Molecular analysis of circulating tumour cells—biology and biomarkers

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Abstract | Growing evidence for intratumour heterogeneity informs us that single-site biopsies fall short of revealing the complete genomic landscape of a tumour. With an expanding repertoire of targeted agents entering the clinic, screening tumours for genomic aberrations is increasingly important, as is interrogating the tumours for resistance mechanisms upon disease progression. Multiple biopsies separated spatially and temporally are impractical, uncomfortable for the patient and not without risk. Here, we describe how circulating tumour cells (CTCs), captured from a minimally invasive blood test—and readily amenable to serial sampling—have the potential to inform intratumour heterogeneity and tumour evolution, although it remains to be determined how useful this will be in the clinic. Technologies for detecting and isolating CTCs include the validated CellSearch® system, but other technologies are gaining prominence. We also discuss how recent CTC discoveries map to mechanisms of haematological spread, previously described in preclinical models, including evidence for epithelial–mesenchymal transition, collective cell migration and cells with tumour-initiating capacity within the circulation. Advances in single-cell molecular analysis are enhancing our ability to explore mechanisms of metastasis, and the combination of CTC and cell-free DNA assays are anticipated to provide invaluable blood-borne biomarkers for real-time patient monitoring and treatment stratification.

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Introduction

The development of personalized medicine for patients with cancer depends on the identification of the molecular drivers of their disease.[1,2] Currently, biomarkers predicting therapy response are most often measured from tumour biopsy samples.[3] However, given that cancer is continually evolving at the molecular level, whether a diagnostic biopsy sample truly represents the patient's disease is questionable, particularly when considering selection of second-line therapies and beyond.[4] Moreover, although targeted therapies matched to the molecular pathology of individual patients' tumour biopsies have demonstrated clear benefits,[5–7] acquired drug resistance and disease relapse is common.[8–12] Thus, there is a pressing need to monitor tumour evolution and reveal—or, ideally, anticipate—the onset of and mechanisms underlying resistance to an expanding repertoire of targeted therapies. Subjecting patients to serial invasive biopsies is often impractical, can be distressing and, in some cases, is anatomically challenging and associated with risk.[13] Importantly, single-site biopsies are unlikely to capture the complexity of the genomic landscape of a patient's tumours owing to profound intratumour heterogeneity.[14–16] Initially described in the 1970s, the concept of clonal evolution as a cause for genetic diversity within tumours has been contemporized by recent pioneering studies that show the remarkable differences in tumour mutational profiles that result from branched evolution.[15,17–21] Tumour heterogeneity is now defined in both 'space and time'[22]—with anatomically distinct regions of the same primary tumour and their respective metastases exhibiting clear differences in genomic architecture.[15,23,24] Although the relevance of heterogeneity in personalized medicine is yet to be defined, a more-comprehensive genomic picture of overall tumour content than a single biopsy can provide is clearly required for optimal therapy selection.

One such avenue that can complement traditional biopsy sampling is the molecular analysis of circulating tumour cells (CTCs). The concept of 'CTC traffic' has been suggested whereby cells migrate between the primary tumour, bone marrow and metastases[25,26] in patients with advanced-stage cancer. If correct, CTCs arising from multiple sites might represent tumour heterogeneity as well as or better than any single tumour biopsy. Also, given the tumorigenic potential of at least a subpopulation of CTCs, as recently demonstrated in breast cancer,[27] these cells are arguably the most important subset of tumour cells to characterize, target and eradicate.[23]

More than 1,500 reports on CTCs have been published in the past decade, spanning topics from technology development (CTC capture, characterization and isolation methods),[28,29] prognostic[30–37] and pharmacodynamic[38] biomarker utility, and CTC-based identification of predictive biomarkers (for example, candidate mutation or receptor status) for therapeutic selection.[39–43] The role of CTCs within metastasis biology has also been explored, and the subset of CTCs with tumour-initiating

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Key points

- Circulating tumour cells (CTCs) can be captured from a simple blood test and their molecular characterization can provide vital insights into tumour heterogeneity
- Advances in single-cell genomic profiling have enhanced the ability to interrogate CTCs for 'actionable' aberrations and emerging resistant subclones, which will assist in patient treatment stratification
- Biological processes during CTC invasion and metastasis such as epithelial–mesenchymal transition and circulating tumour microemboli, typically explored in preclinical models are now being examined in the clinical setting
- CTC and cell-free DNA mutational profiling represent complementary biomarkers for identification of predictive targets and real-time monitoring of disease in clinical practice
- CTC assay validation and clinical qualification is required if CTCs are to become routine tests in the clinic; collaborative efforts between academia and industry are needed to make this a reality

capacity has also been identified.[27,44] With recent technical advances in single-cell profiling,[45,46] DNA and RNA profiles of CTCs can be examined to determine the degree of heterogeneity between individual cells and the degree of overlap with heterogeneity in matched biopsy specimens.

To identify novel therapeutic strategies from CTC research, we need a clear understanding of their role in haematological dissemination, including how these cells 'survive the trip' and eventually seed metastases. In this Review, we discuss the methods for CTC capture, state-of-the-art studies in single CTC profiling and the promise of these techniques for patient treatment stratification and disease monitoring. We describe recent CTC discoveries that illustrate how these cells are involved in haematological spread, with a focus on the epithelial–mesenchymal transition (EMT),[47,48] cellular cooperation,[49–51] circulating tumour microemboli (CTM)[52,53] and the tumour-initiating capability of these cells.[27,44] Finally, we present the current status of CTCs in terms of clinical utility and discuss their complementary role alongside cell-free DNA (cfDNA) as minimally invasive biomarkers for real-time patient monitoring. We focus primarily on lung cancer to illustrate many of the issues, largely because this disease is the leading cause of cancer-related death worldwide and obtaining biopsy samples is particularly challenging in this setting. Furthermore, CTC-based biomarkers are likely to be considerably useful clinically, especially given the late diagnosis commonly encountered for patients with lung cancer. For research avenues in which no lung cancer examples exist, other relevant disease types have been described.

Detecting and characterizing CTCs

The key technical challenge for CTC research is posed by the rarity of tumour cells in blood, with estimates of just one CTC per ~10^7 white blood cells (WBCs) per millilitre of blood. A vast array of technologies has emerged to isolate CTCs,[29,54] mostly using principles of 'enrichment' and 'detection' (Table 1). CTC enrichment—the process of capturing CTCs amongst the vast array of normal blood cells—is achieved by virtue of physical properties of the cells, such as size, density or charge[55–58] or 'specific' biological features, such as tumour cell surface marker expression.[59,60] Detection of CTCs is commonly achieved by immunostaining and microscopy or by PCR-based methods.[29]

Enrichment of CTCs by immunomagnetic capture has been the most successful and widely used approach to date. The semiautomated CellSearch® platform (Janssen Diagnostics, Raritan, NJ, USA) enriches CTCs using ferromagnetic beads coated in epithelial cell adhesion molecule (EpCAM) and defines CTCs according to morphological characteristics, positive expression of cytokeratins and absence of the leukocyte marker CD45.[59,60] CellSearch® is the only CTC enumeration system to have been fully validated with respect to reproducibility and performance characteristics,[59,60–75] and is the only technology to have been deployed in large-scale multicentre trials, leading to its approval by the FDA as an aid to prognosis in patients with metastatic breast cancer, prostate cancer and colorectal cancer (CRC).[32–34] The highly detailed process required for validation and qualification of CTC assays has recently been reported by the Cancer Steering Committee of the Foundation for the NIH Biomarkers Consortium.[76] Although large numbers of CTC technologies are in development, none yet have the same stringent degree of validation and qualification as CellSearch® that permit reliable use in large clinical studies. Promising novel EpCAM-based enrichment technologies include microfluidic devices—notably the CTC-chip,[63] Herringbone chip,[64] iCHIP[65] (all Massachusetts General Hospital Center, USA) and IsoFlux[68] (Fluxion, USA). Another device, the MagSweeper (Stanford University, USA), enriches CTCs using a magnetic rod stirred through a blood sample prelabelled with anti-EpCAM antibody-coated magnetic beads.[61] MagSweeper has a higher CTC purity and ability to isolate live cells. GILUPI Nanomedizin (Germany) have devised an anti-EpCAM antibody-coated functionalized medical wire, which is placed directly into the antecubital vein for 30 min to sample a large blood volume.[62]

The biological relevance of 'epithelial' CTCs (that is, EpCAM-positive and cytokeratin-positive cells) is evidenced by the prognostic significance of CTCs captured by CellSearch® in multiple tumour types.[30–36] However, if at least a subset of CTCs undergoes EMT, whereby epithelial markers are downregulated, technologies reliant on EpCAM and cytokeratin expression for CTC capture might fail to enrich an important subpopulation of cells. Thus, increasing attention has been dedicated to technology platforms that use marker–independent enrichment methods (Table 1).

