Characterization of circulating tumor cells as a reflection of the tumor heterogeneity: myth or reality?

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The current main goal of diagnostic medicine is to detect crucial events in 'infinitely' small samples. The key question now is how to determine whether the rare cell events isolated and characterized from these samples reliably reflect the disease and heterogeneity of the tumor. In this review, we provide a short overview of the most recent methods developed for the isolation and characterization of rare cell events in clinical practice, with a specific focus on circulating tumor cells. We discuss the biological value to studying these cells at the single cell level and how these rare cell events can reflect tumor heterogeneity. The potential biomedical applications are also critically discussed in light of precision medicine.

Introduction

Cellular, genetic, and molecular heterogeneity is a hallmark of cancer. When a patient is first found to have cancer, the tumor mass comprises a couple of thousand to several million cells, including cancer cells functionally linked to noncancer cells in the microenvironment (e.g., endothelial cells or immune cells). This heterogeneity, which has been documented for several decades, initially for morphological and diagnostic assessment, is observed not only spatially in distinct tumor areas, but also at the cell level [1]. Darwin's theory of evolution provides some explanations for this diversity. Cancer evolves along the lines of Darwinian principles, including clonal proliferation, genetic instability with random mutational changes, and epigenetic modifications within the clonal population resulting in genetic diversity (Fig. 1). Paget added to these principles by the 'seed and soil' theory, describing that the combination of multiple genetic events and a favorable microenvironment is required to maintain 'cancer stem-like cells' and drive tumor initiation and growth [2]. Consequently, heterogeneity and cancer development is controlled by the cellular and molecular neighborhood, called the tumor microenvironment, which fuels selection pressure on cancer cells by modulating oxygen levels, and supplying nutriments and growth factors. Cancer is now viewed as a complex, dynamic ecosystem in which evolution within a tumor is driven by selection through the microenvironment [3–6]. Tumors can contain pre-existing resistant clones that evolve and survive genetically as a result of selective pressure; furthermore, these cells are thought to be the 'seeds' responsible for repopulation of the primary tumor and of distant organs [7,8]. Nevertheless, intratumor heterogeneity cannot be limited solely to genetic events. Numerous studies, carried out on cell lines considered to have a high degree of genetic homogeneity, have shown that drug responses are also strongly associated with intercellular epigenetic heterogeneity [9]. Epigenetic mechanisms are defined by numerous processes, including DNA methylation, post-translational modifications of histones, and chromatin remodeling, which are essential for genome organization, gene expression, and cell function [10]. The failure of cancer therapies to achieve durable therapeutic responses is often attributed to intratumor heteroge-

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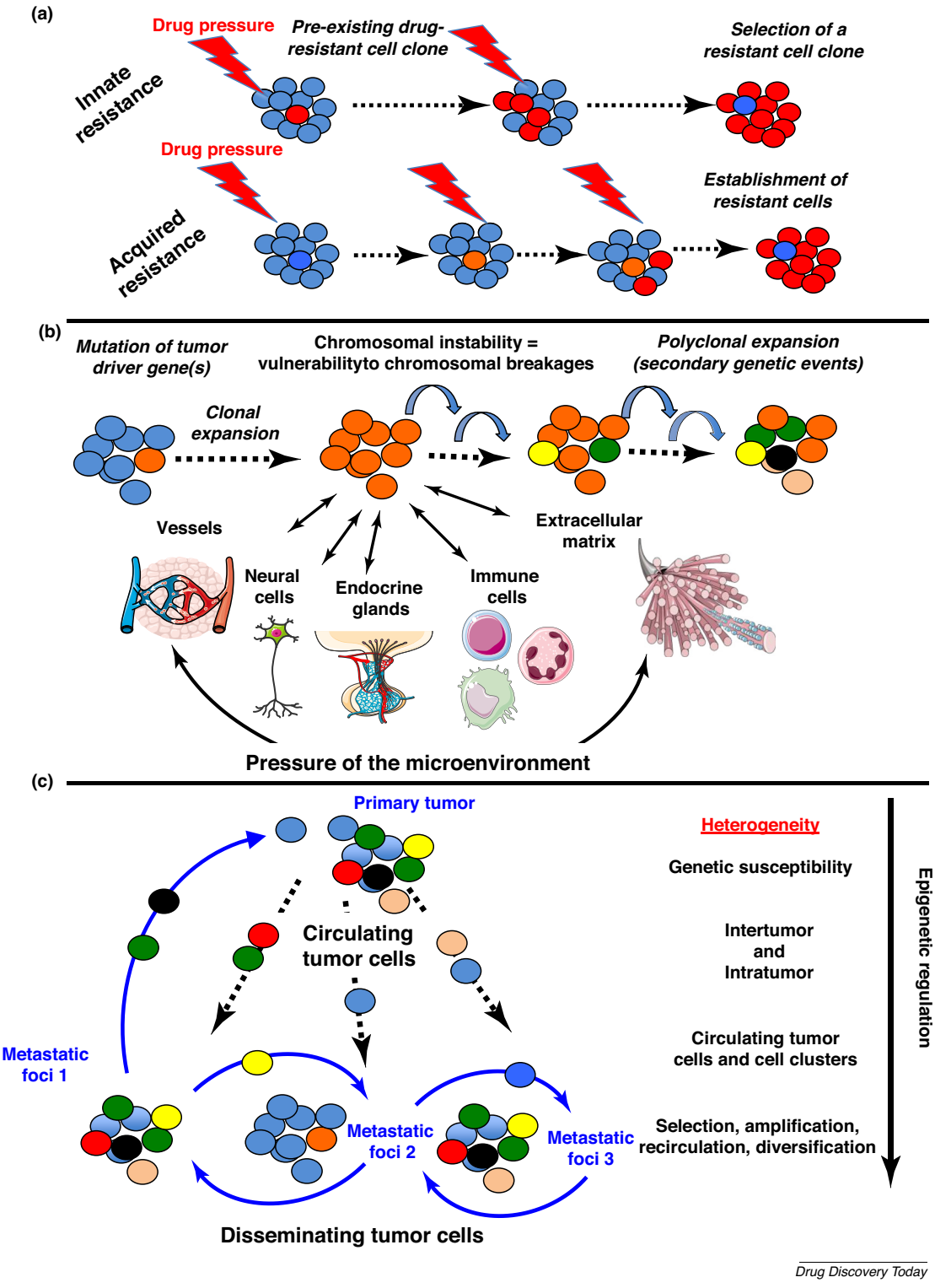

