Reviews

Role of Detecting Circulating Tumor Cell in Clinical Cancer Diagnosis and Therapy

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The concept of existence of circulating tumor cells (CTC) has a history of over 100 years and the settlement of these cells in other organs as disseminated metastatic tumor cells (DTC) has been well accepted[1]. There is a large body of research literatures focusing on CTCs during the past 15 years, and it has been reintroduced as a new method for cancer diagnosis and therapeutic monitoring recently[2].

Metastasis of solid tumors is a cascade event. Initially, tumor cells grow unregulated and lose their ability to adhere to each other. Cancer cells then penetrate to the blood and lymphatic circulations stimulated by their own angiogenesis factors. In the case of the blood circulation, CTCs circulate until they adhere to the vascular endothelia and subsequently settle in secondary or remote organs. Evidence that CTCs are originated from clones in the primary tumor suggests that they may reflect the tumor burden at all stages of tumor progression[3]. It is not known how long CTCs can survive in peripheral blood and the extent that they are able to form metastases[4]. It appears that the potential to grow into metastasis may be restricted to a small fraction of "tumor stem cells"[5]. A group of CTC-associated genes such as AGR2, FABP, S100A13, S100A14 and S100A16 etc. have been identified by gene expression profiling of CTCs from colorectal, prostate and breast cancer patients[6]. Comparing with current clinical methods to evaluate tumor metastasis, they may provide insights that could better understand characteristics of tumor metastasis and lead to development of both noninvasive diagnosis as well as therapeutic targets (Tab 1).

Tumor size and such change are monitored using imaging techniques. Sonography, computerized tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET) are the most commonly used approaches today. Imaging of tumor in patients provides a quick, non-invasive means of assessing the tumor status. However, tumors need to be at least 2-3 mm in size in order to be visualized with even the most advanced imaging technologies. During the imaging process, patients are subjected to radiation and this also creates a potential health hazard. Meanwhile, the high cost of these technologies makes it a significant hurdle to patients and reimbursement companies. Moreover, it is well known that tumor size detected by imaging techniques may not correlate with the malignancy or aggressiveness of cancers[7,8].

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Tab 1. Combinability improves tumor metastatic diagnosis

Tumor diagnosis tests	Advantages	Disadvantages
Serum markers	· Provide reference data in addition to imaging check for diagnosis · Low cost · Cancer screening or risk assessment · Suitable for public screening	· Highly specific marker unavailable · False positives · False negatives · Unknown or indirect correlation with disease status
Imaging	· Allow tumor visualization in patients	· Radiation hazard to patient · High cost · Tumor detection size limit 2 – 3 mm · Tumor size may not correlate to aggressiveness
Histopathology	· Good for final diagnosis · Define the TNM staging system	· Invasive · Cannot be used for monitoring · Incomplete information about metastasis
CTCs detection	· Noninvasive · Quantitative and qualitative · High sensitivity · Good metastasis indicator · Rapid evaluation of chemo and radiation therapy effectiveness · Evaluation of prognosis · Monitoring relapse · Low cost · Suitable for public screening	· Not for early detection · Few tumor specific antigen makers available · Both sensitivity and specificity of current available technologies need to be improved

Until recently the only means of finding metastasis before secondary tumors growing into a visible size by imaging is through histopathological staining and analysis of lymph node tissues excised during surgical removal of the primary tumor. Once nodal spread is confirmed by defining metastasis. However, negative results do not mean that the patient is absolutely free of metastasis. On the other hand, the invasiveness of the procedure precludes it as a means for regular monitoring of tumor progression, therapeutic effect or relapse.

Serum tumor markers such as prostate specific antigen (PSA) and carcinoembryonic antigen (CEA) etc. are widely used as a means to screen the general population for further tests. These antigens are released into the serum from primary or metastatic tumors, and their serum level are thought to be suggestive of tumor mass. However, this concept has been challenged[9]. On the other hand, these markers are not very specific and reliable. Many other pathophysiological conditions of the patient may also cause the same release of these markers and lead to false positive results. Additionally, not all tissue – derived tumors have available markers specific enough for tumor screening. Moreover, many tumor – associated transcripts can sometimes be expressed in normal cells[10,11].