Whatever the basis for CTC enrichment, all single-step enrichment platforms fall short of isolating pure CTCs and result in persistent leukocyte contamination, which is perhaps unsurprising given the immense challenge of isolating small numbers of tumour cells from millions of background leucocytes. Antibody capture systems, for example, enrich a proportion of leukocytes owing to nonspecific binding or physical trapping of cells amongst the magnetic beads; or filtration devices result in residual

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Table 1 | Selected technologies for CTC detection

Technology	Method of CTC enrichment	Method of CTC detection	Notes
Antibody-based capture assays			
CellSearch®[59,60]	EpCAM-coated ferromagnetic beads	Immunocytochemistry for cytokeratin, CD45 and DAPI	FDA approved in advanced breast, prostate and colorectal cancer
MagSweeper[61]	EpCAM-coated magnetic beads enriched using magnetic rod	Microscopic visualization	Live cells can be isolated
GILUPI cell collector[62]	Functionalized EpCAM-coated medical wire	Immunocytochemistry for EpCAM, cytokeratin and DAPI	*In vivo* collection
CTC chip[63] and Herringbone chip[64]	EpCam-coated microposts and chip surface	Immunocytochemistry for cytokeratin, CD45 and DAPI with or without tumour-specific markers, for example, cytokeratins, PSA	Microfluidic microchip technology
CTC iChip®[65]	Magnetic bead capture combined with microfluidic inertial focusing	Immunocytochemistry or RT-PCR	Positive selection by EpCAM or negative selection by CD45 gives a broad population of enriched cells
Ephesia CTC-chip[66]	Functionalized magnetic beads combined with microfluidics	Immunocytochemistry for cell surface and nuclear markers	Flexibility with capture antibody
AdnaTest[67]	Immunomagnetic beads with MUC1-coupled and EpCAM-coupled antibodies	Multiplex RT-PCR for panel of genes (*MUC1*, *HER2* or *EPCAM*)	Cell lysis means quantification of tumour cell number is not possible
IsoFlux[68]	EpCAM-coated magnetic beads combined with microfluidic processing	Immunocytochemistry for cytokeratin, CD45 and Hoechst	Antimouse IgG-coated beads available for user-defined antibody capture
Physical characteristic-based assays			
ISET®[69]	Filtration based on cell size	Immunocytochemistry or FISH	Nonepithelial cells can be isolated
Dean Flow Fractionation[70]	Size-based selection using centrifugal force	Immunocytochemistry for cytokeratin, EpCAM, CD45 and Hoechst	Nonepithelial cells can be isolated
Dielectrophoretic field-flow fractionation[57]	Membrane capacitance	Immunocytochemistry	CTCs selected are viable
Functional assays			
EPISPOT assay[71]	CD45 depletion and short-term culture in plates coated in antibody against MUC-1, PSA or cytokeratin-19	Immunofluorescence secondary antibodies to MUC-1, PSA or cytokeratin-19	Detection of only viable CTCs
CAM[72]	Density gradient centrifugation and cells applied to CAM for short-term culture	Immunocytochemistry for cell-surface markers	Detection of only viable CTCs
Other assays			
ImageStream®[73]	Pre-enrichment by any method of choice	Flow cytometry-based imaging using multi-marker immunofluorescence	Cells can be imaged for up to 10 cell-surface or intracellular markers
High-throughput fluorescent scanning[74]	Red cell lysis and density gradient centrifugation	Immunocytochemistry of cell surface and nuclear markers	Nonepithelial cells can be isolated on a slide
DEPArray™[57]	Requires pre-enrichment step	Fluorescence imaging that enables movement of cells within chip by electric field changes	Isolation of purified single cells for downstream analysis

Abbreviations: CAM, collagen adhesion matrix; CTC, circulating tumour cell; DAPI, 4',6-diamidino-2-phenylindole; EpCAM, epithelial cell adhesion molecule; FISH, fluorescence *in situ* hybridization; HER2, human epidermal growth factor receptor 2; MUC1, mucin 1; PSA, prostate-specific antigen; RT-PCR, real-time PCR.

leukocytes owing to natural variation in leukocyte size (some being larger than the pore size and, therefore, retained on the surface of the filter). To molecularly profile rare cells, the presence of wild-type DNA or RNA from WBCs represents a significant technical hurdle. A number of studies have tried to overcome this limitation by inferring the presence of CTCs through the detection of tumour-specific mRNAs or DNA changes in whole blood or CTC-enriched samples. For example, assays have been developed for cytokeratin-19 (keratin, type I cytoskeletal 19) mRNA detection in breast cancer,[77,78] or more-specific panels of genes have been used to identify carcinoembryonic antigen, cytokeratin-19, cytokeratin-20 and CD133 in colorectal cancer.[79] Furthermore, assays to detect tumour-specific methylation patterns for expression of the breast cancer metastasis suppressor-1 promotor,[80] have been developed. However, these approaches make an assumption that the transcripts are specific to the tumour (which might not be the case) and, importantly, do not provide purified single CTCs for interrogation of genetic profiles beyond the transcript used for detection. The optimal means for obtaining a pure CTC sample is to apply a second isolation step following CTC enrichment, which can be achieved by physical micromanipulation whereby individual cells of interest are visualized under (fluorescent) microscopy and acquired with a pipette or micromanipulator into a separate container for further analysis.[81] A more-recent automated technology—the DEPArray—uses a closed system of moveable electrostatic cages to trap and move cells and has been used for imaging and isolation of single or groups of CTCs.[82,83]

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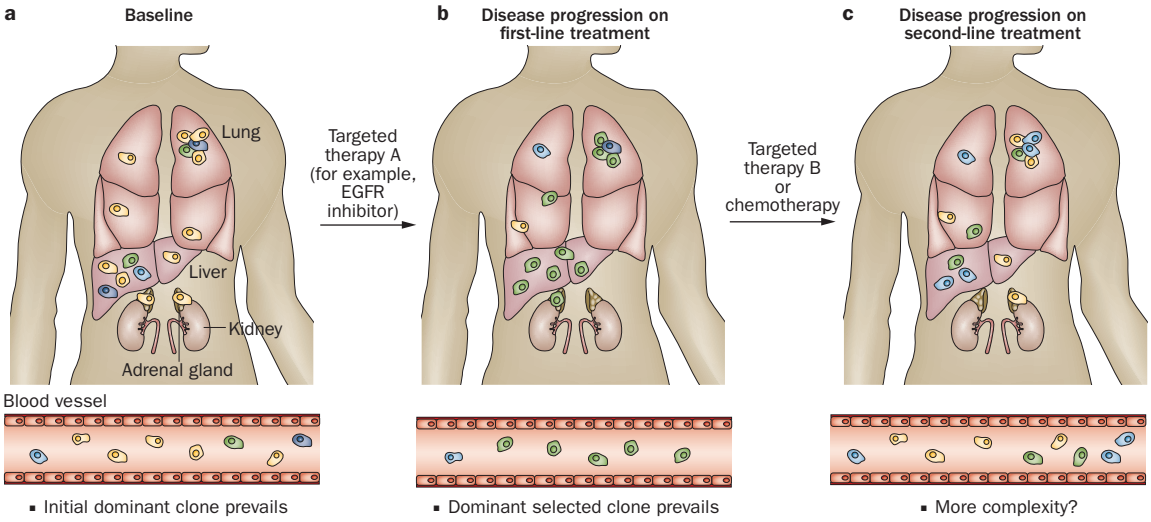

Figure 1 | Potential of single CTC analysis to evaluate tumour heterogeneity and disease evolution. Using a primary NSCLC with pulmonary, liver and adrenal metastases as an example, intratumour heterogeneity is simplistically represented by differently coloured cells. **a** | Blood sampling at baseline reveals CTCs shed from the primary tumour and metastases (and different regions within). The dominant CTC population (yellow cells) represent cells harbouring the driver mutation (for example, EGFR-sensitizing somatic mutation), facilitating selection of targeted therapy (gefitinib or erlotinib). Subclones of cells (green, light blue and dark blue) are present in very low numbers and represent pre-existing mutations that might confer later resistance. **b** | After disease progression on first-line therapy, resistant tumour cells emerge (green cells) after the selection pressure of treatment. **c** | After second-line therapy (usually chemotherapy in the setting of EGFR-mutant NSCLC), whether tumour heterogeneity becomes increasingly complex (and represented in the CTCs) or if the original EGFR-sensitizing mutation (yellow cells) re-emerges remains unclear. Although the usefulness of serial CTC analysis is yet to be confirmed, both cell-free DNA and CTC mutational analysis will be complementary for the purposes presented.[184] Abbreviations: CTC, circulating tumour cell; NSCLC, non-small-cell lung cancer.

