Chapter 2

Profiling Circulating Tumour Cells for Clinical Applications

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Additional information is available at the end of the chapter

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Abstract

Circulating tumour cells (CTCs) refer to cells found in the peripheral blood, which are derived from the primary or secondary tumour. They serve as an alternative to study the biology of the primary tumour especially when tissue biopsy is not available. However, major challenges in CTC analysis are the rarity of these cells and the purity of the isolated population. The advancement in technologies allows detection and enrichment of sufficiently pure CTCs at the single-cell level, facilitating downstream molecular characterisation. Single CTC analysis allows detection of key mutations that may be critical to disease management and helps to address the intercellular differences among tumour cells. In this chapter, we discuss the technologies for CTC isolation and the use of CTCs in achieving early detection and prognosis of cancer, real-time monitoring of cancer therapy and tailoring of personalised treatments.

Keywords: cancer, CTC, single-cell analysis, liquid biopsy, personalised treatment

1. Introduction

Cancer is a leading cause of death in many countries [1]. According to World Health Organisation, approximately one in six deaths is attributable to cancer. The development of cancer is a multistage process. Briefly, normal cells undergo transformation into tumour cells, which are defined by various hallmarks including the ability to sustain proliferative signals, evade growth suppressors, promote replicative immortality, avoid cell death and immune destruction, induce angiogenesis and activate invasion and metastasis [2]. This cellular transformation results in

uncontrolled proliferation and enables tumour cells to migrate from their primary organ to a distant organ, a process known as metastasis. In 90% of cancer patients, death occurs because of metastasis [3, 4].

According to Cancer Research UK, 46% of patients in England are diagnosed in the advance stages of disease, leading to less effective treatment outcomes. To reduce the number of deaths caused by cancer, detection at an early stage of disease development is critical so that clinical intervention can come in place to improve the chances of survival for cancer patients. The diagnosis of cancer involves multiple tests including tissue biopsy, liquid biopsy, imaging scans, genetic tests and an examination of medical history. Tissue biopsy is regarded as the gold standard for the clinical diagnosis of cancer [5, 6], despite the invasiveness and inconvenience of collecting the biopsy sample.

In recent years, liquid biopsy is increasing being used for the detection of cancer because it only requires a routine draw of blood and is less invasive compared to tissue biopsy, which may also not be repeatedly done safely or feasibly [7]. Liquid biopsy includes the analysis of circulating tumour cells (CTCs), circulating cell-free DNA (cfDNA) or exosomes present in the patient's blood [8]. CTCs, in particular, have garnered much attention for its potential clinical utility. CTCs are cells disseminated from the primary or secondary tumour into the peripheral blood and are associated with the development of metastasis. They are precursors of secondary tumour formation and may carry key information relating to the mechanism of metastasis. They are approximately 12–25 μm [9] and present in extremely low numbers (typically 1–10 CTCs per 10 ml of blood or ~1–100 CTCs per 10^9 blood cells) [10–12]. The number of CTCs found in the blood varies with the type and stage of cancer and the treatment provided [13]. Typically, patients in the advanced stages of cancers have higher number of CTCs [14]. CTCs of different cellular morphology may exist in the blood and these include the epithelial CTCs, epithelial-to-mesenchymal (EMT) CTCs and mesenchymal CTCs. Moreover, the mutations found in CTCs are often concordant with the primary tumour [15–17], suggesting that the genetic composition of CTCs is similar to the primary tumour. Thus, CTCs has the potential to be used as a 'surrogate' to study the biology of cancer cells.

In cancer treatment, drug resistance is a major concern. The failure of chemotherapeutic drugs to work in patients lies in the heterogeneity and complexity of cancer cells [18, 19]. Cancer stem cells are resistant to chemotherapy and contribute to the intra-tumoural heterogeneity [20]. Therefore, there is a need for molecular profiling of tumour cells at the single-cell level to better address the intra- and inter-cellular differences in cancer cells and enable clinicians to have a better picture of the disease complexity. While paired tumour/normal tissues is the gold standard for molecular analyses of tumour [21], CTCs may provide information on the dynamic changes in tumour cells when blood is extracted at different times, which cannot be achieved in tissue biopsy. In addition, where tissue is not easily accessible, CTCs may provide a diagnostic window.

However, the main challenges of CTC research are that these cells are extremely rare and the population of isolated CTCs may not be pure due to contamination with white blood cells (WBCs). Therefore, highly sensitive and specific technologies are required to isolate CTCs efficiently. Over the past decade, microfluidics technology has greatly advanced the enrichment and isolation of CTCs from whole blood containing red blood cells (RBCs) and WBCs [22]. Microfluidics deals with the behaviour of fluid passing through the microchannels [23, 24]. It makes use of the laminar flow of fluid in the microchannels to manipulate the fluid to achieve

cell separation. Furthermore, because of the small space and short flow distance in the microdevice, microfluidics-based technologies consume small amounts of reagents and greatly increase the speed and throughput of blood sample processing, allowing clinical adoption. The use of microfluidics facilitates the integration of downstream molecular characterisation of CTCs, which will enhance our understanding on the complexity of cancer development and enable clinicians to develop better therapeutic strategies to eradicate cancer cells and improve the overall survival of patients. In this chapter, we discuss the technologies for CTC isolation and the use of single-cell analysis in achieving early detection and prognosis of cancer, real-time monitoring of cancer therapy and tailoring of personalised treatments.

