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Mini-review

Capturing circulating tumor cells of hepatocellular carcinoma

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ABSTRACT

Early metastases of hepatocellular carcinoma (HCC) may be detected by the isolation of circulating tumor cells (CTCs) in the bloodstream. During the course of therapeutic attempts, monitoring CTC changes in patients with HCC is helpful for the efficacy assessment. Nevertheless, the markers used for the detection, such as α-feto protein, asialoglycoprotein receptor or epithelial cell adhesion molecule, CD133 or CD90, are not specific for HCC CTCs. In spite of these limitations, a timely determination of the existence of CTCs will be beneficial for the monitoring of distant metastases, the evaluation of therapeutic attempts, and the prediction of prognosis.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world; its lethality ranking the third among all malignant tumors. HCC cells invade into vessels and metastasize at an early stage, which contributes to its aggressiveness. However, many patients have been diagnosed only when abnormal symptoms caused by metastatic lesions occur or radiography reveals single or multiple lesions, consequently missing the best time window for surgical resection. Therefore, early detection of metastatic signs and effective measures to block HCC progression may improve the prognosis of patients with HCC.

The invasion and metastases of hepatoma cells represent a multi-factorial, multi-step process, whose mechanism is not fully understood. In the past few years, the function of circulating tumor cells (CTCs) in the process of cancer metastases has been under active investigation [1]. CTCs refer to cancer cells that are disengaged from the primary lesion, and invade into the blood circulation. They are also called disseminated tumor cells (DTCs). As early as 1869, an Australian scholar, Dr. Ashworth, observed the existence of tumor cells in the blood of a patient who died of metastatic cancer, and firstly put forward the concept of CTCs [2]. However, the clinical significance of CTCs had not been widely realized until 1990s. It is now commonly accepted that the existence of CTCs is an intermediate stage of tumor metastases [3], which clearly affects the selection of treatment options and prognosis. One clinical feature of HCC is to invade into vessels, especially in the portal system, generating metastases in the portal vein and other organs. In this context, the in-depth exploration of HCC CTC biology has become an important approach to understand metastasis. On the other hand, clinical detection of CTCs has become a valuable diagnostic tool for the monitoring the efficacy of various interventions (referring Table 1).

2. How to capture CTCs

Although the existence of CTCs in the bloodstream of cancer patients has been recognized for sometime, substantial research has taken place only during the last ten years [1]. The major difficulty

Abbreviations: AFP, alpha-feto protein; ASGP-R, asialoglycoprotein receptor; CSC, cancer stem cells, CTC, circulating tumor cells; CXCR4, CXC chemokine receptor-4; EpCAM, epithelial cell adhesion molecule; FACS, fluorescence-activated cell sorting; HCC, hepatocellular carcinoma; MACS, magnetic activated cell sorting; MMP, matrix metalloproteinase; RT-PCR, reverse transcriptase polymerase chain reaction; TACE, transarterial chemoembolization.

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Table 1
Key points of this concise review.

Methods of CTC isolation
● Isolation of CTCs according to cell density by gradient centrifugation
● Isolation of CTCs by tumor cell size through a polycarbonate membrane
● Fluorescence-activated cell sorting (FACS) for a highly pure cell population
● Isolation of CTCs with magnetic activated cell sorting (MACS), the most commonly used method in current clinical practice
● Micro-fluidic chip isolation for a more sensitive capture of CTCs
● Detection of tumor-specific mRNA, miRNA or cell-free DNA
The significance of CTC detection in patients with HCC
● Monitoring the signs of early metastasis and the concept of "self-seeding"
● Assessing the effectiveness of therapeutic options and signs of recurrence
● Predicting the prognosis, such as tumor-free survival
HCC CTCs and liver cancer stem cells (LCSCs)
● CTCs may be a subset or derivation of CSCs
● Are CTCs equal to circulating CSCs?
Conclusions and perspectives

in the CTC studies is that an extremely small number of CTCs exists in the bloodstream [4] and common serological, imaging and pathological approaches are not sensitive enough to effectively capture CTCs. Approximately less than 10 CTCs may be found among one billion blood cells; therefore highly sensitive methods are required to detect and isolate these cells from the bloodstream [1]. With technological advances, innovative CTC detection and isolation methods have been developed, and are discussed as follows.

