OPINION

Challenges in circulating tumour cell research

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Abstract | During the past ten years, circulating tumour cells (CTCs) have received enormous attention as new biomarkers and the subject of basic research. Although CTCs are already used in numerous clinical trials, their clinical utility is still under investigation. Many issues regarding the detection and characterization of CTCs remain unknown. In this Opinion article, we propose a conceptual framework of CTC assays and point out current challenges of CTC research, which might structure this dynamic field of translational cancer research.

Research on circulating tumour cells (CTCs) is a very active field, with more than 15,190 publications listed under the key phrase "circulating tumor cell" in PubMed in June 2014 (on average, 20 new publications each week in 2013). CTCs are used as biomarkers in >270 clinical trials that are registered at ClinicalTrials.gov. Strong evidence for CTCs as prognostic markers has been documented for breast cancer[1], but CTC detection is also connected to metastatic relapse and progression in other tumour entities, including prostate, lung and colorectal cancer[2–6]. In ongoing interventional studies, the clinical utility of CTCs for treatment decisions is being evaluated[7]. In particular, the use of CTCs as a real-time liquid biopsy has received attention during the past years[8].

However, the diversity of published assays using different principles for enrichment and identification of CTCs is confusing to the cancer research community. CTCs occur at very low concentrations in the peripheral blood, ranging between 1–10 cells per 10 mL in most cancer patients, which poses a serious challenge for any analytical system. This Opinion article addresses two key questions: first, what are the best biological and physical concepts to enrich, detect and characterize all CTCs in cancer patients? Second, what are the unresolved issues in CTC research?

CTC detection approaches

Recent progress has been made in the development of various microfluidic devices to enrich CTCs, but the discovery and validation of new CTC markers is still in its infancy. Classifications of current CTC enrichment and detection technologies are presented in FIG. 1 and FIG. 2, respectively. To define good CTC markers, the biology of CTCs and surrounding blood cells needs to be taken into account. The 'perfect' CTC marker would be expressed on all CTCs but not on autochthonous blood cells (leukocytes, endothelial cells, haematopoietic stem cells (HSCs) and mesenchymal stem cells) and never repressed during the invasion and circulation process. In general, CTC assays start with an enrichment step that increases the concentration of CTCs by several log units and enables an easier detection of single immunostained cells. Cell surface proteins have been used for an antibody-based enrichment step to attach CTCs to columns, microposts or magnetic devices, and markers that are present in all subcellular locations have been used for the subsequent detection and characterization steps. For an unambiguous detection of CTCs, phenotyping by immunostaining or reverse transcription PCR (RT-PCR) might be complemented by additional genomic analyses (for example, fluorescence *in situ* hybridization (FISH) or single cell analysis) to avoid false-positive findings. False-negative findings can be avoided by the use of antibody combinations against various markers that cover the complex heterogeneity of CTCs. RT-PCR-based detection of specific transcripts is quite different from capture and visualization of intact CTCs, and low-level illegitimate expression of the targeted transcript can lead to false-positive results[9]. Subsequently, we will give a brief overview of the technologies used for detection and enrichment of CTCs.

Protein expression-based technologies

Protein-based technologies rely on specific markers that are detected by antibodies. Epithelial markers are expressed on normal epithelia and epithelial tumours (that is, carcinomas) but absent on the mesenchymal leukocytes and have therefore been frequently used to distinguish cancer cells from normal blood cells[8]. Epithelial cell adhesion molecule (EPCAM) is the cell surface marker that is most frequently used for positive enrichment of epithelial CTCs (FIG. 1), and members of the family of cytokeratins (that is, CK8, CK18 and CK19) — cytoskeletal proteins that are specific for epithelial cells — have become 'gold standard' markers for the detection of CTCs with an epithelial phenotype in patients with carcinoma[10–12] (FIG. 2). However, it should be noted that circulating epithelial cells have been reported in patients with benign colon diseases[13] and these cells might be a source of false-positive findings.

Furthermore, carcinoma cells can undergo an epithelial-to-mesenchymal transition (EMT) that results in reduced expression of epithelial markers, and observing these markers might therefore result in false-negative findings[14]. Thus, there is a need for the identification of additional mesenchymal markers that are upregulated during EMT on CTCs. N-cadherin (a membrane protein of the cadherin family) and vimentin (a structural cytoskeletal protein) are both expressed in mesenchymal cells and used for the detection of mesenchymal CTCs. As vimentin is widely expressed in all surrounding normal blood cells, a FISH analysis detecting tumour-specific genomic changes is needed to confirm whether a given cell is indeed a tumour cell[15]. Moreover, commonly used markers for EMT, besides N-cadherin