2. Technologies for enrichment and isolation of CTCs

Many technologies have been developed to enrich and isolate CTCs from the peripheral blood. In most methods, CTCs are separated from the blood cells based on their biological properties and/or physical properties such as size, deformability, density and electric charge. Conventional CTC enrichment systems such as fluorescence activated cell sorters (FACs) have been used to separate CTCs from whole blood based on the expression of cell surface protein markers [25]. Technologies that isolate CTCs based on physical properties also exist. For instance, ISET and ScreenCell use a filtration system to separate the slightly larger CTCs (12–25 μm) from the smaller WBCs (7–15 μm) and RBCs (8 μm) [9]. CTCs and mononuclear cells have a density (<1.077 g/ml) lower than other blood cells (>1.077 g/ml), allowing layered separation of CTCs [6]. CTCs and blood cells exhibit differences in deformability, allowing them to be separated [26]. The dielectric properties of CTCs are different from normal blood cells, allowing separation of CTCs when the cells are subjected to a non-uniform electric field [6, 27]. However, these conventional CTC enrichment methods suffer from limited ability to process large volumes of blood, limited detection sensitivity, inherent losses and poor recovery of viable CTCs, low throughput and insufficient purity due to contamination with WBCs [6].

2.1. Use of microfluidics in CTC enrichment

To overcome these limitations, microfluidics technology offers an alternative platform for isolating CTCs with improved detection sensitivity, high recovery rate, high efficiency and throughput. Each microfluidics platform has its advantages and limitations (**Table 1**). In 2004, US Food and Drug Administration approved the clinical use of CellSearch system (Veridex) for CTC detection in epithelial cancer types such as breast [28], colorectal [29] and prostate cancer [30] for purposes of prognostication. This system uses immunomagnetic and fluorescence imaging technology to enrich and enumerate CTCs from 7.5 ml of whole blood based on the expression of specific proteins in CTCs [31, 32]. CTCs are first separated from other blood cells using magnetic iron nanoparticles coated with antibodies targeted against EpCAM (an epithelial cell adhesion molecule present on the cell surface of CTCs). Subsequently, cells are stained with antibodies targeted against cytokeratin (CK; a protein found in the cytoplasm of CTCs) and CD45 (a cell surface protein found exclusively on WBCs) to differentiate CTCs from contaminating WBCs. DAPI (4′,6-diamidino-2-phenylindole) is also used to stain the nuclei of CTCs and WBCs. Finally, a magnetic field is applied to collect the CTCs, which are identified by positive expression of EpCAM and CK and negative expression of CD45.

System	Separation principle	Strengths	Weaknesses	References
Antibody-based capture				
CellSearch	Positive selection for EpCAM, CK8/CK18/CK19 and negative selection for CD45	• Food and Drug administration (FDA)-approved for clinical use	• Unable to capture tumour cells that lack EpCAM expression • Only applicable to cancers with epithelial origin • Cells are not viable after isolation	[28–30]
CTC-chip	EpCAM-based	• Sample does not require pre-processing • Cells remain intact and viable after isolation	• Unable to capture tumour cells that lack EpCAM expression • Only applicable to cancers with epithelial origin • Unable to recover tumour cells with 100% purity	[33]
CTC-ichip	Size-based separation followed by negative depletion of white blood cells with CD45 and CD66b magnetic beads	• Fast processing time (8 ml/h) • Has the potential to capture CTCs from any cancer type. • Cells remain intact and viable after isolation	• Unable to capture tumour cells that are smaller or similar in size to blood cells. • Unable to recover tumour cells with 100% purity	[51, 52]
IsoFlux	EpCAM-based	• Higher sensitivity of detecting CTCs than CellSearch system	• Unable to capture tumour cells that lack EpCAM expression • Only applicable to cancers with epithelial origin	[53]
Magnetic Sifter	EpCAM-based	• Allows rapid imaging of captured cells on a small area • Cells remain intact and viable after isolation • Reduces sample losses with minimal pre-processing	• Unable to capture tumour cells that lack EpCAM expression • Only applicable to cancers with epithelial origin	[54]
GEDI microdevice	Prostate-specific membrane antigen (PSMA)/HER2-based	• Higher sensitivity of detecting CTCs than CellSearch system • Device geometry reduces capture of WBCs	• Only applicable to prostate cancer, breast cancer, gastric cancer • Unable to recover tumour cells with 100% purity	[43, 55, 56]

