·Review·

Circulating tumor cells and individualized chemotherapy

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[Abstract] Circulating tumor cells (CTC) have been identified in peripheral blood from cancer patients especially those with metastatic lesions. Recently, the analysis of CTC has been developed rapidly and showed good prospects for individualized chemotherapy. The field of CTC research is very important since gene-expression profiling becomes feasible and real time when using CTC as the sample of evaluation. This review was to summarize present CTC detection, enrichment, or both methods and their contribution to individualized chemotherapy.

Key words: neoplasm, circulating tumor cells(CTC), individualized chemotherapy

Since it was proposed, the research on individualized chemotherapy for tumors has yielded a lot of results. Gefitinib has been found to be more applicable to patients exhibiting epidermal growth factor receptor (EGFR) mutation.[1,2] Mutation of V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) has been found to be related to cetuximab monoclonal antibody-resistance.[3] Expression levels of excision repair cross-complementing rodent repair deficiency, complementation group 1 (ERCC1) and breast cancer 1 early onset (BRCA1) genes can be used as platinum-resistant predictors for chemotherapy in patients with lung and stomach cancers.[4-7] However, all of these studies are based on patients whose tumor tissues are available. Because of the heterogeneity of tumors, the expression/mutation of tumor genes would change with medication use.[8,9] A recent large-scale study showed that 21% of the metastatic tumors of patients with breast cancer changed in their expression of the estrogen/progesterone receptor or HER-2 compared with primary tumors.[10] Therefore, monitoring and characterizing tumors in real time is needed to obtain better individualized treatments.

Circulating tumor cells (CTCs), identified more than 100 years ago, make this possible. At the 2008 ASCO meeting, researchers reported the use of CTCs to monitor gene expression in tumor cells and pointed out that gene expression indeed changed after chemotherapy. Of 14 patients with EGFR-positive metastatic colorectal cancer, only 13 were still EGFR- positive after four cycles of chemotherapy and, of 8 patients negative for EGFR, 7 became EGFR-positive after the chemotherapy.[11] As CTCs shed into the blood, it can provide us with potential real-time tumor specimens, so research on the characterization of CTCs has important clinical significance for implementing real-time individualized chemotherapy.

Circulating tumor cells

A CTC is a kind of tumor cell that can survive host immune destruction and present in the peripheral blood of patients with cancer. Although tumor metastasis is a complex process involving many factors, CTCs are still considered an important factor. A study model showed that an average of 1 million tumor cells would be released into the blood daily for every 1 g of tumor tissue,[12] but the survival rate of these tumor cells in circulation was very low.[13] For the vast majority of them, anoikis will occur. Only a few with strong invasive abilities could express inhibitors of apoptosis and survive.[14] These surviving tumor cells were CTCs. CTCs have extremely low levels in the blood, and it is generally believed that only a few of them exist in 1 mL of blood, while the number in blood of patients with advanced cancer is significantly greater.

With researchers focusing on CTCs, there are more and more ways to detect the presence of CTCs. These methods of CTC identification are based on characteristics of the tumor cells from different aspects. Some are identified according to cellular morphology, such as large cell volume, cell diameter greater than 25 μm, irregular nuclei, and a high nucleus/cytoplasm ratio.[15] Some are based on molecular biology, such as CK19, CK20, CEA, and guanylyl cyclase C (GCC) positive expression at the same time.[16] Some methods use immunochemistry, where cells bind with anti-epithelial-cell-adhesion-molecule (EpCAM)

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antibodies and those with cytokeratin-positive, CD45-negative expression in circulating blood are CTCs.[17] Some are a combination of different aspects taking both cell antigen expression and cell morphology into account.[18] Overall, different CTC verifying test methods have different standards, and there are no generally accepted standards for identifying CTCs.