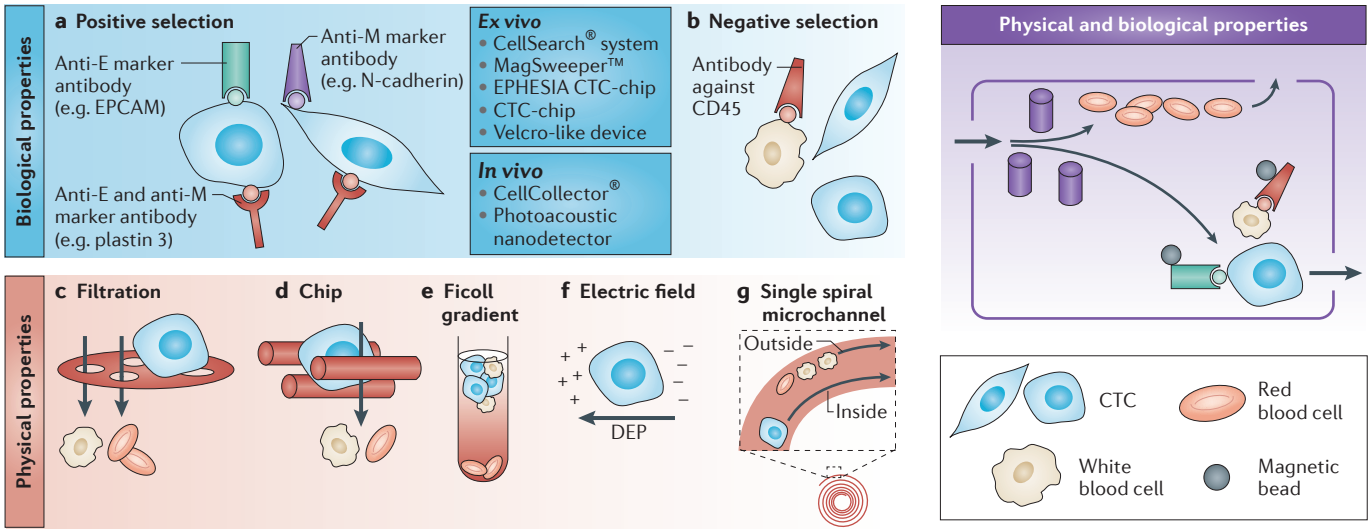

Figure 1 | **Circulating tumour cell (CTC) enrichment technologies.** CTCs can be positively or negatively enriched on the basis of biological properties (for example, expression of protein markers): CTCs can be positively enriched (part **a**) by using an anti-epithelial (E) antibody, an anti-mesenchymal (M) antibody or an anti-E and anti-M antibody, or negatively enriched (part **b**) by using antibodies against CD45 to deplete the unwanted leukocytes. CTCs can also be positively or negatively enriched on the basis of physical properties (for example, size, density, deformability or electric charges) through a membrane and filtration-based system on the basis of the CTC size (part **c**); through posts in a microchip on the basis of CTC size plus deformability (large and stiff CTCs are trapped on a basket of three posts) (part **d**); through a centrifugation on a Ficoll density gradient on the basis of the CTC density (part **e**), through dielectrophoresis (DEP) on the basis of CTC electric charges (under a particular medium conductivity, different types of cells could have different DEP behaviours — DEP can have a high specificity) (part **f**); through a spiral CTC chip on the basis of the CTC size (under the influence of Dean drag forces, the smaller blood cells (red blood cells and leukocytes) migrate along the Dean vortices towards the inner wall, and then back to the outer wall again, whereas the larger CTCs experience additional strong inertial lift forces and focus along the microchannel inner wall) (part **g**). Positive or negative enrichment of CTCs can also be achieved on the basis of physical and biological properties (for example, combination of size (first) and protein expression (second) with the CTC-iChip): CTCs are first selected on the basis of their presumably larger size, whereas the smaller leukocytes are discarded; then, CTCs are immunostained with bead-conjugated antibodies against epithelial cell adhesion molecule (EPCAM), captured in a magnetic field and collected on a glass slide. Another possibility is the depletion of the normal haematopoietic cells by using bead-conjugated antibodies against CD45 and CD15.

and vimentin, also include nuclear localization of β-catenin, and increased production of transcription factors such as SNAI1 (also known as SNAIL), SNAI2 (also known as SLUG), TWIST, zinc finger E-box binding homeobox 1 (ZEB1), ZEB2 and/or TCF3 that inhibit the production of E-cadherin. The expression of EMT markers is associated with an increased capacity for migration and invasion, as well as resistance to anoikis and apoptosis[16,17], all of which might be required for the survival and dissemination of CTCs.

During EMT, different subsets of CTCs can show a range of phenotypes. Thus, there is a need for a broad-spectrum enrichment of all of these CTCs that is based on the use of specific cocktails of cell surface epithelial and mesenchymal markers[18]. However, a large cocktail of different markers covering all potential CTC phenotypes may increase the chance that individual blood cells might also express at least one of these markers, which would lead to false-positive results. One option to overcome this limitation of current epithelial and/or mesenchymal markers could be to target the actin bundling protein plastin 3 — a novel marker that is not downregulated by CTCs during their EMT and not expressed in blood cells[19].

Tumour-specific markers are usually expressed at much higher levels in cancer cells compared with normal cells, and they are specific to certain tumour types. HER2 (also known as ERBB2) and epidermal growth factor receptor (EGFR; also known as HER1) are good examples of tumour-associated markers that are also important in the context of targeted therapies[7,20,21]. Tissue-specific markers, such as prostate-specific antigen (PSA) for prostate cancer[22] and mammaglobin[23] for breast cancer, have a high specificity but can be downregulated during dedifferentiation of tumour cells. At present, cancer testis antigens (for example, the melanoma-associated antigen (MAGE) family) are the most specific tumour proteins, as they are expressed only on cancer cells and in normal testis[24].

Positive selection of CTCs requires an assumption about the unknown nature of CTCs in an individual blood sample. This bias is avoided by negative selection in which the blood sample is depleted of leukocytes using antibodies against CD45 (which is not expressed on carcinomas or other solid tumours) and other leukocyte antigens (FIG. 1). Cells that are positive for both cytokeratins (CK8, CK18 and CK19) and CD45 have been detected in the blood of patients with carcinoma but they might be prognostically irrelevant and could represent artefacts caused by an extended time before sample analysis[25,26]. Alternatively, CK^+CD45^+ cells might also represent circulating tumour-associated macrophages[27]. On the basis of the emerging role of EMT during tumour cell dissemination, a strong focus on technologies based on the depletion of normal $CD45^+$ haematopoietic cells arose to avoid loss of CTCs with high phenotypic plasticity. Using magnetic beads that bind to $CD45^+$ leukocytes, these cells are then removed by placing the sample in a magnetic field[18,28,29]. Another approach is the use of bi-specific antibodies against antigens on leukocytes and erythrocytes that induce the formation of large multicellular rosettes, which can be easily removed from the blood sample by Ficoll density centrifugation[30]. However, not all $CD45^-$ cells in the blood are tumour cells (for example, circulating endothelial cells are $CD45^-$);

thus, subsequent detection and characterization steps are of utmost importance to increase assay specificity.

Physical property-based technologies

A different way to enrich CTCs is to use their physical properties to distinguish them from normal blood cells. For example, tumour cells were initially thought to be bigger (>8 μm in size) and less deformable than haematopoietic cells; thus, different devices based on cell filtration and centrifugal force have been developed during the past years[31–33] (FIG. 1). However, CTCs of various sizes have been identified by the CellSearch® system (Janssen Diagnostics, Beerse, Belgium), as well as other CTC assays, and CTCs that are capable of undergoing EMT might also be as deformable as leukocytes. Thus, more sophisticated label-free approaches (for example, photoacoustic flow cytometry or technologies involving dielectrophoresis (DEP)) have recently been used[34].