As with other biomarker assays, the regulatory requirements for the use of a CTC technology to inform clinical management are necessarily stringent.[76] CTC technologies need to have a reliable, reproducible and robust assay with clinical validation and qualification from prospective studies to be used clinically. This rigorous process requires collaboration between research institutions and industry if CTC-based biomarkers are to become a reality. In addition, assay portability is critical. The CellSave Preservative tube (Janssen Diagnostics) maintains cells for 96 h following collection and was one of the key elements enabling the success and FDA recognition of the CellSearch® system. For successful centralized clinical implementation of new CTC systems, similar blood stabilization approaches will be required, in addition to fully validated assays to ensure standardization of the CTC readout.[76]

Molecular profiling of single CTCs

Once isolated, one can apply a range of genomic profiling technologies to single CTCs. If CTCs are shed randomly from several spatially distinct regions (a hypothesis yet to be tested) a global representation of tumour genomic heterogeneity and clonal dominance can be provided. Longitudinal studies to investigate the evolutionary clonal changes in mutational profiles associated with treatment represent another area of promise for molecular CTC analyses (Figure 1). Most genetic studies of CTCs have, until recently, been performed on DNA or RNA extracted from enriched CTC populations with limited sensitivity because of 'masking' of the tumour profile by wild-type DNA from leukocytes. For example, Punnoose *et al.*[84] analysed the *EGFR* mutation status in CTCs enriched by CellSearch® in patients with non-small-cell lung cancer (NSCLC), revealing detectable mutations in only one out of eight patients who were confirmed to harbour an *EGFR* mutation from their tumour biopsy sample.

Genomic analysis of single CTCs overcomes the considerable limitation imposed by leukocyte contamination, enables the assessment of CTC heterogeneity and aids in identifying co-existing mutations within a cell. As a single cell contains just 6.6 pg of DNA, reliable and uniform whole-genome amplification (WGA) is required for mutational analyses.[85] As yet, no single-CTC profiling studies have been published in lung cancer, but the feasibility of this type of study has been demonstrated in other tumour types (for example, CRC).[85] Following amplification, sensitive and reproducible techniques for genotyping, next-generation sequencing (NGS) and array analyses that include subsequent data interpretation and bioinformatics have been established for single cells.[86] With the degree of amplification required for single-cell analysis, justifiable concerns have been raised about WGA errors masquerading as somatic mutations and amplification bias or allele dropout distorting the genomic profiles.[87] For each amplification approach, a careful empirical assessment is essential to distinguish pre-existing genetic alterations from amplification errors. Reliable identification of somatic changes in CTCs will

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Table 2 | Summary of clinical CTC single-cell molecular profiling studies

Cancer type	Methods	Observations	Reference
Mutational profiling studies			
Breast, prostate and gastrointestinal	Micromanipulation of cytokeratin or EpCAM-positive cells from blood, bone marrow and lymph nodes; CGH and single-stranded conformational polymorphism	Heterogeneity of chromosomal aberrations and *TP53* mutations in minimal residual disease	89
Melanoma	Immunomagnetic enrichment using anti-MCSP antibody and micromanipulation of CMCs; Single-cell CGH	CGH of patient CMCs revealed hierarchical clustering, suggesting a clonal origin	90
Colorectal	CTCs isolated by density gradient centrifugation and manipulation with DEPArray; DNA Sanger sequencing and pyrosequencing	Heterogeneity of *KRAS* mutations between primary tumours, metastases and CTCs	82
Colorectal	CellSearch enrichment and micromanipulation of CTCs; DNA Sanger sequencing	Inter-CTC heterogeneity of driver mutations observed	85
Colorectal	CellSearch enrichment and micromanipulation of CTCs; Array CGH and NGS	Multiple copy number variations in CTCs; Mutations demonstrated in CTCs present in tumours at subclonal level	91
Gene-expression profiling studies			
Breast	Isolated CTCs with MagSweeper and micromanipulation; High-throughput microfluidic-based qRT-PCR array	Demonstrated two major subgroups of CTCs, one with increased metastasis-associated genes and one with high expression of EMT associated genes	92
Melanoma	Isolated CTCs with MagSweeper; Use of Smart-Seq for mRNA sequencing	Reproducible gene-expression assay; Putative CMC gene-expression profiles similar to melanocytes and melanoma cell lines, but distinct from WBCs	46
Prostate	Isolated CTCs with MagSweeper and micromanipulation; RNA-Seq for gene-expression analysis	Despite RNA degradation, prostate-specific and cancer-specific transcripts were identified	93
Prostate	CTCs isolated with filtration and micromanipulation; High-throughput microfluidic-based qRT-PCR array	Heterogeneous upregulation of EMT-associated gene expression, particularly in castration-resistant disease	81

Abbreviations: CGH, comparative genome hybridization; CMC, circulating melanoma cell; CTC, circulating tumour cell; EMT, epithelial–mesenchymal transition; EpCAM, epithelial cell adhesion molecule; MCSP, melanoma-associated chondroitin sulphate proteoglycan; NGS, next-generation sequencing; qRT-PCR, quantitative, real-time PCR; RNA-Seq, RNA sequencing; WBC, white blood cell.

require detection in multiple independent samples and establishing the absence of these genetic features in control tissue, such as the 'contaminating' leukocytes. Despite the daunting challenges of single-cell profiling, such as the limited starting material and the complex and costly downstream processing, the WGA and NGS methodologies have proven robust enough to reliably assess DNA copy number changes acquired over one cell cycle,[86] establishing that obtaining similar detailed informative data from CTCs is feasible. Finally, for each type of cancer examined and methodology employed, how many CTCs are needed to produce a representative 'snapshot' of the disease at the time of blood sampling remains to be determined.[76]

Initial clinical studies of molecular profiling of single CTCs have focused on technical feasibility and comparing mutation profiles of CTCs with primary tumours (Table 2). For example, Gasch *et al.*[85] isolated single CTCs from patients with CRC by manual micromanipulation after enrichment using CellSearch®. Amplified CTC DNA was then examined for common point mutations in *PIK3CA*, *KRAS* and *BRAF* using Sanger sequencing, which are all relevant genes in predicting resistance to anti-EGFR therapy.[88] *KRAS* mutations were identified in CTCs from one out of five patients, with 100% concordance with the primary tumour. *PIK3CA* mutations were identified in subsets of CTCs in four out of five patients, one of whom harboured two different *PIK3CA* mutations in separate CTCs, revealing the ability of single-cell profiling to inform mutational heterogeneity.[88]

In another study, single CTCs were isolated from patients with CRC using the same approach for array-comparative genome hybridization (CGH) and NGS of a panel of 68 CRC-associated genes.[91] Findings were compared with primary tumours and metastases. Multiple copy number changes were identified in 37 CTCs from six patients, with similarities to the primary tumours. NGS of the tumours and corresponding CTCs from two patients was also performed; driver mutations (in *KRAS*, *APC* and *PIK3CA*) were identified in the tumour samples and CTCs, but 20 'branch' mutations were exclusive to the CTCs. Additional sequencing of the original tumours confirmed 17 of these mutations were actually present at a subclonal level in either the primary tumour or metastases.[91] These data support the notion that CTCs can provide a more global overview of tumour genomics and have the potential to be more informative than analyses of single biopsy samples. However, how low-frequency mutations will translate to improving the prediction of response or resistance to targeted therapies remains unclear.