2.1. Isolation by cell density

Because of the various densities of different cell populations in the bloodstream, a gradient distribution will present in cell separation medium after centrifugation. According to this principle, target cells in the patient's blood samples are separated by centrifugation with cell separation medium. The cell gradient medium often used for CTC separation is Ficoll [4–6]. Whole blood with anticoagulants is added directly into the centrifuge tubes. After gradient centrifugation, erythrocytes, neutrophils and mononuclear cells (lymphocytes, monocytes, epithelial cells and tumor cells) are separated into distinct layers in test tubes with tumor cells being enriched to the upper layer. Use of OncoQuick®Plus kits [7] significantly improved the separation efficiency and the sensitivity was improved from a fraction of 1.8×10^7 by Ficoll gradient to 9.5×10^4 mononuclear cells in 10 ml of blood samples by the kit, which is composed of a sterile centrifuge tube, a porous barrier and separation medium. However, a tumor cell-spiked assay with either OncoQuick or Ficoll confirmed a similar tumor cell recovery rate of 70–90% by both methods [8,9]. The limitation of this method is low sensitivity, contamination of other cells, and a considerable loss of tumor cells [10]. Nevertheless, this method could be a pre-enrichment step for further purification by more specific approaches, such as fluorescence-activated cell sorting (FACS) or separation by immunomagnetic beads.

2.2. Isolation by tumor cell size

Based on the fact that the diameter of tumor cells is generally larger than the vast majority of blood cells, Vona et al. [11] developed an isolation method, which could enrich tumor cells by filtrating blood samples through a polycarbonate membrane. The method was useful in separating CTCs from peripheral blood; while tumor cells remained on the membrane. Recently, Lin et al. improved the microfiltration device which is capable of multiplexed imaging and genetic analysis. The microdevice has the potential to enable routine CTC analysis in the clinical setting [12]. ScreenCell is a new device which could separate both fixed cells and live cells at 82–88% recovery rate [13]. The advantage of this filtration method is that isolated cells remain intact with the specific surface antigen or markers without being destroyed, which is crucial for later purification of CTCs, as well as morphologic and genetic analysis. The disadvantage of this method is the lack of specificity as well as a ready loss of tumor cells (26% with ScreenCell device). Similar to the gradient centrifuge discussed above, this filtration method appears to be more appropriate as a pre-enrichment of CTCs for further purification.

2.3. Fluorescence-activated cell sorting

The frequently-used fluorescence-activated cell sorting (FACS) in current CTC research is developed based on flow cytometry. FACS features a high speed and accuracy, and is the combination of technologies including laser scanning, computer science, electronics, fluid mechanics, cellular immunology, monoclonal antibodies and other high-tech methods [14,15]. Whether tumor cells will be detected with this method depends on the number of cells to be analyzed. Given the fact that the number of CTCs in the peripheral blood of patients with liver cancer is often less than 10 per ml [4] or 2–8 per 10^9 blood cells [16], this device has a limited application in the capture of CTCs in a large volume of blood. However, it is a very useful research tool to isolate a highly pure pool of CTCs with specific surface markers, such as EpCAM or CD133 from samples that have been pre-enriched by microfiltration, gradient centrifugation or elutriation [16,17].

2.4. Isolation of CTCs with immunomagnetic beads

The basic principle of immunomagnetic isolation is to coat specific antibodies onto magnetic beads. These immunomagnetic beads bind to cell surface markers via a specific antibody-antigen reaction, and they can be separated by an oriented displacement in an external magnetic field [18]. Bead-based enrichment methods are divided into two categories: positive and negative enrichment. Positive enrichment refers to the method whereby tumor cells are separated directly in a magnetic field. Negative enrichment refers to removing non-specific cells to purify target cells through a magnetic field. Immunomagnetic bead isolation may separate CTCs from the peripheral bloodstream, demanding highly effective and sophisticated equipment, such as CellSearch™ or Isoflux systems.