The significance of CTCs to individualized chemotherapy

CTCs are potentially significant for individualized treatment, mainly due to the inherent deficiencies in the research-primarily, access to tumor tissues. So far, most studies are directed at patients who have had surgery, biopsy, or both. In practice, for patients with advanced cancer and nonsurgical patients, tissues obtained by biopsy are few, making it very difficult to detect gene expression or mutation. More importantly, by taking medication, gene expression or mutation can be changed and result in changes in drug sensitivity.[8,9] For example, some patients with non-small cell lung cancer with a good response to the tyrosine kinase inhibitors no longer respond to the inhibitors 1 year after initiating the therapy.[1,19] A secondary resistance may be due to the emergence of a secondary EGFR mutation, such as T790M.[8,9] So the tumor gene expression/mutation should be monitored in real-time way during treatment. However, in practical work, it's extremely difficult to obtain tumor tissues several times. For most patients, it's very difficult to find measurable lesions after resecting the tumor (unless the disease recurs). Even if there are measurable lesions, it is difficult to get enough tissue. In addition, obtaining tumor tissue requires invasive procedures, hardly applicable for conventional inspection. This is the greatest challenge for individualizing drug treatment.

In recent years, the questions of how to obtain CTCs in a simple and noninvasive way and how CTCs can serve individualized treatment have gradually received attention, and techniques established for CTC detection and collection undoubtedly provide possible solutions to this problem. In 2008, New England Journal of Medicine published a paper about detecting CTCs with the CTC-chip, and the authors detected not only CTC EGFR mutation in patients with lung cancer, but also gene mutations related to drug resistance in patients with drug resistance.[20] It can be predicted that a high sensitivity method for monitoring and characterizing CTCs may be adopted to guide the use of molecular-targeted drugs.

In addition, changes in the number of CTCs in cancer treatment might work as predictors for monitoring early metastasis and evaluating treatment effect.[21] In 2008, a large-scale phase 2 clinical study showed that CTC detection before either surgery or chemotherapy was an independent predictor for early recurrence in patients with cancer.[22] A study by Stathopoulou et al also showed that although patients with breast cancer had other indicators for good prognosis, if CTCs were detected, the patient's lifetime would be significantly decreased, suggesting that CTC detection of patients with early stage cancer may be an important evaluation index for whether adjuvant therapy is needed.[23]

Finally, with the use of highly sensitive detection methods, changes in CTC number may be used to assess response to treatment. Despite a limited number of research subjects, it was observed in the course of treatment that changes in the number of CTCs and the effect of treatment were significantly associated: treatment was effective when CTCs reduced in number and was ineffective when the CTC number increased.[20]

Research on CTC detection and collection technology in individualized chemotherapy

Of the many ways to detect CTCs, polymerase chain reaction (PCR) and reverse transcription-polymerase chain reaction (RT-PCR) are relatively common. Although the PCR technology continues to progress, which greatly increases the detection sensitivity of this method,[24,25] it is still difficult to detect very low levels of CTC gene expression/mutation. We will not go far into PCR technology but focus on technologies that might best serve individualized chemotherapy.

Density gradient centrifugation

CTCs can be enriched using density gradient centrifugation, by removing red blood cells and parts of granulocytes. Rosenberg et al.[26] detected CTCs in patients with gastrointestinal cancer using density gradient centrifugation. The detection rate was 30%, and the rate was significantly associated with distant metastases. The advantages of density gradient centrifugation are that it is simple, cheap, and fast, and the enriched tumor cells could be counted in smears and could also be used for detecting gene expression/mutation by PCR or RT-PCR. Its disadvantages are that the separated tumor cells are impure, and the separation efficiency is not high because there is a considerable number of tumor cell losses. According to the existing literature, this method is only used for CTC detection and comparison of the relationship between the detection rate and tumor stage. Unfortunately, there are no reports on the relationship between detection rate and overall survival in patients, nor are there reports where mutations are detected in tumor cells using this method.

Isolation by the size of epithelial tumor cells (ISET)

This method was first reported by Vona et al.,[27] based on the principle that the diameter of the vast majority of tumor cells is greater than that of blood cells. CTCs are retained by filtering peripheral blood through a polycarbonate membrane with a diameter of 8 μm. RT-PCR might fail to detect a CTC in 1 mL of peripheral blood, but ISET would not, indicating that ISET is more sensitive than RT-PCR. According to in vivo experiments, ISET could not only collect single tumor cells in blood, but could also collect the tumor microemboli. In 2007, Zheng et al.[28] improved the experimental materials, using a more advanced Parylene C membrane to separate CTCs. Compared with the polycarbonate membrane, the Parylene C membrane had a higher density of filtration pores, which were also more evenly distributed than those of the polycarbonate membrane. Besides, there was no fusion between filtration pores, thus providing better sorting, and in vitro experiments showed its recovery rate of

tumor cells were up to 90%.

