Molecular Therapy
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Review

Progress and application of circulating tumor cells in non-small cell lung cancer

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Non-small cell lung cancer (NSCLC) has the highest morbidity and mortality worldwide among malignant tumors. NSCLC is a great threat to health and well-being. Biopsy is the gold standard to diagnose lung cancer, but traditional biopsy methods cannot fully reflect the true condition of tumors. There is growing evidence that a single-point biopsy fails to reveal the complete landscape of the tumor due to intratumor heterogeneity, but it is impractical to complete multiple biopsies that are separated both spatially and temporally. Liquid biopsy heralds that a new era is coming. Circulating tumor cells (CTCs) are tumor cells that circulate in the peripheral blood after being shed from primary or metastatic tumors. CTCs constitute a considerable portion of a liquid biopsy, which contributes to the diagnosis, assessment of prognosis, and therapy of NSCLC. Herein, this review discusses the technologies for detection and enrichment of CTCs as well as clinical applications involving CTCs.

INTRODUCTION

Lung cancer remains the most common malignancy in the world and is a leading cause of tumor-related death. A recent report analyzing mortality data from 1930 to 2017 showed that almost 25% of all cancer deaths are due to lung cancer. The 5-year relative survival rate is 5% for patients diagnosed with advanced disease and 57% for those diagnosed with localized-stage disease.[1] There are many reasons that lung cancer is often diagnosed at an advanced stage, including lack of routine physical examination, lack of early diagnostic biomarkers, and lack of disease surveillance methods. Pathological results obtained by tissue biopsy are the gold standard for the diagnosis of lung cancer. Early diagnosis facilitates prompt treatment and disease monitoring. Bronchoscopy, percutaneous needle biopsy, and surgical resection are the three direct methods for retrieving tumor samples.[2] There are many limitations to tissue biopsy. For instance, during tissue biopsy, the appropriate biopsy method must be selected according to the size and location of the lesion. This becomes challenging for small lesions or poorly located lesions. Single-site biopsy cannot fully reflect the heterogeneity of tumors,[3,4] the procedure is invasive, and biopsy is unrealistic spatially and temporally. Furthermore, the financial situation of the patient must also be considered. Liquid biopsy is a noninvasive approach that has attracted attention from researchers because it allows for the retrieval of multiple samples with low risk.[5] Liquid biopsy will fundamentally facilitate the development of cancer precision medicine.

Circulating tumor cells (CTCs) are a considerable part of a liquid biopsy.[6] CTCs are tumor cells that circulate in the peripheral blood after being shed from primary or metastatic tumors.[7] A liquid biopsy involves the enumeration and characterization of CTCs to provide a clinical message about prognosis, cancer staging, drug selection, and therapeutic efficacy. In early lung cancer, preoperative CTC detection is related to disease-free survival and overall survival (OS). In advanced lung cancer patients, the presence of CTCs and the high persistence rate of CTCs are related to poor prognosis.[8] CTCs play a significant role in the occurrence of metastasis, which is the main cause of cancer-related deaths.[9] In clinical applications, detection of CTCs has many advantages. First, the positive detection rate of CTCs is higher than serum tumor markers in primary lung cancer diagnosis, and CTCs are a reproducible indicator that may provide an advantage over imaging. Furthermore, CTCs can be obtained repeatedly through simple blood samples. Additionally, when compared to traditional biopsy, liquid biopsy is expected to better reflect tumor heterogeneity because the CTCs originate from distinct tumor locations.[10]

CTCs can be divided into single CTCs and CTC clusters,[7] which generally refers to a cell cluster composed of two or more cells.[7] CTC clusters stem from oligoclonal clusters of primitive tumor cells. Compared with single cancer cells, CTC clusters constitute a rare but highly metastatic subcollection of CTCs.[11] CTC clusters have strong viability. Traditional models show that metastasis is seeded by a single cell in the primary tumor, and the aggregation efficiency of tumor cells in the blood or distant organs is very low.[12] Seeding requires the collective role of tumor cells moving along in clusters. A study by Liotta et al.[13] demonstrated that larger clumps produce more metastases than smaller clumps with matching cell numbers. This study also confirmed the importance of tumor mass in the process of metastasis.[13] Tumor cells can be transferred together as clusters. Tumor cells can be retained by cancer cell clusters, require epithelial gene expression, and can switch between epithelial differentiation states to achieve metastatic proliferation and migration components.[12] It has been shown that CTC clusters participate in

