Chapter 11

Importance and Detection of Epithelial-to-Mesenchymal Transition (EMT) Phenotype in CTCs

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Abstract

The current dogma is that epithelial-to-mesenchymal transition (EMT) promotes circulating tumour cell (CTC) formation and is ultimately a driver of metastasis. There is also accumulating evidence that EMT-phenotype changes are commonly associated with therapy resistance. Thus, capturing EMT-phenotype CTCs is expected to yield important clinical information in regard to prognosis and response to therapy as well as allowing the study of metastatic processes. However, the isolation and identification of EMT-phenotype CTCs with commonly used isolation/detection methods are suboptimal, and current efforts on improving the isolation of EMT-phenotype CTCs are associated with pitfalls that need to be overcome. This chapter explores the significance of EMT in CTC formation and the role of EMT in cancer metastasis and resistance to therapy. We also comprehensively review the past and current limitations of evaluating EMT phenotypes in CTC isolation and analysis and discuss how CTCs can be seen in a more holistic fashion as important biomarkers for clinical management.

Keywords: CTC, EMT, vimentin, immunomagnetic cell isolation, metastatic disease

1. Introduction

Circulating tumour cells (CTCs) were first discovered by the Australian pathologist Thomas Ashworth in 1869, who described single cells and cell clusters in a patient's blood and proposed a role for CTCs in the metastatic process [1]. Recently, due to improved CTC detection techniques, these cells, together with circulating tumour nucleic acids (ctNA), are emerging as attractive, accessible, non-invasive biopsies to guide the best therapy for a

patient's cancer. CTC counts are closely related to cancer progression and stage, and there is mounting evidence from studies on prostate-, breast-, colorectal- and other cancers that CTCs have prognostic value (reviewed by Caixeiro et al. [2]).

In essence, CTCs are very rare cells, and usually only between 0 and 30 CTCs can be isolated from a 5–10-ml blood sample of a cancer patient; although for some patients, CTC counts can be considerably higher. Isolation technologies allow enrichment and separation of CTCs from the millions of surrounding blood cells by initial gradient centrifugation or red blood cell lysis followed by further enrichment of CTCs due to their physical properties or by employing antibody-based negative or positive enrichment techniques (reviewed by Yu et al. [3]). Enrichment steps are followed by CTC identification primarily by immunocytostaining. The most common CTC identification pattern relies on positive staining for nucleated cells (4',6-diamidino-2-phenylindole (DAPI) or Hoechst staining) and cytokeratin (CK; positive CTC marker) associated with a lack of CD45 staining (negative CTC marker, expressed on leucocytes). Advances in single cell analysis technology have contributed to maximise the information that can be gained from CTCs isolated from a single blood sample. Tumour biomarkers such as gene amplification, mutation, rearrangement and expression can be successfully analysed while CTC protein levels can be determined. There are high expectations that CTC-based assays will find utility for clinical testing, guiding therapy and monitoring treatment in the not-too-distant future (reviewed by Becker et al. [4]). However, cancer cells, including CTCs, are extremely heterogeneous, and therefore, isolating a representative range of CTCs remains difficult.