Gene-expression studies in CTCs might be essential for determining the nature and extent of tumour heterogeneity, linking phenotypic differences with genetic and epigenetic aberrations. However, preserving RNA is more difficult than DNA and concerns have been raised about the impact of sample processing on CTC expression profiles.[83,94] Deciphering the changes in gene expression that are biologically relevant as opposed to those caused by technical and biological 'noise' remains a significant

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hurdle for single-cell transcriptome studies. To address this issue, Ramsköld *et al.*[46] reported the development of a single-cell mRNA sequencing protocol—Smart-Seq—with improved transcriptome coverage compared with previous mRNA-Seq protocols,[45] and demonstrable high reproducibility and low technical variation. Despite stochastic loss of less-abundant transcripts in single cells, highly expressed transcripts were reliably detected and expression profiles of melanoma CTCs were similar to melanocytes and melanoma cell lines and, importantly, clearly distinguishable from leukocytes. Interestingly, melanoma-associated antigens and cell-cycle markers are expressed highly in CTCs, whereas low levels were observed for genes associated with the regulation of cell death and MHC class I genes, indicating a potential mechanism whereby CTCs avoid immune detection and survive the trip.

A similar workflow was used to perform mRNA sequencing on CTCs isolated from patients with metastatic prostate cancer.[93] This study noted a high rate of RNA degradation consistent with apoptosis amongst CTCs, although prostate tissue-specific and cancer-specific transcripts could still be elucidated. Importantly, MagSweeper processing was shown to have little effect on the gene-expression profile in cell lines.[93] In comparison with normal prostate tissue, 181 genes were overexpressed in the CTCs, with a range of functions that included control of the cell cycle, metabolic processes, apoptosis, cell adhesion and cell communication, demonstrating the potential of this process to provide insight into metastasis biology and ultimately identify novel targets for therapeutic intervention.

Together, these studies demonstrate the feasibility of analysing the genetic and transcriptional profiles of a single CTC. Although the techniques remain in their infancy, the contribution that single-cell profiling of CTCs could make to our understanding of cancer heterogeneity, disease evolution (through serial CTC analysis) and tumour biology is clearly evident. The high cost currently associated with such analyses is a challenge to overcome and collaborative efforts to standardize protocols are needed.

The biology of CTCs in patients

The molecular events underpinning haematological dissemination in patients with cancer are incompletely understood, but advances in CTC detection capabilities and single-cell profiling are beginning to improve our knowledge. A wealth of information derived from preclinical models is beginning to be translated to clinical studies, which have a multitude of important questions to address. For example, the comparative functional significance of single CTCs compared with clusters of CTCs (that is, CTM) is not known. We do not yet have a clear understanding of how CTM—which might be composed of tumour and accessory host cells—enter, navigate or leave the vasculature. The hypothesis that tumour cell survival in the bloodstream is enhanced within CTM has been expressed by some,[30,95] and is supported by evidence from preclinical studies.[95–104] Further evidence from clinical studies suggest that EMT[47] and cell cooperation[49] occur and methods are being explored to identify the unique subpopulation of CTCs with tumour-initiating capacity.

Epithelial–mesenchymal transition

EMT is a concept rooted in embryogenesis and involves ectodermal cell migration within the embryo during gastrulation to produce the mesoderm (type I EMT).[105] EMT also plays an important part in wound healing and fibrosis (type II EMT).[106] The concept has been extended to cancer metastatic progression (type III EMT), whereby epithelial tumour cells are thought to lose their apical–basal polarization and cell–cell contacts, permitting invasion through adjacent tissue as spindle-shaped mesenchymal tumour cells with a motile cytoskeleton.[47,48,107]

Through the molecular analysis of primary tumours and metastatic sites, panels of markers have been used to show the EMT, whereby loss of E-cadherin and gain of vimentin or fibronectin are amongst the most commonly described.[108] Although the concept of EMT remains a topic of debate, emerging evidence derived from the analysis of CTCs supports the occurrence of the EMT phenomenon, and its significance is highlighted by recent correlative studies showing the presence of mesenchymal markers in tumour tissue as a poor prognostic factor in multiple cancers including NSCLC,[109] CRC,[110] breast cancer[111] and others.[112,113] EMT is also increasingly being recognized as an important cause of intrinsic and acquired resistance to chemotherapy.[114–117] However, the extent to which EMT occurs in tumours remains unclear, as is its dynamic interplay between the primary tumour, CTCs and metastases.

Interestingly, metastases often histopathologically resemble the predominantly epithelial primary tumour, rather than being mesenchymal in appearance.[118] It has been proposed that cells transition from epithelial to mesenchymal states to migrate, but revert back to an epithelial state at distant sites by the reverse process, mesenchymal–epithelial transition (MET), as occurs in organ development (Figure 2).[119] If true, the dynamics of the process are difficult to define through analysis of tumour biopsy alone, and so CTCs have the potential to provide the missing link in respect of properties of the travelling cells. Non-epithelial-based CTC capture systems will be essential to isolate the mesenchymal cell population.

EMT and cell cooperation

A proposed alternative model to EMT and MET as a fluid process is cooperation between the cells forming the metastasis. Such cooperation was elegantly demonstrated using a model of epithelial and mesenchymal cells derived from hamster oral keratinocytes.[50] Pure populations of either epithelial or mesenchymal cells formed tumours at the subcutaneous injection site in mice, after which only mesenchymal cells entered the blood stream, with neither cell type alone leading to the formation of lung metastases. When administered

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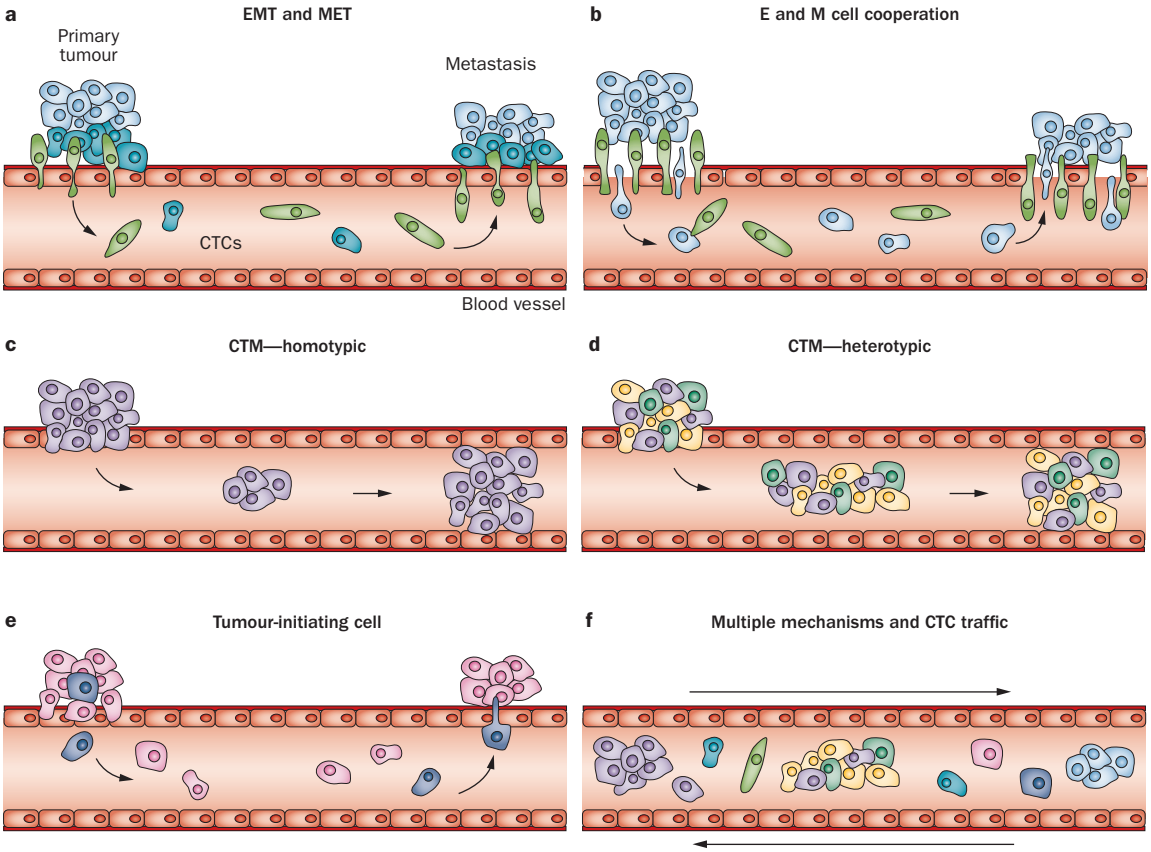