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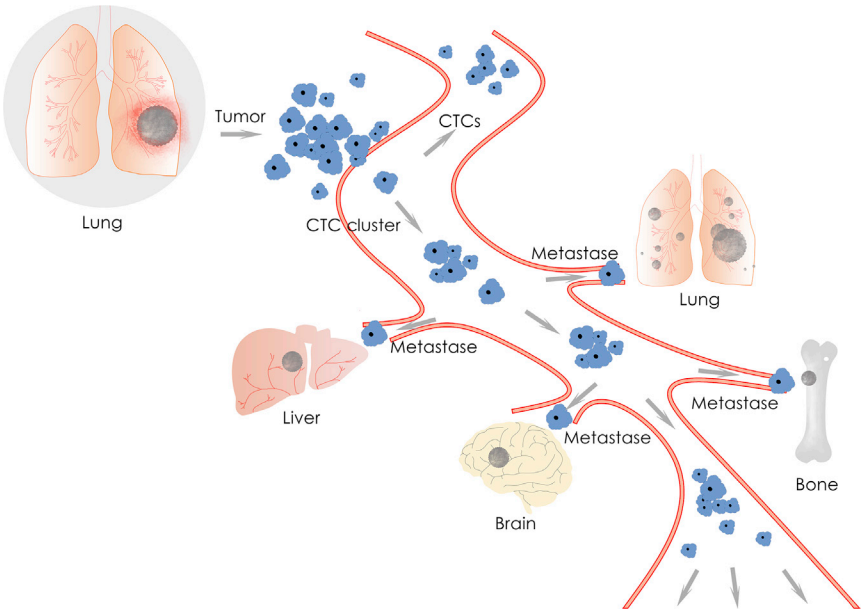

Figure 1. Generation and metastasis of CTCs and CTC clusters

CTCs are divided into single tumor cells and cell clusters. CTCs originate from the primary tumor or metastasis and can spread to nearby tissues or distant organs, such as liver, brain, and bones, with peripheral blood circulation.

epithelial-mesenchymal transition (EMT) through the large amount of TGFβ released by platelets that co-aggregate in these clusters, and obtaining EMT status may enhance chemoresistance.[14] CTC clusters exhibit aggressiveness and predict poor prognosis;[15] the tighter the cell cluster, the shorter the patient survival.[14]

Here, after a brief introduction to CTCs, we discuss the detection and enrichment technology of CTCs, focusing on the clinical application of CTCs, including early diagnosis, prognosis, and treatment (Figure 1).

TECHNOLOGIES FOR ENRICHMENT, ISOLATION, AND DETECTION OF CTCs

CTCs were first discovered by Thomas Ashworth in the 1860s.[16] Because of the rarity and heterogeneity of CTCs, research is ongoing to identify methods for enrichment and separation to improve CTC detection (Table 1). An important process of capturing CTCs is cell enrichment, which is based on both physical and biological properties. Physical properties mainly include size, electric charges, density, and deformability. Biological properties include viability, surface protein expression, and invasion capacity.[17] Biological characteristics use positive selection, targeting tumor-related biomarkers, or negative selection, removing blood cells with common white blood cell biomarkers[18] (Figure 2).

Epithelial cell adhesion molecule (EpCAM) does not exist in normal blood cells but is typically expressed by epithelial cells. Therefore, EpCAM expression can be used to detect CTCs. Although there exists an immunobead assay using anti-EpCAM antibodies to detect CTCs (called CellSearch), EpCAM-based systems for CTC enrichment have limitations. During the EMT process, EpCAM can be downregulated.[19] In addition, EpCAM does not necessarily exist in all tumor types, resulting in a decrease in the detection rate of CTCs.[18] One possible solution is the detection of CD45,[17] which is a common leukocyte antigen. The EPISPOT technique is a functional assay based on a negative enrichment (leukocyte depletion) combined with the detection of proteins secreted by CTCs.[20] In addition to direct detection methods, mRNA-based CTC detection methods use reverse-transcriptase polymerase chain reaction (RT-PCR) to detect epithelial or lung tissue-specific mRNA to infer presence of CTCs.[21] However, nonspecific products can be amplified, which may be misinterpreted as originating from tumor cells, resulting in overestimations of CTC presence.

Separation of CTCs based on cell physical characteristics is common. ISET is a representative way for separating CTCs according to cell size and physical characteristics and does not rely on cell surface biomarkers.[22] CTCs are generally larger than blood cells, and CTCs that have gone through the EMT process can be extracted according to cell size to increase detection rate. However, using a method based on cell size will reduce the detection rate for smaller cells and deformable CTCs.[20] Vortex-mediated deformable cytometry (VDC) can solve the problem of cell deformation.[23] Yagi, Negishi, Hosokawa and their colleagues[24–26] developed a microcavity array (MCA) system that detected CTCs according to differences in size and deformability between normal blood cells and tumor cells. In addition, there are also devices based on dielectrophoresis (DEP), such as the DEPArray system, which is a chip composed of various microelectrodes that can form multiple dielectrophoresis cages to enrich CTCs based on cell surface charge.[20]

Flow control chips, microdevices, and microfluidic platforms have become some of the mainstream technologies in CTC research due to their advantages of miniaturization, portability, high cost performance, single cell analysis, and online separation/detection. Researchers have developed various microfluidic chip platforms for CTC analysis,[27] including a Parsotrix method involving a cassette device that collects CTCs and selects them based on size.[17] Sawada et al.[28] developed a novel EpCAM-independent CTC enumeration system, which is integrated with a sorting system to use a microfluidics chip called Fishman-R. Fishman-R is able to detect the mutation of epidermal growth factor receptors (EGFRs) and *PIK3CA*. At the same time, Zhou et al.[29] investigated a new multi-flow microfluidic device (MFM), which can sensitively provide high purity of separation results without the need to label. MFM is used to separate tumor microemboli based on size inertial migration. CTC chips[30] are based on EpCAM-coated microposts, and the chip surface has high