Beside enumeration of CTCs, further molecular characterization is required to define the nature of these cells. Thus, the number of studies that focus on the characterization of CTCs has increased during the past years (BOX 1).

Functional assays

Functional assays can be used to detect and characterize CTCs, and they are mandatory to discover the biology of CTCs, with particular emphasis on the discovery of the 'metastasis initiator cells' (MICs). At present, such assays are limited by the low concentration and yield of CTCs. To our knowledge, only two different *in vitro* assays have been optimized to detect viable CTCs obtained from cancer patients: first, the EPISPOT assay, which detects specific proteins secreted during the *in vitro* culture of CTCs[6,30] (FIG. 2) and, second, an invasion assay that examines the ability of CTCs to digest a fluorescently labelled cell adhesion matrix[35] (FIG. 2). CTCs are first isolated using other methods before these assays are used to examine their function.

Important *in vivo* information can be obtained by transplantation of patient-derived CTCs into immunodeficient mice: metastases that were grown after xenotransplantation of breast cancer CTCs had an $EPCAM^{low}MET^{high}CD47^{high}CD44^{high}$ phenotype, which may be characteristic of metastasis-initiator cells[36] (FIG. 2). A recent report on patients with small-cell lung cancer showed that CTCs from patients with either chemosensitive or chemorefractory tumours are tumorigenic in immunocompromised mice, and the resultant CTC-derived explants mirrored the response of the donor patient to platinum and etoposide chemotherapy[37]. However, at present, these *in vivo* assays require very high CTC yields in the transplanted blood sample (for example, >1,000 cells per 7.5 mL in breast cancer), which have so far only been achieved in a few patients.

Important questions in CTC research

To stimulate future investigations in the field of CTC research, we will discuss some of the most important questions. This section is based on our current knowledge and our opinion; thus, it is aimed to induce discussion among researchers rather than to represent a dogmatic view.

Can CTCs be used for the early detection of solid tumours? Interestingly, in a mouse model of pancreatic cancer, pancreatic cells with a mesenchymal phenotype and stem cell properties were found to be circulating in the blood and had seeded the liver before any primary tumour was detectable[38]. However, the stage of tumour development at which blood-borne dissemination occurs in cancer patients is still a matter of debate. In cancer patients, CTCs that have homed to bone marrow (disseminated tumour cells (DTCs)) are also detected in patients with pre-invasive lesions (for example, patients with ductal carcinoma *in situ*[39,40]), suggesting that blood-borne dissemination is an early event. However, small

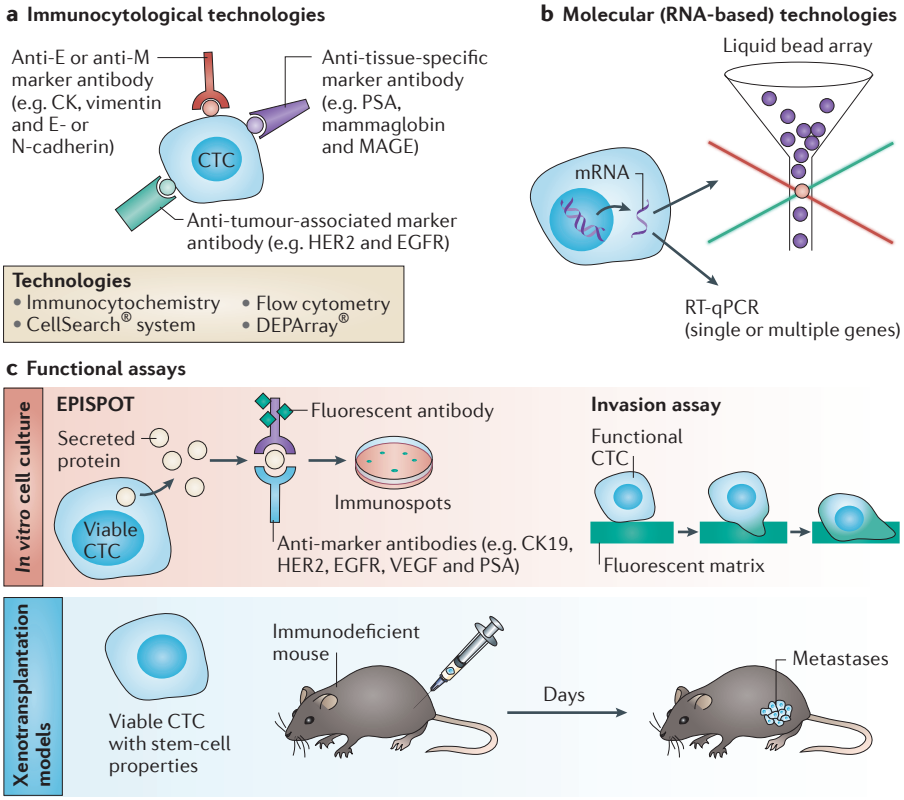

Figure 2 | **Circulating tumour cell (CTC) detection technologies.** CTCs can be detected using immunological, molecular or functional assays. **a** | Immunological technologies. CTCs can be detected by using a combination of membrane and/or intra-cytoplasmic anti-epithelial (E), anti-mesenchymal (M), anti-E and anti-M, anti-tissue-specific marker or anti-tumour-associated antibodies. **b** | Molecular technologies. CTCs can be detected by using RNA-based technologies (multiplexed reverse transcription (RT)-PCR combined with liquid bead array detection allows simultaneous amplification and detection of multiple transcripts, using liquid bead array multi-parameter RT-quantitative PCR (RT-qPCR)). **c** | Functional assays. *In vitro*: viable CTCs can be detected by using the fluoro-EPISPOT technology (CTCs secrete specific tumour marker proteins that are captured by the coating antibody on the bottom of the culture dish). The cells are then discarded and the captured proteins are detected by a second fluorochrome-conjugated antibody or by performing an invasion assay (invasive CTCs ingest the fluorescent matrix and become fluorescent themselves). *In vivo*: CTCs with stem cell properties can give rise to tumour growth in an immunodeficient murine host. The outcome of these experiments depends on the experimental conditions and mouse strains, and potential caveats also include the lack of interaction of these circulating cells with a functional immune system. CK, cytokeratin; EGFR, epidermal growth factor receptor; PSA, prostate-specific antigen; MAGE, melanoma-associated antigen; VEGF, vascular endothelial growth factor.

