Circulating tumour cells: the evolving concept and the inadequacy of their enrichment by EpCAM-based methodology for basic and clinical cancer research


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Increasing evidence suggests that circulating tumour cells (CTCs) are responsible for metastatic relapse and this has fuelled interest in their detection and quantification. Although numerous methods have been developed for the enrichment and detection of CTCs, none has yet reached the ‘gold’ standard. Since epithelial cell adhesion molecule (EpCAM)-based enrichment of CTCs offers several advantages, it is one of the most commonly used and has been adapted for high-throughput technology. However, emerging evidence suggests that CTCs are highly heterogeneous: they consist of epithelial tumour cells, epithelial-to-mesenchymal transition (EMT) cells, hybrid (epithelial/EMT+) tumour cells, irreversible EMT+ tumour cells, and circulating tumour stem cells (CTSCs). The EpCAM-based approach does not detect CTCs expressing low levels of EpCAM and non-epithelial phenotypes such as CTSCs and those that have undergone EMT and no longer express EpCAM. Thus, the approach may lead to underestimation of the significance of CTCs, in general, and CTSCs and EMT+ tumour cells, in particular, in cancer dissemination. Here, we provide a critical review of research literature on the evolving concept of CTCs and the inadequacy of their enrichment by EpCAM-based technology for basic and clinical cancer research. The review also outlines future perspectives in the field.

Key words: circulating tumour cells, circulating epithelial tumour cells, circulating EMT+ tumour cells, circulating hybrid tumour cells, circulating irreversible EMT+ tumour cells, circulating cancer stem cells

introduction

Although the risk of cancer death is linked to the stage of the disease at the time of diagnosis, >90% of cancer deaths result from the development of disseminated metastases [1–4]. Currently, the mechanism of genesis of metastases from solid tumours is not fully understood and remains one of the most enigmatic aspects of cancer biology. New insights are fast causing paradigm shifts in our understanding of metastatic phenomena. For instance, recent evidence indicates that tumour cells disseminate at a relatively early stage of the natural history of tumour growth [5, 6]. Current tumour staging procedures and high-resolution imaging technologies are not sensitive enough to detect micro-metastases or early tumour cell dissemination, the key events in tumour progression. Therefore, detection of disseminated tumour cells (DTCs) might be complementary to the current imaging procedures used for tumour staging and improve the identification of cancer patients at high risk of metastatic relapse [7–10]. This view has spurred interest in the development of sensitive assays that allow the specific detection of single DTC in cancer patients. Since sampling for the detection of DTCs is an invasive procedure, therefore, the focus in recent years has shifted to circulating tumour cells (CTCs) in the peripheral blood.

Over the years, many procedures have been developed for the detection of CTCs in the peripheral blood, yet none has reached the ‘gold’ standard of sensitivity and more importantly, of specificity. Epithelial cell adhesion molecule (EpCAM) is expressed ubiquitously, albeit at variable levels, in epithelial cells and their cancers, but is absent in blood cells [11–13]. This galvanized our group to pioneer the development of first-ever use of EpCAM-based enrichment followed by reverse transcription-polymerase chain reaction (RT-PCR) for detecting CTCs in cancer patients [14]. The use of our anti-EpCAM-labelled microbeads for this purpose was later commercialized as Epithelial Enrich™ (Dynal Biotech, Norway). Among the current EpCAM-based technologies, the automated CellSearch® system (Veridex, Warren, NJ, USA) has gained considerable attention over the past several years [8]. The system has been cleared by the Food and Drug Administration for monitoring CTCs in metastatic breast, colon, and prostate cancers [15–17]. Currently, the EpCAM-based approach is one of the most commonly used in exploring CTCs, and a great deal of effort and resources has been invested in adapting the method for modern automated high-throughput
technologies [18–24]. Numerous studies from our laboratory [6, 25, 26] and others have shown that the presence of elevated levels of CTCs, as determined by the EpCAM-based approach including the CellSearch® system, is negatively correlated with prognosis in patients with various types of cancers. However, the relatively low sensitivity and specificity of the approach is a significant concern [27–29]. Furthermore, increasing evidence suggests that CTCs are highly heterogeneous [10, 30–36]. They consist of epithelial tumour cells, epithelial-to-mesenchymal transition (EMT) cells, hybrid (epithelial/EMT+) tumour cells, irreversible EMT+ tumour cells, and circulating tumour stem cells (CTSCs). The EpCAM-based approach does not detect CTCs with low EpCAM levels and non-epithelial phenotypes such as CTSCs and those that have undergone EMT [37–39]. Thus, the approach may lead to underestimation of the significance of CTCs, in general, and CTSCs and EMT+ tumour cells, in particular, in cancer dissemination.

