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The Selection Strategy for Circulating Tumor Cells (CTCs) Isolation and Enumeration: Technical Features, Methods, and Clinical Applications

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Abstract

The key aim of the proposed chapter is to provide readers a brief description for the most important parts of the field of circulating tumor cells (CTCs): the core techniques, including negative and positive selection-based CTC isolation, and the differences between them. Most importantly, we will also review the clinical applications and important findings in clinical trials. The evidence-based review will not only help clinicians use CTCs to predict recurrence and foresee the disease-related outcomes but also to inspire the researchers in this field to conduct further investigations.

Keywords: circulating tumor cells, negative selection, cancer, stem cell, liquid biopsy

1. Introduction of circulating tumor cells (CTCs)

1.1. Brief history of CTC researches

Circulating tumor cells (CTCs) are cells shedding from primary tumor(s) into the adjacent vasculature and are floating around in the circulation throughout the human body. The cells, as seeds for the subsequent initiation of distant site metastases, are responsible for the cancer-related deaths [1]. For the first time, CTCs were described via the observation in the blood of cancer patient by Dr. Thomas Ramsden Ashworth, who postulated that “cells identical with those of the cancer itself being seen in the blood may tend to throw some light upon the mode of origin of multiple tumors existing in the same person” in 1869 [2]. In 1906, Goldmann reported that visible venous invasion by cancer in approximately 20% of 500 necropsies and

microscopic invasion of vasculature in nearly 10% [3]. In very early 1900s, several reports of observation of free cancer cells by morphology have been discussed in patients with melanoma, gastric cancer [4–6], and lung cancer [7]. However, long being in the technical limitation on isolation of these rare cells in circulation, the realization of CTCs isolated from living cancer patients and analysis of their clinical impacts began since 1930s [8–10]. One of the first systemic surveys in 40 living cancer patients was done by Pool and Dunlop in 1934 [11]. In this period, morphology and cytochemical characteristics remained the most important method to identify “abnormal” or “atypical” cells. Tumor cell embolization was observed [12] and widely accepted to be one of the major mechanisms for dissemination of cancer [13, 14]. Since 1986 with the development of polymerase chain reaction (PCR), investigators began to utilize these nucleic acid-based detection methods to help identify CTCs, including circulating tumor mutated DNA and mRNAs [15–23]. In early 2000s, semi-automated devices appeared and facilitate the advances of CTC testing in clinical study enrolling healthy subjects and patients with various types of cancer [24–26], given the fact that numerous previous methods were relatively operator-dependent and often lacked of validated sensitivity, specificity, coefficient of variation and reproducibility [22, 27–31]. In 2004, CellSearch™ (Veridex, Janssen Diagnostics, USA) got approval from United States (US) Food and Drug Administration (FDA) for testing in patients with breast, colorectal, and prostate cancer [32, 33]. Since 2012, a rapid exploration of number of CTC isolation devices having nearly fully automated design emerged [34–40].

Recently, CTC studies are focusing on devices harboring high sensitivity, high specificity, reduced sample requirement, label-free isolation, and the ability to catch living CTCs for in-vitro culture. Owing to the less invasive nature than conventional cancer tissue biopsy, CTCs, as serum circulating tumor DNA (ctDNA) and microRNA (miRNA), are termed to be “liquid biopsies.”

1.2. Natures of CTCs

It has been a long time after CTCs were noticed and efficiently captured by many methods; however, little is known about the behavior of CTCs [41]. Investigators have observed some phenomenon about what CTCs look like, how CTCs shed, migrate, live, defense human immune system, and initiate distant metastases.

First, CTCs were believed to be larger than normal blood cells, which contributed the development size-based isolation strategy. Marrinucci et al. [42] supported that the fact of CTCs being larger than white blood cells and having high nuclear to cytoplasmic ratios with voluminous cytoplasm. In addition, the morphology of CTCs is highly similar to that of cells from original biopsied cancer tissues. Numerous devices were developed on the basis of this characteristics and collect cells with larger size (often $>15\ \mu\text{m}$), including dielectrophoresis (DEP) [43, 44], optically induced dielectrophoretic (ODEP) [45] force-dependent devices, and filter-based systems [46–61]. However, other investigators found that the size of real CTCs could be greatly differed from cell lines [62], and might even vary interindividually and intraindividually [63, 64]. The size criteria of CTC definition remain in debate.

Second, CTCs exist in almost all staged cancer and could be detected in the course of the disease [27]. In 1995, Hansen et al. analyzed the blood samples drawn from surgical fields during 61 oncologic surgeries and 93.4% samples found tumor cells [65], which suggest one of the possible routes of early dissemination of cancer cells. In 2000, Yamashita et al. found that signals of CTCs (carcinoembryonic antigen messenger RNA, CEA mRNA) from preoperatively negative to postsurgically positive might suggest a specific type of surgery could contribute to the cancer cell dissemination [66]. Similar results were reported by other investigators to support the findings in various types of cancer (but breast cancer mainly) [67–73]. Although CTCs were found in early-stage cancer patients, the cells do not result in metastasis all the times. The clearance of human immune systems and inadequate “soil” of distant organs are one of the plausible explanations. To look on the bright side, CTCs in very early stage cancer could help early diagnosis of cancer and prevent overwhelming dissemination and cancer death [74].

Third, CTCs would form cell clusters, clumps, or circulating tumor microemboli (CTM) and were found to highly correlate to cancer progression [75–79] and resistance to systemic anticancer therapies [78, 80], which was established on the basis of many animal model and preclinical reports [81–85]. Recently, Sarioglu et al. [86] designed a Cluster-Chip for efficient capture of CTC clusters and enable the detailed characterization of the biological properties and role of CTC clusters in metastasis [86]. Studies of cancer metastasis have emphasized the novel concept of “seed and soil” as a key determinant of metastatic propensity [87]. This model matches the importance of mutated genetic drivers within tumor cells conferring proliferative and invasive properties, with that of the microenvironment of the distant organ or “niche,” which may facilitate metastasis occurrence. However, the physical characteristics of single CTCs and CTC clusters may also contribute to metastatic propensity, especially as they impact the ability of epithelial tumor cells to survive the loss of cell adherence and shear forces in the blood stream, i.e., different survival signals among the cancer cell “seeds” may be important. For instance, in a mouse endogenous pancreatic cancer model, noncanonical Wnt signaling is elevated within CTCs, where it appears to suppress anoikis [88], while in a subcutaneous tumor xenograft model, the admixture of tumor and stromal cells within microemboli may contribute to stromal-derived survival signals [77, 89, 90].

Fourth, CTCs could also be detected in cancer patients who underwent curative surgery, indicating minimal residual disease in the circulation [91–99] and suggesting the correlation to disease recurrence in the following months [92, 95, 100, 101]. van Dalum et al. [99] found that the presence of CTC in blood drawn pre and one and two years after surgery, but not postsurgery is associated with shorter Relapse-free survival (RFS) and OS for stages I–III breast cancer, which could partially answer the question of how frequent and how long oncologists should follow patients’ CTCs up and the timing of CTC testing after curative surgery. This phenomenon indicates that postsurgical adjuvant therapy might be required in specific population on the basis of CTC testing which remains uncertain to date.