FIGURE 1

Origin of tumor heterogeneity. All components of a tumor mass are affected by heterogeneity. (a) Tumor tissues comprise diverse cancer cells exhibiting innate resistance to specific drugs, which can be amplified during the course of treatment. In addition, acquired under drug pressure, cancer cells can acquire molecular mechanisms to form resistant clones *de novo*. (b) Tumor evolution associates clonal proliferation, genetic instability with random mutational changes, and epigenetic modifications within the clonal population, resulting in genetic diversity. The tumor microenvironment is a key regulator of these processes. (c) Tumor heterogeneity is amplified by cell reseeding of cancer cells from one site (primary or metastatic) to another site (primary or metastatic). In addition to this autoamplification, epigenetic events strongly increase the cell diversity and strengthen the heterogeneity of cancer cells.

neity. Consequently, better characterization of intratumor heterogeneity would provide a powerful opportunity to track back through the formation of the malignancy and define in detail the evolution of the tumor, from the tumor-initiating events to the subsequent development of malignant clones.

The most recently developed surgical methods of diagnostic sampling, such as needle biopsies, limit the amount of material available for biological analyses. In addition, a biopsy is a partial snapshot of the tumor mass at a given time and does not allow longitudinal studies. Consequently, a source of biological materials representative of the disease in real time, more accessible with low invasive methods for repeated sampling, is mandatory to monitor the clonal evolution of cancer cells during treatments. Unfortunately, distant metastases are the common consequence of most cancer types and cancer cells predominantly spread through the blood vasculature or can be also associated with spread from the lymphatic system into the blood vasculature [11,12]. After a series of consecutive, selective events (e.g., intravasation, survival in the circulation, arrest in distant capillaries; extravasation and instalment of migrant cells in the distant organ), only a few cancer cells will successfully survive all steps of the metastatic cascade. Although the metastatic process can be considered as a relatively inefficient process overall, its high selectivity contributes to tumor heterogeneity and, thus, drug resistance. Circulating tumour cells (CTCs) that have migrated into the bloodstream originate from established tumor masses (e.g., primary or metastatic sites) [13–16]. Based on their location, CTCs are easily accessible; however, the extremely low number generates major challenges for their isolation, thus requiring specific technological approaches.

Technical approaches for isolating CTCs

CTCs are cell events traveling alone and/or in clusters among abundant leukocytes, thrombocytes, and erythrocytes [17–19]. CTC clusters are rare compared with single CTCs, although Aceto *et al*. demonstrated a high metastatic potential, increased 23- to 50-fold compared with single cells [17]. More recently, by using microfluidic and zebrafish approaches, Au *et al*. demonstrated that CTC clusters rapidly and reversibly reorganize into single-file chain-like geometries, reducing their thermodynamic resistance and allowing CTC clusters to pass through capillary-sized constrictions [18]. The authors hypothesized that circulating tumor 'microemboli' by CTC clusters are an important process of tumor dissemination. Furthermore, CTC clusters are considered as hybrid epithelial-mesenchymal transition (EMT) cell agglomerates with higher metastatic potential [19]. Based on their rarity (~1–10 cells per ml of blood), analysis of CTCs requires reliable approaches with high efficiency and specificity. The number of technical tools for the isolation of CTCs has exploded over the past decade [20–24]. The main techniques available are based on the physical or biological properties of CTCs (Table 1). A nonexhaustive list of the most recent methods is presented in Table 1, and these can be used separately or in combination depending on the objectives. Some can be used as a pre-enrichment step and include a range of techniques based on the different properties of CTCs allowing the separation of cancer cells from hematopoietic cells (e.g., negative magnetic bead selection). Others are more adapted for single cell isolation (e.g., DEPArray®). Three types of CTC specificity are conventionally used for their isolation: (i) biological characteristics (e.g., cell surface markers); (ii) physical