Liver cancer CTCs were first isolated by ISET in patients with liver cancer. The corresponding criteria for identifying circulating hepatocellular carcinoma cells were morphological characteristics plus positive α -fetoprotein (AFP) staining. CTC detection was applied to 44 patients with early primary liver cancer by Vona et al.,[15] and 23 patients were identified with CTCs. Furthermore, the detection of CTCs was significantly related to tumor spread, portal vein tumor thrombus, and short survival time. According to the results of nested PCR detecting a total of 60 CTCs isolated from 10 patients, β -catenin mutations were found in 3 patients, while the β-catenin mutations have been reported to be related to tumor progression.[29,30] Vona's research is very valuable in that molecular analysis was implemented after gathering the CTCs, and it shed light on the later detection of gene mutation or expression in CTCs.

Breast cancer The ISET method has also been used to study breast cancer. HER-2 overexpression in breast cancer is an indication of the use of the molecular-targeted drug trastuzumab. Pinzani et al.[31] studied the DNA of CTCs harvested with the ISET and the results showed that the levels of HER-2 amplification of CTCs were consistent with those of pathological tissue sections in all 7 patients on study. Isolating tumor cells by ISET offers promise for individualized treatment-the detection of HER-2 amplification in the CTCs of patients with breast cancer could be used to guide the use of the molecular-targeted drug trastuzumab. Of course, this detection method still needs verification through experimental data and large-scale clinical trials.

The ISET method to obtain tumor cells is simple in principle, and the low cost, high sensitivity, and accessibility to further molecular detection of CTCs make it attractive for individualized cancer therapy. But so far, the tumor types studied by this method are very limited, and some types of tumor cells, such as small-cell lung cancer, with small sizes, do not fit for this method. In addition, the size of the CTCs in blood is not uniform, and some smaller cells can easily go undetected and may limit the application of this method.

Immunomagnetic separation

Immunomagnetic separation is now the most widely used method for CTC separation, and it is superior to density gradient centrifugation.[32] Its principle is that antibodies carried on the surface of magnetic beads can combine specifically with the tumor cells. While the magnetic beads are in a superparamagnetic state, cells combined with them can be separated by putting them in a magnetic field. CellSearch is a semiautomatic CTC instrument developed on this principle, and it has been approved by US Food and Drug Administration (FDA) for predicting disease-free survival and overall survival in patients with breast cancer. In recent years, CellSearch has also been used to detect CTCs in patients with other cancers.

Breast cancer Breast cancer was the first type of cancer studied by CellSearch. CellSearch is the only CTC detection instrument currently approved by the US FDA that can be used to predict disease-free survival and overall survival of patients with breast cancer. Before treatment, more than 5 CTCs detected in each 7.5 mL of blood indicated a poor prognosis for precure patients.[33,34] The latest research results also showed that the detection of CTCs was an independent predictor of early recurrence in patients with breast cancer undergoing surgical resection after neoadjuvant chemotherapy.[22] Another study found that as tumors progressed, HER-2 expression levels in breast cancer would change.[35] Therefore, monitoring the expression of HER-2 levels in CTCs of patients with cancer would likely provide a basis for doctors in clinical settings. In 24 patients with primary tumors with HER-2 negative expression, Meng et al.[35] found that HER-2 gene amplification was detected in the CTCs of 9 patients with disease progression. Of the 9 patients, 4 received Qu Herceptin-based monoclonal antibody treatment, 1 patient achieved complete remission and 2 achieved partial remission.

Prostate cancer Prostate cancer is a cancer with high incidence in Western countries, and the research of CTCs in prostate cancer is relatively rich. Prostate cancer is also a tumor with higher numbers of detected CTCs.[36] Shaffer et al.[17] examined CTCs of 63 patients with progressive hormone-resistant prostate cancer, and the results showed that they had an average of 16 CTCs separated from every 7.5 mL of peripheral blood. The majority of patients (65%) had more than 5 CTCs separated from every 7.5 mL of peripheral blood. As few as 5 CTCs could be used for molecular analysis, including EGFR expression, chromosome multiples, androgen receptor amplification, etc, thus helping us to understand the nature of the tumor to inform individualized treatment selection.