Box 1 | **Molecular characterization of circulating tumour cells**

For genomic analyses, most studies have isolated single circulating tumour cells (CTCs) and carried out whole-genome amplifications (WGAs) to increase the amount of DNA, which is subsequently subjected to the analyses of specific point mutations and copy number variations (including amplifications) using conventional and next-generation sequencing technologies[78,82]. Although substantial advances in single-cell technologies have been made, WGA has the inherent risk of inducing a bias and false findings. Thus, substantial efforts need to be undertaken to show that the result obtained reflects the real status of a single CTC. Pooling of individual CTCs from the same patient sample might decrease this problem to some extent and increase reproducibility, but this will lead to a loss of information on intra-patient heterogeneity. Besides isolation of single CTCs, enrichment by 3–4 log units might be sufficient to obtain a concentration of one CTC in 1,000 blood cells, which is in the range that is suitable for highly sensitive mutation analyses technologies such as digital PCR or BEAMing PCR (beads, emulsions, amplification and magnetics PCR). Another (much simpler) approach is fluorescence *in situ* hybridization (FISH) analysis of single CTCs identified by immunocytochemistry[18,20,55]. Such an immuno-FISH approach can be combined with automated detection of CTCs and might be easier to implement in future clinical diagnostics.

RNA and microRNA analyses have been carried out on epithelial cell adhesion molecule (EPCAM)-enriched CTC fractions that are still contaminated with leukocytes[23,94] (leukocyte-derived RNA is subtracted by the analysis of pure leukocyte fractions) or on single isolated CTCs of patients with breast cancer[95]. Although the analysis of single isolated CTCs is more cumbersome, the screening of impure enriched CTC pools is limited because the surrounding leukocytes might express the target genes of interest and 'contaminate' the result unless genes are targeted that are highly expressed in CTCs but have a low expression in normal blood cells.

concurrent invasive lesions might have been missed in these investigations. Lesions that are detectable in patients by current imaging procedures usually already contain more than 10^9 tumour cells. There is an ongoing debate as to whether these small tumours already contain the variants required for metastasis or whether DTCs will undergo a parallel progression that leads to new variants.

Nagrath *et al.*[41] identified CTCs in more than 90% of patients with different types of cancer; most patients in this study had overt metastases, but the few patients with localized prostate cancer who were included also had detectable CTCs and displayed even higher CTC counts than patients with metastatic prostate cancer. This unexpected finding had induced high hopes that CTC detection could be used for the early detection of cancer. However, subsequent publications of the same group, using improved CTC-chip devices, reported much lower incidences in metastatic[18,42,43] and early stage[44] cancer patients, and other groups have also reported much lower incidences in early stage cancer patients[11]. Increasing the sensitivity of CTC analyses, however, might also lead to false-positive results in healthy controls[42]; even current CTC assays have resulted in false-positives in patients with benign colon diseases[13]. These challenges must be overcome to use CTC assays for the early diagnosis of cancer.

The limited blood sample volumes that are available from cancer patients may impose another serious limitation on the detection of rare CTCs by micro-devices in early stage cancer, in which the CTC counts are very low. One possible way to overcome this limitation is to collect the CTCs directly, by filtering a large volume of a patient's blood; for example, during the 30-minute application of the CellCollector® (Gilupi, Potsdam, Germany) in the peripheral arm vein, up to 1.5 litres of blood pass the 2 cm functionalized area of the collector and enable CTCs to be bound by antibodies against EPCAM[45]. This new device is currently being validated in a multi-centric clinical trial in patients with high-risk prostate cancer (European ERA-NET on Translational Cancer Research TRANSCAN project 'CTC-SCAN'; Principle Investigator: K.P.). An alternative approach is the development of leukapheresis, an elutriation for subsequent *ex vivo* CTC analyses using flow cytometry and real-time PCR for molecular characterization[46]. Leukapheresis is a standard clinical method that is frequently used to isolate mononuclear cells from several litres of blood for various applications, including stem cell harvest. Leukapheresis has recently been applied to non-metastatic cancer patients and has recovered large quantities of CTCs (a median of 7,500 CTCs per patient) for molecular analyses[47]. Although this approach is very interesting for experimental studies, including comparative evaluation of different CTC technologies, it is much more invasive and time-consuming than taking a blood sample, and this may hamper its use in the clinic. With the rapid development of new CTC technologies, it might become possible to test the hypothesis that CTCs are present in most early stage cancer patients.

Can the characterization of CTCs reveal the origin of these cells and identify particular distant organs as potential sites of metastatic relapse? CTCs are released from the primary tumour and/or (micro)metastases into the bloodstream. Most CTCs travel as single isolated cells, but clusters of CTCs (also called 'microemboli') can occur, particularly in patients with advanced disease[48]. At present, there are only a few reports on the half-life of CTCs after they are released into the circulation. In patients with breast cancer, the half-life of CTCs was estimated to range between 1 hour and 2.4 hours, and it was concluded that tumour cells somewhere in the tissues of the patients must refill the pool of CTCs every few hours[49]. Thus far, it is unclear whether CTCs that are derived from primary versus metastatic tumours and between metastases in different organs have a special signature that will reveal their origin. Such information would be very useful in the clinic to identify the organ that harbours an occult metastasis or even the origin of the primary tumour in patients with cancer of unknown origin.