TABLE 1

Main techniques currently used for isolating CTCs

Technology	Name	Advantages	Limitations
Methods based on physical properties			
Density gradient	Percoll; Ficoll-Hypaque; OncoQuick	Low cost; reliable	Low purity (should be considered a pre-enrichment step); risk of losing large CTCs and CTC clusters
Microfiltration	ScreenCell; ISET (isolation by size of epithelial tumor cells); CellCyto	Relative high speed process; biomarker independent; high efficiency	Low purity; risk of losing small CTCs (diameter lower than that of white blood cells); ISET: CTC can be damaged or fragmented
Microfluidic technics (including microfluidic adhesion-based devices)	ApoStream; ClearCell FX; Cluster CTC-Chip; CTC-iChip; Dielectrophoresis (DEP): DEPArray; Ephesia CTC-Chip; OncoCEE (cell enrichment and extraction); OnQChip; Parsortix	Cell viability; efficiency; single cell isolation; high sensitivity; DEPArray: possibility to evaluate the DNA content	Relatively expensive; time consuming; pre-enrichment step required to reduce initial volume; biomarker dependent; the DEPArray requires specific electric properties; CTC-iChip is limited to small clusters of up to four cells
Methods based on biological characteristics			
Antibody-conjugated magnetic nanoparticles or microbeads or functionalized cell collectors	AdnaTest; CellSearch; Dynabeads; EasySep; IsoFlux; MACS; MagniSort; MagSweeper; MojoSort; RosetteSep; CellCollector	Cell viability; high efficiency; high purity; CellSearch is the only FDA-approved system for use in diagnostics (metastatic breast, prostate and colorectal cancers)	Biomarker dependent
Functional properties	EPISPOT; chick chorioallantoic membrane assay; telomerase activity (TelomeScan)	High sensitivity and specificity	EPISPOT: requires efficient antigen binding and specific epitope presentation

properties of cancer cells, including bigger size, lowest deformability, altered density, and electric charges; and (iii) functionalities.

Isolation by density gradient

CTCs, similar to most cancer cells, exhibit abnormal cytological features, including a larger size and heterogenous nuclear shape, leading to differential biomechanical and electric properties compared with healthy cells. These modified physical characteristics form the basis of the methods used for their isolation. Their higher nuclear:cytoplasm ratio allows their isolation by density gradient centrifugation [e.g., Ficoll-Hypaque, Percoll, and OncoQuick® (Greneir Bio-One international, France)] based on differences in their sedimentation coefficients (Fig. 2). Gertler *et al*. compared the density-gradient centrifugation system OncoQuick® with the standard density-gradient Ficoll-Hypaque and showed that both methods led to similar cancer cell recovery rates (range: 70–90%) [25]. However, if such approaches are efficient to enrich biological samples in CTCs and are relatively inexpensive, the recovery of CTC clusters and large CTCs is not efficient and the purity of collected CTCs is low with the presence of numerous leukocytes. In addition, the cytotoxicity of density medium can impact cell viability. Thus, density-gradient centrifugation should be considered a pre-enrichment method and should be combined with another CTC isolation approaches.

Isolation by microfiltration

The cytological characteristics of CTCs have led to the development of microfiltration devices to capture these rare cell events [23,26]. Microfiltration is a simple process allowing the enrichment of large volumes of blood in minutes with a high recovery rate (>85% depending on the device used) and is independent of any biomarkers expressed by CTCs. Small format devices dedicated to targeted downstream analyses have been developed [e.g., ScreenCell (Sarcelles, France) and ISET (RareCells, France)]. ScreenCell devices cover three main filtration systems: (i) ScreenCell® Cyto requires fixed cells and is dedicated to molecular techniques; (ii) ScreenCell® CC allows CTC recovery for cell cultures; and (iii) ScreenCell® MB is adapted for RNA or DNA analyses [27,28]. However, the final purity of CTCs collected is relatively low (~10%), single CTCs and CTC clusters are simultaneously captured, and depending on the flow and pressure used, cells can be damaged. Furthermore, detachment of captured CTCs on the filtration membrane can be difficult, thus limiting the downstream analyses. Furthermore, the background signal on the filters after immunocytochemistry used for the detection of CTCs can restrict their use.