System	Separation principle	Strengths	Weaknesses	References
Label-free capture				
ClearCell FX (spiral chip)	Size-based	• Fast processing time (3 ml/h) • Cells remain intact and viable after isolation • Cost-effective • Has the potential to capture CTCs from various cancer types.	• Unable to capture tumour cells that are smaller or similar in size to blood cells. • Unable to recover tumour cells with 100% purity	[11, 40]
Microfluidic biochip	Size-based	• Fast processing time (7.5 ml of blood in 3 h) • Allows single-cell isolation • Able to isolate viable CTCs with 100% purity • Has the potential to capture CTCs from various cancer types.	• Limited number of cell chambers for imaging • Unable to capture tumour cells that are smaller or similar in size to blood cells.	[16, 17]
Vortex	Size-based	• Fast processing time (7.5 ml of blood in 20 min) • Cells remain intact and viable after isolation • Has the potential to capture CTCs from various cancer types.	• Unable to recover tumour cells with 100% purity • Low CTC capture efficiency • Unable to capture tumour cells that are smaller or similar in size to blood cells.	[57]
Microfluidic device for deformability-based cell classification	Size and deformability-based	• Cells remain intact and viable after isolation • Cost-effective • Has the potential to capture CTCs from various cancer types.	• Unable to recover tumour cells with 100% purity	[35]
DEPArray	Electric charge-based	• Allows single-cell isolation • Able to isolate viable CTCs with 100% purity • Has the potential to capture CTCs from various cancer types.	• Limited throughput • Large amount of sample loses	[58, 59]

Abbreviations used are: CTC, circulating tumour cell; EpCAM, epithelial cell adhesion molecule; CK, cytokeratin proteins.

Table 1. Comparison of selected microfluidics systems used for enriching CTCs.

In 2007, a microfluidics chip developed for CTC enrichment and isolation, known as CTC-chip, was introduced [33]. In this system, CTCs are captured as blood flows through the microchannel containing EpCAM antibody-coated microposts [33]. CTCs are captured with ~50% purity and sample processing takes 1–2 ml/h. Following CTC-chip, a broad range of microfluidic devices were generated to isolate CTCs based on physical size, density, deformability [34–41] or antibody-mediated CTC capture in surface functionalised microchannels [33, 42–47].

Although the affinity binding methods (e.g. CellSearch, CTC-chip) may isolate CTCs of better purity than the physical methods, the strong antibody-antigen interaction in the microdevice may affect the recovery of viable CTCs [11, 48] and hinder subsequent downstream analysis. Additionally, affinity binding methods will lose out on the subpopulation of CTCs that have down-regulated expression of epithelial markers (e.g. EpCAM) such as the mesenchymal CTCs, resulting in an underrepresentation of the actual CTC count in the blood [11, 49]. Therefore, the limitations imposed by affinity binding methods prompt the development of label-free microfluidic devices that can isolate CTCs with increased purity and viability.

To efficiently separate CTCs from the large pool of RBCs and WBCs, a spiral microfluidics chip was introduced in 2013 [11, 50]. The spiral microchannel (500 μm width × 160 μm height) consists of two inlets and two outlets over a length of ~10 cm. Blood (diluted 2–2.5×) is pumped into the outer inlet while sheath fluid is pumped through the inner inlet. The additional sheath fluid in the spiral chip facilitates the Dean migration of large volume of RBCs in a well-controlled manner, thus allowing high haematocrit samples (20–25%) to be processed. In spiral microchannel, CTCs and other blood cells experience Dean drag forces in addition to inertial lift forces and the combined effects cause hydrodynamic focusing of the cells to specific region of the microchannel. The larger CTCs are focused near the inner wall of the channel while smaller WBCs and RBCs are focused along the outer wall, leading to efficient size-based isolation of CTCs [50]. Furthermore, the spiral chip is able to recover >85% spiked cancer cells and deplete almost 100% of WBCs from the blood sample [11, 50]. Trypan blue staining showed that most of the recovered cells (>98%) are viable [11]. Therefore, the spiral chip allows continuous isolation of CTCs in a single step with high sensitivity, recovery and throughput (3 ml of blood can be processed in an hour) [11]. The spiral chip is also designed to have large microchannel dimensions and high flow rate to prevent non-specific binding of CTCs to the walls and eliminate any potential clogging issues [11].

2.2. Microfluidics and single CTC isolation

Most microfluidics systems isolate CTCs in bulk rather than individually. The bulk analysis of CTCs may mask the presence of key mutations that are critical to disease progression, indicating the need for single-cell analysis. To isolate and study CTCs at the single-cell level, approaches such as micropipette aspiration [60, 61] and laser microdissection [62] have been used to manually select the individual CTCs. However, these methods are laborious and suffer from low throughput, making them less suitable for clinical use. Commercial platforms such as DEPArray (Silicon Biosystems), which rely on the dielectric properties of CTC for single-cell isolation, suffer from large amount of sample losses [58].