Although several reviews have been published on the subjects of EpCAM in tumorigenesis [13, 40] and clinical utilization of CTCs for prognosis of cancer, currently, there is no review on the suitability of EpCAM-based enrichment methods for the detection of CTCs. Therefore, our aim was to provide a critical and updated review of research literature on the evolving concept of CTCs and the inadequacy of their enrichment by EpCAM-based methodology for basic and clinical cancer research. We also outline future perspectives in the field.

**circulating tumour cells**

CTCs are defined as tumour cells that originate from primary tumours, recurrences, or metastases, circulate freely in the peripheral blood, and possess antigenic and genetic tumour-specific characteristics [29]. They encompass all types of cells, single or clusters of a few to dozen [41–44]. The escape of cancer cells into the circulation occurs as cell strands or sheets [45, 46]. It is estimated that 1 million cells per 1 g of tumour tissue can spread daily into the blood stream [47].

The presence of CTCs in patients with cancer was first reported >140 years ago in 1869 at autopsy [48] and in 1955 in vivo [49]. The molecular characterization of CTCs is considered a real-time ‘liquid biopsy’ that might contribute to the development of improved individualized therapy and monitoring response to the treatment of cancer patients [7, 10, 50]. Hence, a great deal of effort and resources is invested in developing methods of CTC detection. As several excellent reviews on existing approaches to the enrichment, isolation, and characterization of CTCs have already been published [10, 50–56], a comprehensive overview of these methods is beyond the scope of this article. Broadly, as outlined in Figure 1, the enrichment methods are based on the properties which distinguish CTCs from abundant blood cells: (i) physical properties (size, deformability, density, and electric charge) and (ii) biological characteristics (surface protein expression, viability, and invasive capacity). As shown in Figure 2, biological characteristics are mainly used in immunological procedures with antibodies against either tumour-associated antigens (positive selection) or the common leucocyte antigen CD45 (negative selection). The positive selection using an anti-EpCAM antibody allows isolation of cells that express EpCAM only. The method does not detect CTCs expressing low levels of EpCAM and non-epithelial phenotypes such as CTSCs and those that have undergone EMT and no longer express EpCAM. Also, the isolated CTCs have reduced viability. This is because their release requires relatively strong conditions to break the ionic bond between EpCAM on the cell surface of CTCs with anti-EpCAM antibody. Of note, culturing of CTCs isolated by positive selection without their separation from nano-sized, non-toxic, and biodegradable microbeads, commercially available as CD326 (EpCAM) MicroBeads (Miltenyi Biotec), has been reported [57]. On the other hand, CTCs isolated by physical methods and negative selection contain epithelial and non-epithelial phenotypes, including CTSCs and EMT+ tumour cells, and are relatively more viable. Of note, all the circulating cells isolated by physical methods and negative selection are not necessarily tumour derived. They may represent normal blood vessel or stromal cells, circulating mesenchymal cells or stem cells, or other host cells that normally exist in rare quantities in the circulation [58]. Therefore, unlike positive selection procedures that provide reasonably pure epithelial CTCs, those isolated by physical methods and negative selection are of mixed types and invariably impure and need further purification for downstream applications such as immunophenotyping, genomic profiling, and in vitro culturing. CTCs are characterized, as outlined in Figure 3, with a broad spectrum of procedures. These include immunocytochemical methods (flow cytometry, EPISPOT assay, and immunostaining), molecular biological methods (qPCR, fluorescent in situ hybridization, and comparative genomic hybridization).