Figure 2 | Proposed mechanisms of tumour cell dissemination. **a** | EMT as a complete or partial process.[107] In this model, tumour cells from the primary epithelial tumour (blue cells) undergo EMT either partially (turquoise) or completely (green). These cells then undergo the reverse process of MET at a distant site to form the metastatic lesion.[118] **b** | EMT and cell cooperation. In this model, only mesenchymal cells (green) invade the vasculature, enabling epithelial (blue) cells to pass through as passengers. Both cell types are needed to form metastasis.[49] **c** | CTM (purple cells) have been detected in many patients with solid tumours. CTM are considered to have a survival advantage over single cells, which are susceptible to anoikis and immune attack.[134] **d** | CTM (purple cells) with accessory cells, such as fibroblasts or endothelial cells (yellow and dark green) have been shown to have greater potential to form distant metastases than CTM without accessory cells.[52] **e** | Tumour-initiating cells. In this model, only a minority of circulating cells (tumour-initiating cells or cancer stem-like cells; dark blue) have the ability to survive within the circulation and form distant metastases.[130] Daughter cells (pink) might not have this potential and ultimately undergo apoptosis, releasing cell-free DNA into the circulation. **f** | CTC 'traffic'. The concepts described in a–e are unlikely to be mutually exclusive and all potentially contribute to metastasis. Abbreviations: CTCs, circulating tumour cells; CTM, circulating tumour microemboli; EMT, epithelial–mesenchymal transition; MET, mesenchymal–epithelial transition.

intravenously, only the epithelial cells formed lung metastases. The subcutaneous administration of mixed epithelial and mesenchymal cells resulted in both cell types entering the circulation and lung metastases being formed from the epithelial population. These observations suggest that cellular cooperation might be required (mesenchymal cells invading and enabling 'passenger' epithelial cells to follow) for tumour cells to complete the metastatic cascade (Figure 2). A second mouse model of small-cell lung cancer (SCLC) classified tumour cells as neuroendocrine or mesenchymal and supported the cooperation hypothesis.[51] When either cell type was administered separately by subcutaneous injection into immunocompromised mice, local tumours formed without liver metastases, whereas both cell types administered together led to development of liver metastases. The exact mechanisms underpinning this cellular cooperation remain unclear. If such cellular cooperation does occur in patients, then targeting the mesenchymal cells—or the interaction between epithelial and mesenchymal cells—might prove a novel therapeutic strategy. CTC analysis in patients, by virtue of capturing the EMT properties of cells undergoing metastasis, might confirm whether these preclinical observations hold true in the clinical setting.

Evidence for EMT through CTC studies

A number of studies have interrogated the EMT characteristics of CTCs in patients (Table 3). Most CTC technologies rely on the expression of epithelial antigens (for example, EpCAM) by tumour cells for their capture. However, considerable disparity exists between the numbers of EpCAM-positive CTCs detected in different cancer types, perhaps due to the EMT phenomenon. Non-antigen-dependent techniques might, therefore, capture a higher number of CTCs.

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Table 3 | Clinical studies that have explored EMT phenotype of CTCs and CTM*

Cancer type	Stage	*n*	Enrichment	EMT markers	Reference
Immunohistochemistry					
NSCLC	Metastatic	3	ISET®	E-cadherin, vimentin or N-cadherin	120
SCLC	Metastatic	3	ISET®	E-cadherin, vimentin or N-cadherin	120
Immunofluorescence					
NSCLC	Metastatic	6	ISET®	Cytokeratins, vimentin	121
Breast	Locoregional and metastatic	50	CD45 depletion	Cytokeratins, vimentin or TWIST	122
Breast	Metastatic	16	EpCAM capture	Cytokeratins, vimentin, N-cadherin or O-cadherin	123
Prostate	Metastatic	41	EpCAM capture	Cytokeratins, vimentin, N-cadherin or O-cadherin	123
SCCHN	Locoregional	11	CD45 depletion	Cytokeratins, vimentin, N-cadherin or CD44	124
RNA in situ hybridization					
Breast	Metastatic	11	EpCAM, HER2 and EGFR	Panel of seven epithelial and three mesenchymal genes	125

*Includes all published clinical studies evaluating markers of EMT in CTCs. Abbreviations: CTC, circulating tumour cell; CTM, circulating tumour microemboli; EMT, epithelial–mesenchymal transition; EpCAM, epithelial cell adhesion molecule; NSCLC, non-small-cell lung cancer; SCCHN, squamous cell carcinoma of the head and neck; SCLC, small-cell lung cancer.

One of the first studies to address the EMT phenotype of CTCs used single-marker and dual-marker immunohistochemistry on cells isolated by size using ISET® (Rarecells, USA) in patients with SCLC and NSCLC.[120] The captured CTCs demonstrated heterogeneous expression of EMT markers, consistent with previous reports that describe partial EMT rather than an 'all or nothing' phenomenon.[108] These observations were supported by our further studies using immunofluorescent analysis of CTCs in SCLC (Figure 3) and by others in NSCLC[121] as well as breast,[122,123] prostate[123] and head and neck[124] cancers, revealing heterogeneity in EMT markers. In a study of breast cancer by Kallergi and colleagues,[122] CTCs expressing mesenchymal markers were identified more frequently in patients with advanced-stage disease than in those with localized disease, suggesting an association between EMT and disease progression. In 2013, Yu *et al.*[125] reported on serial EMT profiling of breast cancer CTCs using RNA *in-situ* hybridization for a panel of epithelial and mesenchymal genes in patients undergoing chemotherapy. They identified a spectrum of CTCs, spanning a mixed epithelial–mesenchymal phenotype, at baseline and emergence of a mesenchymal CTC phenotype was observed at times of disease progression (suppressed at times of treatment response), further implicating mesenchymal CTCs in metastatic progression. Although these studies explored mesenchymal markers to profile the EMT phenotype of CTCs (vimentin, N-cadherin, O-cadherin, twist, fibronectin and serpin peptidase inhibitor),[121–124] no defined marker or panel of markers has been identified with which to definitively identify CTCs that have undergone partial or complete EMT. Evaluation of additional markers of mesenchymal CTCs might be guided by the RNA sequencing of mesenchymal CTCs and CTM captured by Yu *et al.*[125] In this study, the authors reported 170 transcripts enriched in CTCs and CTM and, for example, highlighted the transcription factor, FOXC1, along with TGFβ activation as a potentially important contributor to EMT in breast cancer.

These initial studies demonstrate clear heterogeneity in EMT markers both within and between patients. The presence of both epithelial and mesenchymal cells in patients with metastatic cancer supports the preclinical models of cell cooperation, whereas the concurrent expression of both epithelial and mesenchymal markers is more in keeping with the model of partial EMT. We hypothesize EMT occurs to varying degrees in different tumour types, but further studies are required to explore this. Loss of epithelial characteristics provides a plausible explanation for the low yield of CTCs by CellSearch®—particularly in NSCLC—and highlights the need for future studies to include technologies that isolate a spectrum of cell phenotypes (perhaps using more than one technique). Correlative studies are required to assess the prognostic and predictive significance of circulating EMT cells and the therapeutic relevance of dynamic shifts in cell populations upon treatment. Genomic profiling of the different phenotypic cell types at the single-cell level might indicate the metastatic capability of individual cells, and functional studies of the isolated populations could be used to assess metastatic potential in more detail.