Fifth, the captured CTCs according to their method of isolation, sometimes are alive for *in vitro* culture [48, 61, 102, 103] and might play a very important role to continuously obtain primary cancer cell lines in the near future. Furthermore, CTC-derived cell lines and xenografts

might reveal new therapeutic targets and can be used for drug screening [104, 105]. The phenomenon is the main difference of CTCs from ctDNA possibly being released from dead cancer cells. In addition, living CTCs can also colonize their tumors of origin, in a process that is called “tumor self-seeding.” Kim et al. [106] successfully revealed the self-seeding phenomenon in breast cancer, colon cancer, and melanoma tumors in mice model, which was predominantly mediated by CTCs with aggressive features, including those with bone, lung, or brain metastatic tropism. The cancer-derived cytokines IL-6 and IL-8 acted as CTC attractants and the markers MMP1/collagenase-1 and the actin cytoskeleton component fascin-1 as mediators of CTC infiltration into mammary tumors. The important findings of tumor self-seeding phenomenon could explain the relationships between tumor size, anaplasia, vascularity and prognosis, and local recurrence seeded by disseminated cells following ostensibly complete tumor excision.

Sixth, CTCs could represent a merged status of a whole tumor mass, including static and active parts with expression of specific functional markers [107–110] and could serve as a multifunctional biomarker [108, 111]. Functional analyses on CTCs might provide the possibility to identify the biological characteristics of metastatic cancer cells, including the identification of metastasis-initiating cells [104].

1.3. CTCs in cancer progression

1.3.1. Cancer migration, invasion, epithelial-mesenchymal transition (EMT), mesenchymal-epithelial transition (MET) and cancer stem cells (CSCs)

As mentioned above, Hansen et al. [65] found that CTCs exist in the 93% blood samples drawn from surgical fields. That correlated to one of the two common routes of cancer migration: hematologic and lymphatic spreading. In clinical aspect, tumor migration and invasion means tumor growth or progression and can be analyzed via the time from disease-free status to recurrence or time from baseline to enlargement of tumor size. Early in 1999, Palmieri et al. have found a significant correlation among clinical stages, tumor progression, and presence of circulating cancer-associated antigens in stages I–III melanoma patients [112]. In other cancer types, investigators widely agreed with the observation that the higher CTCs signals indicate to higher cancer stage and recurrence rate, suggesting larger number of CTCs might promote cancer progression [113–117]. However, not only CTC count but also the specific properties of cancer cells matter. Two of them have been widely reported are epithelial-mesenchymal transitions (EMTs) or stem-like properties of CTCs [118].

In many animal species, EMTs normally occur during critical phases of embryonic development. The formation of mesenchymal cells (nonepithelial) that are loosely embedded in an extracellular matrix from a primitive epithelium is an important feature of most metazoans [119]. During this transition, mesenchymal cells acquire a morphology that is appropriate for migration in an extracellular environment and settlement in areas that are involved in organ formation, which involves interactions between epithelial and mesenchymal cells. Mesenchymal cells can also participate in the formation of epithelial organs through mesenchymal-epithelial transition (MET) [119]. CTCs may also undergo phenotypic EMT changes, which

allow them to travel to the site of metastasis formation without getting affected by conventional treatment [118, 120, 121]. The acquired molecular changes by CTCs undergoing EMT that facilitates cancer progression and resistance to conventional therapies [122, 123]. EMT markers, including vimentin, twist, ZEB1, ZEB2, snail, slug, and N-cadherin in CTCs, primary HCC tumors and adjacent nontumoral liver tissues were evaluated by Li et al. [123] and the twist and vimentin expression levels in CTCs could be promising biomarkers for evaluating metastasis and prognosis in liver cancer patients. Most importantly, CTCs would abandon their epithelial properties (EpCAM) [88, 124] and escape from CTC capture by positive selection strategy (will discuss below) and become one of the main downsides of the strategy of CTC isolation. Several investigators have noticed that phenomenon and suggested that there is an urgent need for optimizing CTCs detection methods through the inclusion of EMT markers [120, 125–129]. Deeper understanding of those processes is of fundamental importance for the development of new strategies of early cancer detection and effective cancer treatment approaches that will be translated into clinical practice [122].

Stemness features of CTCs, sometimes termed as circulating cancer stem cells (CSCs), have also been getting noticed as EMT of CTCs in recent years. The CSC hypothesis claims that a small subset of cells within a tumor has the ability of both tumor initiation and sustaining tumor growth [130–132]. These cells with expression of stemness markers are capable of forming floating spheres in serum-free medium, a property associated with stem cells and are able to differentiate into an aberrant cell phenotype constituting tumor heterogeneity [133]. Among all the possible molecular markers of stemness feature, CD133, CD44, ICAM-1, and CXCR4 are common used antibodies for labeling the subpopulation from other CTCs and actually technically available [134–136]. These are not the only markers to identify CSCs and depend on cancer types. Sun et al. [137] found that stem cell-like phenotypes (labeled with CD133 and ABCG2) in EpCAM-positive CTCs, and a preoperative CTC of more than 2 cells/7.5 ml blood is a novel predictor for tumor recurrence in HCC patients after surgery, especially in patient subgroups with AFP levels of less than 400 ng/ml or low tumor recurrence risk. Many other studies in various types of cancer have come across with the similar conclusions, including breast, colorectal, gastric, liver, and NSCLC, etc. [120, 123, 138–145].

Therefore, the subpopulation of CTCs, CSCs, and CTC with EMT features is probably the one of the key determinants of future CTC and cancer metastasis investigations.

1.4. The impact of CTCs on multidrug resistance

In 2011, Gradilone et al. reported an interesting study aiming to test the hypothesis that drug-resistant CTCs might have predictive value in metastatic breast cancer (MBC) and possibly retain stem-like properties [146]. As the study presented, the extraction of mRNA from CTCs for multiple drug resistance proteins (MRPs) analysis are most commonly used protocol. They also found the expression status of MRP1 and MRP2 in CTCs was found to correlate to response to anthracyclines (doxorubicin or epirubicin) [147]. In 2013, Nadal et al. found an interesting phenomenon that a relative enrichment of cytokeratin CK(+)/CD133(+) CTCs in triple negative and HER2-amplified tumors was found. While CK(+)/CTCs decreases after chemotherapy when analyzing the whole population, CK(+)/CD133(+) CTCs were enriched in posttreatment

samples in nonluminal BC subtypes. These findings suggest the potential role of CD133 as a promising marker of chemoresistance in nonluminal BC patients [148]. Similar results were also reported in recent years and the authors have come across with the same conclusion that multiple drug resistance profiling (MRPs mainly, sometimes with CD133 [148], ALDH1 [149], and ERCC1 [150]) of CTCs could predict the responses to given chemotherapies [146, 148, 150–152].

One direct proof of CTC exhibiting drug resistance comes from a study in 2014. Pavese et al. observed that CTC and DTC cell lines, established from mice bearing human prostate cancer orthotopic implants, exhibit increased cellular invasion in vitro, increased metastasis in mice, and express increased EMT biomarkers. In addition, CTC cell lines are selectively resistant to growth inhibition by mitoxantrone-like agents. The findings are important and suggested that CTC formation is accompanied by phenotypic progression without obligate reversion. Their increased metastatic potential, selective therapeutic resistance, and differential expression of potential therapeutic targets provide a rational basis to test further interventions [153].

Therefore, developing an in-vitro chemosensitivity test on CTCs is not impossible though it required large-scale clinical trials to test and validate. Yu et al. applied pharmacogenomic (PGx) modeling testing on CTCs, while PGx testing was used on cancer tissue to predict the efficacy of chemotherapeutic agents in preclinical cancer models, and reported the feasibility in 2014. In the report, clinical benefit was seen for study participants treated with chemotherapy regimens predicted to be effective versus chemotherapy regimens predicted to be ineffective with regard to progression-free (10.4 months versus 3.6 months; $P < 0.0001$; HR, 0.14) and overall survival (17.2 months versus 8.3 months; $P < 0.0249$; HR, 0.29) [151]. In another study, thymidylate synthase expression in CTCs could possibly serve as a new tool to predict 5-fluorouracil resistance in metastatic colorectal cancer patients [152]. Other than conventional imaging studies evaluating two-dimensional tumor size every 8–12 weeks for routine tumor assessment during anticancer therapy, CTCs could possibly serve as a rapid responding biomarker to real-time change of cancer cells, including the early response or resistance to given therapeutic drugs [154–156].