Isolation by microfluidic devices

Several microfluidic devices designed to isolate CTCs have been developed [ApoStream® (ApoCell, USA); ClearCell® FX (Clearbridge Biomedics, Singapore); CTC-iChip technology (D.A. Harber, Harvard Medical School, USA); DEPArray® (Silicon Biosystem, Italy); and Parsortix® (Angle, UK)]. A variety of microchips have been developed to fractionate cells in blood according to their size, deformability and/or elasticity, surface electric charges, and/or expression of biomarkers. Such approaches are relatively expensive and require the use of specific cassettes and/or cartridges; however, their main advantages are their high sensitivity and high efficiency. Some devices have the capacity to process or even require low volumes of blood and/or other fluids, explaining the necessity of pre-enrichment methods for reducing the initial sample in case of large volumes. In addition, these systems provide researchers with the opportunity to isolate single cells.

CTC isolation based on microfluidic systems often involves a pre-enrichment step and an isolation and/or capture step. For example, the Parsortix® device enriches CTCs in blood (from 10 ml to ~200 ml) based on their differential size and deformability. A microfluidic cassette is used in which CTCs with contaminating leukocytes can be immunostained and recovered [29]. Pools of CTCs captured by Parsortix® can be processed by DEPArray® or ApoStream® systems for the isolation of single and pure CTCs. These systems work based on the differential electric charge of the cells and have a reported recovery efficiency of >70% combined with excellent cell viability (97%) [30]. The DEPArray® technology provides, in addition to DEP field flow fractionation, an image-based cell selection allowing the isolation of pure, single cell CTCs (stained for selected biomarkers) and an evaluation of cell DNA content by DAPI staining ('DNA index') [30–32]. CTC-iChip technology combines size-based exclusion of erythrocytes and thrombocytes, immunomagnetic depletion of leukocytes, and CTCs positioning in microchannels. More recently, Wu *et al*. developed a system combining microfluidics and acoustics for the isolation of CTCs [33]. High-throughput acoustic separation has a reported recovery rate of >85% and allows the

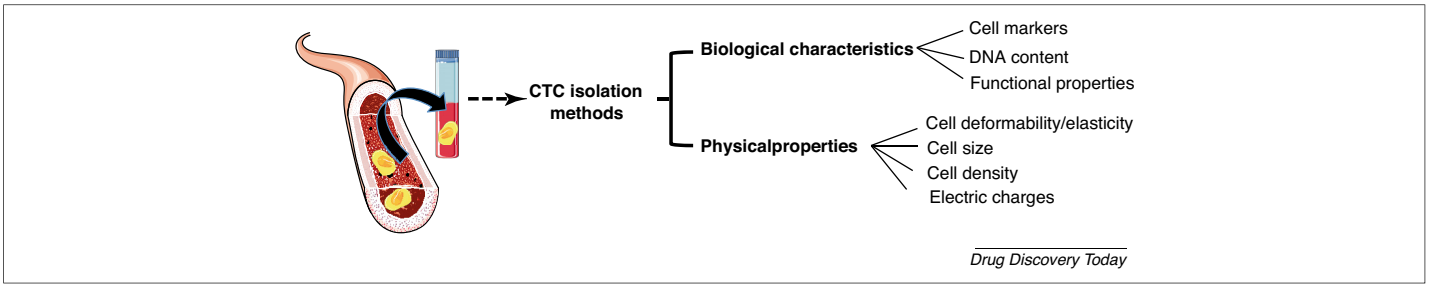

FIGURE 2

Isolation methods of circulating tumor cells (CTCs). Numerous technical approaches have been developed for CTC isolation. These methods are based on the biological characteristics and physical properties of CTCs. CTCs usually require a pre-enrichment step of the biological fluids before their isolation. There is no perfect method: all have advantages and limitations and the choice of the tool used depends on the main objective of the study.

determination of size distribution, phenotypic investigation, and postseparation cell culture. These techniques are relatively expensive (e.g., in terms of both the device and consumables) and time consuming. Overall, microfluidics are characterized by an excellent purity of recovery (>80% depending on the device used), with limited disturbances of the CTCs, and allow the isolation of pure single CTCs and/or clusters from small volumes. The reproducibility is acceptable, but can be affected by the risk of cell loss when loading samples to the cartridges [34]. However, validation of these devices in large clinical trials and establishment of standardized methods are two prerequisites to ensure the acceptance of CTCs as biomarkers in clinical practices; a recent consortium (CANCER-ID EU network; www.cancer-id.eu) aims to address this point.