Tumour-initiating cells

A subset of cancer cells are thought to have the properties of adult stem cells (self-renewal and multipotency) and are capable of initiating tumour growth when transplanted into immunocompromised mice.[126–130] These cells have been referred to interchangeably as 'cancer stem cells', 'stem-like cells' or 'tumour-initiating cells'[131] and have been linked with the mesenchymal phenotype. In a study by Mani and colleagues, immortalized human breast epithelial cells were induced to undergo EMT by ectopic expression of SNAIL or TWIST, transcription factors that regulate mesenchymal cell differentiation.[132] These epithelial cells had a mesenchymal gene-expression profile and were able to form mammospheres in culture, a property associated with tumour-initiating cells.[133] Moreover, they expressed a $CD44^{high}/CD24^{low}$

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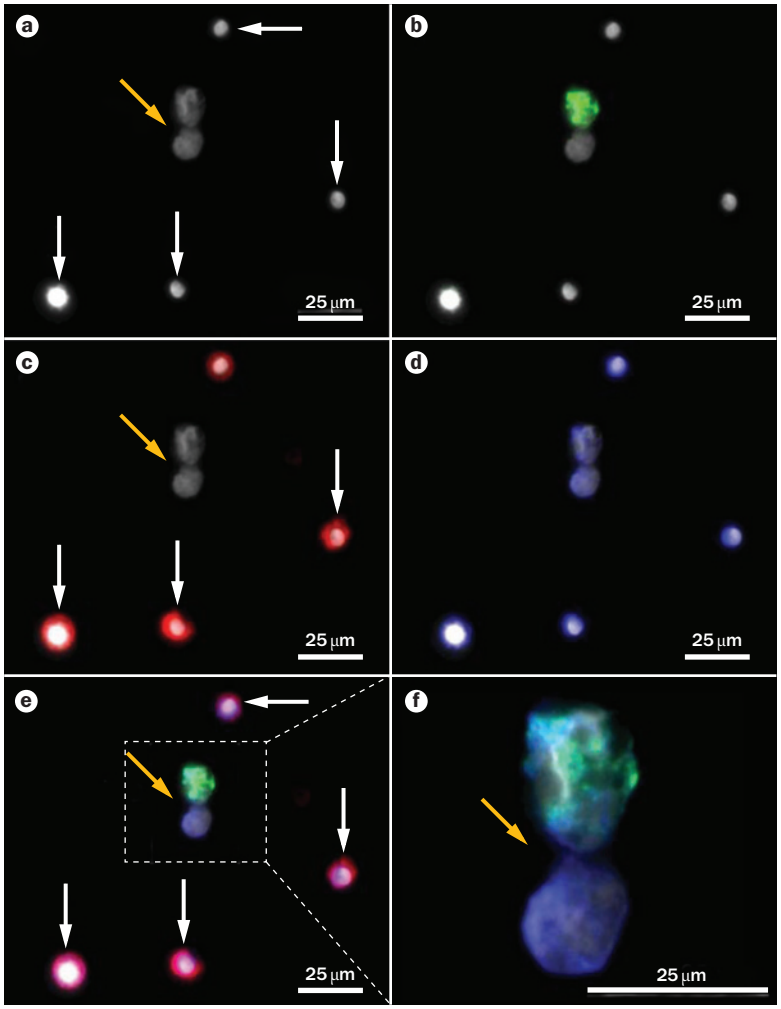

Figure 3 | Examples of EMT cells isolated by ISET®. Epithelial tumour cells were isolated according to size from a patient with small-cell lung cancer and stained by immunofluorescence for **a** | DNA, **b** | various cytokeratins, **c** | CD45 (leukocytes) and CD144 (endothelial cells) and **d** | vimentin. Composite image of staining at **e** | low magnification and **f** | high magnification. White arrows point to CD45+ leukocytes or CD144+ circulating endothelial cells. Tumour cells are indicated by yellow arrows; one cell is positive for both cytokeratin and vimentin (partial EMT) and one cell is vimentin-positive but cytokeratin-negative (complete EMT).

phenotype, consistent with previously reported breast cancer tumour-initiating cell profiles,[126] and were more tumorigenic in immunocompromized mice compared with control human mammary epithelial cells treated with empty vector.

The pioneering work of Isiah Fidler in 1970s showed that the metastatic process is inefficient.[23,134] Recent exploration of metastatic inefficiency using *in vivo* video microscopy following injection of tumour cells into the hepatic circulation in animal models confirmed that although the majority of cells survive within the circulation and extravasate, these cells subsequently fail to initiate tumour growth or to progress beyond early micrometastases.[135,136] The tumour-initiating hypothesis posits that only a minority of cells with metastatic capability possess all the required adaptations to survive passage through the circulation and produce metastases.[137,138] Thus, a major goal of CTC research is to identify these cells in patients, study their genetic makeup and transfer the cells to preclinical models to explore their functional ability, signalling pathways and response to inhibition with drug-screening assays.

Baccelli *et al.*[27] recently reported the first such functional study with CTCs. Blood samples were acquired from patients with metastatic breast cancer and were depleted of WBCs using magnetic beads. The enriched CTC fraction was injected into the femoral medullary cavities of immunocompromised (NSG) mice. Bone, liver and lung metastases developed in CTC-xenografted animals from three patients, all of whom exhibited >1,000 cells per 7.5 ml blood (an exceptionally high number of cells to be detected in patients with breast cancer patients)[34] in corresponding samples enumerated by CellSearch®. By contrast, xenografts did not grow from 106 patients who had <1,000 CTCs in the enriched samples. These promising data provide the first confirmation of a tumorigenic subpopulation of CTCs, albeit the efficiency of the model was low. Using flow cytometry analysis of blood from one patient whose CTCs formed in a mouse xenograft, a subpopulation of cells with the putative tumour-initiating phenotype $CD45^-$, $EpCAM^+$, $CD44^+$, $CD47^+$ and c-MET^+ was identified.[27] This population of cells, when transplanted into NSG mice, gave rise to metastases. However, as the authors acknowledge, other phenotypes including EpCAM-negative cells might also have tumour-initiating properties and give rise to metastases. Indeed, another study in breast cancer revealed EpCAM-negative CTCs with tumour-initiating properties (causing brain metastases) following short-term *in vitro* culture.[44] As more is learnt about the features of tumour-initiating CTCs with *in vivo* models (on the basis of genomic and proteomic analyses), therapeutic targeting of these subpopulations poses the tantalizing possibility of translating short-lived remissions into long-term disease control or cure.

Circulating tumour microemboli

CTM are defined as groups of at least three CTCs that travel together within the circulation.[30] Conceptually, CTM are considered to play an important part in the development of metastases (Figure 2). In the 1970s, preclinical studies demonstrated that CTM (injected in mice as cell aggregates) had a higher propensity to seed distant metastases than single cells,[95–104] suggesting CTM possess a survival advantage. Several groups have subsequently sought to explore explanations for this phenomenon, including avoidance of anoikis,[139] presence of stromal cells providing a unique 'travelling niche'[52] or avoidance of immune surveillance through incorporation of host cells.

CTM and accessory cells—the travelling niche

Preclinical studies have demonstrated that CTM can be composed of groups of tumour cells alone, or tumour cells associated with fibroblasts,[52] leukocytes,[96,140] endothelial cells,[141–143] pericytes[143] or platelets[144–146] (Table 4 and Figure 2). Duda *et al.*[52] presented an elegant *in vivo*

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Table 4 | Preclinical *in vivo* studies of CTM composition*

Cell line	Administration	Model	Findings	Reference
Lewis lung carcinoma‡	Renal capsule	C57BL/6 GFP+ mouse	Tumour cells and fibroblasts	52
T241 fibrosarcoma‡	Intramuscular (femoral)	C57BL mouse	Tumour cells and leukocytes	96
Mel57 melanoma‡	Subcutaneous (flank)	Nude mouse	Tumour cells, endothelial cells, pericytes and laminin	143
MCH66 mammary carcinoma‡	Mammary fat pad	C3H/He mouse	Tumour cells and endothelial cells	142
Morris hepatoma 5123§	Intramuscular (thigh)	Buffalo rat	Tumour cells and endothelial cells	141
MC-38 colon carcinoma‖	Intravenous	C57BL6 mouse	Tumour cells and leukocytes	144
LS180 colon adenocarcinoma T84 human adenocarcinoma	Intravenous	RAG2$^{-/-}$ mouse	Tumour cells and platelets	146

*Including all published preclinical studies identified using the stated search criteria, which contained data on the composition of CTM. ‡Analysis of CTM in the tumour effluent and tumour draining vein. §Analysis of spontaneous CTM in the lungs. ‖Analysis of tumour cell aggregates in the lungs following intravenous administration. Abbreviation: CTM, circulating tumour microemboli.

model, demonstrating that the presence of heterotypic tumour stroma (particularly fibroblasts) increases the viability of tumour cells within CTM and confers an early growth advantage to metastatic colonies. Furthermore, partial depletion of the carcinoma-associated fibroblasts reduced metastatic capability.[52] Promotion of metastasis has also been shown by the association of tumour cells with platelets,[145] leukocytes[140] and endothelial cells.[147]

Whether different tumour cells within CTM have equal metastatic capability is currently uncertain. Intravenous injection of multicellular aggregates composed of two different cell lines (with similar metastatic potential) into syngeneic mice intriguingly resulted in lung metastases with a karyotype unique to one (either) metastatic cell type,[148] in keeping with the concept of clonal evolution.[149] In another study, two cell lines with different metastatic potential were mixed and injected into the flank of nude mice. Polyclonal tumours were produced at the site of primary inoculation and both CTM and ~90% of the resultant lung metastases were polyclonal.[143] When the cell lines were separately injected into opposing flanks, <10% of the metastases arose from the less-metastatic cell line, suggesting higher metastatic capability when incorporated into CTM. Studies of CTM in patients will provide further insight into CTM composition and metastatic potential.