Table 1. Advantages and disadvantages of CTC isolation and detection methods

Technology	Method of CTC enrichment isolation and detection	Advantages	Disadvantages	Reference
Physical property-based assays				
Dielectrophoretic field-flow fractionation (DEP-FFF)	separation by size and polarizability using membrane capacitance	process 30 million cells within 30 min with high recovery rates	requires very specific parameters, such as cell type and electric field frequency	Sharma et al.[17]
ISET	filtration based on cell size	nonepithelial cells can be isolated ISET processed larger volumes and detected higher CTC counts	CTCs can be damaged or fragmented	Tamminga et al.[22]
Dean flow fractionation	size-based selection using centrifugal force	nonepithelial cells can be isolated unprecedented throughput and separation performance		Hou et al.[114]
Parsotrix	size-based selection method	overcoming the detachment limitation ability to capture CTC clusters	CTC heterogeneity regarding size	Sharma et al.[17]
Functional assays				
EPISPOT assay	remove leukocytes via CD45 depletion	detection of only viable CTCs	antigen levels are lower or binding efficiency is reduced	Alix-Panabières et al.[115]
CAM	density gradient centrifugation and cells applied to CAM	detection of only viable CTCs, high sensitivity and specificity	requires over 12 h for isolation and may fail to isolate more heterogeneous cells	Lu et al.[116]
TelomeScan	detect CTCs via a telomerase-specific replication-selective adenovirus the virus replicates in cancer cells only and marks them with green fluorescence protein	more effectively detect EMT or EpCAM-negative tumor cells	may also detect hematopoietic stem cells for false-positive results	Togo et al.[117]
Reactive ion etching (RIE)	primarily due to focal adhesion or Fanconi anemia density	overcomes limitations of cell size heterogeneity	significant nonspecific binding of other blood cells	Sharma et al.[17]
Immunobead assays				
CellSearch	uses anti-EpCAM antibodies	FDA approved in advanced breast, prostate, and colorectal cancer	CTCs that undergo EMT are EpCAM negative and are not isolated by anti-EpCAM assays	Allard et al.[118]
MagSweeper	EpCAM-coated magnetic beads enriched using magnetic rod	live cells can be isolated	the CTCs possessing low or no EpCAM expression will be overlooked (CTCs that undergo EMT are EpCAM negative)	Talasaz et al.[119]
Microdevices and microfluidic platforms				
CTC chip	EpCAM-coated microposts and chip surface	high sensitivity and specificity, 99% success rate in identifying CTCs in the peripheral blood of metastatic pancreatic, prostate, colon, lung, and breast cancer patients	cell viability compromised	Nagrath et al.[120]
Herringbone (HB) chip	EpCAM-coated microposts and chip surface	provides more comprehensive data, primarily through its capture of CTC clusters	cell viability compromised	Stott et al.[121]
Ephesia CTC-chip	functionalized magnetic beads combined with microfluidics	rapid isolation and enumeration of CTCs and non-specific capture rates below 0.4%	lack of increasing the processing capacity of larger sample volumes	Saliba et al.[122]
CTC iChip	integrates size-based enrichment with either EpCAM-based positive enrichment or CD45-negative depletion	high throughput fast processing time more sensitive in detecting low levels of CTCs	limited to single or small 2- to 4-cell clusters, not tumor microemboli	Ozkumur et al.[123]
AdnaTest	immunomagnetic beads with MUC1-coupled and EpCAM-coupled antibodies	the variety of selection markers (antibodies) allows for the possibility of characterizing cells for multiple markers	cell lysis	Normanno et al.[20]

(*Continued on next page*)

Table 1. *Continued*

Technology	Method of CTC enrichment isolation and detection	Advantages	Disadvantages	Reference
GILUPI CellCollector	directly into the peripheral arm vein and captures CTCs with remarkable efficiency	*in vivo* collection, processing 1.5 L of blood in 30 min; increases diagnostic sensitivity of CTC isolation	only used for extraction of CTCs directly from patient's bloodstream, not for use of extraction in blood sample	Talasaz et al.[119]

CTCs, circulating tumor cells; EpCAM, epithelial cell adhesion molecule.

specificity and sensitivity. The success rate of identifying CTCs is as high as 99% in patients with different metastatic cancers. In addition to these, there are many other microdevices and microfluidic platforms to enrich and detect CTCs, such as herringbone chips and, more recently, the CTC-iChip, poly lactic-co-glycolic acid (PLGA)-nanofiber (PN)-NanoVelcro chip,[31] a parallel flow micro-aperture chip system,[32] thermoresponsive NanoVelcro CTC purification system,[33] antigen-specific lysis (ASL),[34] and GenoCTC.[35]

CTCs AS BIOMARKERS FOR EARLY DIAGNOSIS IN LUNG CANCER

The lack of clinical symptoms in early-stage lung cancer is the primary reason for its extremely low survival rate. In addition, physicians generally do not complete a biopsy in patients lacking clinical symptoms. However, early detection of lung cancer patients dramatically improves prognosis. For these reasons, exploring and researching new biomarkers for earlier diagnosis of lung cancer is increasingly important. Recent evidence shows that analyzing CTCs from blood samples can provide reliable early detection and molecular characterization of cancer.