Isolation by immunoaffinity

Immunoaffinity-based CTC isolation (negative or positive selection) is one of the oldest methods used for isolating pure cell populations. The systems [EasySep® or RosetteSep® (CELL Tech, UK), MojoSort® (Biolegend, USA), Dynabeads® (ThermoFisher, USA), IsoFlux® (Fluxion Biosciences, USA), and AdnaTest® (Qiagen, Germany)] are based on the biological properties of CTCs, which express cytoplasmic and/or membrane biomarkers (e.g., EpCAM). Some methods have been improved by adding an activated filter to capture and retain labeled cells [e.g., MACS® (Miltenyi Biotec GmbH, Germany) and MagniSort® (eBioscience, USA)] or by adding a robotic arm with a magnetic rod that binds labeled cells [e.g., MagSweeper® (Jeffrey's Laboratory, USA)]. Their main advantages are their high efficiency, high cell viability and high purity; however, immunomagnetic isolation is dependent on markers expressed by CTCs and the specificity of the targeted antigen, and these systems do not allow the isolation of pure single cells. The CellSearch® device combines antibody affinity for selecting CTCs and an image-based cell selection system [35]. CellSearch® is currently the exclusively US Food and Drug Administration (FDA)-approved device for CTC detection in metastatic breast, prostate, and colorectal cancers.

Several devices combine microfluidic and immunoaffinity isolation through microchannels coated with antibodies against specific targeted antigens expressed by CTCs [e.g., OnQChip® (On-Q-ity Inc., USA); OncoCEEÔ chip (Biocept Laboratories, USA)]. CellCollector® (Gilupi, Germany) has been developed for in vivo CTC isolation and is CE approved. This cell collector is a medical stainless steel wire coated with anti-EpCAM antibody (length: 16 cm; diameter: 0.5 mm) implanted intravenously for 30 min to come into contact with a large volume of blood containing CTCs. In more than 2000 patients, the detection rate was ~70% in early and/or late cancer stages. Its implantation for a duration of 30 min increases the chances of isolating CTCs, but this device is currently restricted to EpCAM-expressing cells.

Regardless of the system used, all are limited by the expression of a membranous marker of interest expressed by CTCs; unfortunately, the phenotypic heterogeneity related to the EMT strengthens caution in the use of single cell biomarker-based methods for the isolation of CTCs.

Functional assessment of CTCs

To overcome some of the limitations mentioned earlier, functional assessment of CTCs can be performed. In addition to the commonly used chick chorioallantoid membrane assay (CAM), two approaches have recently been proposed to identify and characterize CTCs: TelomeScan® (Oncolys Biopharma, Japan) and EPISPOT. TelomeScan® is a genetically engineered adenovirus type 5 with a human telomerase reverse transcriptase (hTERT) gene promoter sequence in the upstream region of the viral E1 gene, which is responsible for replication of the adenovirus. Therefore, viral replication can only occur in cells with active telomerase activity, such as cancer cells. In the E3 region of the vector is a GFP gene under the control of the cytomegalovirus promoter, allowing visual detection of cancer cells containing replicating virus. EPISOPT is an ELISPOT assay dedicated to the detection of proteins secreted from epithelial cancer cells [36]. Isolated CTCs are cultured for a short time on a membrane coated with antibodies that capture the corresponding secreted proteins, which are subsequently detected by secondary antibodies labeled with fluorochromes. Therefore, EPISPOT allows the detection and characterization of CTCs on the basis of their secretome.

CTCs constitute a heterogeneous cell population

CTCs spread from a solid tumor mass and reach distant organs through the bloodstream. Intravasation and extravasation require complex cell modifications to facilitate the motility of cancer cells, which involves the EMT. EMT results in numerous cell rearrangements, including loss of cellular contact junctions, cell adhesion, and cell polarity, and induces marked modifications of epithelial cell morphology corresponding to a modified differentiation profile [37]. CTCs undergo an inverted process (MET) when they settle in the metastatic foci. EMT leads to a loss of epithelial makers for carcinoma cells (e.g., EpCAM) and expression of mesenchymal makers (e.g., vimentin). Similar mechanisms have been described for sarcoma cells. In addition to the genetic instability and mutations described earlier, EMT strongly contributes to the heterogeneity of cancer cells. For instance, inoculation of $EpCAM^+$ breast cancer cells in mice led to the detection of a mixture of $EpCAM^+$ and $EpCAM^-$ CTCs in the blood after 3 weeks and, similarly, an injection of $EpCAM^-$ cells resulted in the detection of both $EpCAM^+$ and $EpCAM^-$ CTCs [23].

Several studies investigated the concordance of HER2 status between primary tumors and CTCs and results differ according the series analyzed. For instance, some studies demonstrated a concordance of ~80–90% of HER2 expression between primary tumors and CTCs [38,39]. By contrast, in a series of 19 patients with $ER^+/HER2^-$ primary tumors, 84% acquired $HER2^+$ CTCs, illustrating the high plasticity of cancer cells to develop drug resistance and the relative value of membranous biomarkers [40]. Similarly, HER2-overexpressing CTCs were observed in ~24.1% of patients, of which eight of 14 patients showed HER2-negative primary tumors [41]. In 62 of 101 patients, Zhang *et al.* [42] and Beije *et al.* [43] showed inconsistent Her2 expression between primary tumors and CTCs, even when there was no significant effect of the HER2 status of CTCs on overall survival; thus, this could change during the course of breast cancer [44].