A particular challenge is the capture of CTCs that have undergone epithelial-to-mesenchymal transition (EMT) [5, 6]. EMT and its reverse, the mesenchymal-to-epithelial transition (MET), are reversible phenotypical changes that allow a cell to form either dense epithelial structures with tight interaction to neighbouring epithelial cells or, by undergoing EMT, to loosen interactions with other cells and become more mesenchymal and migratory. The ability to undergo these changes is important for cells during development to allow the migration of cells and the formation of different tissues. Cancer cells that are able to take advantage of these processes and undergo EMT are proposed to be more motile and consequently are more likely to become CTCs by entering the blood stream [7]. Not surprisingly, EMT-phenotype cancer cells are linked to the presence of metastases. Additionally, cancer cells that have undergone EMT tend to be distinctly more resistant to chemo and radiation therapy [8]. Consequently, the detection and analysis of EMT-phenotype CTCs appear necessary to fully harness CTC information about a given cancer and monitor disease evolution; yet, we are still poorly equipped to detect these cells. Currently, most methods to isolate CTCs, and nearly all current approaches to identify CTCs, rely on the presence of epithelial cell markers. CTC isolation predominantly relies on immunomagnetic targeting of the epithelial cell adhesion molecule (EpCAM), but this epithelial glycoprotein diminishes during EMT, thereby compromising the effectiveness of this strategy [5, 6]. The identification of CTCs usually involves immunocytostaining for epithelial proteins of the cytokeratin protein family, which are similarly downregulated during EMT [9]. Equally problematic is the method of probing for EpCAM, which is frequently used to identify CTCs after size exclusion enrichment [10].

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In this chapter, we summarise the current understanding of EMT in CTC formation, detection of EMT markers in CTCs isolated by common methods and their limitations, and new approaches to better isolate and identify EMT-phenotype CTCs (EMT-CTCs). The clinical relevance of detecting EMT-CTCs is also discussed.

2. EMT in CTC formation

The role of EMT in the metastatic process has been controversial mainly because cells in metastatic tumours often display epithelial rather than mesenchymal characteristics, despite the presence of cells with mesenchymal features in the primary cancer. The recognition that EMT is a reversible process has led to a model adaptation, which postulates that EMT reversal, termed MET, has to occur after extravasation to allow motile cancer cells to resettle and form metastases [11] (**Figure 1**). Regardless of robust *in vivo* data that show increased metastasis associated with an EMT phenotype in the primary tumour, experimentally tracking EMT or MET in the metastatic process remains challenging [12].

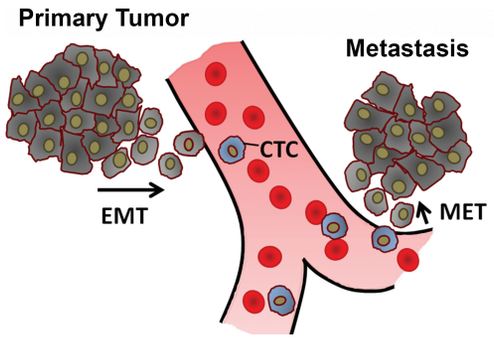

Figure 1. EMT in CTC formation. Simplified illustration of cells in a primary tumour undergoing EMT changes, which enable them to disseminate from the primary cancer, intravasate into the blood stream and travel as CTCs before extravasating the vascular system and, by undergoing MET, regain the ability to form a metastatic tumour.

Several elegant studies and *in vivo* evidence (mainly from mouse models) show that EMT aids tumour cell dissemination and promotes intravasation into the vascular system (CTC formation). MDA-MB-468 breast cancer cells that can be driven to undergo EMT by epidermal growth factor (EGF) exposure were used in a severely compromised immunodeficient (SCID) mouse xenograft model. Xenografts in this model lead to lung and liver metastases, and a peak in CTC counts coincided with the appearance of cells strongly staining for the EMT marker vimentin in the initial xenograft. Vimentin was also expressed in CTCs and CTC clusters, suggesting that EMT promoted CTC formation [13]. Another study, in which KRAS-pancreatic tumour model mice were treated with cerulein to induce pancreatitis and EMT changes in the cancer cells, showed significant increases in CTC counts [14]; however, this finding was not corroborated in a more recent study involving a similar model [15]. A role for EMT in CTC formation was further substantiated when a squamous cell carcinoma prone mouse model with targeted transcription factor Twist1 induction confirmed that Twist1 caused tumour cells