To address these limitations, Yeo et al. developed a microfluidics device capable of performing high throughput, selective isolation of individual viable CTCs with 100% purity amidst a

large population of WBCs [17]. The separation efficiency is high because as few as 1 CTC in 20,000 WBCs can be recovered. Prior to starting the run, the cell suspension is stained with specific fluorophore-conjugated antibodies such as CK or CD45 to facilitate the differentiation of CTCs from WBCs. The device works on the principle of hydrodynamic focusing to restrict cells to flow in a single stream and hold them passively in active control cell chambers that are positioned along the outer curvature of the channel. CTCs and other blood cells flowing through the channel will experience a slight centrifugal force that facilitates their entry into the cell chambers. Because the microfluidics biochip is integrated into a microscope, the cell sitting in the chambers can be observed under the microscope to determine whether it is a CTC or WBC based on size or staining outcome. Each chamber holds 1 cell at a time and each is connected to a control line that can be activated to eject the selected cell back into the main channel and into the collection well. In this manner, the cells isolated in the chambers can be ejected sequentially so that each cell can be recovered individually. To maximise the recovery of rare CTCs, the effluent of each run is recycled back into the device for three times. The device is able to process 7.5–8 ml of blood in 3 h [16], facilitating the enumeration of CTCs in clinical blood samples in a short period of time. This method also allows the collection of single CTCs for downstream molecular profiling.

3. Clinical applications of CTCs

Given the convenience and ease of obtaining blood samples from cancer patients, CTCs are currently being adopted for clinical practice. This is especially the case when tissue biopsy samples are not readily available. With current technologies, CTCs can be isolated in bulk or individually for studies on disease progression and therapeutic treatment. Single-cell analysis offers several advantages over pooled cell analysis. First, single-cell analysis is able to detect critical driver mutations for drug response that are present in low frequency, which may be masked in pooled cell analysis since the mutations may only be present in a subset of clones. For instance, in stage IV non-small cell lung cancer (NSCLC) patients who developed resistance after tyrosine kinase inhibitor (TKI) treatment, sequencing of single CTC revealed the presence of epidermal growth factor receptor (EGFR) mutation T790 M that confers resistance in a subset of CTCs isolated from each patient (0–3 CTCs carry the mutation among <10 CTCs isolated per patient) [17]. Determining the mutation status of EGFR in NSCLC patients is important for patient stratification and treatment (see Section 3.4). Second, single-cell analysis reveals the heterogeneity in gene mutations and chromosomal copy number aberrations (CNA) among tumour cells while bulk cell analysis would have average out the signals. In metastatic breast cancer, a sequencing analysis of 40 single CTCs from five patients demonstrated heterogeneous mutations in four genes, phosphatidylinositol 3-kinase catalytic subunit alpha (*PIK3CA*), tumour protein P53 (*TP53*), oestrogen receptor 1 (*ESR1*) and *KRAS* [63]. Within the same metastatic breast cancer patient, not all CTCs harbour the particular mutation and different patients harbour different gene mutations [63]. In small cell lung cancer (SCLC) patients, chromosomal CNA profiling of individual CTCs obtained at pre-treatment can predict whether the patient is sensitive or refractory to subsequent chemotherapy at an accuracy of 83.3% [64], facilitating clinical decision-making. Third, single-cell analysis facilitates the study of clonal diversity and mutation evolution over the course of chemotherapy, which is difficult to achieve in bulk cell

analysis [65, 66]. In triple negative breast cancer (TNBC) patients, single-cell DNA sequencing revealed that chemoresistant patients carry pre-existing mutations and CNA that were adaptively selected in response to chemotherapy [65]. Single-cell RNA sequencing also found that chemotherapy induces transcriptional reprogramming to favour the resistant phenotype in TNBC patients who develop chemoresistance [65]. Thus, single-cell analysis provides a better resolution of the tumour profile than pooled sample analysis. More importantly, single-cell analysis of CTC can address the heterogeneous profile of cancer cells at the DNA, RNA and protein level and provide insights in the mechanism of metastasis and drug resistance. Here, we discuss the applications of CTCs that aid in the management of cancer and how single CTC analysis can contribute to achieving personalised medicine in the future.

3.1. Biomarker for early detection of cancer

The presence of CTCs in the peripheral blood acts as a biomarker for the early detection of cancer. Thus, the enumeration of CTC is important for cancer screening, especially in patients who at a higher risk of developing cancer due to genetic predisposition or disease state. The effective use of CTC enumeration in the detection of early-stage cancer has been demonstrated in patients with chronic obstructive pulmonary disease (COPD), who are at a high risk of developing lung cancer [5, 67]. Because of the risk, COPD patients are monitored annually for the development of lung cancer using computed tomography (CT) scan. In a subset of COPD patients without cancer diagnosis, CTCs were found in the peripheral blood. After 1–4 years of CTC detection, lung nodules were indeed detected by CT scan, leading to the diagnosis of early-stage lung cancer. Large-scale clinical studies are being done to further validate the use of CTCs as a diagnostic tool for the early detection of cancer [5]. Therefore, CTC can serve as a potential biomarker for the early detection of cancer so that prompt treatment intervention can come in place to improve the overall health of patients.