Various studies have demonstrated the value of CTC quantification as a prognosis factor in patients with metastatic cancer [21,45]. However, recent studies underlined the high cellular heterogeneity within CTCs in breast [46–50], prostate [51–54], hepatocellular carcinoma [55], colorectal [56–58], and lung cancer

[59]. In a series of 290 patients with metastatic breast cancer, Gasch *et al*. observed the presence of CTCs in 61.7% of patients and showed a high heterogeneity concerning PI3K mutations and Her2 expression by CTCs [50]. Similarly, in a short series of patients with inflammatory breast cancer, Bingham *et al*. confirmed the presence of high-frequency mutations (69% TP53, 16% RB, 13% PI3KCA, and 2% ErbB2) in CTCs matching tissue biopsies [48]. Interestingly, the authors found a high heterogeneity of CTCs, as revealed by the isolation of various clones harboring different combinations of mutated and wild-type genes. Similar heterogeneity has been described by Scher *et al*. in patients with prostate cancer [51]. Their study showed that lower heterogeneity scores were associated with a longer median survival of patients treated with androgen receptor-signaling inhibitors compared with a higher heterogeneity of CTCs with shorter median survival of patients treated with taxanes.

An interesting notion of spatial heterogeneity of CTCs was suggested by Sun *et al*. in a series of 73 patients with hepatocellular carcinoma [55]. These authors analyzed CTCs isolated from blood collected from the peripheral vein, peripheral artery, hepatic veins, infrahepatic inferior vena cava, and portal vein before tumor resection. Single-cell characterization demonstrated that the EMT status of CTCs was heterogeneous across the vascular compartments and suggested that CTC heterogeneity influences postoperative lung metastasis and intrahepatic recurrence. In colorectal cancer, using the CellSearch® device, Kondo *et al*. revealed heterogeneity in KRAS status among CTCs [56], and Meassritakis *et al*. described the phenotypic heterogeneity of CTCs in patients with small cell lung cancer on the basis of TTF1, CD56, and EpCAM expression [59]. Thus, CTC heterogeneity is now recognized by the scientific community, strengthening the need to establish a full genomic and molecular profile of CTCs at the single-cell level.

Do CTCs reflect the genetic and/or molecular patterns of primary tumors?

More than 200 clinical trials focused on CTCs are registered in the NIH database (clinical trial.gov) and 79 are currently recruiting (Table 2), illustrating the extraordinary enthusiasm for the biology of CTCs and their potential biological and/or clinical value [21]. CTC count correlates in most studies with disease progression. Thus, in a recent meta-analysis including 21 studies, Bidard *et al*. investigated the clinical value of CTCs in patients with non-metastatic breast cancer treated with neoadjuvant chemotherapy [38]. Data of patients, in particular information on CTCs collected by CellSearch®, were gathered before neoadjuvant chemotherapy (N = 1574) and before surgery (N = 1200). From 861 patients included with full data available, the authors demonstrated that CTC count was an independent and quantitative prognostic factor in patients with early breast cancer treated with neoadjuvant chemotherapy.

However, it has also been demonstrated that the CTC phenotype (e.g., miRNA profile) does not always correlate with CTC count because of their high heterogeneity and that it could be modulated during the course of treatment [58]. Therefore, it necessary to determine whether CTCs could reflect the primary tumor or metastatic foci and how CTCs are modulated during treatments.

Paoletti *et al*. carried out next-generation sequencing (NGS) of somatic mutations and copy number alterations of CTCs from patients with metastatic breast cancer [60]. From 12 patients, they found 85% concordance in at least one more somatic mutation and copy number alteration between paired CTCs and metastatic tissue. These authors also identified the presence of a minority CTC subclone harboring a novel active mutation (ESR1 pA569S). De Luca *et al*. performed NGS of CTCs from four patients with metastatic breast cancer and found a discordance between the mutational status of the primary tumor and CTCs in 3 patients [61], reinforcing the data obtained by Jabokova *et al*., in which HER2 and ESR status of CTCs differed from the status of primary tumors [40]. Similarly, Jiang *et al*. showed that the percentage of clonal mutations and intra- and/or interchromosomal rearrangements identified in CTCs could also be found in primary or metastatic prostate tumors [62]. Carlsson *et al*. compared bone marrow aspirates and blood CTCs from 14 patients with prostate cancer and observed that the proportions of androgen receptor-negative and -positive cells were similar between compartments; however, whole-genome copy number profiling in single cells from three patients identified distinct clonal patterns between both bone marrow and blood [63]. The reports from colorectal and lung cancer show similar results. Lyberopoulou *et al*. observed in 52 patients with colorectal cancer a discordance between primary tumor and CTCs for KRAS, BRAF, CD133, re3130, and Plastin3 rs6643869 [64]. This discordance was confirmed by Kondo *et al*. [56] and by a recent meta-analysis including nine studies and 244 patients [65]. Similar discordance was observed at the epigenetic level [50].