Transcriptome analyses of DTCs in the bone marrow have identified an osteoblast-like phenotype (that is, expression of genes that are normally expressed by osteoclasts or osteoblasts) for human osteotropic breast cancer cells[50–52]. We can speculate that such an adaptation might occur not only in the bone marrow but also each time a CTC reaches a new specific organ, when there may be an acquisition of an 'organ-mimetic phenotype' by the CTCs. In addition, different tumour cells that are present in the primary tumour may have differential abilities to metastasize to different organs[53,54], and this can also shape the characteristics of the DTCs in each organ. Thus, we can imagine that CTCs circulating from metastatic sites will have a specific lung, bone, liver or brain signature that can be detected by molecular analyses. Despite the wealth of information on this issue in animal models, little is so far known about it in cancer patients. A recent study showed that CTCs in patients with breast cancer brain metastases have a specific protein expression signature ($HER2^+EGFR^+$heparanase $(HPSE)^+NOTCH1^+EPCAM^-$), which was required for the formation of metastases that specifically occurred in the brain after xenotransplantation into immunodeficient mice[55]. The authors determined this signature by applying flow cytometry for CTC selection, followed by the establishment of CTC lines that were transplanted into mice. This study encourages future investigations into signatures for other metastatic organs.

What is the relevance of the detection of the EMT in CTCs? One of the key questions is whether current methods are able to capture the MICs. This question has recently been tightly linked to the relevance of the EMT in CTCs. However, we believe that there may be different types of MICs and that the link to EMT might be not mandatory. The EMT enables detachment of tumour cells from a primary site into the circulation, but EMT can be also induced later, after CTCs enter the bloodstream[56]. Research groups have only recently started to apply EMT-related markers in their studies on CTCs in cancer patients and have shown various proportions of CTCs that are mesenchymal[18,19].

However, CTCs that are 'frozen' in a mesenchymal phenotype seem to be unable to form metastases[57]. Recent studies indicate that tumour cell lines that are arrested in a mesenchymal state by expression of EMT-inducing proteins such as SNAI1, TWIST or ZEB1 are more invasive and easily enter the bloodstream, but they are unable to form overt metastases after homing in distant organs[58–60]; these cells may not be able to undergo the reverse process of mesenchymal-to-epithelial transition to establish (micro)metastasis. It is assumed that tumour cells with an intermediate phenotype between epithelial and mesenchymal have the highest plasticity and therefore represent cancer stem cells[61]. Thus, detection of the CTCs with such an intermediate phenotype seems to be of utmost importance. This conclusion is supported by recent data showing that patient-derived CTCs with an intermediate phenotype (that is, low expression of EPCAM and high expression of MET) are able to form metastases after xenotransplantation in immunodeficient mice[36]. CTCs with low EPCAM expression are also detected by the CellSearch system[62]. Further definition of the intermediate phenotype by specific markers is required. In general, this phenotype is characterized by a partial downregulation of the epithelial markers, together with a partial upregulation of the mesenchymal markers discussed above.

Blood-monitoring studies in cancer patients have shown that some CTCs can survive chemotherapy, as postulated for cancer stem cells. Moreover, subsets of breast CTCs have a cancer stem cell phenotype (for example, $CD44^{+}CD24^{-/low}$aldehyde dehydrogenase 1 $(ALDH1)^{+}$)[63] and secrete the stem cell growth factor fibroblast growth factor 2 (FGF2)[64]. A recent xenotransplantation study showed that CTCs expressing CD44, MET and CD47 might represent MICs in patients with breast cancer[36]. In another study, CTCs that were isolated from patients with metastatic breast cancer and that were competent in forming brain metastases after xenotransplantation were $EPCAM^{-}$ but expressed HER2, EGFR, NOTCH1 and HPSE[55]. Thus, MICs of different metastatic sites might have different phenotypes.

However, these studies have been carried out on a limited number of late-stage cancer patients. The MIC phenotypes need to be confirmed in early stage cancer patients and prospectively related to the subsequent occurrence of overt metastases. A potential caveat is that MICs might develop by parallel progression after initial diagnosis of the primary tumour, and serial postoperative blood or bone marrow monitoring might therefore be required to identify MICs.

What is the fate of viable CTCs? The fate of CTCs in the circulation relies on their resistance to anoikis, apoptosis and necrosis. Over-expression of BCL-2 (an anti-apoptotic factor) in CTCs has been recently reported in patients with metastatic breast cancer[65]. Contrary to expectation, higher levels of CTC apoptosis were associated with worse prognosis, and higher BCL-2 levels in CTCs correlated with better outcomes. Further studies are needed to confirm and explain these surprising observations.

Homing of CTCs to distant organs might be influenced by vascular anatomical connection between the primary tumour and the potential site of metastases. For example, CTCs in patients with colorectal cancer are frequently captured in the liver[6], and this observation supports the wealth of information obtained from animal models[57]. Moreover, chemoattraction of CTCs to particular organs may have an additional role. Several mouse studies have previously pointed to the CXC-chemokine receptor

Glossary

BEAMing PCR
A combination of emulsion digital PCR and flow cytometry: beads, emulsions, amplification and magnetics (BEAMing) are combined to achieve the necessary level of sensitivity.

Cancer stem cells
Cancer cells with self-renewing capacity and the ability to create or sustain a tumour cell population.

Castration-resistant prostate cancer
(CRPC). Prostate cancer that no longer responds to androgen deprivation therapy.

CellSearch® system
US Food and Drug Administration (FDA)-cleared technology that allows a sensitive positive capture of CTCs by antibodies against epithelial cell adhesion molecule (EPCAM) coated with ferrofluids: tumour cells are identified by positive immunostaining for antibodies against cytokeratins (CK8, CK18 and CK19), negative immunostaining for the common leukocyte antigen CD45 to exclude leukocytes, and positive DAPI staining as a measure of nuclear integrity.

Clinical utility
The capacity to diagnose and to facilitate a decision to adopt or reject a therapeutic action: the risks and benefits result from test use.

Clinical validity
The predictive value of a test for a given clinical outcome (for example, in cancer, a primary tumour or metastasis will develop in a patient with a positive test): a test identifies the clinical status of a patient.

Dielectrophoresis
(DEP). A phenomenon in which a force is exerted on a dielectric particle when it is subjected to a non-uniform electric field. As biological cells have diverse dielectric properties, DEP can be used to manipulate, transport, separate and sort different types of particles (for example, circulating tumour cells).

Digital PCR
A refinement of conventional PCR methods that can be used to directly quantify and clonally amplify nucleic acids, including DNA, cDNA or RNA that occur at very low frequencies.