The early metastatic relapses cannot be "visualized" until it reaches the minimal tumor size with current conventional image diagnosis. In order to provide more precise information for physicians to evaluate their therapeutic effects, it is, therefore, necessary to develop new strategies for monitoring rare circulation cancer cells. The examination of micrometastases of bone marrow, attention has been focused on the detection of cancer micrometastases from peripheral blood samples. It has been proposed that detecting single or small clusters of CTCs could provide a significant improvement of individual outcome prediction[12,13]. Obviously, detecting cancer cell at the rare or single – cell level in blood is much more sensitive than imaging analysis.

Apparently, detection of CTC can be applied to all metastatic solid tumors of epithelial origin. But how to accurately detect disseminated tumor cells is the most intriguing question which may be divided into two fundamental questions: how to obtain CTCs from peripheral blood and how to identify obtained CTCs.

How to obtain CTCs from blood? Almost all currently available technologies, such as those em-

ployed by Immunicon Corporation and AdnaGen AG are based on positive selection, in which, anti – tumor cell surface marker antibodies coated on magnetic beads recognize and capture the CTCs in patient blood preparation. One limitation of this technique is the availability and the quality of highly specific antibodies against tumor surface antigens. More importantly, tumors cells are well known to be heterogeneous, and since they are in poorly differentiated states, the expression of tissue – specific surface markers are often down regulated to result in theses cells escaping the enrichment process. Allard et al[14] used the CellSearch system to enrich and detect CTCs in 7.5 – mL of peripheral blood. Blood samples spiked with cells from a breast cancer cell line SKBR – 3 were used to establish analytical accuracy, reproducibility, and linearity. The average percentage of SKBR – 3 cells recovered was 85% at each of the spiking levels, a 7.5 – mL blood sample would have to contain, on average, 1.2 ± 0.4 CTCs[14]. It suggests that CellSearch system may be a quite accurate method to enumerate CTCs. However, in real patent, Pantel et al[15] estimated that only 64.5% of patients with breast cancer confirmed with bone marrow metastsis have detectable EpCAM positive tumor cells in their bone marrow preparation[16]. The inability to capture most cancer cells in most patients may lead to false negativity that result in wrong diagnosis.

Alternatively, negative selection has been used for enriching circulating cancer cells derived from different tissues. Processing peripheral blood by porous barrier density gradient centrifugation resulted in a 300 – fold enrichment of breast cancer cells. Real – time RT – PCR analysis confirmed a concomitant reduction in background expression of the CK19 and MUC1. For more sensitive and specific detection, it is desirable to deplete the great majority of background undesired cells including red and white blood cells, and results in an enriched sample comprising rare circulating cells. One such technology from AVIVA Biosciences Corporation (San Diego, USA) based on microchip and immunomagnetic beads has been successfully used to enrich circulating fetal cells from maternal peripheral blood[17]. Recently, this approach has been further optimized by AVIVA on different kinds of cancer cell lines (data not shown). Tab 2 lists different approaches to enrich and detect CTCs in peripheral blood[14,18,2,19].

Tab 2. Different approaches to enrich and identify CTCs in peripheral blood

	Positive selection	Negative selection
Principles	Capture circulating epithelial cells	Deplete peripheral blood cells
Cell enrichment markers	· immunomagnetic beads incubate with anti – epithelia marker (EPCAM, HEA125, MUC – 1, BerEP4) antibodies; · immunomagnetic beads incubate with anti – tumor marker antibodies (Her2 EGFR)	· immunomagnetic beads incubate with anti – leukocyte marker (CD45) antibody
Cell enrichment techniques	· magnetic affinity cell sorting (MACS system) · magnetic beads · ferrofluid – beads system	· magnetic separation · density gradients centrifugation; · microfiltration (Biochip)
Cell identification	· immunocytochemistry detection; · immunofluorescence detection; · nucleic acid based techniques (RT – PCR, real – time PCR)	· immunocytochemistry detection; · immunofluorescence detection; · nucleic acid beased techniques (RT – PCR, real – time PCR)
Sensitivity and Specificity	· False negatives due to heterogeneity · Specificity depends on method of detection and type of cancer	· Improved sensitivity · Specificity depends on method of detection and type of cancer
Reference	Allard et at, 2004; Ring et al, 2004; Racila et al,1998 and Zieglschmid et al, 2005	Baker et al, 2003; Ring et al, 2004; Yamanishi et al, 2002

How to identify CTCs from enriched samples? Following the enrichment, either a specific or combined approaches have been used to identify CTCs. Those methods include: 1) immunofluores-

cence imaging analysis for cell counting; 2) reverse transcription PCR (RT - PCR) to measure the mRNA level of cancer cell genetic markers; 3) analysis of mutations of tumor - related genes such as oncogenes and tumor suppressor genes; 4) DNA methylation assay[20]; and 5) functional activity assay such as telomerase assay[21].

