Cancer Cell Research 24 (2019) 644-649

Available at http:// www.cancercellresearch.org ISSN 2161-2609

Circulating tumor cells in hepatocellular carcinoma: detection techniques, clinical implications, and future perspectives

Yuntai Shen[1,2], Yunjin Zang[1,2]

[1]Department of Medicine, Qingdao University, Qingdao, 266003, China

[2]Organ Transplantation Center, Affiliated Hospital of Qingdao University, Qingdao, 266003, China

Abstract: **Circulating tumor cells (CTCs) are cells released from primary tumor into bloodstream that associated with tumor metastatic and recurrence. Many studies reveal that circulating tumor cells are thought to play an important role in the poor prognosis of patient with hepatocellular cancer (HCC). HCC is a serious health problem worldwide especially in China due to worse prognosis, high incidence and high mortality. It is an urgent task to identify potentially markers for early diagnosis, surgical treatment, improve management of patients and provide personalized therapy. There are many encouraging studies indicate that CTCs has a great potential use as diagnosis markers for patient with HCC. In this review, we are focus on the advances in studies of HCC CTCs, including the detection of CTCs, the clinical implications, the biological characteristic of HCC CTCs and future perspectives.**

Keywords: Circulating tumor cells; hepatocellular cancer; Circulating cancer stem cells

Received 28 June 2019, Revised 25 October 2019, Accepted 28 October 2019

*Corresponding Author: Yunjin Zang, zangyj3657@163.com

1. Introduction

Hepatocellular cancer (HCC) is the most common cancer and causing more than 600000 deaths every year in the world [1]. The advanced medical techniques for treatment of HCC, including liver resection and liver transplantation. However, recurrence rate in patients who undergone liver resection is remains poor and 5 years survival rate for patient with hepatectomy is 12.7% [2]. For patients who taken liver transplantation, 5 years survival rate in patients meet Milan criteria is still remain low [3]. Therefore, it is an urgent task to find high specificity and sensitivity markers to improve early diagnosis rate and predict recurrence during treatment.

CTCs are defined as tumor cells are released by primary tumor or metastatic tumor into bloodstream that might cause tumor metastatic and recurrence [4]. Owing to CTCs are rare in blood circulation and technical limitations, detecting and enumerating CTCs is a challenging task. However, Numerous technologies have been made to improve the specificity and sensitivity of detection and enumeration of CTCs in recent years. CTCs have increasingly drawn attention for its applied value in clinical practice. Recent studies have been indicated that CTCs can be detected in many malignancies including HCC, gastric and colorectal cancers [5-7]. In some studies, CTCs can be used as markers for predict overall survival, risk of metastasis, clinical diagnosis and chemotherapy effectiveness [5-8]. Most recent studies focus on epithelial-mesenchymal transition (EMT), molecular characteristics of circulating tumor cells, recurrence risk and vascular invasion [9-11].

In this review, we focus on the new advancements of CTCs in HCC, including detection and enumeration methods, clinical implications and future perspectives

2. The detection and enumeration methods of CTCs in HCC

Approximately 1 circulating tumor cell is exist in 107 blood cells, it is present a challenging task to enumerate and detect CTCs [12]. Two steps are usually used to identify CTCs, including enumeration and detection of CTCs. According to the features of circulating tumor cells, the enumeration steps including one approach base on physical characteristics (density gradient centrifugation), cell size (isolation by size of epithelial tumor cells, ISET) and electric charge. The detection steps can be classified as nucleic acid-based assays (reverse transcription polymerase chain reaction, RT-PCR) and cell recognition-based assays and other immune-magnetic based approach (magnetic affinity cell sorting).

2.1. The enumeration methods

2.1.1. Density Gradient Centrifugation

Density Gradient Centrifugation is a simple and feasible method. Different cells have a variety of densities in blood. Erythrocytes, leukocytes and CTCs will separate into distinct layer in cell separation medium after gradient centrifugation. To date, there are

many commercially density gradient liquid kits, but these commercially kits remain to enhance the specificity of the enrich procedure. Owing to its lack of specificity, Density Gradient Centrifugation is used as a preprocess step for following process. The particular deficiency in the method is loss of CTCs [13].