The key in the preparation of immunomagnetic beads is to identify specific antibodies corresponding to specific antigens on the target cell surface. Xu et al. recently reported that the immunomagnetic beads coated with a ligand protein (asialofetuin) specific for asialoglycoprotein receptor (ASGP-R) were able to identify CTCs in HCC patients [18]. This approach works when liver cancer cells express ASGP-R, whose natural ligand is asialofetuin [19]. Asialofetuin was first biotinylated to form biotinylated asialofetuin, which was then adhered to HCC cells by specifically binding to the ASGP-R. Meanwhile, magnetic beads were coated with anti-biotin antibodies. Taking advantage of the specific conjugation of "biotin-anti-biotin antibody", HCC CTCs bound to the beads, and then were isolated by magnetic activated cell sorting (MACS). After mixing different number of hepatoma Hep3B cells with blood, they were able to separate Hep3B cells in 61% of blood samples with a known cell number. The authors further confirmed that no CTCs were detected in the blood of healthy volunteers, patients with benign liver disease or non-hepatocellular carcinoma; whereas CTCs were detected in 69 out 85 (81%) of HCC patients. An average of 19 ± 24 CTCs were detected in 5 ml of patient blood samples, and the positive rate and number of CTCs correlated significantly with

the tumor size, portal vein cancerous thrombus formation and the TNM (tumor-node-metastasis) stage, although the TNM system poorly predicts the prognosis in HCC patients [20]. The clinical value of this method requires further verification with other commonly used markers, such as epithelial cell adhesion molecule (EpCAM), in a prospective clinical trial with a large number of cases.

The CellSearch System (Veridex LLC) is a semi-automated device that can detect one CTC among 1 billion or more blood cells in a 7.5 ml blood sample; hence it displays high sensitivity [1]. The principle of the CellSearch System is that the surface of the tumor cells from an epithelial cell source is often positive for EpCAM, whereas blood cells do not express EpCAM [1]. According to these characteristics, CTCs may be isolated by coating magnetic beads with antibodies against EpCAM. The detection process starts with (1) the enrichment of epithelial cells by EpCAM antibody-coated magnetic beads; (2) the fixation of the enriched cells; (3) staining with fluorescence-conjugated antibodies against CK8, CK18, CK19 and CD45; (4) the final detection of CK^+, $CD45^-$ epithelial cells by semi-automatic four-color fluorescence microscopy, CellSpotter Analyzer [21]. Currently this method has been considered as a standard of CTC detection for breast, colon and prostate cancers. However, no gold standard is available for CTC detection in a specific malignancy, including HCC, due to the CTC heterogeneity between cancer types [22].

The CellSearch System has been approved by the U.S. FDA for clinical application, and is mostly utilized to predict the disease-free survival and overall survival of metastatic cancer patients. The clinical application has demonstrated that the system is a beneficial complement to the traditional medical imaging modalities in the detection of CTCs, and it is useful to effectively assess the prognosis of the patients and determine the effectiveness of current treatment options. This system is a simple, non-invasive for monitoring the same patient over time. The system is in clinical trials for detecting CTCs in patients with different cancers, such as colorectal and lung cancers [23,24]. Although a fully automatic device similar to this system, the IsoFlux System, which allows blood to flow through a microfluidic channel, is on the market, no clinical studies using the latter in detecting CTCs have been reported.