2. The strategies for CTC isolation and enumeration

There are hundreds of methods/protocols reported to be able to efficiently detect or isolate CTCs. In a simple way to discuss here, we have several common strategies of CTC isolation could be worthy of development in the future. The first one is **label-free isolation strategy**, including size-based, physical properties-based, morphology-based isolation strategy; the second one is **positive selection strategy**, including positively identification of cancer-specific markers on nucleus or cell surface, or specific DNA mutation(s), mRNA(s) overexpression; and the third one is **negative selection strategy**, consisting of depletion of red and white blood cells by any means. Finally, the fourth one is combination of two or more strategies mentioned above.

2.1. Label-free isolation strategy

Several novel studies using size as a key criterion of CTC identification were reported [157–160]. Early in 2004, ISET system was used for a well-designed clinical trial evaluating 44 patients with primary liver cancer and without metastases, 30 patients with chronic active hepatitis, 39 with liver cirrhosis, and 38 healthy individuals, and all participants were followed up for a mean period of 1 year. Both the presence ($P = 0.01$) and number ($P = 0.02$) of CTCs and microemboli were significantly associated with a shorter overall survival. Beta-catenin mutations could be found in 3 of 60 CTCs which might be suggesting their impact on the initiation of cancer cells invasion [161]. Similar positive findings by size-based CTC isolation were reported in melanoma [162–164], gastric cancer [76, 165], prostate cancer [166, 167], lung cancer [168–170], pancreatic cancer [103], liver cancer [127], sarcoma [171], and breast cancer [172]. Separation by physical properties, i.e., gravity, density gradients, using microfluidic technology [45, 46, 56, 60, 173–186], or microfiltration [53, 172, 187, 188] were also reported to be able to capture CTCs efficiently.

By means of label-free isolation, combined molecular analysis could be easily performed after CTC isolation owing to no chemicals exposure and less procedures done during isolation. For instance, Zheng et al. [189] reported a novel device designed based on membrane microfilter device to isolate CTCs and then send them to PCR-based genomic analysis by performing on-membrane electrolysis with embedded electrodes reaching each of the individual 16,000 filtering pores. Immunocytochemistry and FISH assays following label-free isolation were reported to be successfully performed directly on the filter system [157, 176, 190, 191]. Interestingly, some investigators compared the isolation efficiency of ISET and CellSearch™ systems [76, 93] and one team concluded that a combination of ISET plus CellSearch™ would have better performance in CTC detection in NCSCL patients than ISET or CellSearch™ alone [93].

There are several disadvantages of physical methods should be noticed. First, the isolation process based on physical properties can cause the deformation and damage of CTCs by filter pores [192]. Second, larger size cells could not always be cancer cells and the isolated population often mixed up with megakaryocytes, which are very common to see in the circulation of cancer patients just underwent chemotherapy. Third, small-size CTCs would be inevitably missed by this isolation strategy.

2.2. Positive versus negative selection-based CTC isolation

2.2.1. Positive selection methods

Positive selection strategy is the most commonly used method of CTC isolation in the literature. CellSearch™ is the most evidenced and the only one device having class III approval from US FDA since 2004; therefore, hundreds of clinical trials chose to apply the device for CTC testing for validation [114, 193–204], mainly in patients with breast, colorectal, and prostate cancer. Other representative positive selection platforms are magnetic-activated cell sorting system (MACS) and Isoflux. The main process of positive selection is to label targets cells by anti-CK (AE1/AE3) antibody with ferric beads and immunofluorescence dye. The approximate

sensitivity of detection is 10^{-7} (CTCs/hematologic cells). Another system, MACS used 50–100 nm-sized ferric beads. However, lower sensitivity and lower recovery rate of CTCs were observed. The device was firstly introduced in 1998 [205] and then CTCs obtained by the system could correlated with breast cancer stages [206] and could correlate with progression-free survival in colorectal cancer patients [207]. These systems are all based on immunomagnetic beads technology for CTC isolation and have long been limited by relatively low efficiency of antibody conjugation due to tumor heterogeneity [192]. This limitation further causes the difficulty of molecular analysis [208]. Fortunately, the technique of single CTC isolation and analysis has been much more mature in recent years [166, 209, 210]. However, fewer sampling (CTCs) could greatly contribute to the bias for prediction of target population behavior (the whole tumors in the body). Another downside of positive selection strategy was the limitation of EpCAM-dependent nature. Hyun et al. [124] demonstrated that EMT-induced breast cancer cells maintained in prolonged mammosphere culture conditions possess increased EMT markers and cancer stem cell markers, as well as reduced cell mass and size by quantitative phase microscopy. In addition, EpCAM expression is dramatically decreased in these cells. Moreover, CTCs isolated from breast cancer patients using a label-free microfluidic flow fractionation device had differing expression patterns of EpCAM, indicating that affinity approaches reliant on EpCAM expression may underestimate CTC number and potentially miss critical subpopulations.

In addition to conventional immunomagnetic bead separation methods, density separation and flow cytometry or cell sorting systems have been postulated to be potential tools of CTC isolation and identification considering their high sensitivity and purity since 1998 [27]. This method could be seen as a combination of negative selection strategy and a positive confirmation with surface markers, such as EpCAM or cytokeratins. Later in 2011, leukapheresis and fluorescence-activated cell sorting (FACS) elutriation were also reported to be effective for large volume blood process for CTC isolation with molecular analysis [211]. Recently, many microscale on-chip sorting systems were developed considering the high purity of isolation for CTC culture or tumor related genetic analysis. In 2014, Kim et al. have postulated an on-chip multi-imaging flow cytometry system to obtain morphometric parameters of cell clusters such as cell number, perimeter, total cross-sectional area, number of nuclei, and size of clusters as “imaging biomarkers,” with simultaneous acquisition and analysis of both bright-field and fluorescent images at 200 frames per second [212]. Moreover, laser scanning cytometry is also a novel innovation developed to help identify CTCs [213, 214]. These methods, are mainly based on flow cytometry and sorting techniques, which could possibly yield an extremely high purity of CTCs (more than 80%). However, one of the drawbacks of the cytometric systems is operator-dependent and multiple quality and internal controls are often required when setting a criterion of CTC identification. Another downside of conventional sorting systems is cell damage and decreased viability after sorting process.

Recently, by the advances of nanotechnology, nanoplates [215], nanowires [216], for positively trapping of CTCs are becoming hot devices with theoretically higher sensitive capturing efficiency than conventional ones. These techniques are often developed by biomedical engineers who are good at medicine, biology and engineering; however, the devices seem to

be still in proof-on-concept phase. Hopefully, these new devices would facilitate the development of easy hands-on CTC testing and validation in clinical trials in the near future. In brief, positive selection methods hold the greatest clinical application to date.

2.2.2. Negative selection methods

Negative selection methods are developed on the basis of the disadvantages of positive selection methods-losing non-EpCAM or CK-expressing CTCs and relatively poor recovery rate. The principle of negative selection strategy is to remove all the cells other than CTCs as its first step. Owing to the sequence of isolation has changed, in the negative depletion processing, the cancer information was preserved as possible, which makes the phenomenon that the number of CTCs isolated by a negative method would generally (but not always) larger than those by a positive one. In addition, by the CD45 depletion procedures, CTCs without expression of epithelial markers could be isolated though further clarification of the clinical significance of these cell populations is required [217]. The nature of the isolation strategy increased the sensitivity, recovery rate of CTCs but decrease the specificity with inevitable “background noises.”