In lung cancer, Guibert *et al*. showed a more frequent PD-L1 expression (83%) in CTCs than in tumor tissue (41%) [66]. In this study, high CTC number and pretreatment were associated with increased risk of death and progression. The authors concluded that PD-L1+ CTCs detected before treatment were associated with a poor prognosis in patients treated with PD-1 inhibitors.

Where the spatial heterogeneity of CTCs has been suggested [55], dynamic and/or temporal heterogeneity has also been proposed [67,68]. By using a murine preclinical model of prostate cancer, Kermanshah *et al*. monitored the phenotype changes in CTCs during treatment and showed evidence that CTCs of metastatic mice displayed dynamic and heterogeneous profiles compared with mice with local disease [67]. Interestingly, CTC heterogeneity decreased after chemotherapy and was followed by a significant reduction in metastasis incidence.

Overall, these data reveal the partial genomic overlap of CTCs and primary and/or metastatic tumor foci at a defined time; however, the clonal architecture of advanced disease is a dynamic process, with establishment of new clones gaining dominance in response to treatment.

Concluding remarks

CTCs should be considered as a picture of the tumor tissue at a given time. Indeed, recent studies support cellular heterogeneity within CTCs, which partly reflects the spectrum of mutations in the primary and metastatic tumors. The CTC profile evolves as the disease progresses and, in addition to the mutations detected in tumor tissues, CTCs harbor new sets of mutations reflecting the emergence of minority subclones and/or the evolution of pre-

TABLE 2

Nonexhaustive list of ongoing clinical trials based on the detection of CTCs[a]

Clinical trial identifier	Details	Organs (+treatment)	Outcome	Patients	Study completion
NCT01619111	DETECT III: multicenter, Phase III study comparing standard therapy ± laptinib in patients with HER2-ve MBC versus patients with HER2 + ve CTCs	Breast cancer	CTC clearance rate; overall response rate; clinical benefit rate; overall survival; dynamics of CTC; safety and tolerability of lapatinib; progression-free survival	120	2020
NCT02035813	DETECT IV: study in patients with Her2-negative metastatic breast cancer and persisting Her-negative CTCs	Breast cancer (everolimus, eribulin)	Progression-free survival; overall survival; overall response rate; dynamics of CTCs; quantification of CTC (for everolimus: levels of pS6, change in activation of PI3K/Akt/mTOR pathway; ESR1 mutation; for eribulin: new metastasis-free survival)	520	2019
NCT03070002	Denosumab in patients with ER- and/or PR-positive HER2-negative metastatic breast cancer with bone metastases and detectable CTCs	Breast cancer (denosumab)	Fraction of patients with reduction in CTCs; percentage changes in CTCs; progression-free survival	42	2019
NCT01322750	CTCs: a potential screening test for clinically undetectable breast carcinoma	Breast cancer	Observational study	3125	2023
NCT01961713	CTC analysis in patients with localized prostate cancer undergoing prostatectomy	Prostate cancer	Relationship between CTC quantity and pathological stage; persistent CTC and biochemical recurrence; compare chromosome translocation status	200	2019
NT02997709	Collection and measurement of biomarkers in patients undergoing radiotherapy for prostate cancer	Prostate cancer	Relationship between CTC changes and/or quantitative-imaging parameter changes and patient outcome; comparison of changes in CTCs to endpoint prostate research biopsy status	300	2026
NCT03327662	Utilizing CTC counts to optimize systemic therapy of metastatic prostate cancer	Prostate cancer	Overall survival; CTC-guided switch rates; CTC effects in chemotherapy	1178	2022
NCT01558349	CTCs as biomarkers for prostate cancer detection in patients with gray-zone PSA level	Prostate cancer	CTC detection	500	2021
NCT02456571	CTC immune checkpoint	Prostate cancer	Change in expression of four immune checkpoint biomarkers (PD-L1, PD-L2, B7-H3, and CTLA-4) on CTCs	40	2019
NCT02449837	Investigation of CTCs from patients with cancer undergoing radiation therapy	Non-metastatic disease: head and neck cancer; cervical cancer; non-small cell lung cancer; rectal cancer; metastatic prostate cancer; oligometastatic disease	CTC levels during treatment comparing metastatic and non-metastatic disease	210	2020
NCT03295591	CTCs in mCRC for liver resection	Metastatic colorectal cancer	Cut-off value of CTC counts (time frame: progression-free survival at 6 months) to identify patients with early relapse (<6 months) after liver resection	77	2020
NCT03156777	Application value of CTCs detection for patients with advanced gastric cancer	Gastric cancer	Number of CTCs; profile of CTCs; progression-free survival; overall survival	200	2020
NCT02955173	Significance of CTCs in treatment of gastric and rectal cancer	Gastric and colorectal cancer	Disease-free survival; CTC test	600	2019
NCT02874885	CTCs in patients with rectal cancer	Rectal cancer	Changes in CTC status	520	2023