3.2. Prognostic marker for overall survival and metastasis

The number of CTCs found in the blood can indicate the state of the disease. Patients in later stages of cancer (e.g. metastatic cancer patients) have higher number of CTCs as compared to patients in the early stages of cancer [14, 68, 69]. Furthermore, the number of CTCs can vary with chemotherapy treatment, allowing clinicians to use CTC counts to determine the treatment efficacy and estimate the overall survival and risk of metastatic relapse. A study on early breast cancer patients [70] measured the number of CTCs before and after adjuvant chemotherapy demonstrated that the persistent presence of CTCs after chemotherapy is associated with poor disease-free survival and overall survival. Moreover, prognosis was worst in patients with >5 CTCs per 30 ml of blood [70]. Similarly, studies in lung cancer patients also showed that higher number of CTCs was significantly correlated to shorter survival [32, 71–73]. Specifically, the presence of >8 CTCs per 7.5 ml of blood after treatment strongly correlated with worse survival in small cell lung cancer patients [74]. In prostate cancer, therapeutic treatment resulting in CTC level dropping from >5 to <5 in 7.5 ml of blood is indicative of better overall survival [55]. Thus, the prognostic cut-off value for CTC is dependent on the type of cancer.

Additionally, CTC enumeration can be used for predicting the risk of metastasis. In non-metastatic colorectal cancer patients, those with >5 CTCs per 2 ml of blood were more likely

to develop distant metastasis than those with <5 CTCs [69]. The strong correlation between CTC count and metastasis relapse have been shown in other types of cancers as well including bladder cancer [75, 76], liver cancer [77] and oesophageal cancer [78]. Thus, these patients may benefit more from early treatment.

3.3. Monitoring treatment response and disease progression

Sequential tracking of CTC number during treatment may inform on treatment response and disease progression, providing important information to clinicians on whether the treatment is suitable for the cancer patient. In advanced NSCLC patients, a significant decrease in CTC count after the second cycle of chemotherapy strongly correlated with better overall survival and progression-free survival [32, 79, 80]. However, a lack of decline in the number of CTCs after chemotherapy may suggest that the patient has developed resistance against the specific drug. Therefore, alternative treatment strategies have to be adopted to curb disease progression and improve patient survival. Another potential application may be the detection of early relapse with regular monitoring of the CTC count in post-surgical cancer patients. This may allow early detection and timely clinical intervention to treat the disease when the disease burden is less.

3.4. Identification of therapeutic targets and drug resistance

Given the continuous improvement in microfluidics technology, Khoo et al. demonstrated that patient-derived CTCs could be cultured into CTC clusters *in vitro* using a microfluidic culture device [81]. Prior enrichment of CTCs and supplements of growth factors are not required, thereby shortening the processing time. Using blood samples from the same patient, CTCs are co-cultured with immune cells in specially formulated microwells to promote the formation of CTC cluster within 2 weeks. The success rate of CTC cluster formation is approximately 50%. With the development of this platform, drug screening can be readily conducted and this can facilitate the discovery and testing of novel drugs that are more efficacious in the treatment of cancer. Furthermore, drug responses can be monitored with varying doses of drug to determine the optimal dose for individual patient.

With the advent of next-generation sequencing, the molecular profile of single CTCs can be obtained at the DNA, RNA and protein level. The genomic profile of single CTCs can be compared to normal or non-malignant cells to identify genes that are differentially expressed, which may mediate the process of metastasis and become potential therapeutic targets. The whole genome sequencing of individual CTCs can reveal genetic alterations such as mutations, copy number variations and single nucleotide polymorphisms that may confer selective advantage to tumour cells [82]. The presence of specific gene mutations in CTCs confers drug resistance and determines the type of treatment to be given to patients. This information is particularly useful when the mutation profile of CTC is concordant with the primary tumour. For example, the CTCs of NSCLC patients harbouring the EGFR T790 M mutation confer resistance to TKI treatment (e.g. gefitinib) [83]. Tracking changes in the CTC count and mutation frequency over the course of treatment allows real-time monitoring of treatment sensitivity and resistance. The early detection of these mutations may provide alternative treatment strategies for NSCLC patients and optimise disease management, leading to improved clinical outcomes [17]. Thus, determining the mutation status of EGFR is crucial since it allows

clinicians to select patients who will benefit from TKI treatment. Furthermore, understanding the key mutations behind drug resistance may help to decipher the mechanism and signalling pathways involved in resistance.