In 2005, CTCs were first detected in the pulmonary veins of non-small cell lung cancer (NSCLC) patients by RT-PCR. Manjunath et al.[36] investigated a prospective pilot trial in which CTC clusters were detected in all NSCLC patients, but the study did not identify CTC clusters in high-risk screening subjects. Using a new ligand-targeted PCR detection technique, the diagnostic value of CTCs for distinguishing NSCLC from lung benign disease was investigated. The level of CTCs in NSCLC patients was meaningfully higher than that of benign lung diseases and healthy donors. Ilie et al.[37] designed an experiment that showed CTCs could be detected in patients with chronic obstructive pulmonary disease (COPD) without clinically detectable lung cancer. A total of 245 subjects without cancer were enrolled, and CTCs were detected in 3% of COPD patients. Annual surveillance of CTC-positive COPD patients by computed tomography (CT) scan detected lung nodules 1–4 years after CTCs were detected, leading to prompt surgical resection and histopathological diagnosis of early-stage lung cancer.[37] In the diagnosis of tumors, at present, early tumor screening mainly relies on serum tumor markers and CT examination; traditional detection requires the lesion to reach a certain size before it can be resected. CTCs circulate in the peripheral blood after being shed from primary or metastatic tumors and, therefore, detection does not depend on tumor burden, giving CTCs higher sensitivity and specificity. The positive detection rate for CTCs in the diagnosis of primary lung cancer is higher than

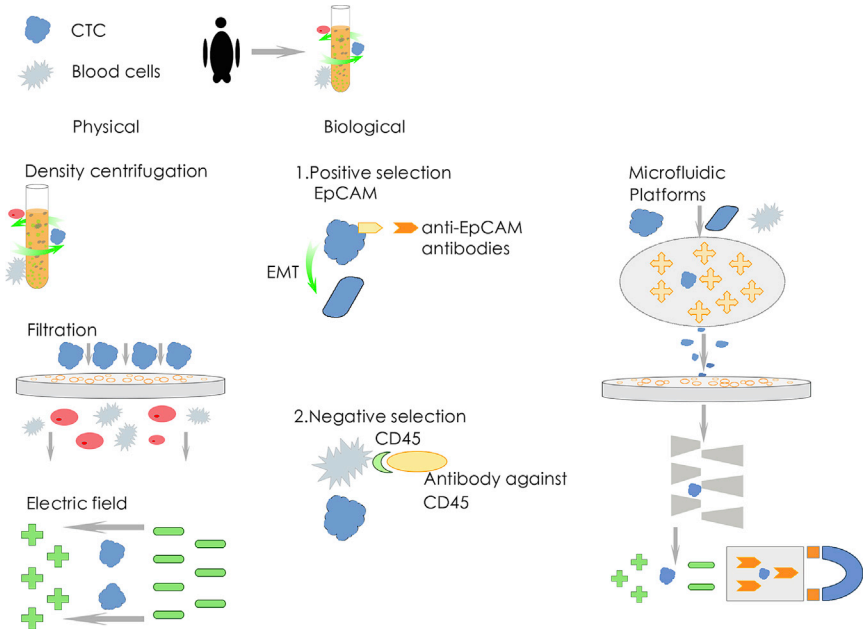

Figure 2. Technologies for detection and enrichment of CTCs

Cell enrichment involves capturing CTCs based on physical properties and biological properties. Physical properties include size, density, and electric charges, whereas biological properties involve surface protein expression and viability. Biological characteristics use either positive selection for targeting tumor-associated biomarkers or negative selection for removal of blood cells with common leukocyte biomarkers. Microfluidic platforms integrate physical and biological characteristics.

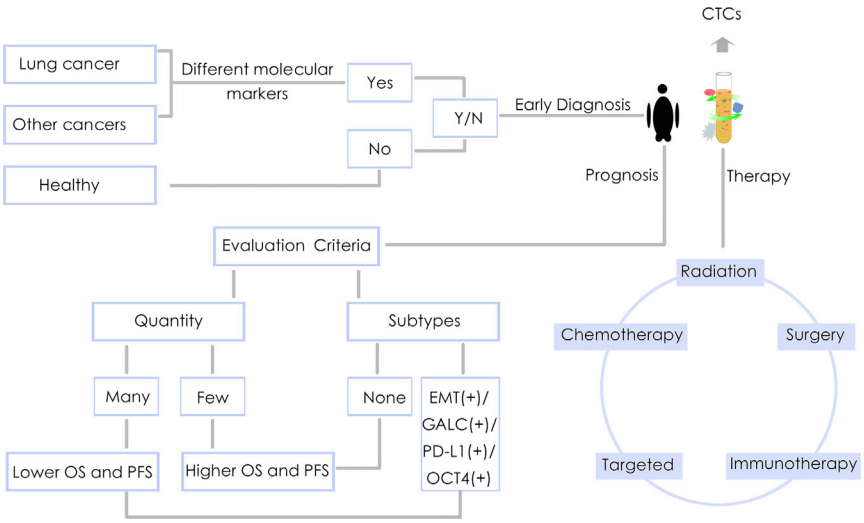

Figure 3. Clinical application of CTCs

CTCs are useful in the diagnosis, prognosis, and treatment of NSCLC. CTC levels are significantly higher in patients with NSCLC than those with benign lung disease or healthy controls. Tumor types can be clarified according to different molecular markers on CTCs. The CTC counts are negatively related to overall survival (OS) and progression-free survival (PFS). Different molecular phenotypes also predict different prognoses. EMT(+)/GALC(+)/PD-L1(+)/OCT4(+) were significantly related to adverse outcomes. The current treatment of tumors, including lung cancer, involves a combination of surgical treatment, chemotherapy, targeted therapy, immunotherapy, and radiation therapy.