In the developing history of the negative selection methods, Naume et al. [29, 218] have proposed to use CD45 (a common antigen of leukocytes) coated beads to remove white blood cells from tumor cells and red blood cells depleted by lysis buffer or density separation processing for CTC isolation. Based on the concepts, Balasubramanian et al. [219] also successfully demonstrated positive staining images for cytokeratin-positive CTC identification after negative selection processes in 32 cancer blood samples. Among all the negative selection systems, one of the representative systems is epithelial immunospot (EPISPOT) [220–224]. By the procedures, CTCs in the blood sample are enriched by anti-CD45 immunomagnetic beads. The isolated CTCs are then cultured in tissue culture plates precoated with antibodies which capture cathepsin D, MUC1, or CK19 protein [225]. After the incubation period, cells are washed out and the released protein spots are detected by the incubation with a fluorochrome-conjugated antibody and counted. Each spot corresponds to one viable CTC. The device focused on the expression of CK19-expressing cells, which were found to be detectable in up to 65 and 70% of colorectal cancer and breast cancer patients, respectively, and correlated with status of metastasis and poor survival in breast cancer [226, 227]. However, a single sample processing in EPISPOT system requires three days for analysis, which prohibits of its clinical use considering the time-consuming problem.

Nevertheless, the background cells in a negatively isolated sample are often mixed with numerous white blood cells and red blood cells if the process is not well-performed, thus prohibit the following molecular analyses. For this disadvantages of conventional negative selection method, a better depletion process for red and white blood cells depletion are warranted. Another device developed by Wu et al. [228], CanPatrol™ CTC enrichment, they reported a recovery rate more than 80%. Interestingly, FISH assay could be successfully performed for ALK gene rearrangement from CTC samples; however, further validation in prospective clinical trials was still required.

Similarly, Lin et al. [229–231] postulated a protocol and a device (PowerMag) to perform red blood cell lysis and immunomagnetic beads conjugation for CD45-positive cells and identify EpCAM-positive cells (defined as CTCs) from the blood samples. The protocol was proven to effectively isolate CTCs from patients with colorectal, head and neck cancer and thyroid cancer. Furthermore, the CTCs isolated by this negative selection method are further proven to be alive and are capable of being cultivated for at least several weeks [229].

2.3. Other methodologies

In addition to pure positive and negative or label-free methods, some investigators proposed their prototypes for CTC isolation. For example, Qin et al. [50] performed CTC isolation by the size and deformability based separation from castrate resistant prostate cancer patients using resettable cell traps. Compared with CellSearch™, the method could capture more than 10 times of CTCs for subsequent analyses. Basically, it is a label-free method and could be rapidly processed.

Synchrotron X-ray microimaging techniques, high-resolution images of individual flowing tumor cells, and nanotechnology were also proposed to help identification of CTCs. Positively charged gold nanoparticles (AuNPs) which were inappropriate for incorporation into human red blood cells were selectively incorporated into tumor cells to enhance the image contrast, which was reported by Jung et al. [232]. This new technology for in vivo imaging of CTCs would contribute to improve cancer diagnosis and cancer therapy prognosis. Moreover, new chemical materials using a refined carbon-coated pure iron-based immunomagnetic nanoparticle-enriched assay, and nested-RT-PCR was also reported to successfully isolate CTCs efficiently.

Furthermore, not only for general population of CTCs, Hosseini et al. [233] postulated an integrated nano-electromechanical chip (NELMEC) to isolate CTCs and CTCs with EMT features from white blood cells. These new technologies hold great promising on automation, which might greatly ameliorate current problems in CTC field.

2.3.1. Comparison of different strategies

In comparison between positive selection strategy and negative selection methods, the former is most commonly used CTC isolation platform and widely validated by prospective clinical trials. Several articles of meta-analysis confirmed the clinical impacts of CTCs obtained by CellSearch™ [107, 234–237]. However, it is relatively costly and device-dependent. Interestingly, some investigators compared the ISET and CellSearch™ systems for their performance on CTC isolation [76, 93] and one team concluded that a combination of ISET plus CellSearch™ would have better performance in CTC detection in NCSCL patients than ISET or CellSearch™ alone [93]. That intriguing conclusion supports the combination method in the following eras; however, a long processing time of combined platforms also causes cell damage or loss which is a problem the combined systems should be noticed.

In comparison between positive selection method and label-free strategy, Konigsberg et al. [238] compared the efficiency of CTC isolation of MACS (positive selection system) with

OncoQuick (label-free system) and found EpCAM-negative CTCs cannot be detected by EpCAM-dependent enrichment methods. EpCAM-independent enrichment technologies seem to be superior to detect the entire CTC population.

Enrichment strategy	System	Detection markers	Pros	Cons
Positive selection	CellSearch	EpCAM, CKs, CD45, DAPI	FDA cleared; reliable; reproducible; visual identification; clinical relevance in metastatic breast, colorectal and prostate cancer; semi-automated processing; capable of detecting smaller CTCs; standardized kits	EpCAM-positivity dependent, expensive; cells losing EpCAM could not be detected; limited number of markers
	CTC chip	EpCAM, CKs, CD45, DAPI	High detection rate; visual identification	EpCAM-positivity dependent, cells losing EpCAM could not be detected; require clinical trial validation
	Ariol system	EpCAM, CKs, CD45, DAPI	High detection rate (versus CellSearch)	EpCAM-positivity dependent
	Laser-scanning cytometer		Automated microscopic procedure; high detection rate	EpCAM-positivity dependent
	Adna test	EpCAM, MUC1, mucin-1, HER2	High sensitivity; rapid processing	No morphology confirmation; EpCAM and MUC1-positivity dependent
Negative selection	EPISPOT assay	CD45, CK19, mucin-1, cathepsin-D	Can detect viable CTCs	Lack of enough clinical trials for validation
	PowerMag	CD45 depletion for 4 repeated times, EpCAM, Hoechst	Clinically validated in several cancer types; viable CTCs	Background noise, subjective judgment of CTCs, labor-intensive; limited markers can be used for a sample
	Negative + flow cytometry, FACS	CD45, EpCAM, CKs, CD133, CD44, Syto62	High sensitivity for multiple markers, high purity of isolation	Controls, cell aggregations, laser compensation, operator-dependent
Label-free (size)	CTC-filtering devices;	Size, CKs, Her2/neu, ALDH1, CD44, CD24	Rapid processing; multiplexed imaging and genetic analysis	Limited by size of CTCs variation
	ISSET	Size, CKs, EGFR, VE-cadherin, ki67	Rapid processing; non-antigen dependent; able to isolate CTM; cell illustrated by IHC staining, able to	Size-dependent (may miss cells less than 8 μ m); require more clinical validation trials; manual processing

Enrichment strategy	System	Detection markers	Pros	Cons
			perform FISH, DNA/RNA analysis	
	DEP force	Size, surface electricity, viability	Rapid processing, can isolate single cell very precisely	Low throughput; time-consuming
	ODEP force	Size, surface electricity, viability	Rapid processing, can isolate single cell very precisely; can differ viable from dead cells	Relatively low throughput
	Tracheal carina-inspired bifurcated (TRAB) microfilter system	Size	High recovery rate, acceptable purity; viable isolation	Require clinical trial validation
Label-free (gradient)	Ficoll + RT-PCR	CK-19, HER2, h-MAM, CEA, maspin, GABA A, B726P	High sensitivity	No morphology confirmation; not really capture CTCs
	Ficoll + RT-qPCR	CK-19, BST1, PTPRC	High sensitivity; quantification	No morphology confirmation; not really capture CTCs
	OncoQuick	CCNE2, DKFZp762E1312, EMP2	High sensitivity; quantification	No morphology confirmation; not really capture CTCs
		MAL2, PPIC and SLC6A8, hMAM, and EpCAM	High sensitivity; quantification	No morphology confirmation; not really capture CTCs

Table 1. Overview of analytical methodologies for the detection and molecular characterization of CTCs.