to undergo EMT, and this was associated with a doubling of CTC counts as well as increased metastasis [16]. Recently, an innovative model of endothelial cells that form vascular-like structures *in vitro* was used to show that SW620 colorectal cancer cells could migrate into these 'vessels', especially when hypoxia-induced EMT was triggered [17]. A concept supporting the notion that mesenchymal properties afford cancer cells some protection in circulation suggests that any cells shedding from a tumour without undergoing EMT might undergo stressful, traumatic events required for the interruption of the strong epithelial cell-cell interactions resulting in reduced viability [18]. Additionally, EMT-phenotype changes are generally thought to reduce sensitivity to stress signals that would normally lead to apoptosis [8]. Taken together, these observations underpin the emerging opinion that EMT-CTCs may comprise a more viable, aggressive tumour cell population than epithelial CTCs, and go some way to explain the association of EMT-CTCs and increased metastasis. In that regard, it is worth noting that transforming growth factor β (TGFβ), which is commonly released by platelets, may promote or maintain EMT in CTCs while in the circulation and promote extravasation [19]. The current understanding of the role of CTCs in establishing distant metastatic sites was recently reviewed and is beyond the scope of this chapter [2, 20].

3. EMT phenotypes in CTCs isolated by EpCAM targeting

It is important to emphasise that, despite the epithelial nature of EpCAM, CTCs isolated by EpCAM targeting can display markers of EMT. This is due to the fact that changes between epithelial and mesenchymal phenotypes are dynamic, and cells can be found in intermittent stages and express mesenchymal as well as epithelial markers at the same time. Accordingly, mesenchymal markers have been successfully detected in CTCs after EpCAM-based isolation. For instance, intermittent EMT phenotype characterised by co-expression of mesenchymal proteins vimentin, N-cadherin and CD133 with epithelial markers EpCAM, CK and E-cadherin was shown in breast cancer and prostate cancer CTCs isolated by EpCAM targeting [21]. EpCAM-based breast cancer CTC isolation also yielded cells with common gene expression of the EMT markers TGFβ1, FOXC1, CXCR4, NFKB1, VIM and ZEB2 [22]. Moreover, higher breast cancer staging correlated with mesenchymal vimentin and fibronectin expression in EpCAM-enriched CTC samples. Interestingly, vimentin and fibronectin expression was also detected in 31 of 92 (34%) of patient samples, which were CTC negative according to the common CTC definition ($DAPI^+$, CK^+, $CD45^-$) but not in samples from healthy control individuals, suggesting the presence of CTCs lacking CK in some patients [23].

4. EMT phenotype in CTCs isolated with alternate strategies

The inability of EpCAM-based CTC isolation to optimally account for EMT-CTCs with EpCAM loss has led to the targeting of alternative, EMT-associated cell-surface markers, for CTC enrichment, or by avoiding these methods altogether and focussing on CTC enrichment due to physical cancer cell properties, mainly size exclusion. Not surprisingly, when EpCAM-

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based immunomagnetic CTC isolation was directly compared with size exclusion CTC enrichment of parallel blood samples from 40 NSCLC patients, CTCs were isolated from a higher proportion of patients (80 vs 23%) by size exclusion, and as expected the isolated cells tended to lack EpCAM; however, they expressed CK and had elevated levels of the EMT-associated epithelial growth factor receptor (EGFR) and thus likely were tumour cells [24].