2.5. Micro-fluidic chip isolation

A micro-fluidic silicon chip, known as CTC-Chip, was developed in 2007 to detect a small number of cancer cells in the bloodstream [16,25]. The surface of this chip was coated with various antibodies at 78,000 microspots. When blood flows through the chip, coated antibodies recognize and bind to surface antigens on tumor cells, and the results depend on the number of cells binding to the chip. This method may detect a single tumor cell over 1 billion blood cells, but the device was initially designed for laboratory use. A second-generation CTC-Chip, known as HB-Chip (herringbone-chip) was developed in 2010 [26]. Compared to the first generation CTC-Chip, HB-Chip possesses the following advantages: (1) the shape of the chip surface changed from the smooth surface to herringbone grooves, so that when the blood sample flows through the chip, microvortices are formed, increasing the opportunity for contact between tumor cells and the coated antibodies on the surface. This change allows an effective capture of rare CTCs (386 ± 238/ml) in 14 out of 15 (93%) patients with metastatic malignancies [26]. Preclinical studies [16,26] have demonstrated that HB-Chip captured more than 90% of cancer cells in blood samples; in comparison with 25% by CTC-Chip; (2) HB-Chip is able to capture clumps of tumor cells which were not separated by CTC-Chip or by other CTC isolation technologies. Further studies of these clumps of tumor cells may provide a deeper understanding of the tumor metastatic process. (3) A standard glass slide is installed on the chip; therefore one may identify cancer cells by traditional pathologic examination. (4) The device is easy to operate, and may handle a large number of blood samples in a high throughput fashion. Thus, it may be used for large-scale clinical studies, and has been a new platform for the analysis of tumor metastases.

Because both the CellSearch System and microfluidic chip capture CTCs from blood samples for the evaluation of metastases, it is unnecessary to obtain solid tumor tissues by an invasive means, therefore, their use is also known as "liquid biopsy" [16,22]. Thus, there is the possibility of wide clinical application. Currently, when the CellSearch System and the microfluidic chips are employed to isolate CTCs of an epithelial cell origin, both use EpCAM antibodies to capture CTCs. However, recent studies have suggested that tumor cells may undergo epithelial mesenchymal transition (EMT) before they gain the ability to enter the bloodstream for distant metastases. Thus, they may lose this epithelial marker, and exhibit more mesenchymal features, such as expression of vimentin and enhanced nuclear translocation of transcription factors modulating EMT, such as snail, Zeb1, SLUG and Twist [3,15]. The cancer cells with acquisition of EMT appear to gain motility and invasiveness, and display morphologic and genotypic characteristics of mesenchymal cells. Therefore, it is conceivable that CTCs derived from epithelial origins are not always EpCAM-positive in the bloodstream, and that capturing CTCs with EpCAM antibodies may fail to recognize a significant portion of CTCs. Based on this assumption, researchers choose a combination of antibodies against a variety of surface markers on CTCs in order to prevent the escape of CTCs during separation. For non-epithelial tumors that do not express EpCAM, the search for other markers, such as CD24/44, CD146, vimentin, and SNAIL/SLUG, is a continuous effort [15,22]. Among these markers, vimentin is an intermediate filament that is expressed in mesenchymal cells; whereas both SNAIL and SLUG are the transcription factors controlling EMT, and exist in both cytoplasm and nuclei. They may not be suitable for cell surface labeling and isolation by MACS.

2.6. Detection of tumor-specific mRNA, miRNA and tumor cell-free DNA

In addition to the methods discussed above which directly detect CTCs by cell surface antigens, researchers also determine specific mRNA expressed in cancer cells by RT-PCR in peripheral blood samples or after an initial enrichment [4,27]. If the results are positive, they indirectly suggest the presence of CTCs. The specificity of mRNA primers that are chosen as liver cancer CTC markers is crucial to the reliability of the results. Marker molecules which have been employed to detect HCC CTCs include: α-fetoprotein (AFP) [4,16], glypican-3 (glypican-specific cytotoxic T lymphocytes) [28], human telomerase reverse transcriptase (hTERT) [29], cytokeratin 18 or 19 [16], snail [30], etc. There exists false positivity in the results due to the existence of free DNA in the bloodstream and highly sensitive RT-PCR assays (1–10 cells out of 10^{6-7} blood mononuclear cells). Although positivity of a specific mRNA, such as AFP, in the peripheral blood of HCC patients indicates the possibility of distant metastases or recurrence after liver transplantation, this method has less value in capturing CTCs directly, as well as in morphological examination [27,31]. In addition, circulating miRNAs may serve as surrogates of remission or progression, metastasis and prognosis, which has been covered in a recent review [32]. In contrast to RNA detection, serum levels of circulating cell-free DNA (cf-DA) were correlated to serum aminotransferase levels and to blood counts of neutrophils and leukocytes, which reflects an inflammatory process in HCV patients with HCC [33]. A separate study found that cf-DNA levels predicted distant metastases after curative treatment in HCV patients with HCC [34]. Thus,