CTCs have now been reported in a majority of epithelial cancers, including those from head and neck [59], breast [60], lung [61], colorectal, gastric, pancreatic [62], renal cell, urinary bladder, and prostate cancers [63]. Their presence has been demonstrated to be an independent adverse prognostic factor in metastatic [15, 64–68] and early breast cancer [69], metastatic [16, 67, 70, 71] and early colorectal cancer [72], metastatic [67, 73] and castration-resistant prostate cancer [17, 74, 75], resectable non-small-cell lung cancer [76, 77], and squamous cell carcinoma of the head and neck [78]. These findings are confirmed in recent meta-analyses, which
also include our three studies [79, 80]. Although patients with metastatic lesions are more likely to have CTCs in their blood, these have also been reported in some localized cancers [54]. All these studies support a critical role for CTCs in tumour progression and metastases. As a result, it is now hypothesized that targeting CTCs during their haematogenous transport could lead to effective interruption of the metastatic cascade and ultimately, reduction of cancer morbidity and mortality [81].

Despite huge endeavours, detection of CTCs is still technically challenging. This is mainly due to their paucity, plasticity, and genotypic and phenotypic alterations [9, 56, 82], which might be caused by multiple intrinsic and extrinsic factors. CTCs are quite rare [83–85]. Their identification and characterization requires extremely sensitive and specific analytical methods, which are usually a combination of enrichment and detection procedures. Our understanding of biological characteristics of CTCs has also been hampered by the non-availability of techniques capable of isolating them in sufficient numbers and under conditions that are compatible with detailed molecular and functional experiments. Currently, there are many unresolved issues towards the routine clinical use of CTCs in cancer patients [29, 55, 56]. Briefly, these include accuracy, sensitivity, and specificity of the techniques, the optimal cut-off for CTC enumeration, optimal makers for CTC identification, and the ability to determine the cell condition (viable or apoptotic, dividing or non-dividing). Unfortunately, there is a great inter-laboratory variability...
in the methods employed for CTC investigations [10, 50–56], and no tumour maker identified so far is specific enough to detect all these rare cells.

**EpCAM for the detection of CTCs: short-lived forays**

Originally discovered in 1979, EpCAM was first described as a dominant antigen in human colon carcinoma [11]. Subsequent studies identified EpCAM to be a type I trans-membrane, 314 amino acid long, 39-42 kDa glycoprotein that functions as a homophilic, epithelial-specific intercellular cell-adhesion molecule [40, 86, 87]. Although the precise function of EpCAM remains largely unknown, recent data suggest that its role is not limited to cell adhesion as it is also involved in cellular signalling, cell migration, proliferation, and differentiation [13, 88–91].

