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Circulating tumour cells in breast cancer: Prognostic indicators, metastatic intermediates, or irrelevant bystanders? (Review)

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Abstract. Circulating tumour cells (CTCs) have been of considerable interest for many years. The rarity of these cells presents the main challenge associated with their analysis. Current detection methods use antibody and nucleic acid techniques and are sensitive for CTC detection but limited in their utility by the occurrence of false-positive results. Despite this, there are a number of clinical studies which show that the presence of CTCs is an important prognostic indicator, particularly in the metastatic setting. Current efforts to phenotype CTCs may provide a valuable insight into the metastatic process and may also allow the development of specific CTC-targeted treatment strategies in the future.

Contents

1. Introduction

Despite the substantial improvements in the development of effective treatments for breast cancer that have occurred over the last few decades, up to 30% of breast cancer patients still succumb to the disease in the ten-year period following diagnosis (Cancer Research UK, 2005). The development and growth of distant metastases are the major causes of these deaths. Although a great deal of research has been conducted, it remains difficult to accurately predict which patients will develop metastatic disease and over what time scale. The risk of metastasis is usually estimated by factors such as tumour size and grade, oestrogen and progesterone receptor status, Her2 overexpression and the number of axillary lymph nodes containing cancer cells. Numerous studies have shown this final factor to be the most important prognostic indicator, as a large number of metastatic nodes is significantly associated with poor disease-free and overall survival (1-4). These data were extrapolated to support the belief that metastasising breast cancer cells first disseminate to the lymph nodes before reaching the peripheral blood and distant sites. However, it is now clear that the dissemination of breast cancer cells occurs in up to 50% of patients with lymph nodes that appear to be tumour-free; consequently, up to 30% of patients with lymph node-negative disease develop metastases within five years (5-7). Additional pathways of metastatic spread exist, and many studies have demonstrated the presence of circulating tumour cells (CTCs) in the peripheral blood of patients with early-stage breast cancer (8,9). These observations suggest that breast cancer cells with metastatic potential may be shed from the primary tumour into the blood early in disease progression. Dissemination of these cells in the haematogenous system, bypassing the lymphatic system, may represent an important metastatic mechanism. This is especially, but not only, the case in node-negative breast cancer patients. However, the clinical significance of detecting such CTCs in the peripheral blood of breast cancer patients is still unclear. The purpose of this review is to discuss the suitability of the different methods available for detecting CTCs. This is a critical issue for the reproducibility of such analyses, the clinical insights their detection may give, and the evidence for their role as metastatic intermediates.

2. Detection of CTCs: Rare needles in large haystacks

A review of the literature from 1996 to 2008 reveals approximately 400 publications reporting on CTCs in breast cancer. The vast majority of these are concerned with methods of detection, with only a few addressing the clinical utility of CTCs and even fewer investigating their mechanistic role in metastases. CTCs - even in patients with the highest levels - are rare, occurring at a frequency of up to ten cells per millilitre of blood. Typically, this equates to one CTC per

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$1x10^{(5-7)}$ peripheral blood mononuclear cells (10). The rarity of CTCs presents the main challenge in their analysis. Methods must permit the detection of these rare epithelial tumour cells with sufficient sensitivity, while maintaining the specificity to disregard the vast excess of haematopoietic cells. A wide range of detection methods, utilising each of the main characteristics that separate CTCs from haematopoietic cells, have been attempted, including those focusing on the use of cell morphology, differential cell densities and tumour cell-specific markers.

In the 1950s and 1960s, identification relied on morphology using light microscopy (11). However, this was associated with an unacceptably high false-positive rate (12). In addition, such microscopic examination of individual samples allowed little potential for the development of high-throughput assays. Physical separation of CTCs from the blood is possible for the enrichment of CTC samples, thus aiding in their detection. Separation on the basis of differential cell densities resulted in the recovery of 10-65% of cultured tumour cells spiked into whole blood (13). Immunomagnetic separation techniques allowed a recovery of up to 85% (14). However, the variability in these rates of recovery, and the fact that many CTCs were potentially lost, suggests that these techniques are impractical in terms of the quantification of CTCs, although their use may reduce false negatives if only a positive or negative readout is required (15).

Immunohistochemistry is currently used to identify isolated tumour cells in the lymph nodes of breast cancer patients. However, this routine practice is not transferable to the detection of CTCs, which are several orders of magnitude less common within samples of involved peripheral blood than tumour cells are within the lymph nodes. It is impossible to designate a blood sample as CTC-negative without examining hundreds of slides. Techniques based on flow cytometry or immunofluorescent microscopy have an advantage in that a larger volume of blood can more conveniently be examined. However, their use is limited by false-positive results due to the non-specific staining of haematopoietic cells (16); a false-positive rate of 1-3% occurs depending on the antibody used (7). Polymerase chain reaction (PCR) techniques, with their obvious potential for increased sensitivity, provide attractive alternatives. Reverse transcriptase-PCR has been used to detect CTCs by means of the epithelial- or breast cancer-associated mRNA transcripts they express. These transcripts include those for cytokeratins (17), mammoglobin (18), mucins (MUC-1) (19) and carcinoembryonic antigen (20). However, the high sensitivity of PCR confers an inherent tendency to produce false-positive results.