2.1.2. CTC-chip

CTC chip is a novel method to detect CTCs with high purity, which help us to precisely isolate CTCs. In 2007, Nagrath was designed CTC-chip to identified CTCs in the peripheral blood. A variety of antibodies were coated with the surface of chip. When blood flows a series of antibodies-coated posts, the antibodies can bind to surface antigens on the CTCs [14]. Another novel method is microvortex-generating herringbone (HB) chip combined use of micro-scale vortices and inertial focusing, allowing cells bind to antibody-coated microplots for high-purity extraction of CTCs [15]. Yu was firstly adopted a two-stage strategy to enhance the efficiency and purity, known as PN chip [16]. Compared with other CTC chip, PN chip consist of a polydimethylsiloxane (PDMS) herringbone structure and a substrate of Ni micropillar arrays. The PN chip can increase the capture sensitivity and decrease nonspecifically bounded cells by combining on-chip purification and off-chip enzymatic treatment. The on-chip introduces a stirring flow to continuous magnetic separation. The off-chip enzymatic treatment make the CTCs were released sufficient from the attached magnetic beads.

2.1.3. ISET

According to the different size between CTCs and other cells. Membrane microfilters are designed to isolate CTCs from peripheral blood [17-18]. Vona was first used polycarbonate material to isolated circulating tumor cells based on different sizes between blood cells and circulating tumor cells [18]. It is called isolation by size of epithelial tumor cells (ISET). Recently, Chen et al used a novel one-stop ISET device with automatic isolation and staining procedure. The one-stop ISET device is able to isolate and identify CTCs and CTC-clusters from cancer patients. Meanwhile, the device has the potential application in clinical diagnosis and improve cancer management [18].

2.1.4. Immunological Enrichment

Immunological enrichment is widely used in enumerate CTCs by targeting CTC cell surface markers. The epithelial cell adhesion molecule (EpCAM), the most widely epithelial surface antigens [19]. CTCs are fished out from the leukocyte background with immunomagnetically labeled monoclonal antibodies targeted to EpCAM. The sensitivity is usually reinforced by applying an anti-CD45 selection of leukocytes [20]. Epithelial markers were expressed in non-epithelial cells or non-tumor epithelial cells may result in false positive. Down regulation of EpCAM expression of CTC may happened in epithelial-mesenchymal transition (EMT). The EpCAM-based strategies have limitations in detecting EpCAM-negative CTCs.

2.2. The detection methods

2.2.1 RT-PCR

A number of tumor-specific mRNA were used to identity the presence of circulating tumor cells in patients with HCC. However, these mRNA such as EpCAM IGFBP1, and AFP mRNA can exist in normal cells and lacking the specificity and sensitivity of detection [21-23]. Among the tumor-specific mRNA, although the AFP is a well-accepted tumor marker, but the AFP can be expressed by normal cells and decrease the specificity of RT-PCR. Better biomarkers expressed in CTCs are critical to detect the existence of CTCs.

2.2.2. The cell search system

U.S.A Food and Drug Administration (FDA) has been approved The CellSearch system due to the high specificity and sensitively enumeration of HCC [5]. The CellSearch system is a semi-automated methodology can detect and enumerate CTCs. Magnetic iron nanoparticles coated with anti-EpCAM antibodies are mixed with peripheral blood to separate the epithelial cells. In order to exclude leukocytes, EpCAM (+) cells are mixed with anti-CK and anti CD45 fluorescent antibodies. DAPI is used to identify the relevant cell fraction. Identify the features of CTCs: positive for CK, positive for DAPI, negative for CD45. Several studies have been detected the presence of CTCs in patient with HCC by this method [5]. Owing to epithelial-mesenchymal transition (EMT), the CellSearch system have limitations in detecting CTCs that have undergone EMT.