An elegant approach to account for the CTCs missed during EpCAM-based capture in HER2-positive breast cancer patients utilised CD45 immunomagnetic depletion of blood cells after an initial EpCAM-based CTC capture, to further enrich the remaining EpCAM-negative CTCs. The EMT-linked transcription factors SNAI1 and ZEB1 were more commonly expressed in these EpCAM-negative cells that were likely tumour-derived cells compared to the EpCAM-isolated counterparts [25]. Vimentin, best known for its functions as a cytoskeletal support protein, can also be present on the cell surface of mesenchymal cells and has been successfully targeted in immunomagnetic isolation of CTCs from colorectal cancer patients and breast cancer patients. After CD45 immunodepletion, CTCs were positively selected with cell-surface vimentin (CSV) targeting. The authors suggest that CSV expression is restricted to cancer cells, and CSV targeting isolates significantly more CTCs from colorectal cancer patients with progressive disease than those with stable disease; moreover, higher CSV-CTC counts were more commonly found in therapy-resistant patients. In a direct comparison of CSV- and EpCAM-based CTC isolation (CellSearch CTC platform) in breast cancer patients, CTCs isolated with CSV targeting were a more reliable marker for progressive disease compared to stable disease. In both CSV-isolated breast and colorectal cancer CTCs, the EMT markers FOXC2, SNAIL, Twist-1 and Slug tended to be highly expressed while E-cadherin and EpCAM levels were low. The CSV antibody is currently not commercially available, thus limiting its

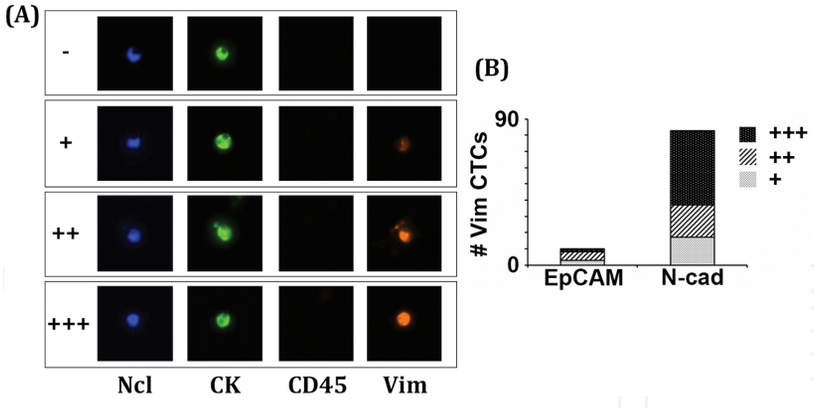

Figure 2. EMT analysis in isolated CTCs. Quadruple staining to detect CTCs with EMT phenotype according to levels of the EMT marker vimentin. (A) CTCs were identified by nuclear Hoechst staining 'Ncl' (blue, Fluxion enumeration kit) and cytokeratin (CK) staining (green; FITC-conjugated anti-cytokeratin antibody: clone c-11, Sigma-Aldrich) as well as exclusion of CD45 expression (red, antibodies: CTC enumeration kit, Fluxion). Vimentin staining, 'Vim' (orange, AF647-conjugated anti-vimentin antibody: clone V9, Abcam), in CTCs, was scored as indicated: Staining of a representative cell for each category, negative (−), weak (+), positive (++) and strongly positive (+++), is depicted. (B) Cell counts for vimentin-positive EMT-CTCs isolated from a representative patient sample using EpCAM- versus N-cadherin- 'N-cad'-based isolation of CTCs using the quadruple staining assay. CTCs were isolated with the IsoFlux CTC isolation platform using EpCAM-based or N-cadherin-based immunomagnetic isolation with the Rare Cell Isolation Kit, Fluxion.