An important development for detecting CTCs was the discovery in mid-1970s that while EpCAM was expressed at variable levels in epithelial cells and their cancers, it was absent in blood cells [11–13]. This galvanized our group to pioneer the development of the first-ever use of EpCAM-based enrichment followed by RT-PCR for detecting CTCs in cancer patients [14]. Furthermore, clinical trials using anti-EpCAM monoclonal antibodies slowed progression and prolonged survival in patients with metastatic colorectal carcinoma [92]. This paved the way for further development of immuno-affinity enrichment methods for the purification and detection of CTCs in the management of cancer patients. At present, the USA Food and Drug Administration approved [15–17] automated EpCAM-based CellSearch® system has widespread acceptance in the clinic [8] and is considered the ‘gold standard’ for all new CTC detection methods [77, 93]. Currently, as shown in Figure 4, there is focus on anti-EpCAM antibody in the development of microfluidic devices (‘chips’) for the single-step isolation of CTCs from unprocessed blood samples [18–24]; validation of these assays is still ongoing. The main advantage of the microfluidic devices is that they can handle cell numbers and sample volumes at least 10 times smaller (<1 ml of blood) than those used in flow cytometry, minimizing time and the use of expensive staining reagents. However, the detection of CTCs, as rare events, is always hampered by the problems of the Poisson statistics, and the analysis of larger blood volume (at least ≥7.5 ml) might be preferable, particularly, in early stage cancer patients with a small burden of CTCs. Thus, technologies that can handle larger blood volumes still occupy a special place in routine diagnostics.

Recently, based on a Seldinger guidewire [94], a novel EpCAM-based single-step method for the isolation of CTCs from unprocessed blood samples was reported [95]. The device was successfully used to isolate CTCs across all tumour stages, including early stage cancer in which distant metastases were not yet diagnosed [95]. This in vivo method has the advantage of fishing for CTCs in a much larger volume instead of using only a small blood sample. Although it is an exciting development, further studies from independent laboratories are required to ascertain its biocompatibility and sensitivity and specificity for the isolation of CTCs.

Numerous studies from our laboratory and others have shown that the presence of elevated levels of CTCs, as determined by the EpCAM-based approach including the CellSearch® system, is negatively correlated with prognosis in patients with early [96] and metastatic breast [64, 97–99], colon [6, 16, 25, 26], prostate [17, 100, 101], non-muscle-invasive bladder [102], hepatocellular [103], ovarian [104], neuroendocrine [105], small-cell [106, 107], and non-small-cell lung [77, 108, 109] cancers. Although the CellSearch® system is the most standardized of any current technology and is now being tested for clinical applications, it has not yet been shown to have a predictive capacity to drive therapeutic decision-making. The CellSearch® system suffers from relatively low sensitivity: only a fraction of patients with metastatic cancers score positive for any CTCs, with a median yield of ~1 CTC per ml and typically low purity [50, 110, 111]. A 3.3-fold loss of EpCAM+ CTCs as detected by the CellSearch® has been attributed to the enrichment and staining procedure [112]. The problem is compounded as only one CTC in 40 establishes metastatic foci, and only 1 in 100 micro-metastases form a tumour [113, 114]. This metastasis inefficiency of CTCs has been attributed mainly to anoikis [42, 115–118] and destruction by immune system cells either in the blood stream or after extravasation [119, 120]. The relatively low sensitivity and specificity of the current EpCAM-based detection of CTCs is a significant concern [27–29]. Thus, while the potential of using CTCs to guide patient treatment remains promising, the rate-limiting step for their widespread use in the clinic remains the lack of robust and high-throughput technologies for the detection and isolation of these rare cells.

**CTCs: variations in the expression levels of EpCAM**

There is now increasing evidence that CTCs remain undetected by conventional EpCAM-based methods in one-third of