Circulating tumour microemboli in patients

Several CTC technologies can reliably isolate CTM from patients, albeit with varying sensitivities (Table 5). Although the possibility that tumour cells form 'clumps' during blood sample processing cannot be entirely excluded, some evidence suggests this is not the case. Specifically, while CTM have been observed using various different methods of isolation (Table 5), not every patient studied has them and CTM are not typically observed when cell lines are 'spiked' into the blood of healthy controls.[150] Moreover, CTM are often found in lymphovascular spaces surrounding the primary tumour in resected specimens of multiple cancer types, suggesting that clusters of cells do break off from primary tumours and this observation argues against the *ex vivo* formation of CTM by sample processing.[151–153] Additionally, initial correlative studies have revealed the prognostic significance of CTM in SCLC,[30] and the unique molecular characteristics of clusters of cells are beginning to be unveiled. For example, in patients with lung cancer, cells within CTM were nonproliferative (based on absence of Ki-67 expression), but up to 62% of single cells had high Ki-67 expression,[30] suggesting tumour cells within CTM are in a state of cell-cycle arrest—perhaps contributing to chemotherapy resistance and enhanced cell survival and resulting in worse prognosis. These hypotheses warrant further investigation.

Although CTM are rarely captured by CellSearch® in the majority of patients with solid tumours, they are frequently detected in SCLC and confer a poor prognosis.[30] In general, studies have been observational and explore the molecular features of CTM. Notably, microfiltration (non-ISET®) has shown CTM from patients with renal cancer were partially covered by endothelial cells and pericytes,[158] mirroring findings from preclinical models.[147] Heterogeneity in EMT markers has also been demonstrated within CTM with partial EMT phenotypes, as is the case for single cells.[120,121] Interestingly, the mesenchymal marker vimentin is expressed in contiguous cells within CTM, which argues against an obligatory association of EMT with loss of cell–cell contacts.[120]

In summary, clear evidence has shown marked heterogeneity in CTCs and CTM, which is perhaps unsurprising given the heterogeneity of underlying tumours. Much data support that only a small population of cells has the ability to seed distant metastatic disease. Although considerable progress has been made in CTC research, our efforts now need to focus on the analysis of cells with metastatic potential to identify novel therapeutic strategies.

Clinical use of CTCs—lung cancer

Alongside the insights into the biology of metastasis and technology development, a host of studies have evaluated the clinical utility of CTCs, from prognostic and predictive use to detecting minimal residual disease, screening applications and disease monitoring.[163,164] With respect to lung cancer, several exciting clinical applications are being developed.

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Table 5 | Clinical studies identifying CTM in patients with cancer*

Cancer type	Stage	*n*	Enrichment method	Frequency of CTM (%)	Reference
***Peripheral venous blood*‡**					
HCC	Localized	44	ISET®	52.3	69
Melanoma	Locoregional and metastatic	79 and 8	ISET®	NS	154
SCLC	Locoregional and metastatic	31 and 66	ISET®	32	30
NSCLC	Locoregional and metastatic	41 and 60	ISET®	43	31
NSCLC	Metastatic	14	Blood plate and immunofluorescence	43	155
Breast	Metastatic	24	Blood plate and immunofluorescence	43	155
Prostate	Metastatic	15	Blood plate and immunofluorescence	43	155
Pancreas	Metastatic	18	Blood plate and immunofluorescence	43	155
Colorectal	Locoregional	>200	Centrifugation and cytopathology	NR	156
***Tumour-draining vein*‡**					
NSCLC	Locoregional	208	ISET®	NR	157
Renal	Locoregional	51	75 μm microfilter	33	158
Renal	Locoregional and metastatic	7 and 3	2 μm microfilter	50	159
***Peripheral venous blood*§**					
SCLC	Extensive	3	CellSearch® (EpCAM)	NR	120
Prostate	Metastatic	15	Herringbone chip (EpCAM)	NR	64
NSCLC	Metastatic	4	Herringbone chip (EpCAM)	NR	64
Prostate	Locoregional and metastatic	8 and 2	Immunomagnetic (cytokeratin)	80	160
Colorectal	Locoregional and metastatic	19 and 13	Immunomagnetic (cytokeratin)	68.8	161
Prostate	Metastatic	25	Immunomagnetic (CD45 depletion)	NR	162
SCCHN	Locoregional	11	Immunomagnetic (CD45 depletion)	NR	124

*Including all published clinical studies that reported CTM identified using the stated search criteria. ‡Marker-independent enrichment. §Marker-dependent enrichment. Abbreviations: CTM, circulating tumour microemboli; HCC, hepatocellular carcinoma; NR, not reported; NS, not specified; NSCLC, non-small-cell lung cancer; SCCHN, squamous cell carcinoma of the head and neck; SCLC, small-cell lung cancer.

Prognostic and pharmacodynamic biomarkers

The FDA approval of CellSearch® extends to prognostic utility in breast cancer, prostate cancer and CRC, for which the presence of CTCs above a cut-off level—five cells in breast and prostate cancers per 7.5 ml blood, three cells per 7.5 ml blood in CRC—is associated with shorter survival.[32–34] Prognostic utility has also been demonstrated with CellSearch® in NSCLC (cut-off level of five CTCs per 7.5 ml blood)[31] and in SCLC, in which CTCs are more abundant than in any other disease type thus far reported (range 0–44,896 cells per 7.5 ml blood, with a cut-off level of 50 CTCs per 7.5 ml blood).[30]

Beyond use as prognostic markers, CTCs can be applied clinically as pharmacodynamic biomarkers. A decrease in either total CTC number[30–33,165–168] or of a molecularly determined subgroup of CTCs (for example, those expressing common targets such as HER2,[169] insulin like growth factor-1 receptor[170] or EGFR[171]) has been reported in response to therapy. In SCLC, the total CTC number is substantially reduced in the majority of patients after a single cycle of chemotherapy,[30,166,167] in keeping with high response rates commonly observed with platinum-based treatment;[172] this change was shown to be independently associated with good prognosis.[30] Conversely, an increase in CTC number from favourable to unfavourable levels after chemotherapy is associated with shorter overall survival in patients with breast cancer, prostate cancer and CRC.[32,33,168] The narrow dynamic range of CellSearch® (that is, the ratio of highest to lowest number of CTCs detected) in most tumour types might preclude its application as a routine pharmacodynamic biomarker or surrogate end point. However, trials addressing this issue are currently in progress;[173] for example, the SWOG 0500 trial (NCT00382018) randomly assigned women with metastatic breast cancer to a switch in chemotherapy if CTCs remained elevated (≥5 cells as per the prognostic cut-off value) after one cycle of treatment or a continuation of the same treatment. Results of this trial are awaited and similar studies are needed in the other major solid tumour types. Newer technologies (such as the iCHIP platform[65]) are anticipated to increase sensitivity and broaden the use of pharmacodynamic measurement in clinical practice. Another anticipated use of CTC enumeration is in the early stage cancer setting, whereby presence of CTCs might help select patients who are most likely to benefit from adjuvant therapy. However, for any proposed clinical application, prospective, randomized controlled trials are first needed to validate and qualify their clinical use.

Providing proof of mechanism (POM) for novel agents is a key objective in early phase trials. Ideally, this is assessed in tumour biopsies, but more commonly peripheral blood mononuclear cells are used as accessible

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surrogates. Beyond the evaluation of CTC numbers, determining the apoptotic fraction by measuring M30 antigen[174,175] and other CTC characterization assays are now being considered as POM pharmacodynamic biomarkers.[176] For example, Wang *et al.*[177] assessed γ-H2AX expression (a marker of DNA damage) in CTCs from patients treated with DNA-damaging chemotherapeutic agents, finding that the percentage of γ-H2AX-positive CTCs increased following treatment. CTC assays demonstrating phosphorylation of EGFR,[178,179] HER2,[179] PI3K,[178] AKT[178] and FAK[180] have also been developed, although these are yet to be applied as pharmacodynamic biomarkers in early phase trials.