Another report addressed the differences between positive and label-free method was reported by Qin et al. [50]. They designed a micropore filtration platform (using resettable cell traps) to perform CTC isolation by the characteristic of CTCs (size and deformability) from patients with castrate resistant prostate cancer. Compared with CellSearch™, the method could capture CTCs 10 times more than CellSearch™ can achieve. The method was also proven to be able to perform subsequent molecular analyses.

Interestingly, some investigators compared the isolation efficiency of ISET and CellSearch™ systems [76, 93] and one team concluded that a combination of ISET plus CellSearch™ would have better performance in CTC detection in NCSCL patients than ISET or CellSearch™ alone [93].

One question which is often and needed to be asked is that how to choose a best platform for upcoming studies and trials. Before answering the question, the readers/investigators should fully understand the differences, pros and cons among these methods. Then you should choose

a platform wisely according to future directions of investigations (i.e., clinical trial, CTC culture, patient-derived xenograft model from CTCs) (i.e., genetic analysis, single cell, physical properties) and the requirements of the study materials (i.e., cells with high purity, with high cell numbers, viability, expression with specific marker, etc.). **Table 1** demonstrated the brief comparison among novel platforms. In our opinion, for genetic analysis and future personalized medicine, we need a large number of CTCs captured for cultivate, whole genome or transcriptome sequencing and avoid sampling errors by hyper-selection of few cells to represent the whole populations of cancer. Therefore, negative selection as first step is currently most suitable strategy among all the methods.

3. The main elements in negative selection plus microfluidic CTCs isolation

3.1. Immunomagnetic beads-based methods

The method is in fact derived from conventional cytological diagnostics for bone marrow and hematologic malignancies. However, when investigators attempted to apply this method to CTC filed, they faced a big problem—the CTCs were so rare to identify in thousands of blood smear slides. Therefore, an alternative method was to exam samples after series of centrifugation, density separation (i.e., in buffy coat or peripheral blood mononuclear cells, PBMC layer), and red blood cells removal. The vast majority of the following detection techniques of CTCs in these prepared samples has long been based on sensitive immunocytochemical (ICC) analysis using antibodies against different epithelial antigens [29, 31, 239–242]. Whether positive selection or negative selection procedures using immunomagnetic beads before ICC analysis are both helpful and critical for efficient CTC identification [29, 31]. Zigeuner et al. [31] found that immunomagnetic cell enrichment significantly improves the sensitivity of detection of CTCs cells added to mononuclear cells compared to immunocytochemistry method.

Although the exact procedures of immunomagnetic beads separation protocol was variable with the beads and antibiotics but they have general principles and we would take the procedures of Dynabeads as an example (modified from Naume et al.'s work in 1997 [29]). The main procedures are preparation of beads, incubation with samples and beads, using a magnetic field or column (depends on chosen systems) for target cells isolation by washing out other cells which did not conjugated with beads. If the target cells are those we do not want to analyze, the procedure is defined to be a negative selection. Conversely, if the cells are the targets in the study, it is a positive selection.

3.1.1. Preparation of the magnetic beads

Rat antimouse (RAM) IgG1-coated M280 Dynabeads coupled to BerEP4 mAb (Product No. 112.07), M450 Dynabeads coated with an anti-CD45 mAb that recognizes all isoforms of CD45 (Product No. 111.19), and Neodymium Magnetic Particle Concentrators were supplied by Dynal (Oslo, Norway). Coating of the M280 Dynabeads with antiepithelial mAb was per-

formed according to the manufacturer's instructions. Briefly, the RAM M280 Dynabeads were incubated with either BerEP4, 9189, or MOC31 mAbs at a concentration of 1/ μ g/10⁷ beads for 30 min at 4°C under gentle rotation, followed by three magnet washes in PBS/0.1% HSA and then stored at 4°C. Before use, the Dynabeads were washed once with separation medium.

3.1.2. Positive immunomagnetic separation technique

A total of 1×10^7 peripheral blood mononuclear cells (PBMNC) were resuspended in cold separation medium to a concentration of 2×10^7 MNC/ml (0.5 ml volume) and incubated with RAM IgG1 M280 Dynabeads coated with either BerEP4 317G5, or MOC31 mAb. The bead concentration varied from 2.5 to 40×10^6 b/ml, as described in individual experiments. The bead/cell suspension was incubated under gentle rotation for 30 min at 4°C. The sample was then diluted to 3 ml and placed against a magnet for 7 min to recover the rosetted cells, followed by two additional washes as follows. The supernatant was removed, and the rosetted cells were resuspended in 3 ml separation medium, followed by treatment with the magnet (7 min). To facilitate ICC TC detection, the positive LMS product was finally resuspended to contain $5\text{--}7 \times 10^6$ beads/ml, and 0.5 ml aliquots were centrifuged onto each cytopsin slide for further immunocytochemical analysis.

3.1.3. Negative immunomagnetic separation technique

A total of 1×10^7 peripheral blood mononuclear cells (PBMNC) were resuspended in cold separation medium to a concentration of 2×10^7 MNC/ml (0.5 ml volume) and incubated with anti-CD45-conjugated M450 Dynabeads at a bead/cell ratio of 2.5:1, 5:1, or 10:1. The bead/cell suspension was incubated under gentle rotation for 45 min. The solution was then diluted to about 30 ml, and the magnet was applied for 5 min, with initial rotation of the tubes onto the magnet to reduce trapping of tumor cells. The supernatant was collected and centrifuged at 450g for 10 min, counted, and resuspended in 10% FBS in PBS to 1×10^6 cells/ml. Then, cytopsin containing 5×10^5 cells were prepared. All the slides were air-dried overnight and stained by immunocytochemistry.

3.2. Microfluidic-based methods for the high purity CTC isolation

In novel era of huge advances of microfluidic devices as mentioned in the section of "Label-free isolation strategy" [45, 46, 56, 60, 173–186]. In fact, the vast majority of microfluidic devices were designed based on EpCAM- or CK-identifying mechanism, which is positive selection method. The CTC-Chip [25, 243], and the herringbone chip [244, 245] have been proven effective to isolate CTCs with both high CTC purity (50–62%) [25, 245] and high recovery rate (90–95%) [244, 245]. There are several microfluidic devices designed to use positive selection strategy for proof-of-concept purpose [34, 57, 59, 124, 184, 210, 246–269] and for specific cancer in clinical trials, (e.g., breast [270, 271], pancreas [272, 273], ovarian [274], prostate [275], esophageal cancer [270], gastric [271], colorectal cancer [276], cancer of unknown primary [277]) and for mutational analysis [278]. Moreover, combined preparation using positive, negative, or label-free selection methods with microfluidic devices for better performance is also feasible and have been reported [93].

However, there are several drawbacks or limitations of microfluidic devices reported [267]. First of all, reports in literature, however, have revealed that EpCAM or CKs are not expressed in all cancer cells (e.g., sarcoma, melanoma, or CTCs bearing EMT), and therefore some kinds of CTCs cannot be targeted via the positive selection-based microfluidic device [279]. Secondly, several microfluidic chips could identify with microtubes or micropoles with or without EpCAM conjugation. It seems to be difficult to release captured CTCs from the chips. The efficiency of identification will not be equal to recovered cells for further molecular or genetic analysis. Thirdly, almost more than 80% of microfluidic devices are still in proof-of-concepts phase and comes from a single team or laboratory. It might be because that the advances of new innovation always come up faster than validation reports. However, we do need well-designed and well-conducted prospective clinical studies to critically elucidate the clinical impacts of the microfluidic devices. The investigators could consider to learn from the developing history of CellSearch™ system.