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**Figure 4.** Schematic representation of microfluidic-based affinity capture of circulating tumour cells (CTCs) from the peripheral blood of cancer patients. Anti-epithelial cell adhesion molecule antibodies are functionalized on microposts to capture CTCs using a microfluidic chip while blood cells flow through freely.
Recent findings support the hypothesis that EMT-like shifts on tumour cells and CSCs occur in CTCs well before pre-invasive lesions [147–149] and metastatic spread [78, 149–160]. For instance, CTCs with a hybrid (epithelial/EMT+) phenotype were reported in patients with metastatic non-small-cell lung cancer [161], early and MBC [149, 152], and advanced prostate cancer [152]. Higher incidence of CTCs expressing EMT-related proteins, such as vimentin and TWIST1, was found in metastatic disease compared with early stage breast cancer patients [149]. This over-expression of EMT markers on CTCs was often accompanied by the presence of stem cell markers ALDH1 and CD44 in breast [162–164], colorectal [165], and renal cell carcinomas [166]; CD133 in castration-resistant prostate cancer [152] and CD44 in squamous cell carcinoma of the head and neck [78]. The existence of a subpopulation of CTCs with stem cell-like CD44+CD24− phenotype and ALDH1 expression was described in MBC [167]. CTCs with stem cell phenotype CD133+CXCR3+, CD26+, or CD133− were reported in metastatic prostate [158] and colorectal cancers [160, 168]. In addition, CTCs from patients with primary or MBC were shown to express receptors and activated signalling kinases of the EGFR/HER2/P13K/Akt pathway [169], one of the major signalling pathways involved in the regulation of mammary stem/progenitor cells, promoting the proliferation and inhibition of apoptosis [170]. Furthermore, CTCs in primary breast cancer patients, like their tissue stem cell counterparts [171], were reported to be mostly triple-negative estrogen receptor−, progesterone receptor−, and HER2+ [172].

From a clinical point of view, EMT markers on CTCs occur more frequently in metastatic than in early stage breast cancer [149] and allowed more accurate prediction of worse prognosis than the expression of epithelial markers alone [39, 121, 163, 173]. EMT markers are also prognostic in MBC patients undergoing high-dose chemotherapy followed by autologous haematopoietic stem cell transplantation [174].

CTCs: presence of irreversible EMT+ tumour cells

Recently, a subpopulation of CTCs that has undergone EMT and became fixed in the mesenchymal lineage, such that it can no longer return to the epithelial phenotype, has been reported [175]. The subpopulation represented a more differentiated CSC EMT phenotype and its role in tumour progression remains uncertain [175]. Therefore, it is very important to differentiate between these two classes of EMT+ cells to ensure that the correct phenotype is being characterized for cancer research and for any other studies aimed at determining the prevalence of EMT+ cells in CTCs.

CTCs: presence of organ-specific mimetism

To add another layer of complexity, transcriptome analyses reveal that each time DTCs and CTCs reach a new niche (distant organs, for example, bone, liver, or lung), they undergo an organ-specific mimetism and may leave the site with a new organ-specific signature [176]. Therefore, as shown in Figure 5, CTCs shed in the peripheral blood either from primary tumour or from metastatic organs are highly heterogeneous, potentially with a variable capacity to establish overt metastases.
It remains unclear whether subpopulations of cells in DTCs and CTCs with epithelial, hybrid, EMT+ tumour cell, or CTSC properties are the actual founder cells of overt metastases. Further studies are needed to clarify this issue using \textit{ex vivo} expansion, cell-tracing, and xenotransplantation.

concluding remarks

Although the precise composition of CTCs remains largely unknown, overwhelming evidence suggests that they are highly heterogeneous. Epithelial markers such as EpCAM are down-regulated in more plastic EMT+ tumour and CTSCs. Therefore, EMT+ tumour and CTSCs in CTCs will be lost using immuno-affinity enrichment (such as used in the CellSearch® System, the AdnaTest, or other EpCAM-based procedures) that relies solely on the level of EpCAM expression on the cell surface. This necessitates that more strategies need to be developed for the isolation of CTCs and more importantly, the automated CellSearch® system cannot be considered as the reference method for all new CTC detection technologies. This view is supported by the observations that (i) epithelial and dedifferentiated (EMT+, stem cell phenotypes alone or both) phenotypes of CTCs were detected in early breast cancer patients [177], (ii) bi-phenotype (epithelial/EMT+) and EMT+ phenotypes of CTCs were detected in a mouse model of pancreatic cancer [147], (iii) EpCAM+ and cytokeratin+ CTCs were isolated from the peripheral blood of cancer patients [178], (iv) CTSCs were enriched in EpCAM−CD45− fraction of CTCs [179], (v) EMT+ tumour cells were detected in patients with undetectable CTCs using the CellSearch® system [179], and in addition (vi) significantly higher numbers of CTCs were detected in cancer patients using EpCAM-independent compared with EpCAM-dependent enrichment technology [39, 44]. All these suggest that a mixture of EpCAM+ and EpCAM− tumour cells circulate in the blood of cancer patients. Therefore, studies using anti-EpCAM antibody-based methods may lead to underestimation of the significance of CTCs, in general, and EMT+ tumour and CTSCs, in particular, in cancer dissemination.