CTCs as predictive biomarkers

Ideally, CTCs will be used as a liquid biopsy to select a specific personalized therapy on the basis of the molecular features of a patient's tumour. This scheme was exemplified in a study of *EGFR*-mutation-positive NSCLC in which an expected activating *EGFR* mutation (on the basis of tumour biopsy analysis) was identified in 19 out of 20 patients using a microfluidic CTC enrichment platform and PCR-based assay.[40] Furthermore, serial analysis of four patients during EGFR-directed therapy revealed emergence of the T790M resistance mutation, demonstrating that CTCs can be used to identify resistance biomarkers.

Another example is in the analysis of the *EML4–ALK* rearrangement, which occurs in 3–5% of patients with NSCLC. Approximately 60% of these patients exhibit impressive responses to ALK inhibition.[5,181] Two studies using ISET® microfiltration have demonstrated the feasibility of *ALK*-fusion FISH testing in CTCs, with high correlation between the CTC *ALK*-fusion status and that of the underlying tumour.[39,182] In the first study, Pailler *et al.*[39] demonstrated that all of 18 ALK-positive patients had at least four *ALK*-rearranged CTCs per millilitre of blood. Furthermore, all CTCs exhibited a unique pattern of *ALK* gene rearrangement with break apart of the 3'5' *ALK* gene probes, whereas the tumours demonstrated heterogeneity in rearrangement patterns. These data suggest a unique population of cells that preferentially enter the blood have greater invasive and migratory properties. In keeping with this notion, the authors demonstrated that *ALK*-rearranged CTCs expressed a mesenchymal phenotype.[39] In a second study by Ilie *et al.*,[182] *ALK* rearrangements by FISH and strong *ALK* protein expression by immunocytochemistry, were detected in CTCs from five patients with NSCLC with corresponding *ALK*-rearranged tumours. In 82 patients with NSCLC whose tumours did not harbour *ALK* rearrangements, FISH and immunocytochemistry testing were negative in all CTCs. Moving forward, this test could be applied as a noninvasive method to select patients who should receive ALK inhibitors, with repeat samples on disease progression being used to interrogate mechanisms of resistance.

Others have evaluated HER2 expression in CTCs in breast cancer[183] and androgen receptor expression and *ERG* rearrangement in prostate cancer,[42,184,185] which have potential use as predictive biomarkers. Indeed, some women with breast cancer have been shown to have discordant HER2 statuses for their CTCs (positive) and primary tumour (negative), but still benefit from trastuzumab therapy.[186] This finding supports the notion that CTC characteristics might be most pertinent to target because these cells are ultimately responsible for metastasis. In general, robust studies assessing the predictive value of CTCs have been few and far between; well-designed, prospective randomized studies using CTCs to stratify patients are needed. Furthermore, the relevance of CTC heterogeneity in response to or in developing resistance to targeted therapy is yet to be unveiled. Indeed, single-cell profiling techniques are anticipated to contribute to the next generation of clinical studies aimed at determining the predictive value of driver molecular aberrations in CTCs and emergence of resistant subclones on targeted therapy (Figure 1).

cfDNA and CTCs as complementary biomarkers

Circulating tumour-derived cfDNA (ctDNA) is undergoing evaluation alongside CTCs for its utility in clinical practice.[187,188] Remarkable advances in sequencing capability for ctDNA have sparked debate as to which of CTCs or ctDNA will be the 'better' biomarker for use in the clinic.[189,190] Head-to-head comparison of ctDNA and CTCs was undertaken in 30 patients with breast cancer harbouring somatic mutations in *PIK3CA* and *TP53*.[189] Detectable ctDNA was found in 97% of these patients whereas CTCs were isolated from 87% of the cohort. Increasing levels of ctDNA were apparent in 17 of the 19 patients with progressive disease and levels increased, on average, 5 months prior to radiologically confirmed disease progression in 10 patients. By contrast, only seven of 19 patients had a demonstrable increase in CTC number and nine of 18 patients had increases in cancer antigen 15-3 levels at disease progression. Both ctDNA and CTC levels correlated with treatment response during therapy, but ctDNA was present in higher numbers of patients and demonstrated a higher dynamic range for pharmacodynamic monitoring.[189] In NSCLC, the presence of activating *EGFR* mutations and mutations that confer resistance to EGFR-directed therapies have been identified in ctDNA using highly sensitive single-molecule PCR (BEAMing; Inostics GmbH, Germany).[191] Using whole-exome sequencing of ctDNA, serial sampling in patients with NSCLC identified an activating *EGFR* mutation at baseline and the emergence of the T790M resistance mutation following treatment with gefitinib,[192] demonstrating the potential for ctDNA profiling to guide stratified therapies in the clinical setting.

Although ctDNA might indeed provide advantages in terms of simplicity and sensitivity compared with CTCs, the emergence of new CTC enrichment platforms are expected to provide increased CTC yield and simplicity. Uniquely, CTC molecular analysis can provide additional information—such as cell morphology, immunocytochemical phenotype and presence of diagnostic epitopes—and the ability to establish the

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presence of multiple mutations within the same cell, which will help decipher tumour heterogeneity and map clonal evolution. Single CTC analysis also provides the opportunity to combine genomic mutational analysis and mRNA profiling,[89] providing a potential linkage between mutational status and pathway activation. For exploratory research, the most striking advantage of CTC analysis is the ability to perform *in vivo*[27] functional testing to provide the opportunity to address issues of metastasis biology.

We suggest that CTCs and ctDNA will be complementary in their biomarker utility. In practice, ctDNA might currently be preferred for mutational analysis, but CTCs provide an unparalleled means to interrogate metastatic biology and determine the functional relevance of novel targets for therapeutic inhibition.

Conclusions

CTCs are an exciting area of research that hold promise as biomarkers to aid in treatment selection for patients, and as a research tool to increase our knowledge of metastasis and identify novel therapeutic targets. The translation of CTCs to a routine clinical test is hindered by the multiple different technologies that exist for CTC isolation and the lack of validation and qualification of assays. Although CellSearch® has been through this extensive process and is a useful prognostic biomarker, more sensitive technologies and flexibility for characterization are still needed. For novel CTC technologies to gain a position in clinical practice, assessment in large-scale clinical trials is required and comparison with the 'gold-standard' CellSearch® technology should be considered. A small number of studies have demonstrated the feasibility of CTC assays for predictive biomarkers, such as identification of *ALK*-rearrangement from CTCs (by ISET®) in patients with *EML4–ALK* fusion-positive NSCLC,[39,182,193] but further validation and qualification are required in collaborative efforts for these assays to progress to the clinic.

Key issues in CTC research must be addressed to maximize the potential of CTC profiling for therapy selection. For example, we must ensure that CTC isolation and characterization technologies have increased sensitivity to accommodate CTC heterogeneity. We also need a greater understanding of CTC biology, including an unravelling of which CTCs (or CTM) 'survive the trip' and how. How many CTCs are needed to optimally capture heterogeneity must also be determined. Robust approaches to mitigate random error in single CTC analysis should also be adopted.

The field of minimally invasive biomarkers in oncology has come of age with the realistic expectation that patient survival will be improved through real-time disease monitoring and stratification of therapy. With continued research, it is anticipated more-informative blood tests (for molecular analysis of CTCs/ctDNA) will become as integral to therapeutic decision-making as routine imaging assessment.

Note in proof

Since submitting our final manuscript, Ni and colleagues have reported single-cell analysis of CTCs in patients with lung cancer. The authors demonstrate reproducible CNV patterns between CTCs within an individual and between patients harbouring the same histological subtypes of lung cancer (NSCLC or SCLC). The authors hypothesize CNV changes are a key event driving metastasis and that CTC CNV profiling could be used as a diagnostic tool.[194]

Review criteria

For this Review, studies were identified through a PubMed search using the terms "circulating tumour cell", "circulating tumour cells", "circulating tumor cell", "circulating tumor cells" and "single cell DNA/RNA", with no start date and an end date of October 2013. Lists of references of selected articles were cross checked to find further relevant studies.

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Author contributions

M. G. Krebs, R. L. Metcalf and L. Carter researched the data for the article, and discussed its content with C. Dive and F. H. Blackhall. All authors wrote the manuscript and edited it before submission.

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