Epithelial-to-mesenchymal transition
(EMT). Conversion from an epithelial to a mesenchymal phenotype, which is a normal component of embryonic development. In carcinomas, this transformation results in altered cell morphology, the expression of mesenchymal proteins and increased invasiveness.

Leukapheresis
A process by which a large amount of blood is withdrawn from a vein, white blood cells and circulating tumour cells are selectively removed, and the remaining blood (red blood cells in platelet- and leukocyte-poor plasma) is transfused back into the donor.

Microfluidic devices
The integration of one or different laboratory functions on a single chip of only millimetres to a few square centimetres in size, in which extremely small fluid volumes (down to < picolitres) are handled.

Parallel progression
Tumour cells leave the primary tumour and home to secondary sites many years before the diagnosis and surgical resection of the primary tumour. These disseminated tumour cells can develop mutations that are independent from the mutational landscape of the primary tumour.

Photoacoustic flow cytometry
The irradiation of individual cells in blood and lymph flow with one or a few focused laser beams operating at different wavelengths, followed by the use of an ultrasound transducer attached to the skin to record laser-induced acoustic waves.

Tumour, node, metastasis cancer staging
(TNM cancer staging). A staging system for classifying cancers that was originally developed by the American Joint Committee on Cancer (AJCC) and that grades cancer by tumour, lymph node and metastatic status.

type 4 (CXCR4)–stromal cell-derived factor 1 (SDF1) axis as important for the homing of CTCs to the bone marrow in breast cancer[66]. Expression of CXCR4 has also been reported in CTCs[67] and DTCs in bone marrow[68] in patients with solid tumours. However, the correlation between CXCR4 expression on CTCs and metastatic patterns needs to be established.

After homing, the DTCs need to adapt to the new (and frequently hostile) conditions of the new microenvironment. Certain distant organs can provide specific niches that allow the DTCs to survive and eventually proliferate. In this context, the recent experimental finding that CTCs can occupy HSC niches in the bone marrow[69] of mice carrying prostate carcinomas is of utmost importance. Moreover, after occupying the niche, tumour cells reduced HSC numbers by driving their terminal differentiation[69]. If this were also true in cancer patients, it might have important clinical implications. First, chemotherapy affects the bone marrow tissue structure and has been associated with an increased release of HSCs into the circulation[70]. This release might clear HSC niches and thereby give CTCs a better chance to seed in the bone marrow. Secondly, CTCs could be mobilized out of the niche and back into the circulation using HSC mobilization protocols that have been used in the clinic for many years[71].

Interestingly, tumour cells from various different cancer types are retained in the bone marrow, even if they do not grow out in this organ[72]. Bone marrow has been implied as a dormancy-inducing organ[73], but it is also a frequent site of overt metastases in breast, prostate and lung cancer. However, the bone marrow is more easily accessible than other organs and it is, therefore, still debated whether this tissue is a special reservoir for CTCs (a 'tumour sanctuary') or whether other organs in which metastases occur provide similar conditions.

Further molecular characterization of CTCs may give more insights into the fate of these cells, which could help to distinguish relevant CTCs from irrelevant CTCs[74].

What is the nature and clinical relevance of CTC clusters? The detection of clusters of CTCs has attracted attention during the past years[75]. In lung cancer, CTC clusters have been associated with a worse prognosis[3] but, in general, much more needs to be learned about the nature and clinical relevance of these clusters. Their detection depends on the technology used and it cannot be excluded that some clusters may represent artefacts generated during the CTC capture process.

Recent data also indicated that CTCs cluster together with other cell types that are present in the bloodstream, such as platelets[18,56] and leukocytes[76]. Although platelets are thought to have a protective effect on CTCs, leukocytes might have either protective or cytotoxic effects on CTCs. Tumour cells in the primary tumour might also cluster together with stromal cells (for example, cancer-associated fibroblasts) and carry them during their journey through the bloodstream, which may help them to form a metastatic niche at a distant site[77].

In summary, the true frequency and nature of CTC clusters in cancer patients are still under investigation, and these studies may reveal important information on metastasis in humans.

How can genotypic and phenotypic characterization of CTCs help to guide therapy? The development of sophisticated single cell analysis technologies have allowed for new insights into the genetic make-up of CTCs and have demonstrated a marked inter- and intra-patient heterogeneity of CTCs. Below, we focus on the question of how the molecular analyses of CTCs can contribute to a better understanding of therapy resistance and to improvements in personalized therapy.

Current therapy decision-making is based on the analysis of the primary tumour, although restaging of metastatic lesions that occur many years after the diagnosis of the primary tumour has started to become more acceptable. However, biopsies of metastatic lesions are invasive procedures and, therefore, the analysis of CTCs as representatives of metastatic lesions (liquid biopsy) might be a good alternative that also allows for an assessment of resistance to therapy in real-time. Recent findings from comparative genomic analyses of CTCs, primary tumours and metastases in patients with colorectal or prostate cancer showed that mutations found in CTCs resemble those detected in both the primary tumour and metastases if sensitive deep-sequencing technologies were applied[78,79]. These data have important implications for the use of CTCs as a liquid biopsy, and they challenge the proposed parallel progression model[80].

Mutations in therapeutic targets or proteins downstream of the target can affect the efficacy of drugs against these targets. For example, mutations in EGFR affect therapies that target EGFR in lung cancer[81], and mutations in KRAS — a protein downstream of EGFR — block the efficacy of therapies that target EGFR in colorectal cancer[54]. Two separate single cell analyses of hundreds of CTCs obtained from patients with colorectal cancer recently revealed a high intra- and inter-patient heterogeneity of KRAS mutations[82,83]. The early detection of CTCs with mutated KRAS might help to guide therapy in individual patients.