these nucleotide detection methods may have clinical values in reflecting progression, metastasis or prognosis; however they are less helpful in the detection of HCC CTCs. When CTCs are enriched by other methods, specific mRNA levels may be used for the further validation of malignancy or the phenotypic characteristics of isolated tumor cells.

3. The significance of CTC detection in patients with HCC

Most of cancer cells that were disseminated from the primary lesion into the blood circulation are eliminated by immune cells, and only a few of them survive in the circulation system. These cells may further develop into small cancerous thrombus in the bloodstream, and under certain conditions they develop into distant metastases. Many studies have shown that before resection of HCC or liver transplantation, tumor cells have detached from a primary lesion and entered into the blood circulation, which is an early event of HCC metastases through the bloodstream [11,35]. A recent prospective study by Fan et al. found that the number of cancer stem cells ($CD45^-/CD90^+/CD44^+$) in the bloodstream had a good correlation with post-hepatectomy intrahepatic recurrence and lower recurrence-free survival of HCC, and that circulating CSCs > 0.01%, tumor stage and tumor size were independent factors in predicting recurrence-free survival [35]. This study suggests that the local tumor recurrence after resection is closely associated with CTC counts. Even though a localized lesion is completely resected and surrounding lymph nodes are tumor-free, and after a period of time, a new cancerous lesion may recur in the original site [35]. The recurrence in the original site is probably due to the returning of the surviving CTCs from the bloodstream; and this process is known as tumor self-seeding [36]. Tumor reseeding, which is similar to self-seeding in the original tissue after resection, in the explants (implanted grafts) after liver transplantation indicates the recurrence, and affects the overall survival and requirement of re-transplantation. The frequency of tumor recurrence in explants was about 5.7% in 60 cases of liver transplantation within 40 months [37] and 7 out of 22 within 3 years after living donor liver transplantation characterized with microvascular invasion [38]. Self-seeding tumor cells grow preferentially in the original site by promoting angiogenesis and matrix formation. The mechanisms as for why tumor cells are self-seeded preferentially in their original sites or explants are not fully understood. The speculations include: (1) the original tissue type has the ability to attract CTCs, (2) has a leaky vasculature, (3) CTCs possess the innate capability to re-seed in the original site or explants due to a familiar microenvironment [39]. The first hypothesis is supported by the interleukin-6 (IL-6) and IL-8 secretion of tumor tissue, whereas the third hypothesis is suggested by the expression of matrix metalloproteinase-1 (MMP-1), collagenase 1 and actin cytoskeleton component fascin 1 by CTCs [36]. Exploring the mechanisms of tumor self-seeding may lead to a better understanding of molecular regulation of primary HCC mass growth, dissemination (metastasis) and reseeding after tumor resection or orthotopic liver transplantation, and aid in the development of new therapies targeting these three separate events [40]. So far, no study is available regarding the relationship of the occurrence of CTCs, HCC cell reseeding and recurrence in the explants after liver transplantation in HCC patients. Further investigation of CTCs in this clinical setting would provide valuable information to predict the HCC recurrence after liver transplantation.

