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Review

Challenges in circulating tumor cell detection by the CellSearch system

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ABSTRACT

Enumeration and characterization of circulating tumor cells (CTC) hold the promise of a real time liquid biopsy. They are however present in a large background of hematopoietic cells making their isolation technically challenging. In 2004, the CellSearch system was introduced as the first and only FDA cleared method designed for the enumeration of circulating tumor cells in 7.5 mL of blood. Presence of CTC detected by CellSearch is associated with poor prognosis in metastatic carcinomas. CTC remaining in patients after the first cycles of therapy indicates a futile therapy. Here we review challenges faced during the development of the CellSearch system and the difficulties in assigning objects as CTC. The large heterogeneity of CTC and the different approaches introduced in recent years to isolate, enumerate and characterize CTC results in a large variation of the number of CTC reported urging the need for uniform definitions and at least a clear definition of what the criteria are for assigning an object as a CTC.

1. Introduction

Circulating tumor cells (CTC) are cancer cells that detach from their primary site during the process of cancer metastasis. They enter the circulatory system, migrate through the body and can form secondary tumors at distant sites. If CTC are present, can be isolated and characterized they represent a minimally invasive source of spreading tumor cells and may serve as a liquid biopsy for management of cancer patients. CTC are however rare events compared to the number of hematopoietic cells, therefore, their detection and enumeration is technically challenging.

At present the CellSearch system is the only validated method for CTC detection that has been cleared by the U.S. Food and Drug Administration. The CellSearch system, designed for the enumeration of CTC in 7.5 mL of blood, was first introduced in 2004 where the analytical accuracy, reproducibility, and linearity of the system was shown (Allard and Terstappen, 2015; Allard et al., 2004). There are various challenges when isolating and enumerating CTC, in this review

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these challenges will be discussed using the CellSearch system as an example.

2. Early evidence for circulating tumor cells

Q3 Circulating tumor cells were first reported by Ashworth (1869). He described the presence of tumor cells with similarities to the cells from the primary tumor, in the blood of a man with metastatic cancer. Engell (1955) described the occurrence of cancer cells in peripheral blood and in the venous blood that drained the tumor during operation and observed a larger frequency of tumor cells in the draining vein as compared to the peripheral blood. Evidence for CTC in the blood from patients with metastatic and primary carcinoma was found by immunohistochemistry staining several decades ago. Moss and Sanders (1990) found evidence for CTC in 7 out of 10 neuroblastoma patients with known disseminated disease by immunostaining. In 1993, CTC were identified with conventional cytology and cytokeratin staining in patients with colorectal cancer by Leather et al. (1993). They isolated tumor cells from 42 patients undergoing resection for colorectal cancer, using a density gradient followed by cytospin and showed immune histological evidence for CTC in 4 of these patients. In the 1990s, peripheral blood progenitor cells were increasingly used for autografting after high-dose chemotherapy. Brugger et al. (1994) made the observation that tumor cells were detected in blood of a portion of breast cancer, small cell and non-small cell lung cancer patients before mobilization of peripheral blood hematopoietic progenitors and discovered an increase after the mobilization. Braun et al. (2000) reported that the presence of tumor cells in bone marrow was associated with poor prognosis. These studies provided important information that tumor cells could be detected by traditional immunochemistry techniques but also lacked the sensitivity to be used in larger multi-center studies.

3. Challenge of rare events detection

Tumor cells in blood are present in a high background of hematopoietic cells and are found in frequencies in the order of 1–10 CTC per mL of whole blood in patients with metastatic disease (Miller et al., 2010). One of the problems one faces in the development of assays to detect these rare cells is that one does not know whether tumor cells are present, and if so at what frequency. To test whether the developed methods are working, known numbers of cells from cancer lines are spiked in blood and the efficiency of the method is than evaluated by the determination of the number of cells observed after the procedure. A variety of cell lines should be tested in optimization of the methods. For example, cell lines with different densities of the target antigen, such as the epithelial cell adhesion molecule (EpCAM), for methods based on immune selection. Or a range of sizes, stiffness and densities when methods based on physical differences between hematopoietic cells and tumor cells are used. A frequent oversight is the challenge to accurately detect the "rare cell" among all the others. This is visualized in Figure 1, which shows the probability distribution of two cell populations. A lognormal distribution for both staining intensities was assumed. Panel A shows two cell populations present in equal numbers and they can be easily discriminated from each other. In panel B the number of stained cells is reduced to 1 in 1000 and 48.9% of the "rare" cells can no longer be discriminated. In panel C this ratio is changed to 1:10.000 and 70.3% of the cells can no longer be discriminated. In panel D this ratio is changed to 1:1.000.000 and in this case 96.2% of the cells can no longer be discriminated. To improve the separation one could improve the staining intensity. The use of for example Phycoerythrin (PE) instead of Fluorescein isothiocyanate (FITC) conjugated antibodies will improve the separation from autofluorescence due to higher quantum yield of the PE fluorochrome as compared to FITC. The limitation will however still be the number of antigens present on the cell. Amplification of the signal by for example increasing the antibody concentration or adding a secondary antibody to boost the signal will however also give rise to an increase in the background. Consideration of the frequency of the cell, that one needs to identify, is thus of utmost importance for the approach taken with the identification of the cells (Shapiro, 2003; Terstappen, 2000; Tibbe et al., 2007).

The use of multiple markers is therefore a requirement for "rare" cell detection. One of the first techniques used for the detection of CTC in whole blood was flow cytometry. Gross et al. (1995) reported a flow cytometric assay, which allowed for the detection of cancer cells in blood by using multiple markers, each containing a different fluorophore. They showed that detection of cells, down to a frequency of 1 in 10^7, is possible if 4×10^8 peripheral blood mononuclear cells (PBMCs) are analyzed. They used an approach to stain the unwanted subpopulation of the cells with one exclusion color and stain the rare cells of interest with one, two, or three different remaining colors. The drawback of this method is the large sample volume that needs to be analyzed, thereby limiting the number of samples that can be analyzed. In addition, the instrument has to be stable, the parameter settings have to be set in advance and cell settling and clumping must be avoided during the measurement.

The problems due to a large sample volume can be avoided by the enrichment of the tumor cells by either depletion of the leukocytes or selection of epithelial cells targeting for example the EpCAM antigen. The latter approach was reported by Racila et al. (1998). In this study, ferrofluids were labeled with antibodies targeting the EpCAM antigen, incubated with 20 mL of whole blood and immunomagnetically enriched, followed by fluorescent labeling with a nucleic acid dye, PE-conjugated anti-cytokeratin (CAM5.2) and peridinin chlorophyll protein (PerCP)-labeled CD45 and analyzed by multiparameter flow cytometry. It was shown that cells of epithelial origin defined as nucleic acid$^+$, CD45$^-$ and cytokeratin$^+$ could be detected in patients with metastatic and organ confined breast and prostate cancer, whereas only few epithelial cells were detected in healthy controls. Using this assay a first indication was obtained that the presence and changes in these "epithelial cells" related to the clinical status of the patient and response to therapy (Moreno et al., 2001; Racila et al., 1998; Terstappen et al., 2000, 1998).

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