EMT markers	Main finding/clinical correlation [reference]
EpCAM-based immunomagnetic isolation:	
EpCAM, CK, E-cadherin, vimentin, N-cadherin, CD133	CTCs with intermittent EMT phenotype are common in advanced prostate- and breast cancer [21]
CK	Low CK level CTCs were correlated with receptor negative metastatic breast cancer and with poorer OS [30]
Vimentin	Vimentin expression in prostate cancer CTCs is associated with decreased OS [31]
TGFß1, FOXC1, CXCR4, NFKB1, VIM, ZEB2	Gene expression of EMT markers shown in CTCs [22]
HER2, VIM, FN1	Presence of EMT-CTCs correlates to disease stage [23]
CK, VIM, FN1	The presence of EMT-CTCs correlates to shorter PFS [32]
EpCAM-based isolation combined/compared with other methods:	
Cell-surface vimentin (CSV)	EMT-CTC numbers were a more reliable progressive disease marker for breast cancer patients when isolated targeting CSV versus EpCAM [27]
Vimentin, twist, ZEB1, ZEB2, snail, slug and E-cadherin	The expression of both twist and vimentin in CTCs was significantly correlated with portal vein tumor thrombus in liver hepatocellular carcinoma [33].
CK, EpCAM	Most CTCs from metastatic breast cancer patients showed intermittent phenotype while 16% of patients had EpCAM- only CTCs and 33% EpCAM-null CTCs [34]
CK, EpCAM, CDH1, FN1, CDH2	Combined EpCAM/EGFR/Her2-based CTC isolation was linked to increased EMT-CTC numbers in metastatic breast cancer patients with disease progression [35]
TWIST1, SLUG, SNAIL1, ZEB1, FOXC2	EMT-CTCs were more common in primary breast cancer patients with poorer prognostic markers (ie needed neoadjuvant treatment) [36]
Targeted immunomagnetic isolation (non-EpCAM):	
Cell-surface vimentin (CSV)	>5 >5 EMT-CTCs more common in progressive colorectal cancer [26]
CD45-based immunomagnetic blood cell depletion:	
Vimentin, twist, CK	EMT -CTCs more prevalent in CTCs from metastatic breast cancer patients [37]
EpCAM, CK, VIM	CTCs of 'CTC-negative' NSCLC patient by EpCAM based isolation were tumorigenic in mice [29].
Isolation by cell size:	
Vimentin, CK	Pancreatic cancer CTCs without CK or vimentin are more commonly in patients with lymphnode metastasis [38].
CK, EpCAM,VIM, TWIST	Proportion of EMT-CTCS is linked to response to therapy in gastric cancer [39]
EpCAM, CK, VIM, TWIST	Intermittent phenotype and EMT CTCs were predominant in hepatocellular carcinoma and correlated with metastasis [40]

OS: overall survival, PFS: progression-free survival. Dependent on their nature, EMT markers follow protein or gene nomenclature

Table 1. Detection of EMT biomarkers in CTCs.

use and confirmation of the data by others [26, 27]. A study that investigated gene expression in ovarian cancer CTCs showed the expression of EMT markers in most individual CTCs from three patients, while only 30% of these cells also expressed epithelial CK5 or CK7. However, all CTCs expressed epithelial MUC1 [28]. In a patient with non-small cell lung cancer who was CTC-negative according to EpCAM-based (CellSearch) enrichment, it is worth noting that CTCs enriched by CD45 depletion were tumourigenic in mice and CTCs isolated from the same patient by size exclusion showed predominant EMT or intermittent phenotype [29].

In our laboratory, we embarked on the isolation of CTCs from advanced ovarian cancer patients using N-cadherin-based immunomagnetic isolation and captured approximately three times more $CTCs^-$ when using N-cadherin-based versus EpCAM-based CTC isolation (data not shown). We also developed an assay to probe CTCs for vimentin as marker of EMT, which showed that N-cadherin-based CTC isolation from advanced ovarian cancer patients increased the capture of EMT-CTCs (**Figure 2**). Studies that investigated EMT markers in CTCs, isolated by various strategies, are compiled in **Table 1**.

