumor stem cells in the whole tumor tissue did not exceed 5% . In vivo experiment showed that 1×10^3 $CD133^+$ liver cancer cells were enough to form tumor in NOD/SCID mice. Most cells in transplanted tumor were $CD133^-$ cells, and $CD133^+$ cells were less than 1%, consistent with that in human liver cancer specimens. $CD133^+$ liver cancer cells can also be induced to differentiate into non-liver cells in vitro, while $CD133^-$ cells do not possess such differentiation ability. The results of gene analysis showed that $CD133^+$ liver cancer cells express a high level of genes related with self-renewal and proliferation of stem cells, such as β -catenin, Oct-3/4, Bmi, SMO and Notch-1, and so on, which are also involved in signaling pathways correlated with liver cancer. The clinical significance of the presence of $CD133^+$ liver cancer cells in tumor tissues of liver cancer patients has also been investigated preliminarily. Song et al.[26] found that the increase in the percentage of $CD133^+$ liver cancer cells was consistent with an increased level of serum alpha fetoprotein, relatively higher pathological grading and poor prognosis of patients with liver cancer. While another study[27] showed that the amount of $CD133^+$ liver caner cells was unrelated with the pathological grading of patients with liver cancer, suggesting that further evidences are needed to support the presence of $CD133^+$ liver cancer cells as a prognostic indicator for liver cancer.

Although $CD133^+$ has not been detected in many liver cancer cell lines,[23,25] its percentage is 65% in Huh7 liver caner cell line and as high as 90% in Hep3B.[25] Furthermore, $CD133^-$ liver cancer cells can also form tumors in immune deficient mice, although relatively more cells are needed.[25] According to the report of Ho et al.,[28] CD133 is also the marker of circulating endothelial progenitor cells (EPCs) in patients with liver cancer. Taken together, as a marker of liver CSCs, CD133 still has some problems in sensitivity and specificity. Using two-dimensional electrophoresis and mass spectrum technology, Ma et al.[29] identified aldehyde dehydrogenase (ALDH), which was specially expressed in $CD133^+$ liver cancer cells but not in $CD133^-$ liver cancer cells. In addition, $CD133^+$ liver cancer cell population had heterogenicity in that

ALDH was only expressed in a part of the cells and the tumor formation ability of $CD133^+ALDH^+$ liver cancer cells was significantly stronger than that of $CD133^+$ $ALDH^-$ cells, suggesting that $CD133^+ALDH^+$ is a more specific candidate phenotype of liver CSCs compared with $CD133^+$.

The study of Yang et al.[30, 31] indicated that CD90 may be another important candidate marker of liver CSCs. $CD90^+$ cells isolated from the human liver cancer cell line can form tumors in immune deficient mice, and the percentage of $CD90^+$ cells in various liver caner cell lines was positively correlated with the tumor formation and metastatic ability of these cell lines. $CD45^-CD90^-$ cells were isolated from all liver cancer specimens and blood samples of 91.6% liver cancer patients, and all of these cells can form tumor in immune deficient mice. The number of $CD45^-CD90^+$ cells in liver cancer tissues was positively related with the number of $CD45^-CD90^+$ cells in paired blood specimens. $CD90^+$ cells isolated from transplanted tumors can form tumors at the second or third time in immune deficient mice. Further investigation found that $CD90^+CD44^+$ cells were more malignant than $CD90^+CD44^-$ cells, since they can form tumors in situ in the liver of immune deficient mice and form pulmonary metastatic tumors. CD44 antibody can block the activity of CD44, induce in vitro apoptosis of $CD90^+$ cells, and inhibit tumor formation of $CD90^+$ cells in immune deficient mice. The above results suggest that CD90 is an ideal candidate marker of liver CSCs, and CD44 is a potential treatment target aiming at $CD90^+$ liver CSCs.

After successful attempt to identify and isolate liver CSCs, several investigation groups have extended their studies on the biological characteristics of liver cancer stem cells and related signaling pathways. Ma et al.[32] found that the drug resistance of $CD133^+$ liver cancer cells was realized by activating AKT/PKB and Bcl-2 survival signaling pathways, suggesting that blocking above pathways may increase the sensitivity of cancer cells to chemotherapy. Yamashita et al.[33-35] reported the role of the Wnt/β -catenin pathway in regulating the self-renewal ability of normal liver stem cells and liver CSCs. This investigation group believes that epithelial cell adhesion molecule ($EpCAM^+$) liver caner cells possess all characteristics of liver CSCs. EpCAM is the target gene of the Wnt/β -catenin pathway and blocking this pathway can inhibit $EpCAM^+$ liver cancer cells. In China, using OV6, a marker of liver precursor cells, Yang et al.[36] isolated $OV6^+$ cells from liver cancer cell lines and found that $OV6^+$ liver cancer cells had more potent tumor formation ability in vivo and anti-chemotherapy ability compared with $OV6^-$ liver cancer cells. In addition, they demonstrated that activation of the Wnt/β -catenin pathway plays an important role in the self-renewal and anti-chemotherapy ability of $OV6^+$ liver cancer cells. The above studies indicate that therapies that targeting at blocking the Wnt/β -catenin pathway may help to enhance the therapeutic effect for liver cancer. In addition, Tang et al.[37] reported that deactivation of the TGF-β pathway and/or activation of the IL-6 pathway may cause dysdifferentiation and hepatocarcinogenesis.