that of serum tumor biomarkers. CTCs are a reliable indicator of a disease state and may be more effective than imaging.[38] CTC level can be used as a conducive biomarker to diagnose NSCLC.[38] The molecular phenotype of the detected CTCs can be used to distinguish tumor types. Folate receptor-positive CTCs (FR+-CTCs) showed the highest diagnostic potency[38,39] in lung cancer. The diagnostic efficacy of distinguishing lung cancer from benign lung diseases can be improved significantly with the combination of FR+-CTCs along with other tumor biomarkers.[40] In particular, squamous cell lung cancers do not usually express folate receptor[41,42] (Figure 3).

CTCs AS BIOMARKERS TO GUIDE THE TREATMENT OF LUNG CANCER

The current treatment of tumors, including lung cancer, is a combination of surgical treatment, chemotherapy, targeted therapy, immunotherapy, and radiation therapy. Changes in baseline CTC counts are correlated with clinical outcome, and CTC analysis may provide information about the biology of lung cancer, since it can help fine-tune therapy for each patient. Heterogeneity within and between tumors is a major obstacle to successful treatment. Liquid biopsy of blood sampling of cells such as CTCs from lung cancer patients can be easily and repeatedly obtained, providing a basis for lung cancer pathology and patient treatment options.

APPLICATION OF CTCs IN NSCLC SURGERY

Early-stage lung cancer can be treated by surgery, which can reduce tumor burden. Even with a successful operation, 30%–50% of patients with resectable NSCLC relapse within 5 years of the operation. Preoperative assessment of CTCs can identify patients with non-metastatic lung adenocarcinoma (NMLA) who have a higher risk of disease recurrence. In the age of precision oncology, CTC enumeration may help select the most suitable patients for surgery. In a prospective study, more than half of the patients evaluated were CTC-positive at baseline, and the positive rate of CTCs in these patients decreased 1 month after operation. The average CTC counts were 3.16 per 10 mL before surgery and 0.66 per 10 mL after surgery.[43] However, intraoperative procedures can lead to blood-borne spread of tumorigenic CTCs and circulating tumor microemboli (CTM).[44,45] In order to reduce the spread of blood-borne CTCs caused by surgery, differences in the order of intraoperative blood vessel ligation have been compared. Manjunath et al.[45] first reported that ligating the outflow vein during surgery could reduce the spread of tumor cells and improve progression-free survival (PFS) and OS.[46]

APPLICATION OF CTCs IN NSCLC RADIATION THERAPY

Radiation therapy is a recognized treatment for common epitheliomas, and CTC counts decrease after radiation therapy. The median CTC counts were 9.1 CTCs/mL in patients before radiotherapy, which was higher than that after radiotherapy (0.6 CTCs/mL).[47] CTCs can be used as a prognostic biomarker for the risk of recurrence after stereotactic body radiation therapy. Higher pre-radiotherapy CTCs and the existence of CTCs after radiotherapy are significantly related to the increased risk of recurrence outside the targeted therapeutic sites.[48] This means that if the CTC counts are negative after treatment, cancer is controlled.[49] In locally advanced cancer, distant metastasis after surgery and radiotherapy is common. In a study by Martin et al.,[50] g-H2AX (a biomarker for radiation-induced DNA double-strand breaks) was used to measure the level of DNA damage. G-H2AX is the phosphorylated form of histone H2AX and can function as a sensitive marker for double-strand breaks, which signify genomic instability and can potentially contribute to cancer initiation and progression. It has been found that elevated levels of gH2AX are present in a number of human cancer model systems.[51] Increased g-H2AX signal in blood samples after radiation therapy indicates the presence of CTCs from radiated tumors. The study reported that after the start of radiation therapy, seven of nine patients who received palliative radiation therapy and four of eight patients who received radical radiation therapy showed an increased CTC counts.[50] This demonstrates that radiotherapy can mobilize living tumor cells into the blood circulation of patients with NSCLC. If the release of CTCs during reverse transcription is conducive to metastasis, an understanding of the nature and dynamics of this phenomenon may put forward new treatment strategies,

such as early enhancement or acceleration of reverse transcriptase, or new combination therapies. CTCs and CTM mobilized by radiotherapy represent potential therapeutic targets.

APPLICATION OF CTCs IN CHEMOTHERAPY OF NSCLC

A change in the number of CTCs reflects the efficacy of chemotherapy. A study by Wei et al.[52] analyzed blood samples from patients receiving four to six rounds of chemotherapy, in which researchers collected blood samples from patients before each cycle was performed. The average number of CTCs decreased from 5.8 in the first cycle to 2.4 in the fourth cycle, and the number remained nearly the same in cycles four through six.[52] Chemotherapy can reduce the number of CTCs and can also reduce tumor burden. But drug resistance may develop during chemotherapy,[53] and there was no difference in CTC number in cycles four through six. After the fourth cycle of chemotherapy, CTC counts were not changed significantly. Another possible reason for this is that the differences are subtle and cannot be measured, which demonstrates the need for more sensitive detection methods.