5. The pitfalls of non-EpCAM-based CTC isolation

The clear advantage of EpCAM-based CTC isolation is the observation that EpCAM is only rarely found on cells circulating in the blood stream of healthy individuals, resulting in a limited number of false-positive 'CTCs'. In our hands, using the IsoFlux CTC platform and EpCAM-based enrichment, the average $Hoechst^+$, CK^+, $CD45^-$-false positive 'CTCs', obtained from 10 healthy blood donors is 1.8 per 9 ml of blood with a range of 0–5 cells. By contrast, the greatest problem with the use of EMT markers for CTC isolation or CTC identification, or with CTC isolation techniques relying on physical cell properties such as size and plasticity, is the increased risk of detecting false-positive 'CTCs'. This is the case because some rare cells found in normal blood can express a number of epithelial and mesenchymal markers. For instance, circulating endothelial cells (CECs) can be found at varying numbers in blood samples of healthy individuals (0–29/ml blood) [41] and increased numbers in cancer patients [42]. CECs do not only express cytokeratin, but typical EMT markers such as N-cadherin, EGFR, vimentin and fibronectin [43–48]. Moreover, circulating endothelial cells tend to be above 10 μm in diameter [49], and some endothelial cells might therefore not be excluded from size-based CTC enrichment. There are currently limited data evaluating potential CEC contamination in either filter-enriched CTC samples or samples enriched by positive or negative immunotargeting. However, it is likely that the inclusion of CECs in CTC counts in the literature (i.e. false-positive CTCs) has inadvertently led to overestimation.

A particularly interesting approach to avoid the issues surrounding EpCAM is the use of CD146 (MCAM)-based immunomagnetic CTC isolation. Elevated expression of CD146 has been reported for melanoma, breast-, ovarian- and prostate cancer [50], and CD146-based immunomagnetic CTC isolation was reported for breast cancer and melanoma patients [44, 51]. However, CD146 is also an endothelial marker used to define and target CECs [41]. Thus, CD146-based CTC isolation needs to be complemented by cancer-specific CTC identification,

such as Melan-A for melanoma CTCs, for example. Alternatively, there is a need to distinguish co-purified CECs from CTCs using specific endothelial markers not expressed on cancer cells. The endothelial marker CD34 has been used to distinguish CD146-enriched breast cancer CTCs from CECs [44]. Whether CD34 is the most reliable or specific marker to distinguish true CTCs from false positives still needs to be confirmed.

Our preliminary data suggest that the accumulation of false positives, most likely endothelial cells, is also an issue when using N-cadherin-based immunomagnetic CTC isolation. While CTC numbers isolated from advanced ovarian cancer patients were approximately four times higher than EpCAM-isolated CTC numbers, we also detected more Hoechst$^+$, CK$^+$, CD45$^-$ false-positive 'CTCs' in the blood from seven individuals without any history of cancer (data not shown). N-cadherin, EGFR and cytokeratin expression of endothelial cells suggest that targeting these proteins in CTC isolation or identification might lead to similar problems. Moreover, other cells in the circulation, such as monocytes, macrophages and neutrophils, also express the EMT markers EGFR, vimentin and N-cadherin. Further, tumour-associated macrophages of breast cancer and prostate cancer patients were also shown to express cytokeratin and therefore could be confused with CTCs [52–55]. Thus, while non-EpCAM-based CTC isolation techniques appear to produce higher CTC counts and favour isolation of CTCs with EMT features, they also may enrich for false-positive cells, and as long as identification solely relies on CK and CD45 staining of nucleated cells, these cannot be sufficiently well discriminated from CTCs. Advances in identifying CTCs and distinguishing them from false positives, in particular endothelial cells, will refine CTC detection and help avoiding diagnostic errors when progressing CTC-based assays into the clinic.

