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Research progress on circulating tumor cells of hepatocellular carcinoma

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ABSTRACT

Circulating tumor cells (CTCs) are the cells released from the primary tumor and found in the peripheral blood, which can colonize and develop at a distance through blood circulation. At present, the commonly used separation and detection methods of CTCs are mainly divided into physical methods, biological methods, and microfluidic chip-based methods. Monitoring CTC count and cell phenotype is of great significance for early screening and diagnosis of hepatocellular carcinoma (HCC). Moreover, the CTC count and cell phenotype are related to assessing the clinical efficacy of the treatment of HCC and the clinical stage of HCC patients. The CTCs count is also closely related to the overall survival, progression-free survival, and postoperative recurrence of patients with HCC.

1. Introduction

Primary liver cancer (PLC) is one of the most common malignant tumors worldwide. In 2018, PLC ranked sixth in global incidence and fourth in mortality rates.[1] China accounts for 46.71% of new PLC cases in the world. The main risk factor is chronic infection by the hepatitis B virus.[2] Hepatocellular carcinoma (HCC) is the most common type of liver cancer, accounting for about 90% of PLC.[3] The early clinical symptoms of patients with HCC are not obvious. Several patients have been definitely diagnosed while in the middle or advanced stages, resulting in negative side effects from treatment and poor prognosis. Therefore, early detection, diagnosis, and treatment have great significance in the treatment of HCC. Circulating tumor cells (CTCs) are cells released from the primary tumor and found in peripheral blood, which can colonize and develop at a distance through blood circulation.[4] CTCs play an important role in early diagnosis, concomitant diagnosis, disease monitoring, drug resistance monitoring, immunotherapy monitoring, and postoperative follow-up, etc. Based on current studies[5–7], the level of CTCs in patients with HCC is significantly higher than that in patients with benign liver lesions. The CTC level is related to the degree of liver injury in patients with HCC. We present a review of the research progress on CTCs of HCC.

2. Separation and detection of CTCs

Although millions of tumor cells are shed and released into the blood from the primary tumor, only a small proportion successfully affect distant organs after escaping the immune system.[8] Therefore, the amount of CTCs in peripheral blood is very low, accounting for only $1/10^7$–$1/10^6$ of the number of blood cells. The focus of CTC research is on effectively separating CTCs from peripheral blood cells, enriching them, and detecting them even at small amounts.

2.1. Separation by physical characteristics

The common physical separation methods are density gradient centrifugation and filtration. Separation by density gradient centrifugation depends on the different densities of each blood cell. After adding a specific separation medium (such as Ficoll-Paque PLUS) and centrifugation, CTCs and mononuclear cells are suspended above the separation solution, while the smaller white blood cells and red blood cells are deposited under it. The filtration works by setting a threshold, e.g., using the 8 μm pore size filter membrane, so that blood cells that are smaller than 8 μm can be filtered. The cells separated by physical characteristics have a complete structure and good biological activity; this method is cost effective. The cells, however, have poor sensitivity and specificity, easily resulting in false negatives and false positives.

Separation using the CanPatrol system is based on filtration. Blood is passed through the filter membrane with a pore size of 8 μm after red blood cell removal and other pretreatments. The cell samples are obtained after filtration. The detection is based on multiple RNA *in situ* hybridization. Fluorescent probes (containing epithelial cell markers EpCAM, CK8/18/19, mesenchymal cell markers vimentin, twist, and leukocyte markers CD45) are added to the sample to hybridize with target mRNA after which the nuclei are stained with DAPI. After

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incubation, the cells expressing epithelial cell markers^{+}, DAPI^{+}, and CD45^{-} are considered epithelial CTCs, cells expressing mesenchymal cell markers $^{+}$, DAPI^{+}, and CD45^{-} are considered mesenchymal CTCs, and those expressing epithelial cell markers^{+}, mesenchymal cell markers^{+}, DAPI^{+}, and CD45^{-} are considered mixed CTCs.[9]

2.2. Separation by biological characteristics

The most common biological separation method is immunomagnetic separation, which is divided into positive and negative separation. In the positive separation, the epithelial cell adhesion molecule (EpCAM) antibody is bound to the surface of magnetic beads, the surface antigen of the CTCs is specifically bound to the EpCAM antibody and directly separated under a strong external magnetic field. In the negative method, the CD45 antibody is wrapped on the surface of the magnetic beads, specifically bound with the white blood cell surface antigen, and the white cells are separated under a strong external magnetic field. The remaining samples contain a higher concentration of CTCs.[10]