2.2.3. Asialoglycoprotein receptor (ASGPR)–Based Magnetic Separation

Novel cell enrichment approach being developed for CTC detection in patient with HCC. Asiaglycoprotein receptor (ASGPR) is glycoprotein expressed on HCC cells and hepatocytes membranes with its ligand [24]. Li and his colleagues developed a novel magnetic cell separation system to detect CTCs in HCC patients. In this system, HCC cells were bound by ASGPR antibody and magnetically separation by second antibody-coated magnetic beads. The isolated HCC cells were identified by immunofluorescence staining using anti-P-CK and anti-carbamoyl phosphate synthetase 1 (CPS1) antibodies. Normal hepatocytes do not exist in blood circulation unless they become

cancer cells. The result showed that 89% of HCC patients were detected CTCs and no CTCs were found in the healthy, benign liver disease or non-HCC cancer subjects [24].

3. Biology of CTCs in patient with HCC

3.1. Circulating cancer stem cells (CSCs)

Cancer stem cells (CSCs) are consider participating in initiation, progression, metastasis of tumor [25-26]. It is accepted that cancer stem cells play an important role in cancer metastasis and relapse. CD44 has been identified as a CSC marker in hepatocellular carcinoma [27]. Hou found that CTC-positive patient with CD44 positive were likely to developed metastasis. Moreover, patient with CD44 positive CTCs were more likely to have highly metastatic ability than patient with CD44 positive CTCs. Thus, CD44 positive CTCs are related with HCC invasive risk [28]. In a prospective study, Yang found that compared to CD90 positive and CD44 positive CTCs, CD90 positive and CD44 negative have a slower capacity of metastasis, indicating CD90 positive and CD44 positive CTCs have stronger aggressive ability [29]. CD44 could be used as marker for predicting invasive and metastasis in patient.

3.2. Epithelial to Mesenchymal Transition (EMT)

Most patients with HCC are die from metastasis. The epithelial to mesenchymal transition (EMT) is a multi-step biological process that participate in metastasis and progression of many cancers [30]. The tumor cells could enter into bloodstream to exhibit distant metastasis after they may undergo epithelial to mesenchymal transition. The liver is an epithelial-derived organ, but EpCAM expression pattern of liver have been found different from other epithelial-derived organs. In liver tumorigenesis, there are a few HCC cells positive for EpCAM and harbor more mesenchymal features [31]. Thus, these EpCAM-based enumeration methods (e.g. The CellSearch system) cannot detect the epithelial-mesenchymal transitioned circulating tumor cells.

Liu detected CTC for HCC patients and healthy volunteers by adopted a novel strategy to explore the relationship between EMT process and HCC. They were combined filter-based method with RNA in situ hybridization (RNA-ISH) technique to detect epithelial mesenchymal mixed CTCs. Their result shown that mesenchymal CTCs have associations with metastasis [32]. The epithelial mesenchymal mixed CTCs may have the potential to predict extrahepatic metastasis.

4. Clinical implications

Detecting the presence of CTCs from peripheral blood is gradually get attentions for the clinical implication of CTCs. Many studies have been revealed the clinical correlation between CTCs and HCC patient since 1990s. These studies shown that CTCs may play an important role in predicting prognosis of cancer, monitoring tumor resistance and metastatic and improve early diagnosis rate.

4.1. CTC detection in patient with HCC could be predicting patient survival

Vona used ISET method to detect CTCs in 44 patients with HCC and microemboli in 23 patients (52%) from 44 HCC patients in 2004. while, patients with chronic active hepatitis, patients with cirrhosis or healthy volunteers were not detected CTC [17]. Vona also found that the presence and number of CTCs and microemboli were significantly associated with a shorter survival [17]. Furthermore, a recent study reported that $\geqslant 2$ EpCAM-positive CTCs is independent predicating factor for postoperative recurrence [33]. It suggested that EpCAM-positive CTCs have the potential to monitor treatment response. Schulze detected $\geqslant 1$ EpCAM-positive CTCs in 18 HCC patients among 59 HCC patients, indicating a strong association between EpCAM-positive CTCs and survival [34]. These studies indicate that CTCs result in metastasis and recurrence, may be used as potential therapeutic target for HCC patients after hepatectomy and liver transplantation.