3.3. Perspective for future of CTC technology: combinations of several methods

Our perspectives for future CTC isolation is mainly combined methodologies instead of conventional ones based on a single isolation strategy.

Yamamoto et al. [49] displayed a combination of size-based filtration plus a magnetic column method for CTC isolation. The combined use of the column and filter decreased the required time for the spiked cancer cell capture, and the recovery rate of the spiked cancer cells from blood was significantly higher using the combination process (80.7%) than that using the filter alone (64.7%). Moreover, the recovered CTCs are more abundant by the combination process. Another combination was ISET and CellSearch™ systems [76, 93] and the combination had better performance in CTC detection in non-small cell lung cancer (NSCLC) patients than ISET or CellSearch™ alone [93]. Furthermore, density separation plus flow cytometry or cell sorting systems have been postulated to be potential tools of CTC isolation and identification considering their high sensitivity and purity since 1998 [27]. This method could be seen as a combination of negative selection strategy and a positive confirmation with surface markers, such as EpCAM or cytokeratins. Later in 2011, leukapheresis plus fluorescence-activated cell sorting (FACS) elutriation were also reported to be effective for large volume blood process for CTC isolation with molecular analysis [211]. These studies illustrated the possibility and better efficacy the combination can achieve, therefore, in our opinion, to find a suitable combination of CTC isolation protocols considering the balance of efficiency, time, sample and costs is very important in the future CTC field.

Intriguingly, several liquid biopsies, as aforementioned, could be combined to be tested in a single sample and at the same time. To realize the goals and minimize the blood sample required, Chudziak et al. [248] reported a novel device, Parsortix system, could negatively select CTCs and perform cfDNA analysis simultaneously. The system recovered more CTCs than CellSearch™ system in the comparison.

4. The applications of CTC testing in clinical cancer researches

As aforementioned, CTC testing are designed to help the diagnosis, early detection and monitoring for response and disease status of cancer patients. Clinical trials to evaluate and validate are inevitable during the developing of any CTC testing. Here, we introduce several important clinical validated studies for the clinical impacts of CTC testing in different cancer types.

4.1. Breast cancer

One meta-analysis reported by Liao et al. [107], 14 studies with 2336 patients were enrolled and found that presence of CTCs in peripheral blood was significantly associated with the size of tumor [OR 0.68, 95% confidence interval (CI) (0.54, 0.87), $P = 0.002$], tumor grade [OR 0.71, 95% CI (0.55, 0.91), $P = 0.006$], estrogen receptor (ER) status [OR 0.72, 95% CI (0.57, 0.91), $P = 0.007$], and progesterone receptor (PR) of tumor status [OR 0.78, 95% CI (0.61, 0.98), $P = 0.04$]. In addition, the presence of CTCs is highly correlated with tumor size, tumor grade, ER, and PR status in patients with breast cancer. Although the analysis did not consider the method of isolation which might be one of the downsides and biases of the analysis, the results suggested a trend of physical (tumor size), functional (tumor grade) and status of drugable targets (ER, PR status), which are very useful clinically.

In Zhao et al. [234] performed a meta-analysis collecting 24 trials with 4013 breast cancer patients and 1333 controls. Poor overall survival was found to be associated with the positive CTC detection (HR = 3.00 [95% CI 2.29–3.94], $P < 0.0001$) and recurrence-free survival as well (HR = 2.67 [95% CI 2.09–3.42], $P < 0.0001$). CTC-positive breast cancers were significantly associated with high histological grade (HR = 1.21 [95% CI 1.09–1.35], $P < 0.0001$), tumor size (>2 cm) (HR = 1.12 [95% CI 1.02–1.22], $P = 0.01$), and nodal status (≥ 1) (HR = 1.10 [95% CI 1.00–1.21], $P = 0.037$). The studies, different to that of Liao et al. [107], mentioned about prognostic values of CTC testing. However, the two reports did not mention about the isolation methods and might neglect the biases from CTC number is highly correlated to the method of isolation.

For the purpose of technical standardization, Janni et al. [235] conducted a pooled analysis of individual data from 3173 patients with nonmetastatic (stages I–III) breast cancer from five breast cancer institutions. The prevalence and numbers of CTCs were assessed at the time of primary diagnosis with the FDA-cleared CellSearch System. Results confirmed that ≥ 1 CTC(s) were detected in 20.2% of the patients and CTC-positive patients had larger tumors, increased lymph node involvement, and a higher histologic tumor grade than did CTC-negative patients (all $P < 0.002$). Multivariate Cox regressions confirmed that the presence of CTCs was an independent prognostic factor for disease-free survival [HR, 1.82; 95% confidence interval (CI), 1.47–2.26], distant disease-free survival (HR, 1.89; 95% CI, 1.49–2.40), breast cancer-specific survival (HR, 2.04; 95% CI, 1.52–2.75), and overall survival (HR, 1.97; 95% CI, 1.51–2.59). The study addressed the clinical impacts of CellSearch™ system in breast cancer patients and it has confirmed the positive results from a large pooled database.

For a subset in breast cancers, Rack et al. [91] addressed the role of CTCs isolated by CellSearch™ in a prospective trial enrolling 2026 early average-to-high risk breast cancer patients and found an independent prognostic relevance of CTCs both before and after adjuvant chemotherapy. The study successfully proved the prognostic role of CTCs in adjuvant settings. The next direction of future studies should be designed to answer the question that whether if extended adjuvant therapy is needed and whether if the extended therapy did reduce the risk of recurrence or not.

For the role of CTCs in a novel and specific therapy in breast cancer, Paoletti et al. [280] reported that heterogeneous mechanisms of resistance to fulvestrant, including estrogen receptor alpha gene (ESR1) mutation. CTC enumeration, phenotyping, and genotyping might identify patients who would benefit from fulvestrant dose escalation versus switching to alternative therapies. The CTCs could possibly help find the resistance genes during the therapy and warn the clinicians to change therapy in time before the tumor already gets progression.

In triple negative breast cancer (TNBC) who lacks of drugable targets (hormone therapy) in breast cancer, Hall et al. (2015) enrolled 44 TNBC patients using CellSearch™ for CTC testing and found that ≥ 1 CTC in each sample was identified in 30% of patients completing neoadjuvant chemotherapy (NACT). Multivariate analysis demonstrated that detection of ≥ 1 CTC predicted decreased RFS (log-rank $P = 0.03$, HR 5.25, 95% CI 1.34–20.56) and OS (log-rank $P = 0.03$, HR 7.04, 95% CI 1.26–39.35). The results suggested a modification of clinical management for TNBC patients with positive CTC detection after NACT, including extension of NACT or adding another anti-cancer therapy before tumor recurs.

4.2. Lung cancer

In a meta-analysis reported in 2013, pooled results from a total of 20 studies, comprising 1576 nonsmall cell lung cancer (NSCLC) patients showed that CTCs were associated with lymph node metastasis (OR = 2.06; 95% CI: 1.18–3.62; $Z = 2.20$; $P = 0.027$) and tumor stage (OR = 1.95; 95% CI: 1.08–3.54; $Z = 2.53$; $P = 0.011$). CTCs were significantly associated with shorter overall survival (relative risk [RR] = 2.19; 95% CI: 1.53–3.12; $Z = 4.32$; $P < 0.0001$) and progression-free/disease-free survival (RR = 2.14; 95% CI: 1.36–3.38; $Z = 3.28$; $P < 0.0001$) [281]. Another study reported the ability to recurrence prediction after curative surgery is positive [282].