6. EMT-phenotype CTCs, do they have clinical relevance?

Circumstantial evidence linking EMT changes to advanced disease and increased metastasis is strong. EMT phenotype in patient tumour tissue is often prognostic and correlates negatively to overall survival and disease progression. Most notably, a switch from the expression of the epithelial cell-cell adhesion molecule E-cadherin to the mesenchymal N-cadherin is thought to be central to EMT, and it is commonly found in association with disease progression in various cancers including melanoma, pancreatic-, bladder- and colorectal cancer (reviewed by Cavallaro et al. [56]). Other studies found elevated expression of a number of E-cadherin transcriptional repressors such as Twist, Slug, ZEB1/2 and Snail1 linked to poorer prognosis in endometrial-, colorectal-, hepatocellular-, bladder-, gastric- and lung cancer [57–63]. Equally, increased expression of vimentin was associated with poorer outcomes for patients with gastric-, colorectal-, bladder- and breast cancer [58, 64–66]. However, the correlation of EMT with poorer overall or disease-free survival is not universal. A recent study, which established a comprehensive EMT gene expression signature in tumour tissue, found that poorer disease-free survival was associated with an EMT gene expression pattern in ovarian and colorectal cancer but not in breast cancer; therefore, it will be important to better define the context in which EMT gives cancer cells a selective advantage [67]. As detailed above, EMT marker gene or protein expression has also been studied in CTCs, and the overall emerging

evidence suggests that increased EMT-phenotype detection in CTCs correlates with more advanced disease stages and is the predominant phenotype found in the blood of patients with metastatic disease (see **Table 1**).

Cancer cells frequently undergo EMT when exposed to stress, and this makes them significantly more resistant to a variety of therapies. However, it is not well understood whether the range of phenotypic EMT changes that cause increased mobility and metastasis is instrumental in resistance or merely associated with it. Mounting *in vitro* evidence suggesting that EMT confers drug resistance has been thoroughly reviewed previously [8]. More recently, *in vivo* data reaffirm the link between EMT and therapy resistance. For instance, the loss of E-cadherin expression in erlotinib-treated non-small cell lung cancer tumour tissues correlated with poorer progression-free patient survival [68], and EMT gene expression signatures in ovarian or prostate cancer patient tissue were associated with resistance to platinum therapy or docetaxel and androgen deprivation, respectively [69, 70]. Interestingly, two recent studies suggested that in breast- and pancreatic cancer mouse models, the majority of cells that metastasised to the lungs did not undergo EMT. Nevertheless, EMT was involved in drug resistance and conditional metastatic outgrowth when mice were treated with the drugs cyclophosphamide and gemcitabine, respectively [15, 71]. These data add to the controversy regarding the role of EMT in the metastatic process and warrant further research. The data do support the survival/therapy resistance functions associated with EMT and it is plausible that cancer cells may 'escape into EMT' to render themselves resistant to drug treatment. Data are starting to emerge suggesting these changes might be detectable by CTC analysis as subtyping CTCs as possessing epithelial, intermittent or mesenchymal characteristics showed that gastric cancer CTCs of a patient progressing on therapy were all of EMT phenotype. However, and confusingly, any remaining CTCs detected in gastric cancer patients that responded to therapy were more epithelial in nature [39].

Regardless, it is possible that therapy inadvertently induces cells that survive drug exposure to change into more mobile, viable and aggressive clones. Due to their EMT phenotype, these cells may be ideally equipped to leave the primary tumour, become CTCs, prevent anoikis and potentially form distant metastases sites. Additionally, the survival advantage of EMT cancer cells might allow time to acquire alternative resistance mechanisms such as mutations. In turn, that would allow EMT-CTCs to undergo MET after extravasation to enable the resettling and formation of proliferating metastases. The underlying mechanisms need to be more thoroughly investigated, and the ability to accurately isolate EMT-CTCs will prove central to clarifying the role of EMT in therapy resistance, disease relapse and metastatic processes. Efficient EMT-CTC isolation and identification may also allow the development of diagnostic tests that monitor escape into EMT as part of therapy response to inform improved patient management.

7. Conclusion

Despite open questions regarding how EMT contributes to cancer progression and drug resistance, there is strong evidence that EMT changes, per se, are useful prognostic markers.

Consequently, EMT-CTC isolation and analysis have the capacity to progress EMT research and importantly allow the development of feasible, non-invasive diagnostic tests to predict and monitor the effectiveness of specific therapies. More reliable identification of these cells will permit the translation of EMT and CTC research into clinically relevant tests to guide therapy.

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