The existence of CTCs is a prerequisite for the formation of portal vein cancerous thrombi. Li et al. has found that the expression level of CXC chemokine receptor type 4 (CXCR4) in the portal vein cancerous thrombus tissues was significantly higher than in the original HCC tissue [41]. They also found that down-regulation of CXCR4 expression by RNAi significantly reduced the ability of growth, invasion and metastasis of HCC cells separated from the portal vein cancerous thrombus [41]. A separate study has confirmed that hepatoma cells with higher CXCR4 expression exhibited more lymphomatic invasion through induction of active MMP-9 and MMP-2 secretion [42]. The ligand of CXCR-4 is stromal cell-derived factor-1 (SDF-1), and activation of the receptor allows actins to be polymerized, forming pseudopodia, thus induces tumor cell migration directed to specific target organs. Therefore, enhanced CXCR4 expression in HCC cells may imply possible contribution to its invasion and metastasis to the portal lumen [41,42]. However, the mechanisms of the preferential formation of HCC thrombus in the portal vein remain to be investigated.

In-depth understanding of CTC pathobiology may aid in the development of anti-cancer drugs. Scientists have begun to determine whether a patient is sensitive to a particular medication by determining the alterations in gene expression profile in CTCs. In addition, in evaluating the efficacy of anti-metastatic treatments, the change of CTC counts in the bloodstream before and after the treatment is a reliable parameter. With the availability of this application, there is no need to determine the changes in metastatic lesions, which may greatly shorten the research cycle [22]. In animal models of implanted hepatoma cells, removal of an implanted xenograft significantly reduced detectable CTC numbers in host animals [43]. It is not difficult to understand that there is a significant drop in CTC counts after surgical resection of the original tumor mass in patients with various tumors, and that

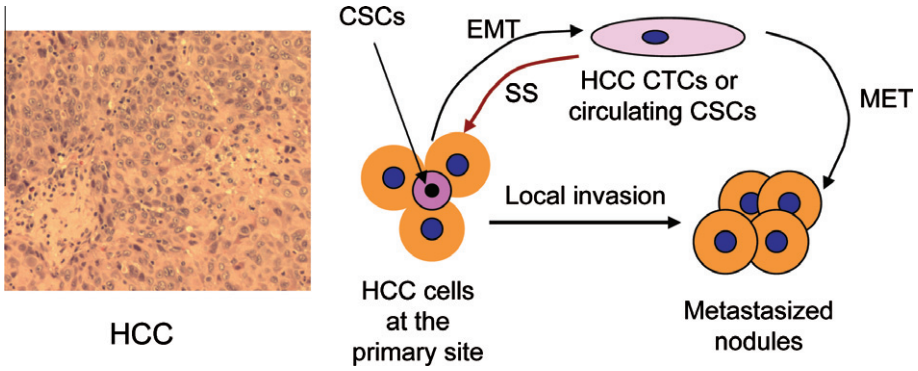

Fig. 1. Schematic illustration of circulation tumor cells and circulating cancer stem cells in HCC. On the left is a representative micrograph of HCC developed in an HBV-infected patient. Hematoxylin and eosin stain, 100×. The right panel intends to provide a schematic illustration of CTCs, and their relationship with EMT when CTCs enter the bloodstream and MET when they are able to form ectopic metastasis. CSCs, cancer stem cells; CTCs, circulating tumor cells; EMT, epithelial mesenchymal transition; MET, mesenchymal epithelial transition. SS, self-seeding of CTCs from circulation to the primary site if the primary was removed.

the rebound of CTC counts in patients with HCC after radiofrequency ablation, transarterial chemoembolization (TACE) or liver transplantation indicates the recurrence and metastasis of HCC [44]. In summary, the enumeration and characterization of CTCs may become an indispensable biomarker for monitoring the efficacy of treatments in clinic.