Significance of study on liver cancer stem cells

First of all, the theory of CSCs has updated our understanding on the genesis, development, metastasis and recurrence of liver cancer. The investigators have been trying to reveal the molecular and cellular mechanisms of tumorigenesis. With the uninterrupted progress in stem cell biology in the past 30 years and unceasingly deepening in research on tumor stem cells, we have gradually recognized that cancer may be a kind of stem cell disease. CSCs are the initiator cells of tumor formation and help maintain the growth of tumorS. Most cells in cancer can not self-renew and would ultimately die after limited proliferation. Therefore, spread of these cells would probably not lead to the metastasis and recurrence of tumors. On the contrary, CSCs are the root of metastasis and recurrence of tumors. in the past a few years, evidences on the presence of liver CSCs are discovered uninterruptedly, providing more reasons for the hypothesis that liver cancer may

be a kind of stem cell disease.

Secondly, the theory of tumor stem cells has greatly impacted the traditional treatment idea for liver cancer. The metastatic and recurrent rate of liver cancer after surgery is high. In addition, liver cancer cells are insensitive to radiotherapy and chemotherapy with poor long-term therapeutic effects. At present, all clinical treatments for cancer are targeting at all tumor cells and the evaluation on efficacy is generally based on regression or relief of tumor. According to the theory of CSCs, most cells in cancer only possess limited proliferation ability. Killing these cells can delay or relieve tumor progression, but the tumor could still recur if CSCs leading to tumor formation and maintaining tumor growth can not be killed. Therefore cancer treatment should target at those CSCs.[38] Similar to other CSCs, liver CSCs also express drug resistance related protein, such as ABCG2,[21] which are at the resting stage most of the time. Traditional chemotherapeutics mainly target at tumor cells at a rapid proliferative stage, therefore, they can not kill all tumor cells and recurrence can not be avoided. Accordingly, it is necessary to perform in-depth investigation on the biological features of liver CSCs and explore specific molecular markers and signaling pathways to provide ideas for the treatment targeting live CSCs, thereby changing the treatment of primary liver cancer radically.

The theory of CSCs also has great importance for making a diagnosis, judging prognosis and determining treatment regimen of liver cancer. Clinical study showed that disseminated breast cancer cells can be detected in the bone marrow in approximately 30% of breast cancer patients at clinical diagnosis and only 50% of these patients had clinical manifestation of osseous metastasis five years later, suggesting that quite a lot of patients with disseminated tumor cells being detected in the bone marrow did not form obvious osseous metastasis.[7] According to the theory of CSCs, a possible explanation is that there are no CSCs within the disseminated tumor cells in patients without osseous metastasis, since metastasis would happen only when CSCs are disseminated. It is supposed that similar phenomenon exists in liver cancer patients, which was preliminarily supported by the results of some studies.[23,24] Therefore, exploration of specific and sensitive markers for liver CSCs and developing corresponding diagnosis reagents for disseminated liver stem cells to detect the presence of disseminated liver CSCs in the blood and bone marrow of liver cancer patients can help predict metastasis and/or recurrence more accurately and establish more reasonable individualized treatment regimens.

Problems and study direction

In general, there is still little progress in the study on liver CSCs compared with studies on stem cells of other types of solid tumors. Our knowledge about liver cancer stem cells is only limited to a few literatures, we even have not obtained confirmatory results for the isolation and identification of liver CSCs. Evidences on the presence of liver CSCs are still questioned. Even reported results need to be validated by more studies. It is believed that it will take a long time to recognize liver CSCs.

Since there is still no accepted markers for liver CSCs at present, the problem to be resolved right now is to determine the specific marker for liver CSCs. There have been reports on considering SP, $CD133^+$, $CD90^+$, $EpCAM^+$ and $OV6^+$ as phenotypes of liver CSCs, and Huh7 cells at G_0 phase were even considered as liver CSCs.[39] However, all of these candidate phenotypes have their deficiency in specificity or sensitivity,[21,22,25,30] or need more experimental validations. Among the above candidate markers, only the crossover expressions of CD90 and CD133 were reported.[30] Yamashita et al.[35] found that there is no difference in the expression of CD133 between $EpCAM^+$ and $EpCAM^-$ Huh1 liver cancer cells. However, only $EpCAM^+$ cells have tumor formation ability and CD90 is only expressed in $EpCAM^-AFP^-$ liver cancer cell lines. Facing such a confusing phenomenon, a possible explanation[40] is that different markers may represent different differentiation phases of liver CSCs. Other similar point of view[35] indicate that

different activated signaling pathways in normal cells from which liver cancer stem cells are originated lead to expression of different markers in liver CSCs. These problems need to be further considered and investigated.

In addition, studies on liver CSCs in the future should resolve the following important problems: (1) Identifying the relationship between liver CSCs and liver stem cells. The study on liver stem cells started a long time ago. Currently, it is believed that liver is an organ without vigorous proliferation and differentiation, and liver stem cells would only be present when the liver is subjected to serious chronic injury. Now there are different points of view on the origin of liver CSCs. Obviously, identifying whether liver cancer stem cells originate from liver stem cells and identifying possible mechanisms of hepatocarcinogenesis that involving stem cells would no doubt have a very important significance; (2) Identifying biological features and signaling pathways of liver CSCs that are different from normal liver stem cells and liver cancer cells; (3) Identifying drug resistance of liver CSCs and elucidating its mechanisms; (4) Research and development on the treatment targeting at liver cancer stem cells. We believe that with the deepening of investigations, specific markers of liver CSCs and related signal transduction pathways will be discovered, and the important role of liver CSCs in the genesis, development, metastasis, recurrence and prognosis of liver cancer will be evaluated, which will have important practical importance in the diagnosis, treatment and prognosis judgment of liver cancer.

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