TABLE 2 (*Continued*)

Clinical trial identifier	Details	Organs (+treatment)	Outcome	Patients	Study completion
NCT02335151	CTCs in pancreatic adenocarcinoma	Pancreatic cancer	Peak in CTCs during postoperative phase after curative tumor removal, kinetics of CTCs up to day 7; months to tumor recurrence; number of surviving patients	56	2019
NCT02072616	Detection of CTCs for diagnosis of pancreatic adenocarcinoma	Pancreatic cancer	Sensitivity of CTCs for diagnosis; performance of ctDNA (KRAS) for the diagnosis; prognostic impact of CTC/ctDNA (KRAS) and/or CA19.9; time to first recurrence or death	142	2021
NCT03340844	Role of CTC spread during pancreaticoduodenectomy in patients with pancreatic and periampullary tumors	Pancreatic cancer	CTC detection; local tumor recurrence; metastasis; patient survival	62	2022
NCT03295591	CTC in mCRC for liver resection	Liver cancer	Cut-off value of CTC counts; progression-free survival; overall survival	77	2020
NCT02973204	CTC and tumor DNA in hepatocellular carcinoma and neuroendocrine tumors	Hepatocellular carcinoma; neuroendocrine tumors	Flow cytometry for detection of CTCs in peripheral blood (absolute and relative count); correlation between mutations found in ctDNA and CTC and survival/treatment response according to RECIST criteria	130	2020
NCT02812680	Utility of CTCs and plasma miRNA in esophageal adenocarcinoma	Esophageal cancer	CTCs and miRNA as biomarkers of cancer and predictive markers for neoadjuvant therapy by using CTC chips	100	219
NCT02951897	Application of detecting CTCs in accurate treatment of early stage lung adenocarcinoma	Lung cancer	Disease-free survival	120	2019
NCT02630615	CTCs in lung cancer	Lung cancer	Assess activity of novel DNA repair inhibitors as function of DNA repair mutations detected in CTC samples	80	2020
NCT03479099	Liquid biopsy in lung cancer	Lung cancer	Diagnostic sensitivity, accuracy, and specificity of combined CTC and ctDNA	130	2019
NCT02499458	Prospective validation of CTC and circulating endothelial cells as biomarkers in renal cancer	Renal cancer	Sensitivity and/or specificity of CTC enumeration (microfluidics versus CellSearch); progression-free survival; overall survival; molecular characterization of CTCs	70	2019
NCT02246738	Initial evaluation of telomerase-based CTC assay in cohorts of patients with bladder cancer	Bladder cancer	Number of adverse events	66	2018
	Detection of CTC in patients with sarcomas	Sarcoma	Progression-free survival	20	2018

[a] Total recruiting studies: 79 (clinical trials.gov).

existing clones under drug pressure. In addition to this temporal evolution fueling CTC heterogeneity, spatial heterogeneity contributes to amplify the process in a dynamic manner. CTCs are rare cell events frequently masked by the background of peripheral leukocytes and, in such context, the molecular characterization of CTCs in the blood remains challenging. Genomic, global transcriptomic analyses and epigenetic profiling are informative and complementary approaches for characterizing CTCs and defining their mutational profile, as shown in a recent report that analyzed androgen receptor splice variant 7 (AR-V7) or KRAS status of CTCs in patients with castration-resistant prostate cancer [69].

Expansion of patient-derived CTCs as 3D organoids or in specific microfluid culture devices could be a way to obtain enough biological material for molecular phenotyping and/or drug screening [70]. Similarly, circulating tumor cell-derived xenograft (CDX) models are potentially powerful methods for expanding CTCs and studying their biological properties and disease progression, as well as as a drug development platform [71,72]. Liquid biopsies also include circulating tumor DNA (ctDNA), which is mainly released after tumor cell death. Although ctDNA can allow assessment for the emergence of specific cancer cell subclones characterized by a specific new set of mutations, ctDNA cannot give a full picture of tumor evolution

combining dynamic genetic and phenotypic and/or epigenetic changes that result in the modification of cancer cell properties.

Thus, CTCs are an easily accessible biological material and a useful tool in the functional study of tumor progression. However, the lack of standardized procedures for the isolation and characterization of CTCs restricts current CTC investigations to research. A combination of CTC isolation with full genomic and downstream RNA analyses is a promising approach to better reflect the therapeutic response, to anticipate drug resistance, and to adapt treatment day by day, patient by patient [54]. A kinetic profile of CTCs compared with tumor tissue will complete the full picture of disease progression and could be the basis of future personalized medicine in oncology.

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