The baseline number of CTCs can predict the treatment efficacy of pharmaceutical medicine. Krebs et al.[54] studied baseline CTC values in blood samples from 101 patients who had not previously received advanced treatment and measured CTCs before standard chemotherapy began. In univariate analysis, the PFS of patients with less than five CTCs and those with five or more CTCs before chemotherapy was 6.8 and 2.4 months, respectively; OS was 8.1 and 4.3 months, respectively.[54] Muinelo-Romay et al.[55] conducted a prospective study that included 43 patients who were diagnosed with NSCLC. Blood samples were obtained before cycles one, two, and five of chemotherapy and were analyzed by CellSearch technology. At baseline, 18 patients (41.9%) were positive for CTCs, of whom 10 patients (23.2%) had CTCs $\geq$ 5. The PFS and OS were worse in the group with baseline CTC counts > 5 compared to the patients with baseline CTC counts $\leq$ 5.[55] Thus, baseline CTC values can predict treatment efficacy.

Chemotherapy can affect the EMT process in CTCs, and cisplatin-based adjuvant chemotherapy prolongs survival. However, there is accumulating evidence that chemotherapy can induce EMT in diffuse tumor cells (DTCs) and in CTCs, leading to recurrence and metastasis.[56] Gemcitabine has therapeutic benefits for the survival of patients with NSCLC. Gemcitabine treatment can reduce EpCAM-positive CTCs in NSCLC patients and inhibit EMT through the HGF/cMET pathway, thereby inhibiting metastasis and regeneration.[57]

APPLICATION OF CTCs IN TARGETED THERAPY OF NSCLC

Targeted therapy is the standard treatment for advanced NSCLC, but it remains a challenge to obtain tumor tissue for genetic testing. CTCs provide a solution to a limitation of tissue biopsy, in which the biopsy cannot fully reflect the heterogeneity of tumor cells. CTCs are more conducive to the dynamic monitoring of disease progress than traditional detection methods. A more profound understanding of the genomic pattern of lung cancer has recently led to advances in targeted therapies for NSCLC. Researchers have identified principles of acquired drug resistance to targeted therapies and have made full use of available treatment choices to improve patient prognosis.

For advanced NSCLC patients with *EGFR* mutants, the standard therapy involves treatment with tyrosine kinase inhibitors of epidermal growth factor receptors (EGFR-TKIs). In the past, the standard first-line treatment was first-generation TKIs (gefitinib, erlotinib), or second-generation TKIs (such as afatinib).[58] Based on results from the FLAURA study, osimertinib is now the standard first-line treatment in the United States and Europe.[59] A large percentage of NSCLC patients will eventually develop resistance to EGFR-TKIs.[58] Repeated invasive procedures, such as surgical procedures or biopsies to assess the development of EGFR-TKI resistance, are impractical. Therefore, there is an urgent need to explore convenient and less-invasive techniques to monitor EGFR-TKI efficacy and resistance.[60] Mutation analysis at the CTC level has the potential to identify heterologous mutations in a noninvasive manner.[61,62] Sensitive detection of low-abundance *EGFR* mutations in plasma by microfluidic digital PCR with accurate quantification will provide an effective method to predict treatment response, monitor disease progression, and predict acquired resistance.[63]

The level of CTC expression predicted treatment response. A study by He et al.[64] investigated the correlation between the efficacy of EGFR-TKI and the level of CTCs in advanced NSCLC patients. In this study, 66 patients with stage III/IV NSCLC were divided into two groups based on median CTC count (median CTC counts were 68.5, and patients had CTC counts ranging from low to high). All 66 patients received EGFR-TKI treatment. The response rates of the low and high CTC groups were 53.3% (16/30) and 27.8% (10/36), respectively, and this was a statistically significant difference. The median OS of the low CTC group was 22.8 months, and the median OS in the high CTC group was 18.3 months. The median PFS of the low and high CTC groups was 11.5 months and 5.6 months, respectively, and this difference was statistically significant. In conclusion, CTC counts can be used to evaluate the efficacy of EGFR-TKI therapy.[64] In addition, certain CTC phenotypes can predict treatment efficacy. For example, folate-receptor-positive CTC counts can be used to predict the outcome of NSCLC patients who are treated with EGFR-TKIs and can be used as an alternative or supplement to computed tomographic scanning.[65]

The *T790M* mutation in *EGFR* is acquired in some cases of NSCLC as tumor cells become resistant to selective TKIs.[66] Osimertinib was the third-generation EGFR-TKI to receive US Food and Drug Administration (FDA) approval for *EGFR*-mutant NSCLC patients who acquired the *EGFR T790M* resistance mutation.[67] With the rise of the third-generation EGFR-TKIs that overcome *T790M*-related drug resistance, *T790M* detection became key to guiding management. Mok et al.[68] conducted an international, randomized, and open-label phase III trial in which 419 patients who were *EGFR T790M*-positive were divided into two groups. One group received oral osimertinib, and the other group received intravenous pemetrexed plus carboplatin or cisplatin. After first-line EGFR-TKI treatment, some patients developed *T790M*-positive transformation. The efficacy of osimertinib

was significantly better than that of platinum-based drugs plus pemetrexed.[68] The *T790M* mutation status observed through CTC analysis is consistent with the *T790M* mutation status observed through tissue biopsy, which can be used to evaluate the therapeutic efficacy of targeted therapy after drug resistance.[66,69]