For small cell lung cancer, a relatively aggressive subtype with poor prognosis population, a total of seven papers covering 440 SCLC patients were combined in the final analysis. The meta-analysis revealed that CTCs were significantly associated with shorter overall survival (HR = 1.9; 95% CI: 1.19–3.04; $Z = 2.67$; $P < 0.0001$) and progression-free survival (HR = 2.6; 95% CI: 1.9–3.54; $Z = 6.04$; $P < 0.0001$) [283].

Interestingly, in a molecular era nowadays, cancer therapy often relies on genetic or molecular information from cancer tissues, CTCs as well. Das et al. [105] checked the status of ERCC1 expression on captured and found that low expression of ERCC1 on CTCs correlates with progression-free survival (PFS) in patients with metastatic NSCLC receiving platinum-based therapy. ERCC1 expression was conventionally checked on NSCLC cancer tissue to predict the response to platinum therapy, which has been the first line standard chemotherapy in

patients without active EGFR mutation responding to tyrosine kinase inhibitors (TKIs). The impacts of the study suggested that analysis of ERCC1 expression on CTCs in lung cancer patients could predict the chemotherapy responses. It is a predictive role could possible direct therapy in the future if the findings were confirmed in another large-scale phase III clinical trials. In addition, Yanagita et al. [284] evaluated CTCs and cfDNA in EGFR-mutant NSCLC patients treated with erlotinib until progression. Among the enrolled 60 patients, rebiopsy was performed in 35/44 patients (80%), with paired CTC/cfDNA analysis in 41/44 samples at baseline and 36/44 samples at progression. T790M was identified in 23/35 (66%) of tissue biopsies and 9/39 (23%) of cfDNA samples. At diagnosis, high levels of cfDNA but not high levels of CTCs correlated with progression-free survival. Therefore, cfDNA and CTCs are complementary, noninvasive assays for evaluation of acquired resistance to first-line EGFR TKIs. Recently, *ALK* rearrangement on CTCs are successfully performed and compared with cancer tissues [51, 285]. Chromosome instability and *ROS-1* rearrangement on CTCs were also proved to be successful [51]. Immune cells analysis, tumor-associated macrophages (TAMs) accompanied with CTCs analysis were also proven to be possible and CTCs are competent to specifically manipulate TAMs to increase cancer invasiveness, angiogenesis, immunosuppression and possibly lipid catabolism in lung cancer patients [286]. These studies pointed to the driven mutation detection and would directly benefit to NSCLC patients under targeted therapies.

4.3. Gastrointestinal tract cancer

In 2014, a meta-analysis comprised 26 studies with peripheral blood samples of 1950 cases for final analysis. The pooled results showed that gastric cancer (GC) patients with detectable CTCs (including circulating miRNAs) had a tendency to experience shortened RFS (HR = 2.91, 95% CI [1.84–4.61], I² = 52.18%). As for patient deaths, we found a similar association of CTC (including circulating miRNAs) presence with worse OS (HR = 1.78, 95% CI [1.49–2.12], I² = 30.71%, *n* = 30). Additionally, subgroup analyses indicated strong prognostic powers of CTCs, irrespective of geographical, methodological, detection time and sample size differences of the studies [287]. In addition, the role of EMT status on CTCs correlates with poor treatment outcomes was also revealed. CTCs expressing CD44 were also found to be prognostic and indicated to malignant behaviors of gastric cancer [288].

For pancreatic cancer, a prospective study addressing the role of CTCs, CTMs in 63 pancreatic ductal adenocarcinoma (PDAC) patients before treatment using anti-EpCAM (epithelial cell adhesion molecule)-conjugated supported lipid bilayer-coated microfluidic chips. CTM was an independent prognostic factor of overall survival (OS) and progression free survival (PFS). Patients were stratified into unfavorable and favorable CTM groups on the basis of CTM more or less than 30 per 2 ml blood, respectively. Patients with baseline unfavorable CTM, compared with patients with favorable CTM, had shorter PFS (2.7 versus 12.1 months; *P* < 0.0001) and OS (6.4 versus 19.8 months; *P* < 0.0001). Differences persisted if we stratified patients into early and advanced diseases. The number of CTM before treatment was an independent predictor of PFS and OS after adjustment for clinically significant factors. Therefore, in conclusion, the

number of CTM, instead of CTCs, before treatment is an independent predictor of PFS and OS in patients with PDAC [272].

In molecular analysis to predict treatment response, Abdallah et al. [152] found that thymidylate synthase expression in circulating tumor cells can be useful tool as a 5-FU resistance predictor biomarker in patients with colorectal cancer while other studies elucidate the prognostic and predictive roles of CTCs [198, 289–291]. Recently, *KRAS* and *BRAF* were successfully detected on CTCs by high-resolution melt (HRM) and allele-specific PCR (ASPCR) and *KRAS*-codon 12/13- and *BRAF*-codon 600-specific assays. Comparing tumor tissues and CTCs mutation status using HRM, Mohamed Suhaimi et al. [292] reported that a 84.1% concordance in *KRAS* genotype ($P = 0.000129$) and a 90.9% ($P = 0.174$) concordance in *BRAF* genotype. Another report utilized ISET system plus PCR for *KRAS* codons 12 and 13 mutation with a 71% concordance between cancer tissue and CTCs from colorectal cancer patients [293]. In gastrointestinal stroma tumor, Li et al. [294] conducted a trial to elucidate the role of CTCs expressing ANO1(DOG1) in GIST. ANO1s were more frequently detected in unresectable patients. Tumor size, mitotic count, and risk level were associated with ANO1 detection in resectable GIST patients. The presence of ANO1 significantly correlated with poor disease-free survival (15.3 versus 19.6 months, $P = 0.038$). Most patients turned ANO1-negative after surgery and inversely, all 21 patients with recurrence turned ANO1-positive with high ANO1 expression levels. Moreover, in the neoadjuvant setting, decline of ANO1 expression level correlated with the response of imatinib. In the near future, these results would possibly promote the genetic analysis on mutation-driven cancer therapies although they have not become routine screen tools in CRC patients to date.

4.4. Head and neck cancer

Grobe et al. [92] used CellSearch™ for CTC isolation in 80 oral cavity cancer patients and found that 12.5% patients harbored CTCs in peripheral blood, whereas in 20.0% patients DTCs in bone marrow could be detected. Significant correlations could be found for CTCs and tumor size ($P = 0.04$), nodal status and DTCs ($P = 0.02$), and distant metastasis with CTCs ($P = 0.004$) and DTCs ($P = 0.005$). Univariate and multivariate analyses revealed that CTCs and DTCs were significant and independent predictors of recurrence-free survival ($P < 0.001$) as well as in other findings in HNSCC, including the ability of prediction 6-month death [231]. In 2015, Oliveira-Costa et al. reported that immunohistochemistry was performed in cancer tissues and in CTCs by immunofluorescence and Nanostring. Correlation was shown between PD-L1 and tumor size and lymph node metastasis, HOXB9 and tumor size, BLNK and perineural invasion, and between ZNF813 and perineural invasion. PD-L1 positivity was an independent prognostic factor in this cohort ($P = 0.044$, HH = 0.426) in OSCC patients [295]. The results could possibly apply to current immune-oncology studies.