Separation and detection of the CellSearch system are based on the immunomagnetic positive method and immunohistochemical staining. The cells are observed and analyzed using a microscope. They are labeled with DAPI fluorescent dye, the CD45 fluorescent antibody, a cytokeratin fluorescent antibody, and identified as CTCs when the cells are $\geq$4 μm, DAPI^{+}, keratin^{+}, and CD45^{-}.[11] Although hepatoma cells are epithelial-derived cells, epithelial-mesenchymal transformation (EMT) is prone to occur in the process, which makes these cells acquire the mesenchymal cell or stem cell phenotypes, as well as a stronger ability to migrate and invade.[12] The CellSearch system depends on the expression of EpCAM, which can only detect epithelial CTCs. It poorly detects mesenchymal and epithelial-mesenchymal mixed CTCs, which limits its relevance in the diagnosis of liver cancer.

Studies have revealed that the asialoglycoprotein receptor (ASGPR) as a marker that clearly detects liver cancer CTCs.[13] ASGPR is a specific transmembrane protein, which is often expressed on the surface of hepatocytes. In this method, liver cancer CTCs are bound to biotinylated ASGPR ligands, labeled with antibiotic-coated magnetic beads, and finally separated under a magnetic field.

2.3. Separation by microfluidic chips

Microfluidic technology and its microchannel network are widely used in the chemical, biological, and medical fields. Their ability to analyze or process microlevel fluids and suspensions is superior to that of other methods. Recently, a variety of microfluidic platforms have been developed for the separation of CTCs. Unlike traditional technology, microfluidic technology has the characteristics of small sample requirements, a rapid processing speed, a strong multiplexing ability, and a large surface-volume ratio.[14] The separation technique of the microfluidic platforms is similar to that of traditional methods, including physical separation, biological separation, and the combination of both. Physical separation uses various outside forces (such as light, sound, electricity, and magnetic force) to separate cells according to cell size, density, and compressibility. For example, the ClearCell Cx and ClearCell Fx systems[15] use cell size, deformability, and cell inertia in a spiral channel to separate CTCs. Biological separation uses the modified antibody distribution on the chip surface according to the CTCs surface antigen to separate cells. For example, the EpCAM antibody is wrapped on the surface of the microcolumn on the CTC chip. The CTCs are enriched according to the microcolumn location distribution and antigen-antibody reaction.

3. CTC and the diagnosis of HCC

At present, the diagnosis of HCC mainly depends on the detection of serological tumor markers, i.e., alpha-fetoprotein (AFP), AFP variants, des-gamma-carboxy-prothrombin (DCP) and imaging examination (ultrasound, computed tomography [CT], and magnetic resonance imaging [MRI]), while the gold standard of diagnosis is a pathological examination.[16] For patients in the early stage, imaging examination proves difficult to identify tumor lesions smaller than 2 mm, while the pathological examination makes it difficult to obtain the sample. When the diagnosis was confirmed by imaging and/or pathology, most of the patients were in the middle and late stages. AFP is the most used diagnostic marker for liver cancer with a sensitivity of 57.9–66.3%.[17–19] The sensitivity of AFP combined with DCP can reach >82%.[19] When the traditional diagnosis is made, most patients lose out on the best time for treatment.

Due to the low level of CTC in patients with early-stage tumors, the diagnosis of HCC solely using CTC is likely to be falsely negative. Nowadays, the combined tests of several serological tumor markers are often used to improve the positive rate. The combined CTCs tests and other serological tumor markers have been studied by many scholars. Yan[20] et al. obtained the sensitivity and specificity of 82.01% and 90.70%, respectively, for the diagnosis of HCC by using the CTCs test combined with the cell free DNA test. Feng[7] et al. found that the sensitivity and specificity for the HCC diagnosis were 83.3% and 82.6%, respectively, by a combination of CTCs and AFP tests. The positivity rate of CTCs was 61.3% in patients with HCC with a negative AFP test result.

Some experimental models[8] have shown that cells from the primary tumor can spread to the blood and distant organs while in the early stage. Therefore, the presence of CTC can be detected in the blood of patients with HCC at the early stage. Patients with metastasis are more likely to carry mesenchymal CTCs and mixed CTCs[21] compared to patients at the early stages of HCC. Therefore, monitoring CTC count and cell phenotype is of great significance for early screening and diagnosis.