Anaplastic lymphoma kinase (*ALK*) rearrangement was observed in CTCs, and CTCs can be available for complementing tissue-based *ALK* testing in NSCLC to guide *ALK*-targeted therapy.[70] *ALK* rearrangements were detected in CTCs of *ALK*-positive NSCLC patients,[71] and NSCLC patients with *ALK* rearrangement inevitably acquire resistance to *ALK* inhibitors.[72] Pailler et al.[73] isolated CTCs from patients with disease progression at the single-cell level to investigate resistance mutations after treatment with crizotinib (n = 14) or lorlatinib (n = 3). The genetic heterogeneity and clinical utility of CTCs is beneficial for identifying treatment resistance mutations in patients with *ALK* rearrangement. CTC sequencing may provide a unique method to evaluate heterogeneous resistance mechanisms, which helps clinicians personalize treatment and create choices for patients who acquire resistance to *ALK*-targeted therapies.[73] Echinoderm microtubule-associated protein-like 4 anaplastic lymphoma kinase (EML4-ALK)-positive CTCs can predict therapeutic efficacy and early detection of resistance to *ALK* inhibitors.[74,75]

To evaluate whether *ROS1* rearrangement occurs in CTCs, *ROS1* rearrangement was evaluated in CTCs of four patients treated with crizotinib. The *ROS1* mutation was compared in CTCs at baseline and during crizotinib treatment in patients with *ROS1* rearrangement. DNA content quantification and chromosome counting were used to evaluate the chromosomal instability (CIN) of CTCs.[76] The CTCs of NSCLC patients with *ROS1* rearrangements showed extensive heterogeneity of *ROS1* gene aberrations, accompanied by high levels of chromosomal instability, which also created the potential for tumor metastasis.[77]

Detectable genetic alterations of NSCLC patients include not only common mutations, such as *EGFR*, *ALK*, and *ROS-1*, but also rare driver mutations, such as mesenchymal-epithelial transition (*MET*) amplification, rearranged during transfection (*RET*) rearrangement, and human epidermal growth factor receptor-2 (*HER-2*) insertion. These mutations are widely used to identify therapeutic targets in NSCLC patients, to explore therapeutic resistance, and to further guide treatment.[7] Researching these gene targets through CTC analysis provides an avenue to predict treatment efficacy, monitor disease progression, and predict resistance.

APPLICATION OF CTCs IN NSCLC IMMUNOTHERAPY

In addition to surgery, chemotherapy, and targeted therapy, immunotherapy is a novel treatment for NSCLC.[78] The FDA confirmed immune checkpoint inhibitors as first- or second-line treatments for NSCLC. Checkpoint inhibition of programmed death protein 1(PD-1)/programmed death ligand 1 (PD-L1) interaction is a successful immunotherapy in the treatment of metastatic lung cancer.[79,80] The level of PD-L1 expressed in CTCs can be used as an indicator to track the adaptive changes of immunotherapy targets. Improvements in PFS and OS are mediated by the immune checkpoint blockade targeting the PD-1/PD-L1 axis for patients with advanced NSCLC. The FDA has approved two antibodies that block PD-1 in NSCLC, nivolumab (Opdivo, Bristol-Myers Squibb) and pembrolizumab (Keytruda, MSD Sharp and Dohme), and an antibody that targets PD-L1, atezolizumab (Tecentriq, Roche).[81] Ipilimumab and nivolumab are approved as first-line treatments for PD-L1-expressing lung cancers.[82] Separately, ongoing studies are evaluating atezolizumab as a third-line treatment in NSCLC. Researchers are committed to exploring the potential additional clinical applications of these therapeutic agents. Current studies are investigating whether monitoring the expression of PD-L1 can effectively assess tumor response to treatment with immune checkpoint inhibitors.[83]

In advanced NSCLC patients, PD-L1 is a potential biomarker to select patients for immunotherapy, and the expression of PD-L1 in tumor cells or tumor-infiltrating immune cells is related to the improvement of the efficacy of anti-PD-1/PD-L1 inhibitors. The level of expression of PD-L1 in CTCs can help screen patients and can supplement the results of tissue PD-L1 biopsy by testing the expression of PD-L1 at the metastatic tumor site. Ilié et al.[84] assessed the expression of PD-L1 in CTCs in blood samples from 106 patients with advanced NSCLC. The results of this study indicated that the expression of PD-L1 in CTCs was associated with the status of PD-L1 in tumor tissues, which revealed a potential use of CTCs for assessing PD-L1 expression through a noninvasive real-time biopsy.[84] In another study, Chen et al.[85] reported processing 2 mL of peripheral blood from 51 patients with NSCLC to analyze the level of PD-L1 in CTCs. CTCs were detected in 86% of newly diagnosed NSCLC (I–IV) patients, and the PD-L1-positive rate ($\geq$1 PD-L+ CTCs) was 67%. The study demonstrated that the positive rate of PD-L1 in CTCs was higher than that in tumor tissues.[85] When a MCA system was utilized to detect CTCs and PD-L1 expression, PD-L1 expression was found to be positive in 73% of patients by CTC detection, and the positive rate of CTCs expressing PD-L1 ranged from 3% to 100%. This demonstrated heterogeneity of CTC PD-L1 expression in patients with NSCLC.[83,86] Monitoring the expression of PD-L1 on CTCs can be used to predict the efficacy of inhibitors.