The transcriptome of single CTCs also provides information on the identification of therapeutic targets and drug resistance. In prostate cancer, analysis of the mRNA profile in CTCs is required to determine drug sensitivity or resistance. Specifically, the expression of Arv7 mRNA, a truncated form of androgen receptor that remains constitutively active, in CTCs is predictive of anti-androgen therapy failure with enzalutamide and abiraterone [84, 85]. In prostate cancer patients with Arv7 expression, alternative drugs such as taxanes are used for treatment [86–89]. Additionally, RNA-seq of CTCs can reveal miRNAs that are dysregulated in cancers, making these miRNAs potential targets for cancer therapy.

The expression profile of proteins in single CTCs also plays a role in determining the anti-cancer treatment. For example, oestrogen receptor (ER) is a primary target in the treatment of breast cancer patients. Thus, primary tumours of breast cancer patients are stratified as ER+ or ER- and hormonal therapy is given based on the status of ER expression in the primary tumour. However, breast cancer patients with ER+ primary tumours can harbour ER- CTCs in the blood, which may escape the hormonal therapy [90]. Similarly, metastatic breast cancer patients with HER2+ primary tumours can carry HER2- CTCs in the blood [91–93]. Because of this discordance, HER2-targeted therapies may only be effective against the primary tumour but not the CTCs. Thus, cancer cells will not be fully eradicated and this may lead to metastatic cancer relapse. In this situation, additional treatment strategies have to be adopted to target the CTCs, on top of the primary tumour. Therefore, the monitoring of genetic aberrations is important in identifying acquired mutations that confer resistance to drug therapy.

The expression status of programmed death ligand 1 (PD-L1) in CTCs aids in identifying the groups of patients who are likely to benefit from the immunotherapy as well as predicting the response to the immunotherapy. Tumour cells express PD-L1 that binds to PD-1 receptor found on the surface of activated T cells and B cells to induce an immunosuppressive effect by reducing cytokine production and immune cells proliferation [94, 95]. It was previously shown that metastatic tumour cells have higher PD-L1 expression than primary tumour cells [96]. In breast cancer, the detection of CTCs expressing PD-L1 indicates that patients carry metastatic cells that have the potential to evade immune destruction [94]. Breast cancer patients with a high frequency of PD-L1(+) CTCs are more likely to benefit from anti-PD-L1 immunotherapy than patients with PD-L1(−) CTCs [94]. In a study on NSCLC patients, PD-L1 expression on CTCs was monitored throughout the course of immunotherapy [97]. After 6 months of therapy, patients with PD-L1 expression in CTCs had poor prognosis while patients without PD-L1 expression in CTCs benefitted from the therapy [97].

Given the intra-tumoural and inter-tumoural heterogeneity and dynamic nature of cancer, single-cell analysis of CTC in circulation may provide information on the evolution of tumour and how they evade drug therapy and immune response (**Figure 1**). This enables clinicians to have a more holistic view of the disease complexity and more efficient targeting of cancer cells, moving towards the development of personalised therapy for individual patients.