4. CTC and the treatment of HCC

At present, there are many methods for the treatment of HCC including hepatectomy, liver transplantation, transcatheter arterial chemoembolization (TACE), radiofrequency ablation, molecular targeted therapy, and immunotherapy.[16]

Many studies have shown that the detection of CTCs is of great value in assessing the clinical efficacy of the treatment of HCC. Mu[22] et al. collected 50 preoperative and postoperative peripheral blood samples of patients with HCC who solely underwent hepatectomy and used the Captor and negative immunomagnetic beads to separate and enrich the CTCs. The results showed that in 96% of the patients, CTCs were detected before the operation and the CTC count decreased after the operation. Wu[23] et al. collected peripheral blood samples of 120 patients with HCC who underwent TACE, used density gradient centrifugation and negative immunomagnetic beads to separate and enrich the CTCs. The CTC count decreased after TACE. However, some scholars hold different views. Fang[24] et al. collected the peripheral blood of 42 patients with HCC treated with TACE and found that the CTC count increased after TACE. He also found that there was no difference in the progression time of patients regardless of whether the CTC count increased after TACE.

The CTC is also closely related to the clinical stage of HCC. CTC was detected in 69 of 85 (81%) patients with HCC by Xu[13] et al. The positivity rate of the CTC was highly correlated with the TNM stage (66% in stage I and 100% in stage IV). Luo[6] et al. used the CanPatrol system to detect the different phenotypes of CTCs. He found that mixed and mesenchymal CTCs were associated with Barcelona Clinic Liver Cancer, which may be related to the stronger invasive ability of the CTC after EMT. Thus, the patients with mixed and mesenchymal CTCs have a higher clinical stage. Rau[25] et al. analyzed the blood samples of 81 patients with a definite disease status and found that the CTC count in patients with progressive disease (PD) was significantly higher than that in the partial response (PR) and stable disease (SD). The median CTC counts in PD and PR + SD were 50/ml and 15/ml, respectively.

CTC can guide HCC treatment to a certain extent. Zhou[26] et al. collected peripheral blood samples and pathological samples from 117

patients undergoing hepatectomy. Compared to the CTC-negative patients, they found that CTC-positive patients had more microvascular invasions (mVI) and a longer spread distance of mVI. By comparing the early recurrence rate and overall survival time of patients with different resection margins, it is believed that the distance from the resection margin to the tumor in CTC-positive patients should be larger than 1 cm.

5. CTC and the prognosis of HCC

Besides intrahepatic dissemination, hematogenous metastasis is also a common route for metastasis. A CTC count is, therefore, closely related to the overall survival (OS), progression-free survival (PFS), and post-operative recurrence of patients with HCC.

Sun[27] et al. collected the CTCs of 123 patients with HCC who underwent radical resection using the CellSearch technology. They considered that when the preoperative cell count in 7.5 mL blood ($CTC^{7.5}$) $\geq$2, the recurrence of tumors in these patients was earlier than that in patients with $CTC^{7.5}$ <2. They also considered that preoperative $CTC^{7.5}$ $\geq$2 is an independent prognostic factor for tumor recurrence. Luo[28] et al. collected CTCs and CTC clusters from the peripheral blood of 214 patients with HCC. They considered that the presence of CTC clusters in peripheral blood indicated a poor prognosis of HCC and was an independent predictor of OS and PFS.

However, Wang[29] et al. had different opinions. After studying 47 patients with HCC who underwent liver transplantation, they found that the proportion of CTC phenotypes changed, with the increased proportion of epithelial and mesenchymal CTCs. However, the changes in CTC counts and phenotypes were not significantly correlated with HCC recurrence.

6. Conclusion

As a form of liquid biopsy, CTCs hold great potential in facilitating the implementation of precision medicine in patients with HCC. CTC is also frequently used for early diagnosis, guiding treatment, and monitoring recurrence because of its non-invasive, comprehensive, and real-time features.

However, there are some limitations in CTC detection. Firstly, detection of CTCs in HCC is currently challenging. In recent years, many experts and scholars have studied and applied CTC in breast cancer, lung cancer, prostate cancer, colorectal cancer, and other diseases. Its application in HCC research is, however, not as relevant as in the aforementioned diseases. The main reason is the heterogeneity of liver cancer CTC and the occurrence of EMT in the process of metastasis, leading to the lack of highly sensitive and specific markers in the detection of liver cancer CTCs. Secondly, compared to tumor markers (represented by AFP), the cost of CTC detection remains high, which restricts its promotion to clinical application. Thirdly, there is no gold standard for the detection of CTCs and a variety of detection methods also lead to differences in data and conclusions because of the variance among the recovery rates.[30] In addition to looking for more efficient markers and effective detections, exploring the gene expression of CTC and its clinical significance may be one of the research directions for liver cancer CTCs in the future.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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