Wu et al. [296] reported a meta-analysis conducted a computerized retrieval of literatures. Twenty-two retrieved studies were eligible for systematic review, of which nine conformed for the diagnostic test meta-analysis and five for the prognostic analysis. Subgroup analysis showed 24.6% pooled sensitivity and 100% pooled specificity of detections by using positive selection strategy, which moreover presented low heterogeneity. The presence of CTC was

significantly associated with shorter disease free survival (DFS, HR 4.62, 95% CI 2.51–8.52). The presence of CTC indicates a worse DFS.

4.5. Liver cancer

Early in 2004, Vona et al. have reported that the presence ($P = 0.01$) and number ($P = 0.02$) of CTCs and microemboli (CTMs) were significantly associated with a shorter survival [161]. Fan et al. [297] reported a meta-analysis consisting of 23 trials and found that CTC positivity was significantly associated with RFS (HR 3.03, 95% CI: [1.89–4.86]; $P < 0.00001$) and overall survival (OS) (HR 2.45, 95% CI: [1.73–3.48]; $P < 0.00001$). CTC positivity were also significantly associated with TNM Stage (RR 1.30, 95% CI: [1.02–1.65]; $P = 0.03$), Tumor size (RR 1.36, 95% CI: [1.09–1.69]; $P = 0.006$), Vascular invasion (RR 1.99, 95% CI: [1.43–2.77]; $P < 0.0001$), Portal vein tumor thrombus (RR 1.73, 95% CI: [1.42–2.11]; $P = 0.0001$), Serum alpha-fetoprotein (AFP) level (RR 2.05; $P = 0.01$) [297]. Sun et al. found that Stem cell-like phenotypes are observed in EpCAM⁺ CTCs, and a preoperative CTCs of ≥ 2 is a novel predictor for tumor recurrence in hepatocellular carcinoma (HCC) patients after surgery, especially in patient subgroups with AFP levels of ≤ 400 ng/ml or low tumor recurrence risk. EpCAM⁺ CTCs could serve as a real-time parameter for monitoring treatment response and a therapeutic target in HCC recurrence [137]. The prognostic value of overall survival of CTCs in HCC patients has been also revealed [298].

4.6. Genitourinary tract cancer

Rink et al. (2012) found that using CellSearch™, CTC were detected in 23 of 100 patients (23%) with nonmetastatic urothelial carcinoma of urinary bladder. CTC-positive patients had significantly higher risks of disease recurrence and cancer-specific and overall mortality (P values ≤ 0.001). After adjusting for effects of standard clinicopathologic features, CTC positivity remained an independent predictor for all end points (hazard ratios: 4.6, 5.2, and 3.5, respectively; P values ≤ 0.003). HER2 positivity was found in 3 of 22 patients (14%). There was concordance between CTC, primary tumors, and lymph node metastases in all CTC-positive cases (100%).

4.7. Skin cancer and melanoma

Conventionally, melanoma cells lack of cytokeratin or EpCAM expression and CTCs by definition are very difficult to identify. However, investigators broke through the strait by combination with CTCs plus cfDNA. Salvianti et al. [299] enrolled 84 melanoma patients and 68 healthy controls for CTC and cell-free DNA (cfDNA) testing to assess the diagnostic performance of a tumor-related methylated cfDNA marker in melanoma patients and to compare this parameter with the presence of CTCs. The percentage of cases with methylated RASSF1A promoter in cfDNA was significantly higher in each class of melanoma patients (in situ, invasive and metastatic) than in healthy subjects ($P < 0.001$). The concentration of RASSF1A methylated cfDNA in the subjects with a detectable quantity of methylated alleles was significantly higher in melanoma patients than in controls. When the CTCs plus RASSF1A cfDNA are jointly considered, a higher sensitivity of the detection of positive cases in invasive

and metastatic melanomas could be obtained. A similar finding was obtained to suggest combine cfDNA (GNAQ/GNA11 mutations) and CTCs to identify uveal melanoma patients with poor prognosis [300]. In another reports, a phase III trial of adjuvant immunotherapy after complete resection of stage IV melanoma, quantitative real-time reverse-transcriptase polymerase chain reaction (qPCR) for expression of CTC-specific MART-1, MAGE-A3, and PAX3 mRNA biomarkers were found to be not associated with known prognostic factors or treatment arm. In multivariate analysis, pretreatment CTC (>0 versus 0 biomarker) status was significantly associated with disease-free survival (DFS; HR 1.64, $P=0.002$) and overall survival (OS; HR 1.53, $P=0.028$). Serial CTC (>0 versus 0 biomarker) status was also significantly associated with DFS (HR 1.91, $P=0.02$) and OS (HR 2.57, $P=0.012$) [301]. The report suggested CTCT could be a new risk factor other than any conventional known factors, which might change the staging systems if the evidence gets solid and validated.

4.8. Other cancers

For ovarian cancer, Romero-Laorden et al. [302] performed a meta-analysis enrolling 14 studies. Results showed the presence of CTCs and DTCs is associated with adverse clinicopathological characteristics and poor clinical outcomes in ovarian cancer patients. They noticed that different CTC number obtained by different devices could not be compared. Using size-based isolation strategy (MetaCell®) in 118 ovarian cancer patients, CTCs might have add-on values on current staging system and the cells could be cultivated after isolation [303, 304]. Furthermore, in another meta-analysis, eight studies of 1184 ovarian cancer patients were included in the final analysis. In the PB group, it showed that patients with positive CTCs had significantly shorter overall survival (OS) and disease-free survival (DFS) than patients with negative CTCs (HR, 2.09; CI, 1.13–3.88 and HR, 1.72; CI, 1.32–2.25, respectively). The same result was shown with DTCs in the BM group (HR, 1.61; CI, 1.27–2.04 and HR, 1.44; CI, 1.15–1.80, respectively) [305].

For carcinoma of unknown primary (CUP), Matthew et al. [306] used a real-time, single-cell multiplex immunophenotyping of CTCs to inform diagnosis of tissue of origin in CUP patients. CellSearch™ plus multiplexed Q-dot or DyLight conjugated antibodies were used for cytokeratin 7 (CK7), cytokeratin 20 (CK20), thyroid transcription factor 1 (TTF-1), estrogen receptor (ER), or prostate-specific antigen (PSA) expression. The feasibility of staining multiple markers in CTCs presented in this work suggested CTCs could possibly have a non-inferior role as that of cancer tissues in diagnostics.

5. Unanswered questions in the field of CTCs, technically and clinically

The unanswered question is that the optimal protocol or device has not been found or validated. Many investigators have clearly realized that the number of CTCs cannot easily compare with that counted by another system, but some are not. Recently, Fina et al. [307] compared two CTC isolation methods in a clinical trial. AdnaTest EMT-1/ and EMT-2/Stem CellSelect/Detect kits, and ScreenCell Cyto devices were both performed for all samples.

Higher CTC detection rates were obtained with the AdnaTest approach when using for CTC-enrichment antibodies against ERBB2 and EGFR in addition to MUC1 and the classical epithelial surface marker EPCAM (13% versus 48%). When the physical properties of tumor cells were exploited, CTCs were detected at higher percentages than with positive-selection-based methods. The results supported that different approaches for CTC detection probably identify distinct tumor cell subpopulations. Technical standardization before clinical validity would be the most urgent issue we have to solve.

6. Concluding remarks

We suggest the investigators to combine different isolation methods to achieve the most optimal performance of CTC isolation and clinical trials for solid validation. The cooperation among medical oncologists and biomedical engineers are critically important for the future advances in CTC field. Genetic or molecular analysis, such as PCR for epigenetics or mutation of specific gene(s) or next-generation sequencing for whole genome, whole exon, or chosen targeted genes will be the major directions for personalized cancer therapies. The advances of microfluidic devices will quickly solve the conventional problems of time-consuming, sample-consuming, operator-dependent, and marker-dependent limitations.

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