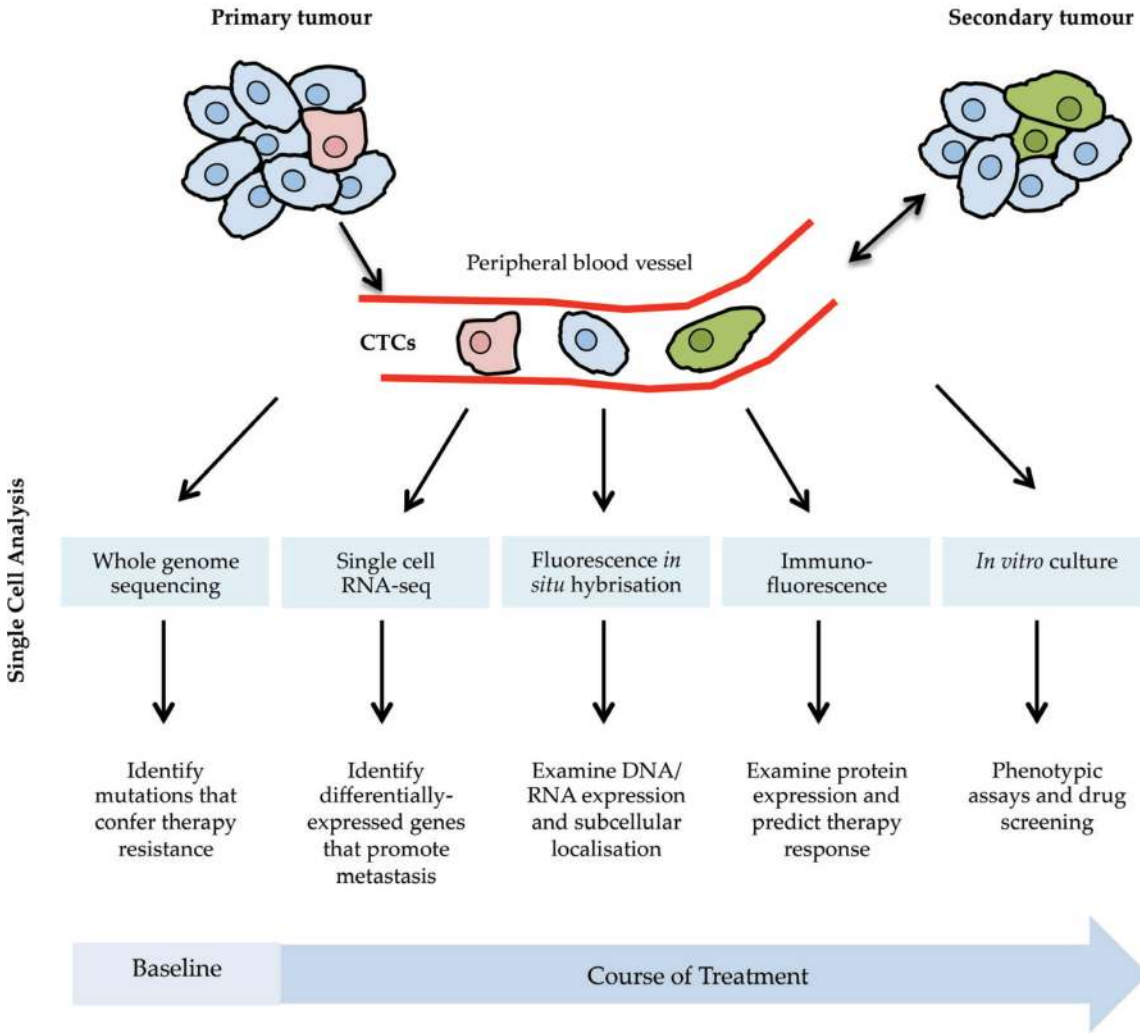

Figure 1. A proposed scheme of how single-cell analysis of CTCs may address tumour heterogeneity. Heterogeneous tumour cells are colour-coded. Heterogeneity stems from genetic or epigenetic changes that confer selective advantage to the tumour cells. Several strategies of single-cell analysis can be adopted to dissect the heterogeneity of tumour cells. Sequential analyses of individual CTCs aids in the monitoring of therapeutic response, tumour evolution and detection of treatment-resistant cells.

3.5. Comparing CTC with cell-free DNA and exosome

Apart from CTCs, other liquid biopsy markers that confer diagnostic and prognostic relevance include the cell-free DNA (cfDNA) and exosomes. Each marker has its own strengths and weaknesses (**Table 2**). cfDNA refers to DNA released from necrotic and apoptotic cells and they can be found in blood plasma. Cancer patients usually have higher concentration of cfDNA compared to healthy individuals [98, 99]. Most cfDNAs in the blood plasma are around 70–200 bp long [100, 101]. cfDNA includes the circulating tumour cell DNA (ctDNA), which are released from CTCs. The fraction of ctDNA contributing to cfDNA is small although usually higher in late stage cancer patients (>5–10%) than patients in the early stages of cancer

Liquid biopsy	Strengths	Weaknesses
Circulating tumour cell (CTC)	• Allows downstream molecular analysis and *in vivo* or *in vitro* functional studies • Able to evaluate the DNA, RNA and protein profile of tumour cells • Able to study the cellular phenotype, morphology and protein localisation	• Extremely rare and challenging to isolate • Heterogeneous population of CTCs may lead to false positive and false negative results
Cell-free DNA (cfDNA)	• High sensitivity in detecting genetic aberrations	• Challenging to isolate pure population of cfDNAs • Molecular characterisation is limited to genomic DNA and unable to evaluate the RNA and protein profile • Unable to perform phenotypic and functional studies
Exosome	• Abundant in the plasma • Allows downstream molecular analysis and functional studies • Able to evaluate the DNA, RNA and protein profile of tumour cells • Allows analysis of inflammatory, stromal and other systemic changes	• Challenging to isolate pure populations of tumour-derived exosomes • Unable to perform phenotypic studies

Table 2. Comparison of the liquid biopsy markers.

(<1%) [98, 102, 103]. A large fraction of cfDNA comes from non-malignant cells that contain wild type DNA. Because of this issue, highly specific and sensitive technologies are required to isolate cfDNA that originates from tumour cells.

The isolated cfDNAs are subjected to sequencing analysis using next-generation sequencing platforms to identify tumour-associated genetic mutations or epigenetic changes. For example, in a large-scale study of NSCLC patients on gefitinib treatment, cfDNA was used as a surrogate of tissue biopsy to identify the EGFR mutations, demonstrating its clinical utility [100, 104]. A comparison on the frequency of mutation detection between cfDNA and CTC revealed that cfDNA showed a higher frequency of the mutation from the same patient [100, 102], suggesting that cfDNA is more effective in detecting these genetic changes. Another advantage that cfDNAs have over CTCs is that cfDNAs can be obtained from bio-banked fluids such as frozen plasma whereas CTCs can only be obtained from peripheral blood [100]. However, the use of cfDNAs as a liquid biopsy marker also poses several limitations. First, because cfDNA can originate from any cell type including normal cells and tumour cells, there will be a high background of wild type DNA and thus isolating a pure population of the rare tumour-derived cfDNA is technically challenging. The abundance of wild type DNA may mask the detection of low copy genetic mutations that could be important for early detection of cancer or drug resistance. Second, molecular profiling of cfDNAs is restricted to the DNA level as the characterisation of the transcriptome and proteome is not possible [100].