4.2. CTCs can assist in selection of treatment

The number of CTCs is growing may indicate that changes happened in the cancer or treatments are not available for patient, the tumor cells are getting resistant to therapy. Detection of CTCs could provide a more effective therapeutic schedule to treatment. Detection of CTCs may be used as a rather rapid marker to diagnosis tumor in advanced tumor earlier than traditional methods (e.g. CT or PET). the peripheral blood samples were analyzed to elevated the relationship between the number of CTC and transarterial chemoembolization therapy (TACE). Huang found the numbers of CTCs could reflect the therapeutic effect of postoperative transarterial chemoembolization therapy (TACE). The count of CTCs in patients received with TACE less than the patients received no management [8].

4.3. Measurement of CTCs could be useful for predict metastasis and recurrence

People doubt if uncompleted hepatectomy caused postoperative metastasis and recurrence. Thus, extended resections were employed in surgical operation. but the extended resections were unsuccessful. A study indicated that $\geqslant 1$ EpCAM-positive CTCs in HCC patients and patients with cirrhosis or benign hepatic tumor. Compared with HCC patients without CTCs, The HCC patients with

EpCAM-positive CTCs are more prone to metastasis and recurrence. EpCAM-positive CTCs are associated with aggressively tumor type and appear to play an important role in distant metastasis [35]. EpCAM play a role in both cell migration and proliferation [30]. Guo performed a study to investigate the clinical significance of EpCAM mRNA-positive CTCs using a novel platform based on different negative enrichment and qRT-PCR. EpCAM mRNA-positive CTCs were detected in patients with higher recurrence rates and shorter time to recurrence [21]. In the epithelial-mesenchymal transition, the decreased expression of EpCAM are related to reduced migration and decreased proliferation in breast cancer patients [21]. For gastric cancer, Imano et al have found up-regulated EpCAM resulted in peritoneal metastasis [36]. Taken together, CTCs could be a diagnostic marker in early metastasis and recurrence.

5. Future perspectives

Targeted therapies, hepatectomy and liver transplantation are the main treatment for patient with HCC. However, HCC recurrence rate of patient after surgical operation is still a clinical problem. Earlier detection accurately of CTCs is great valuable to assess the effects of HCC therapy and guide targeted therapies. The detection of CTCs is adopted to evaluate the treatment outcome and valuable to individualized treatment. Although many advanced technologies have been used for detection of CTCs. there are no available methods meet the practical demand of clinical diagnosis. The clinical diagnosis could be required a high-specificity, high sensitively and low-cost technique. It is essential to create a simple and sensitivity detection method. As a result, it can prompt cancer biology research and improve the life quality of patients.

References

[1] Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries[J]. CA Cancer J Clin, 2018, 68(6): 394-424.

[2] Lin S, Hoffmann K, Schemmer P. Treatment of hepatocellular carcinoma: a systematic review[J]. Liver Cancer, 2012, 1(3-4): 144-58.

[3] Sanchez Antolin G, Garcia Pajares F, Perez E, et al. Milan criteria versus San Francisco criteria in hepatocellular carcinoma: our center's experience[J]. Transplant Proc, 2009, 41(3): 1012-3.

[4] Sun Y F, Guo W, Xu Y, et al. Circulating Tumor Cells from Different Vascular Sites Exhibit Spatial Heterogeneity in Epithelial and Mesenchymal Composition and Distinct Clinical Significance in Hepatocellular Carcinoma[J]. Clin Cancer Res, 2018, 24(3): 547-559.