Cytokeratins, a family of genes encoding ~30 separate structural proteins, are the markers of choice for CTC detection using RT-PCR as they are expressed at relatively high levels in epithelia and epithelial tumours, but rarely in other tissues. Cytokeratin 19 (CK19) is the most commonly used marker for the detection of CTCs in breast cancer patients as, of all the cytokeratins, it seems to be the most frequently expressed in breast tumour cells (6,18,21). However, reports vary regarding the specificity of CK19, and a number of potential sources of false positivity have been identified in RT-PCR studies. CK19 may be induced in certain peripheral blood cells by cytokines and growth factors, which circulate at higher concentrations under inflammatory conditions and neutropenia (21). In addition, two CK19 pseudogenes, CK19a and CK19b, have been identified (22,23). Pseudogenes are non-functional copies of genes arising from the integration of reverse transcripts of mRNA into the genome. The pseudogenes CK19a and CK19b are potential sources of false positivity for CTC assays based on the detection of CK19 transcripts using RT-PCR, as any genomic contamination of mRNA preparations may lead to the amplification of pseudogene DNA. In addition, we have found that CK19a may be expressed as an apparently non-functional mRNA (Hogan and Hughes, unpublished data). Primers used for RT-PCR analysis need to be carefully designed to avoid the detection of these pseudogenes. In the case of CK19, a great deal of work was invested in order to design an assay with sufficient specificity (24).

However, the limitations of single marker assays are now recognised and, more recently, multimarker analyses are being performed to identify complementary markers. These, when used in combination, increase the sensitivity of CTC detection. For example, in 2007 Xi *et al* found that the combination of MGB2 and either CK7 or CK19 provided a very high sensitivity/specificity assay (25). Similarly, in the same year, Nakagawa *et al* developed a multimarker assay using three target mRNAs: stanniocalcin-1 (STC-1), N-acetylgalactosaminyl transferase (GalNacT) and melanoma antigen gene family-A3 (MAGE-A3) (26). The presence of CTCs was significantly associated with the stage of disease, and the assay was demonstrated to be sufficiently sensitive for the detection of CTCs in early-stage breast cancer. Previously, CTCs in this setting were rarely detected reproducibly. The use of multiple markers may help compensate for both tumour cell heterogeneity in marker expression and for the infrequency of CTCs in blood (especially in early-stage breast cancer).

Most recently, a new technology called the CellSearch System has been developed. This uses a combination of methods for the detection of CTCs, involving two independent layers of detection for the enhancement of specificity. Initially, circulating epithelial cells are marked with immuno-iron particles used for magnetic separation. Fluorescently-labelled monoclonal antibodies specific for leukocytes (CD45-allophycocyan) and epithelial cells (cytokeratins 8,18,19-phycoerythrin) distinguish epithelial cells from leuckcytes. The CellSearch System was originally approved by the FDA in January 2004 as a diagnostic tool for identifying and counting CTCs in blood samples to predict progression-free and overall survival in patients with metastatic breast cancer. In November 2007, it was approved by the FDA as an aid in monitoring metastatic colorectal cancer and, in February 2008, this was extended to include patients with metastatic prostate cancer. To date, there is no evidence to support its use in diagnosing breast cancer or in monitoring patients with early stages of the disease.

In summary, as each of the many different assays published has some drawbacks, a clear consensus on which is the most reliable for CTC detection remains to be drawn. It is interesting to note that a recurring issue with many of the techniques is the frequent detection of false positives: the apparent detection of CTCs in control individuals who lack (known) tumours. While it is reasonable to assume that these are false positives - that

ividuals did not actually have circulating tumour evidenced by the fact that they did not subsequently present with tumours - it may be that they did in fact have circulating epithelial cells. It remains to be seen whether certain normal physiological processes or non-cancer pathologies can result in healthy individuals displaying a low number of circulating epithelial cells. Consequently, many of the assays described may accurately detect their targets, but may lack the required specificity for cancer cells as opposed to epithelial cells.