Several tumor markers have been frequently used for CTC detection. Those markers are generally categorized by different cancer types, such as EpCAM, cytokeratin 8, 18, and 19[14], EGFR[22], mammaglobin[23], MUC - 1[24], HER - 2[25], Pan - cytokeratin[26], Cytokeratin c - erbB2[27], β - HCG, c - Met, GalNAc - T, MAGE - 3[28], Telomerase[29] and/or HER - 2/neu[14] have been used for breast cancer selection; uPAR for gastric cancer[30]; EpCAM, cytokeratins, PSA and PMSA for prostate cancer; and CEA, cytokeratin 20[31,32] and EGFR for colon cancer selections. Pantel et al concluded a list of different CTC makers[15,33,34,35,36,37,38,39] (Tab 3). Many epithelial cancer cells share some common epithelial markers such as EPCAM (epithelial - cell adhesion molecule) and GA733 - 2[40]; they have also been used as detection markers for epithelial cancer cells in blood. Since 90% of solid tumors are of the epithelial origin, these markers, once detectable in circulation are considered from tumor cells, especially for those who have already been diagnosed as cancer patients. Development of cancer type - related patterns of selection markers would likely promote CTC application. Furthermore, it will not be sufficient to simply characterize the primary tumor as a therapeutic target, but it is essential to include disseminated tumor cells into therapeutic target. Additional molecular and functional description of these cells will be essential to develop and select more efficient forms of systemic therapy[15].

Tab 3. CTCs magnetic separation markers

Primary tumor	CTC separation makers*
Breast cancer	Cytokeratin, c - erbB2, Pan - cytokeratin, Telomerase, mammaglobin, CK8, CK9, CK19 EPCAM, EGFR, β - HCG, c - Met, GalNAc - T, MAGE - 3, MUC - 1, HER - 2, p53 mutations
Colorectal cancer	CK7, CK8, CK19, CK - 20, KRAS mutations, MUC, guanylyl cyclase, CEA, CD44 splice variants
Prostate cancer	PSA, PSM
Pancreas cancer	RAS mutations
Skin cancer (melanoma)	p97, MAGE3, MUC18, Tyrosinase
Lung cancer	Microsatellite alterations, CK19
Stomach cancer	CK20
Live cancer	α - Fetoprotein, hTERT
Head and neck cancer	Microsatellite alterations

* CEA, carcinoembryonic antigen; CK, cytokeratin; EGFR, epidermal growth factor receptor; HCG, human chorion gonadotropin; MAGE3, melanoma - associated antigen 3; PSA, prostate - specific antigen; PSM, prostate - specific membrane antigen; hTERT, human telomerase reverse transcriptase; EPCAM, epithelial - cell adhesion molecule.

Recent studies performed by means of positive selection technologies indicated that in the case of breast cancer, the length of survival was directly correlated with the number of detectable CTCs in patients' peripheral blood[14,41]. Five CTCs per 7.5 ml blood has been established as a cut - off point. Patient at the point of diagnosis or after the first therapeutic regimen having more than 5 CTCs per 7.5 ml blood had a poorer prognosis of survival than those having less CTCs[41]. Since 2004, AdnaGen AG (Langenhagen, Germany) and Immunicon Corp. (Pennsylvania, USA) have developed products to detect and monitor CTC forcertain type of cancers after regulatory approval in Europe and US respectively. Normally breast cancer patients with HER - 2 - negative primary tumors are not suggested for Herceptin treatment. In a recent study, HER - 2 specific amplification from CTC preparation was found in a subset (9/24) of such patients. When these patients acquired Herceptin - containing therapy (4/9), one was found to have a complete response and 2 others had a partial response[42]. Such strategy may be used to personalize chemo - or radiotherapy regimens, and predict prognosis and

monitor metastasis relapse.

In summary, evaluation of circulating tumor cells has emerged as a highly informative diagnostic tool to effectively assist cancer diagnosis and therapeutic monitoring by providing valuable prognostic reference. Widespread usage of this technology is predicted as technology improvements make it more specific, sensitive and reliable, and suitable for both quantitative and qualitative evaluation for individualized tumor diagnosis and therapy.

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