Cells from breast cancer cell lines that lack EpCAM frequently express CD146, a subset marker of primary breast cancers which correlate with poor prognosis [180]. CD146+ CTCs were also detected in MBC patients [181]. Therefore, in attempts to detect EpCAM− CTCs, the combined use of anti-CD146 and anti-EpCAM antibodies has been advocated [181]. Likewise, combining anti-EpCAM and anti-cytokeratin antibodies has been demonstrated to compensate for CTCs, which have low or missing expression of either EpCAM or cytokeratins [182, 183]. More recently, adding anti-CD49f to anti-cytokeratin antibodies was demonstrated to enhance the detection of CTCs involved in EMT-associated processes, such as drug resistance and metastasis [184]. Other well-documented cell surface markers of circulating epithelial tumour cells are CD176 [185], EGFR [35, 78, 169], HER2 [35, 163, 164], and MuCl [124, 162, 164, 186]. CTSCs are also characterized by surface expressions of CD26 [160], CD44 [78, 155, 157, 167, 187–189], CD133 [152, 158, 168, 190–192], CXCR4 [157, 158, 160], and nestin [193]. Likewise, circulating EMT+ tumour cells exhibit surface expression of
N-cadherin [78, 152], O-cadherin [152], vimentin, [78, 149, 152, 157, 163, 194], and fibronectin [163]. Therefore, future studies may also involve combinations of anti-EpCAM with antibodies against the above-mentioned surface markers of circulating epithelial tumour cells, CTSCs, and EMT+ tumour cell phenotypes.

More recently, denasking of EpCAM by treatment with Tween\textsuperscript{1} has been demonstrated to improve the recovery of CTCs with low EpCAM levels in breast cancer patients [195]. Since the sensitivity of the capture of CTCs is significantly dependent on the anti-EpCAM antibody clone [126], a simultaneous use of different anti-EpCAM antibody clones has also been suggested [196]. While all these approaches are very promising, future results from several independent laboratories will determine their use, if any, for basic and clinical cancer research.

**future directions**

It is abundantly clear that EpCAM-based positive selection for enrichment of CTCs is inadequate for basic and clinical cancer research. This raises an important question: has the time come to substitute the technique with unbiased negative selection as has been advocated [39, 78, 197, 198] or perhaps simply employ strategies that isolate CTCs based on size, density, surface charge, deformability, or other physical characteristics [199–208]. Future studies investigating CTCs should also explore denasking of EpCAM and combinations of anti-EpCAM antibody clones with other antibodies against surface markers of circulating epithelial tumour cells (such as CD176, EGFR, HER2, and Muc1), CTSCs (such as CD26, CD44, CD133, CXCR4, and nestin), and circulating EMT+ tumour cell (such as N-cadherin, O-cadherin, vimentin, and fibronectin) phenotypes. We believe that the sensitivity and more importantly specificity of EpCAM-based approach could be significantly improved by including surface biomarkers of epithelial tumour, CTSC, EMT+, and hybrid cell subpopulations. This approach is worth pursuing as there is plethora of literature, suggesting that elevated levels of CTCs, as determined by the EpCAM-based approach including the CellSearch\textsuperscript{2} system, are negatively correlated with prognosis in patients with various types of cancers. Similar literature for the physical methods and negative selection approach is eagerly awaited.

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