Another example is the *HER2* oncogene, which is amplified in approximately 20% of primary breast cancers and has become a key target for therapies with antibodies and tyrosine kinase inhibitors. There is increasing evidence that overt distant metastases and CTCs have discrepant HER2 statuses compared with the primary tumour in up to 30% of cases[84]. At present, it is not known how this discrepancy arises. HER2 expression might be truly different in the primary tumour versus the metastases (that is, there is some kind of differential selection) or, alternatively, tumour heterogeneity might lead to an incorrect classification because small subclones are missed. In particular, the presence of $HER2^+$ CTCs in patients with $HER2^-$ primary tumours[20,21] has induced multi-centre trials that aim to investigate whether these patients will benefit from HER2-targeting therapies such as lapatinib (DETECT-III study; ClinicalTrials.gov identifier: NCT01619111) or trastuzumab (TREAT-CTC study; ClinicalTrials.gov identifier: NCT01548677).

Another important biological target in breast cancer is the oestrogen receptor (ER). Interestingly, only 1% of tumour cells need express ER to be considered ER^+. Thus, it is conceivable that ER^+ tumours shed ER^- CTCs, which are the source of ER^- metastases that may arise after years of ER-targeting therapies[85]. Indeed, ER^- CTCs were found in patients with breast cancer who had ER^+ primary carcinomas[85]. Ongoing follow-up studies will show whether these CTCs can escape ER-targeting therapies and cause relapse during or following endocrine therapy in patients with breast cancer.

In prostate cancer, PSA and prostate-specific membrane antigen (PSMA) are upregulated following androgen receptor (AR) activation and AR suppression, respectively. PSA and/or PSMA-based measurements as a surrogate for AR signalling in CTCs might indicate whether AR-based therapy is likely to be effective[22]. In 140 blood samples from patients with castration-resistant prostate cancer (CRPC), amplifications of the *AR* gene locus could be detected in 30–38% (REFS 86,87). Mutations in *AR* were very recently

identified in CTC-enriched peripheral blood samples from patients with CRPC[88]. *AR* amplifications enable the tumour cells to profit from the minute amounts of residual androgens in patients receiving drug-induced castration therapy, and *AR* mutations can result in tumour cells that are refractory to androgen blockade[89]. Thus, both types of genomic aberrations support the growth of prostate cancer cells in patients with CRPC[88].

These investigations exemplify how molecular CTC analyses may have an important future impact on better understanding treatment resistance in cancer patients. Thus, CTC enumeration and characterization might become an important new companion biomarker in drug development. To what extent CTC analyses can be replaced or complemented by analysis of circulating tumour DNA (ctDNA) is a subject of ongoing investigations (BOX 2).

What is the clinical utility of CTCs? There is a wealth of information on the clinical validation of CTC detection and enumeration (for example, large-scale pooled analysis of thousands of patients with breast cancer[1]), which resulted in the inclusion of CTCs in the new edition of the tumour, node, metastasis cancer staging (TNM cancer staging) manual in 2010 as classification $cM_0(i+)$ (that is, no clinical signs of overt metastasis but the detection of isolated tumour cells in blood, bone marrow or lymph nodes). Many publications deal with patients in advanced disease stages, but there is also an increasing number of publications on patients at earlier disease stages without clinical and radiological signs of overt metastases, particularly in breast cancer[1,90], but also in other tumour entities[91]. These publications show a significant correlation between the CTC counts and the prognosis of cancer patients, suggesting that CTCs are either surrogates of metastatic activity or causally involved in the metastatic process.

However, CTC measurements have not been included into the clinical guidelines (see, for example, the American Society of Clinical Oncology (ASCO) guidelines) because their clinical utility (that is, their capacity to facilitate a decision to adopt or to reject a therapeutic action) is still unclear. Thus, interventional studies are required to demonstrate which treatment changes need to be made according to the enumeration and/or characterization of CTCs. Examples of studies based on CTC enumeration are the SWOG SO500 study (ClinicalTrials.gov identifier: NCT00382018) and the METABREAST study. The SWOG SO500 study is a randomized Phase III trial. Its objective is to determine whether women with metastatic breast cancer and elevated CTCs (≥5 per 7.5 mL of whole blood) after 3 weeks of first-line chemotherapy derive increased overall survival from changing to an alternative chemotherapy regimen (standard practice once there is clinical evidence of progressive disease) at the next course, rather than waiting for clinical evidence of progressive disease before changing to an alternative chemotherapy regimen. One potential problem might be, however, that elevated CTC counts are indicators of worse prognosis but the applied therapy may not change the course of the disease, as indicated by the results of the SWOG SO500 study[92]. The reverse approach is to identify patients who may not need an aggressive treatment based on their low CTC count. In the METABREAST study, patients with metastatic breast cancer who have not previously been treated will be randomized between the clinician choice and CTC count-driven choice. In the CTC arm, patients with ≥5 CTCs per 7.5mL of whole blood will receive chemotherapy, whereas patients with <5 CTCs per 7.5mL will receive endocrine therapy as first-line treatment.

In all of the clinical studies mentioned in this section, the CTCs were detected with the CellSearch technology and are $EPCAM^+$. Future studies will include the detection of $EPCAM^-$ CTCs and the characterization of CTCs for expression of specific therapeutic targets (for example, HER2) for stratification of therapies with antibodies or small molecule inhibitors (see above). These studies will eventually identify specific tumour types and disease stages in which CTC measurements will be clinically useful.

Conclusions and outlook

The presented framework of CTC biology and classification of CTC assays might help to structure this dynamic field of translational cancer research. Better insights into the biology of CTCs will further improve CTC assay development. The cellular and molecular characteristics of CTCs are summarized in FIG. 3. On the basis of the known heterogeneity of tumour cells derived from different tissues, we may need tumour-specific CTC assays rather than one technology for CTC detection in all cancer types. Even within one cancer type, such as breast cancer, CTCs that are derived from primary tumours with different molecular subtypes or distinct distant sites (for example, the brain) may require specialized assay conditions.

The heterogeneity of CTCs also poses a problem for the liquid biopsy approach, and future studies with defined clinical endpoints need to address the question of how many CTCs should be profiled to account for heterogeneity. The direct comparison of the biology of CTCs and the primary tumour is hampered by the fact that a small biopsy is also unlikely to reflect heterogeneity within the primary tumour. A recent paper has shown that most 'private' CTC mutations could be detected by high-resolution targeted sequencing in

Box 2 | **Circulating tumour cells and circulating tumour DNA**

Tumour cells are released from the primary tumour and metastases into the blood as viable or apoptotic cells (circulating tumour cells (CTCs)), whereas circulating tumour DNA (ctDNA) is mainly released as fragments from necrotic and apoptotic tumour cells. Detection of CTCs requires more cumbersome enrichment and detection methods, whereas the detection of ctDNA can be done using blood plasma or serum. Thus, recent publications have suggested that analyses of ctDNA may replace CTC detection for monitoring cancer progression in the future[96–98].