Other tumors Allard et al.[36] applied the CellSearch method to a larger sample across multiple diseases. In 2183 blood samples collected from 964 patients with metastatic cancer, an average of 60 CTCs were detected in each 7.5 mL of blood. Their article reported the average numbers of CTCs in a variety of patients with metastatic cancer (7.5 mL of blood for each patient): prostate cancer, 75; ovarian cancer, 6; breast cancer, 84; gastric cancer, 24; colorectal carcinoma, 4; bladder cancer, 42; kidney cancer, 1; lung cancer, 30; pancreatic cancer, 2; and unknown primary tumor, 16. Unfortunately, the article only reported the average CTC numbers in different cancer patients and did not go deep into the molecular analysis of those CTCs.

As a semiautomated measurement tool, CellSearch can reduce human error and can standardized results across multiple laboratories,[36] making this method conducive to clinical applications. Besides, CTCs more than a certain number can also undergo molecular analysis, thus CellSearch currently shows the most potential for clinical applications. However, tumor cell loss is considerable using CellSearch, and the separation efficiency is not high, with the purity of CTCs very low (0.01% −0.1%).[37] Therefore, it doesn't seem appropriate for individual patient tests.

Microchip technology (CTC-chip)

CTC-chip is first reported by Nagrath et al.[18] in 2007. They made a small chip of microcolumn containing EpCAM antibodies. Tumor cells can be retained when peripheral blood, without any treatment, pass through the chip with a specific device. The obtained cells were then stained with 4,6-diamidino-2-

phenylindole (DAPI), anticytokeratin antibodies conjugated with Rose Red, and a fluorescence-binding anti-CD45 antibody. Cells stained positive for anticytokeratin were CTCs , and CD45-positive cells were polluted blood cells. The detection sensitivity of this method was more than 99%, and the purity of obtained CTCs was up to 50%, two orders of magnitude higher than that of the CellSearch method, making this more conducive to molecular analysis.

Lung cancer CTC-chip approach has been studied most deeply in lung cancer. In 2008, CTCs of metastatic non-small cell lung cancer in peripheral blood were separated with the CTC-chip, and the detection of EGFR mutation, tyrosine kinase inhibitors resistance-related gene mutation T7908, were then applied to the isolated CTCs.[27] Due to its high CTC number (74 CTC/mL blood) and high purity, the detection rate of EGFR mutation was 92%. Furthermore, it could detect the tyrosine kinase inhibitor resistance-associated secondary mutation, which could serve to guide the use of molecular-targeted drugs. The changes in CTC number were significantly correlated with treatment effect during treatment, suggesting that this highly sensitive method to monitor changes in the number of CTCs may be used to evaluate the effect of cancer treatment.

Other tumors Though the CTC-chip is a new detection method lacking detailed reports, quantities of CTCs were isolated from peripheral blood from patients with metastatic lung, prostate, pancreatic, breast, and colorectal cancers.[18] Although there are no follow-up reports about the molecular analysis of these CTCs, in view of the fact that the feasibility of molecular analysis was affirmed, we have reason to believe that CTC-chip has enormous potential in guiding individualized drug treatment.

CTC-chip can separate tumor cells from the whole blood at one time and is more advanced to obtain the highest number of tumor cells with high purity than other technologies. It's the most conducive to use as an individual detection method among the existing technologies.

Problems and prospects

There is a considerable amount of published research on the study of CTCs in patients with cancer, yet few papers focus on CTCs for individualized treatment. This is mainly because individualized treatment is still a relatively new concept in the field of cancer treatment, with literature published only in recent years. Therefore, we only have a small number of researchers concerned with CTCs in the role of individualized chemotherapy. CTCs play a role in individualized treatment mainly through real-time detection of gene expression/mutation, which may be used to select an individualized course of treatment for patients with cancer. At the same time, the prognosis and treatment effect can also be predicted by detecting changes in CTC numbers. However, because there are only a few studies, more large-scale experimental and clinical research is needed to verify the status of CTCs in individualized chemotherapy. In addition, the role of CTCs in individualized treatment would also benefit from more published reports on individualized treatment overall.

Although the CTC detection methods vary, only CellSearch has entered clinical trials. Other methods are still in the research stage. What's more, not even CellSearch is popularized. There is still a long way to go if CTCs are to guide individualized chemotherapy. It's undeniable that CTCs have enormous research potential for individualized medicine in the future. We hope that CTC detection can serve clinical intervention and benefit an enormous number of patients with cancer in the near future.

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