[5] Qi L N, Xiang B D, Wu F X, et al. Circulating Tumor Cells Undergoing EMT Provide a Metric for Diagnosis and Prognosis of Patients with Hepatocellular Carcinoma[J]. Cancer Res, 2018, 78(16): 4731-4744.

[6] Abdallah E A, Braun A C, Flores B, et al. The Potential Clinical Implications of Circulating Tumor Cells and Circulating Tumor Microemboli in Gastric Cancer[J]. Oncologist, 2019.

[7] Germano G, Mauri G, Siravegna G, et al. Parallel Evaluation of Circulating Tumor DNA and Circulating Tumor Cells in Metastatic Colorectal Cancer[J]. Clin Colorectal Cancer, 2018, 17(1): 80-83.

[8] Shen J, Wang W S, Zhu X L, et al. High Epithelial Cell Adhesion Molecule-Positive Circulating Tumor Cell Count Predicts Poor Survival of Patients with Unresectable Hepatocellular Carcinoma Treated with Transcatheter Arterial Chemoembolization[J]. J Vasc Interv Radiol, 2018, 29(12): 1678-1684.

[9] Chen Y, Li S, Li W, et al. Circulating tumor cells undergoing EMT are poorly correlated with clinical stages or predictive of recurrence in hepatocellular carcinoma[J]. Sci Rep, 2019, 9(1): 7084.

[10] Ou H, Huang Y, Xiang L, et al. Circulating Tumor Cell Phenotype Indicates Poor Survival and Recurrence After Surgery for Hepatocellular Carcinoma[J]. Dig Dis Sci, 2018, 63(9): 2373-2380.

[11] Von Felden J, Schulze K, Krech T, et al. Circulating tumor cells as liquid biomarker for high HCC recurrence risk after curative liver resection[J]. Oncotarget, 2017, 8(52): 89978-89987.

[12] Yu J J, Xiao W, Dong S L, et al. Effect of surgical liver resection on circulating tumor cells in patients with hepatocellular carcinoma[J]. BMC Cancer, 2018, 18(1): 835.

[13] Tan S J, Yobas L, Lee G Y, et al. Microdevice for the isolation and enumeration of cancer cells from blood[J]. Biomed Microdevices, 2009, 11(4): 883-92.

[14] Nagrath S, Sequist L V, Maheswaran S, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology[J].