The analysis of whole CTCs can be done at the DNA, RNA (mRNA or microRNA) and protein levels, whereas the analysis of ctDNA can be done only at the genomic level. CTCs can be characterized by immunocytological and molecular assays at the DNA, RNA and protein levels and, most importantly, functional *in vitro* and *in vivo* assays can be carried out, whereas ctDNA can be analysed by molecular DNA assays, including next-generation sequencing. Pre-analytical conditions for both CTC and ctDNA analyses must be standardized. Both biomarkers are currently evaluated for the following clinical applications: prediction of the risk for metastatic relapse or progression; stratification and real-time monitoring of therapeutic efficacy; and identification of therapeutic targets and resistance mechanisms. The prognostic relevance of CTCs has been shown with large cohorts of thousands of cancer patients, particularly in breast cancer[1,99], whereas the clinical validation of ctDNA analyses with next-generation sequencing technologies has, so far, been focused on monitoring therapy resistance in selected small cohorts of advanced stage cancer patients[96,97,100]. Both CTC and ctDNA approaches are complimentary as biomarkers. In addition, the detection and characterization of viable CTCs might provide more insights into the biology of cancer metastasis than ctDNA analyses.

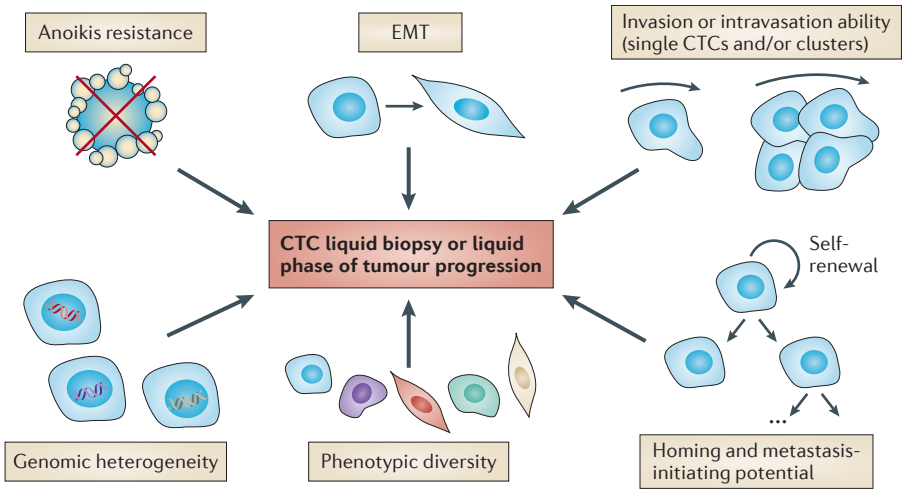

Figure 3 | **Current concept of cellular and molecular characteristics of circulating tumour cells (CTCs).** The scheme summarizes the characteristics of CTCs in cancer patients on the basis of the current knowledge. 'Liquid biopsy' describes a new diagnostic approach based on CTC analysis, whereas 'liquid phase of tumour progression' refers a particular disease stage that is characterized by the presence of CTCs. These characteristics may not be definitive but may change once more information on the biology of CTCs becomes available, and there is more information on some aspects than others, as discussed in the main text. For example, the mechanisms by which viable CTCs avoid cell death in the circulation and the conditions required for the homing and metastasis-initiating potential of CTCs are still under investigation. Phenotypic plasticity or diversity relates not only to epithelial-to-mesenchymal transition (EMT) but also includes the expression of proteins related to apoptosis, proliferation, invasion and chemotaxis, as discussed in the main text. Genomic heterogeneity has been reported to include the genes discussed in the main text (*HER2*, oestrogen receptor (*ER*), androgen receptor (*AR*) and *KRAS*) and other genes, such as adenomatous polyposis coli (*APC*), *TP53*, *PIK3CA*, neurofibromin 1 (*NF1*), *MLH1*, neuron navigator 3 (*NAV3*) and the gene encoding β-catenin (*CTNNB1*), as well as the copy number variations found in individual CTCs[78].

small subclones of the primary tumour[78]. Thus, multiple biopsies might be required to obtain reliable results.

Important goals of further molecular characterization of CTCs are the identification of MICs and treatment-resistant clones. The use of real-time monitoring of CTCs to analyse mutations that are relevant to cancer therapies might lead to considerable improvements in cancer therapy. Moreover, we can now evaluate whether diagnostic or therapeutic interventions contribute to the release of CTCs and, if so, whether this induced release is relevant for the outcome of the patient. New, surprising findings may challenge our current paradigms and medical practice. For example, it can be speculated that the tumour biopsy itself might induce the release of viable tumour cells in some patients.

In conclusion, it is hoped that this Opinion article will stimulate further developments in new technologies for the detection and characterization of CTCs and highlight some directions for the clinical validation of CTCs as new biomarkers. Thus far, only the CellSearch system has made the transition as a technology from exploratory to clinical decision-making status, and only for a few types of cancer. The newer devices still face the challenges of development as accredited methods for decision making. Thus, new assays have to be validated in clinical trials to achieve clinical validity and, more importantly, clinical utility[93].

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Competing interests statement

The authors declare competing interests: see Web version for details.

DATABASES

ClinicalTrials.gov: http://clinicaltrials.gov

FURTHER INFORMATION

American Society of Clinical Oncology (ASCO) guidelines: www.asco.org/quality-guidelines/guidelines
DETECT-III study: www.detect-studien.de
European ERA-NET on Translational Cancer Research TRANSCAN project 'CTC-SCAN': www.transcanfp7.eu
METABREAST study: http://cancerres.aacrjournals.org/cgi/content/meeting_abstract/72/24_MeetingAbstracts/OT3-4-06

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