Exosomes are membrane-bound microvesicles derived from multivesicular bodies (MVBs) and secreted into the extracellular environment through fusion of MVB to the plasma membrane [8, 100]. They mediate cell-cell communication by transferring biomolecules such as DNA, RNA, proteins and lipids from the donor cell to the recipient cell [105]. Exosomes can originate from many cell types including tumour cells, epithelial cells, fibroblasts, neuronal cells, haematopoietic cells and adipocytes [8, 106]. Most exosomes are around 30–200 nm and are present in large quantities in biological fluids including serum, plasma, urine and saliva [8, 100]. Tumour cells release tens of thousands of exosomes in a day, resulting in hundreds of billions of exosomes per ml of plasma [100, 107]. Since exosomes are abundant, they are easier to isolate compared to CTCs and cfDNAs. However, there is a lack of efficient tumour exosome enrichment method [108] because the isolation of exosomes is largely based on exosome-specific surface markers [100, 109], which do not distinguish between exosomes derived from tumour cells and normal cells.

Because exosomes carry the DNA, RNA and protein content from tumour cells (i.e. cell of origin), they are useful diagnostic and prognostic tools of cancer. Similar to CTCs and cfDNAs, exosomes can be subjected to DNA analysis to determine the genetic aberrations and mutational landscape of tumour cells. Additionally, the RNA and protein profiles of tumour exosomes can be characterised to provide insights into the biology of tumour cells. For example, analysis of exosomal mRNA and proteins allows real-time monitoring of therapeutic response and drug resistance in glioblastoma patients [110, 111]. The up-regulated expression of a panel of serum-derived exosomal miRNAs (miR-1246, miR-4644, miR-3976, miR-4306) serves as a biomarker for the diagnosis of pancreatic cancer [112]. The down-regulated expression of miR-92a is associated with high risk of cancer relapse in hepatocellular carcinoma patients [113]. Furthermore, previous reports showed that tumour exosomes play a role in suppressing immune response, promoting tumour cell growth, angiogenesis and metastasis [100, 114–116]. Similar to CTCs, molecular profiling of exosomes provides insights into the mechanism of metastasis and drug resistance.

3.6. Barriers to adoption of CTC as a clinical test

Although CTCs have numerous potential clinical applications, the incorporation of CTCs into routine clinical practice still faces several challenges. First, there is a lack of reproducibility in CTC enumeration when different measurements were taken from the same patient [117]. This variation is likely caused by the extremely low frequency of CTCs in peripheral blood, where >90% of patients with localised diseases and up to 30–40% of patients with metastatic disease do not have >5 CTCs per 7.5 ml of blood [117, 118]. A difference of 1 CTC may lead to different stratification and prognostic outcome [117]. A possible solution to improve the CTC yield is to process larger volume of blood sample from cancer patients [117, 119]. Second, there is a lack of standardisation across the myriad of CTC detection platforms in defining and isolating CTCs [120, 121], resulting in variability of CTC count. Studies comparing EpCAM-dependent and EpCAM-independent CTC enrichment methods using blood samples from the same patient showed that EpCAM-independent methods generate a higher CTC count compared to the CellSearch system [117]. Thus, a universal quality control system is required for detecting and isolating CTCs across the various platforms so as to benchmark the reliability of these methods [117, 120]. Cross-validation studies on CTC enumeration from different laboratories

will also minimise the inter-observer variation [117]. Lastly, the current CTC isolation technologies have limited sensitivity and are not applicable to all types of cancer [117]. Therefore, before CTCs can be fully adopted for routine clinical use, well-designed appropriately powered validation studies are required.

4. Conclusion

Late clinical diagnosis and chemotherapy resistance are the main factors leading to reduced chances of survival for cancer patients. To combat cancer, CTCs provide invaluable information on the status of the disease and the likely outcome of chemotherapeutic treatment. The enumeration of CTCs allows early detection of cancer, prognosis and real-time monitoring of chemotherapy treatment. The single-cell analysis of CTCs provides a wealth of genetic information that enables better understanding of the disease complexity for individual patients and provides the opportunity for the development of personalised treatment. To aid in delivering better therapeutic medicine, current technologies allow CTCs to be cultured *in vitro* for the identification of novel therapeutic targets and optimal drug dosage for individual patients. Therefore, the molecular characterisation of CTCs is important for improving the clinical outcomes in cancer patients.

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