Nature, 2007, 450(7173): 1235-9.
[15] Stott S L, Hsu C H, Tsukrov D I, et al. Isolation of circulating tumor cells using a microvortex-generating herringbone-chip[J]. Proc Natl Acad Sci U S A, 2010, 107(43): 18392-7.
[16] Yu Xiaolei, Zhang Lingling, Guo Shishang. Capture and Release of Cancer Cells by Combining On-Chip Purification and Off-Chip Enzymatic Treatment [J]. ACS Appl. Mater. 2015, 7, (43):24001-24007
[17] Vona G, Estepa L, Beroud C, et al. Impact of cytomorphological detection of circulating tumor cells in patients with liver cancer[J]. Hepatology, 2004, 39(3): 792-7.
[18] Vona G, Sabile A, Louha M, et al. Isolation by size of epithelial tumor cells: a new method for the immunomorphological and molecular characterization of circulatingtumor cells[J]. Am J Pathol, 2000, 156(1): 57-63.
[19] Yamada S, Utsunomiya T, Morine Y, et al. Expressions of hypoxia-inducible factor-1 and epithelial cell adhesion molecule are linked with aggressive local recurrence of hepatocellular carcinoma after radiofrequency ablation therapy[J]. Ann Surg Oncol, 2014, 21 Suppl 3: S436-42.
[20] Wang L, Li Y, Xu J, et al. Quantified postsurgical small cell size CTCs and EpCAM(+) circulating tumor stem cells with cytogenetic abnormalities in hepatocellular carcinoma patients determine cancer relapse[J]. Cancer Lett, 2018, 412: 99-107.
[21] Guo W, Yang X R, Sun Y F, et al. Clinical significance of EpCAM mRNA-positive circulating tumor cells in hepatocellular carcinoma by an optimized negative enrichment and qRT-PCR-based platform[J]. Clin Cancer Res, 2014, 20(18): 4794-805.
[22] Nel I, Baba H A, Weber F, et al. IGFBP1 in epithelial circulating tumor cells as a potential response marker to selective internal radiation therapy in hepatocellular carcinoma[J]. Biomark Med, 2014, 8(5): 687-98.
[23] Simon F, Bockhorn M, Praha C, et al. Deregulation of HIF1-alpha and hypoxia-regulated pathways in hepatocellular carcinoma and corresponding non-malignant liver tissue--influence of a modulated host stroma on the prognosis of HCC[J]. Langenbecks Arch Surg, 2010, 395(4): 395-405.
[24] Li J, Chen L, Zhang X, et al. Detection of circulating tumor cells in hepatocellular carcinoma using antibodies against asialoglycoprotein receptor, carbamoyl phosphate synthetase 1 and pan-cytokeratin[J]. PLoS One, 2014, 9(4): e96185.
[25] Tian B, Luo Q, Ju Y, et al. A Soft Matrix Enhances the Cancer Stem Cell Phenotype of HCC Cells[J]. Int J Mol Sci, 2019, 20(11).
[26] Huo Y, Chen W S, Lee J, et al. Stress Conditions Induced by Locoregional Therapies Stimulate Enrichment and Proliferation of Liver Cancer Stem Cells[J]. J Vasc Interv Radiol, 2019.
[27] Asai R, Tsuchiya H, Amisaki M, et al. CD44 standard isoform is involved in maintenance of cancer stem cells of a hepatocellular carcinoma cell line[J]. Cancer Med, 2019, 8(2): 773-782.
[28] Lee D, Lee J W. Self-renewal and circulating capacities of metastatic hepatocarcinoma cells required for collaboration between TM4SF5 and CD44[J]. BMB Rep, 2015, 48(3): 127-8.
[29] Fan S T, Yang Z F, Ho D W, et al. Prediction of posthepatectomy recurrence of hepatocellular carcinoma by circulating cancer stem cells: a prospective study[J]. Ann Surg, 2011, 254(4): 569-76.
[30] Thiery J P, Sleeman J P. Complex networks orchestrate epithelial-mesenchymal transitions[J]. Nat Rev Mol Cell Biol, 2006, 7(2): 131-42.
[31] Li Y M, Xu S C, Li J, et al. Epithelial-mesenchymal transition markers expressed in circulating tumor cells in hepatocellular carcinoma patients with different stages of disease[J]. Cell Death Dis, 2013, 4: e831.
[32] Liu Y K, Hu B S, Li Z L, et al. An improved strategy to detect the epithelial-mesenchymal transition process in circulating tumor cells in hepatocellular carcinoma patients[J]. Hepatol Int, 2016, 10(4): 640-6.
[33] Sun Y F, Xu Y, Yang X R, et al. Circulating stem cell-like epithelial cell adhesion molecule-positive tumor cells indicate poor prognosis of hepatocellular carcinoma after curative resection[J]. Hepatology, 2013, 57(4): 1458-68.
[34] Schulze K, Gasch C, Staufer K, et al. Presence of EpCAM-positive circulating

tumor cells as biomarker for systemic disease strongly correlates to survival in patients with hepatocellular carcinoma[J]. Int J Cancer, 2013, 133(9): 2165-71.

[35] Xu W, Cao L, Chen L, et al. Isolation of circulating tumor cells in patients with hepatocellular carcinoma using a novel cell separation strategy[J]. Clin Cancer Res, 2011, 17(11): 3783-93.

[36] Imano M, Imamoto H, Itoh T, et al. Impact of intraperitoneal chemotherapy after gastrectomy with positive cytological findings in peritoneal washings[J]. Eur Surg Res, 2011, 47(4): 254-9.