3. Prognostic significance of CTCs

Despite the difficulty involved in reliably detecting CTCs, there have been a number of convincing studies in which their levels provided prognostic insights before or during treatment of primary and metastatic breast cancer cases. In 2003, Garforio *et al* reported the detection of CTCs in 92 patients using antibodies directed against cytokeratin 7/8 (8). The presence of cytokeratin-positive cells in patient blood before chemotherapy correlated with poor disease-free (p=0.058) and overall (p=0.003) survival at a median follow-up of 21 months. Weigelt *et al* (2003) investigated the presence of circulating tumour cells in metastatic breast cancer patients by studying the mRNA expression of CK19, p1B, PS2 and EGP2 by quantitative PCR (27). Patients with detectable CTCs had poorer disease-free and overall survival at 2 years than CTC-negative patients (17 vs. 36%; p=0.0053). In 2004, Cristofanilli *et al* reported the use of the CellSearch System to quantify CTCs in patients with metastatic breast cancer. The presence of five or more CTCs in 7.5 ml of blood before any treatment was administered was correlated with a shorter disease-free (2.7 vs. 7.0 months; p<0.001) and overall (10.1 vs. >18 months; p<0.001) survival than the presence of less than 5 CTCs (28). In support of this conclusion, in 2006 Hayes e*t al* demonstrated that the detection of a similar threshold of CTCs at any time during treatment served as an accurate indication of subsequent rapid disease progression and mortality for metastatic breast cancer patients (29). While these reports are somewhat consistent, in that each suggests that the presence of CTCs is a marker of poor prognosis, there are striking differences between the studies in the proportions of patients determined as positive for CTCs and in the actual levels of CTCs detected. Whether these differences are related to the technologies employed or to the cohorts selected remains difficult to determine.

While the detection of CTCs is a prognostic indicator in metastatic breast cancer, its clinical utility in early-stage disease remains uncertain. In 2008, Pachmann *et al* reported the detection of CTCs in 90% of non-metastatic breast cancer patients using laser scanning cytometry (30). CTCs were quantified in whole blood taken before or during adjuvant chemotherapy. The authors concluded that CTCs are strongly influenced by systemic chemotherapy and, importantly, that an increase of >10-fold in their levels at the end of therapy is a potent predictor of relapse. Using a quantitative RT-PCR assay based on the expression of a number of markers, in 2007 Nakagawa *et al* detected CTCs in 43% of patients with early-stage breast cancer (26). The detection of CTCs correlated significantly with the stage of the disease (p=0.0007) and predicted the presence of axillary metastases (p=0.012). Also in 2007, Ignatiadis *et al* reported an investigation of the prognostic value of cytokeratin 19 mRNA-positive circulating cells in early-stage breast cancer, focusing on clinically relevant subgroups based on oestrogen receptor and Her2 expression (31). They found that the presence of detectable CK19 mRNA in the blood was correlated with the development of early metastasis within the first five years of disease onset in patients with ER-negative, but not ER-positive, tumours. The reasons for this are not immediately apparent, but may be linked to both biological factors and responses to therapy. Further prospective clinical trials are needed to determine the clinical usefulness of CTCs, especially in the early-stage breast cancer setting.

It is worth noting that the current guidelines of the American Society of Clinical Oncology (2007) explicitly state that the measurement of CTCs should not be used to reach a diagnosis of breast cancer or to influence any treatment decisions in patients with the disease. Partly as a consequence of this, there are no studies showing that the routine use of a CTC test has an impact on prolonging survival or improving the quality of life of breast cancer patients.

4. Are CTCs pre-metastatic cells?

The mechanisms by which CTCs become true metastatic cells are poorly defined at best. What is known is that if this process occurs, it is highly inefficient. An estimated $1x10^6$ tumour cells are released into the bloodstream on a daily basis (32). The majority of these are destroyed by the immune system (33), while many more are destroyed by haemodynamic forces (34). A very small proportion of extravasated CTCs (~2%) are capable of dividing and forming micro-metastases, while an even smaller number (0.02%) can evolve into fully-fledged distant tumours (35). This highlights a key obstacle in the clinical use of CTC detection. Current technologies make no attempt at differentiating between CTCs that have metastatic potential, as opposed to those that are merely detectable. Recent advances in phenotyping and genotyping CTCs have allowed some investigation of heterogeneity among CTCs and also between CTCs and primary tumours. Of particular note is the observation that ErbB2-positive CTCs are detected within the blood of patients with primary tumours having low ErbB2 scores (36). Continuing this line of investigation, Barok *et al* (2007) showed that ErbB2-positive CTCs may be sensitive to ErbB2-targeted (trastuzumab) treatment, even when the primary tumour is not responsive. (37). Traditionally, management and treatment options for breast cancer, particularly in the adjuvant setting, have been determined by the characteristics of the primary tumour itself. These new studies offer the prospect of specifically targeting the circulating, and therefore potentially metastatic, population of tumour cells.

5. Concluding comments

Our understanding of the biology of CTCs and their contribution to the development of metastases is currently very limited. This is in large part due to the difficulties associated with reliable CTC detection. A potential clinical role for their

routine detection was demonstrated in the monitoring of response to treatment in the metastatic setting. However, this is not likely to become common practice unless the assays become more robust and less expensive. The development of new detection methods with high sensitivity and specificity will be critical in making CTC detection a useful clinical tool for the analysis of early breast cancer, and may even present a novel instrument for screening women at high risk of breast cancer. However, further large-scale prospective clinical trials are necessary before CTC detection can be incorporated into routine clinical practice. On a mechanistic level, recent advances have been made in phenotyping CTCs. These may provide detailed insight into the metastatic process, and may permit direct exploration of CTC-targeted treatment strategies (4).

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