4. CTCs and cancer stem cells

The theory of cancer stem cells (CSCs) suggests that CSCs, although small in number, are the origin of all cell types in tumors and are responsible for oncogenesis, progression, invasion and metastasis [15,45]. CSCs grow slowly and may renew themselves, thus they often are insensitive to chemotherapy or radiation therapy, constituting a cell source for tumor recurrence and metastases [45]. A new generation of CSCs may be induced under the circumstances of microenvironmental changes, chemotherapy or radiotherapy [46]; however CSCs are not equivalent to CTCs due to the fact that not all CTCs are able to form ectopic metastasis [3]. Thus, only those CTCs that form ectopic metastases have the CSC characteristics, and are known as circulating CSCs [3,15,35] (Fig. 1). It is thought that only when CSCs in a primary lesion are eradicated, can we stop tumor metastases and recurrence, and truly improve the therapeutic outcome of the malignancy. A number of studies have demonstrated the existence of liver cancer stem cells (LCSCs) [5,15,47,48], and CTCs may be a sub-class or derivation of CSCs [3]. Further study of the microenvironment and the intrinsic factors which cause tumor cells to acquire the capability to invade into vessels, to be disseminated and seeded to distant sites will be a critical step towards controlling metastases.

The markers for the identification of LCSCs are overlapping with those for tissue stem/progenitor cells. They are not specific for CSCs or CTCs, and have less diagnostic value if they are employed to detect malignancies in patient tissue or body fluid samples [47,49]. When these markers are used individually or in combination for identifying CSCs in tumor tissue or CTCs in the bloodstream, the isolated CSCs or CTCs should be verified by cancer stem cell biology, such as oncogenicity and resistance to chemotherapy [15]. Previous studies suggest that CD13, CD133 and EpCAM are surface markers of LCSCs [15,48,50,51]. CD13 has been confirmed to be a marker for slowly growing CSCs [48]; whereas, $CD133^+$ plus $EpCAM^+$-cells are thought to be tumor-initiative in HCC [15,51]. Yang et al. [52] found that $CD45^-/CD90^+$ cells could be detected in tumor and blood samples of all patients with HCC; whereas they were undetectable in the liver tissues or blood samples of the control group or in patients with liver cirrhosis [5,52]. When $CD45^-/CD90^+$ cells were isolated from liver cancer tissue and blood, and inoculated into immune-deficient mice, this subpopulation of cells formed xenografts. Moreover, when $CD90^+$ cells were further isolated from the xenograft tissue of the host mice, and re-transplanted cells subsequently formed tumor xenografts. This xenograft-reforming experiment demonstrated the existence of CSCs in liver cancer, and that $CD45^-/CD90^+$ were reliable markers for liver and circulating CSCs. In fact, the isolation of $CD45^-/CD90^+/CD44^+$ CSCs from the bloodstream of HCC patients before hepatectomy predicted the recurrence [35].

5. Conclusions and perspectives

Research of HCC CTCs and CSCs provides a new paradigm for the diagnosis and monitoring of liver cancer; however there are still unknown issues that require in-depth basic studies and clinical investigations. The basic research will be likely to focus on how tumor cells are able to enter the bloodstream, migrate to distant sites and form metastases. Early HCC thrombus formation in the portal vein, self-seeding after surgical resection or reseeding after liver transplantation are of particular interests for further investigations. There has not been a consensus as to which method or combination of methods are sensitive and specific for capturing CTCs, nor does there exist a gold standard for the selection of cell surface markers. In this context, the verification and comparison of CTCs isolated by various centers with different methods may better define which cells are "true" CTCs. Translational and clinical investigations should aim to develop or improve technologies to efficiently enrich, purify and characterize HCC CTCs or circulating CSCs in the bloodstream, to identify specific molecular markers of these two types of tumor cells, and to define their biological characteristics and the relationship to each other. For clinical applications, automatic devices with a high throughput capability and a low cost permissive for general affordability are welcome. When multicenter clinical studies have verified the usefulness of a particular device or approach, CTC examination should be performed as a routine diagnostics in determining the efficacy of the established algorithms of HCC management, such as surgical resection, radiofrequency ablation, percutaneous ethanol injection or transarterial chemoembolization (TACE), as well as treatment with chemotherapy agents, such as sorafenib, bevacizumab and cetuximab [44,53]. Hopefully, with continued improvement of the sensitivity and specificity of technologies in the detection of CTCs and frequently monitoring the changes of CTC counts in HCC patients, this non-invasive examination will become a valuable tool in the detection of early metastases, the evaluation of therapeutic efficacy (response to specific treatment or recurrence), and the prediction of prognosis.

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