By analyzing the level of PD-L1 on CTCs, treatment efficacy can be predicted. In patients with positive CTC PD-L1 expression during initial treatment, PFS is shorter than in patients with negative CTC PD-L1 expression.[87] Guibert et al.[88] assessed PD-L1 expression in blood samples collected from patients with advanced NSCLC before treatment with nivolumab and during cancer progression. The study demonstrated that in patients treated with PD-1 inhibitors, the presence of PD-L1+ CTCs before treatment was related to poor prognosis.[88] The persistence of PD-L1-positive CTCs treated with nivolumab is related to poor treatment efficacy in NSCLC patients.[83] Monitoring overall CTC counts and PD-L1+ CTC counts allows for assessment of treatment response.[89,90] In a study of 89 NSCLC patients previously treated with nivolumab, baseline CTC counts were significantly correlated with OS.[91] Anti-PD-1/PD-L1 treatment significantly improved the prognosis of patients with NSCLC. Janning et al.[10] used the Parsortix system to evaluate the expression of PD-L1 in CTCs from NSCLC patients. All patients showed an

Table 2. Sample outcomes using CTCs in immunotherapy research

Authors (year)	Isolation method	Staging	Evaluation criteria	Therapy	Outcome	Reference
Nicolas Guibert et al. (2018)	ISET	advanced metastatic NSCLC	CTC numbers and PD-L1 positive rate	Nivolumab	high CTC counts experienced worse outcomes compared to those with a low CTC load. Median CTC number was higher at progression compared to pre-treatment (52.5/7.5 mL (7.5–228) versus 30/7.5 mL; p = 0.03). Patients who have PD-L1-positive CTCs are resistant to therapy	Guibert et al.[88]
Angela Alama et al. (2019)	ScreenCell CYTO (ScreenCell)	advanced NSCLC	CTC numbers and PD-L1 expression	Nivolumab	high CTC counts experienced a worse outcome compared to those with a low CTC load the presence of PD-L1-positive CTCs had no significant prognostic impact. Benefited from nivolumab treatment	Alama et al.[91]
Yen-Lin Chen et al. (2019)	CellMax	I–IV NSCLC	PD-L1-positive rate	ICI	CTCs are more likely to be positive for PD-L1 than the corresponding tumor tissue. PD-L1 expression status on CTCs can be determined serially during the disease course	Chen et al.[85]
Yasuhiro Koh et al. (2019)	MCA system	II–III/IV NSCLC	CTC numbers and PD-L1 expression		PD-L1 expression was detectable on CTCs in patients with lung cancer, and intra-patient heterogeneity was observed. No correlation was observed between PD-L1 expression in tumor tissues and CTCs	Koh et al.[86]
Angelo Castello et al. (2020)	ISET	advanced NSCLC	CTC numbers and metabolic parameters	ICI (nivolumab, pembrolizumab, combination of nivolumab and ipilimumab, atezolizumab)	CTC number is modulated by previous treatments and correlates with metabolic response during ICI. CTC counts and metabolic parameters were prognostic factors for PFS and OS	Castello et al.[94]
Lina Zhang et al. (2019)	SE-iFISH strategy	advanced NSCLC	aneuploid CTCs and expressing PD-L1	Nivolumab	increased CTCs might be relevant to nivolumab resistance and progression of disease in post-immunotherapeutic patients	Zhang et al.[95]
Mao Lin et al. (2017)	MACS, FACS, qRT-PCR	IV NSCLC	CTC numbers and CTC-related gene expression	NK cell therapy	NK cells reduced the number of CTCs in the patients' peripheral blood; CTCs can be used to evaluate the efficacy of NK cells.	Lin et al.[93]
Elizabeth Punnoose et al. (2012)	CellSearch	advanced NSCLC	CTC enumeration	pertuzumab	decreased CTC counts were associated with longer PFS	Punnoose et al.[96]
Chiara Nicolazzo et al. (2016)	CellSearch	IV NSCLC	CTC numbers and PD-L1 expression	Nivolumab	the presence of CTCs and the expression of PD-L1 on their surface were found associated with poor patient outcome. The persistence of PD-L1-positive CTCs might mirror a mechanism of therapy escape	Nicolazzo et al.[97]

CTCs: circulating tumor cells; PD-L1: programmed death-ligand 1; NSCLC: non-small cell lung cancer; ICI: immune checkpoint inhibitor; PFS: progression-free survival; OS: overall survival

increasing level of PD-L1+ in CTCs with disease progression, and the increase in PD-L1+ CTCs could be used to predict resistance to PD-1/PD-L1 inhibitors.[10] EMT is a key process that allows cancer cells to escape from a primary tumor. EMT-related pathways can induce PD-L1 expression in tumor stem cells. EMT progress of cancer cells is upregulated with PD-L1 expression, and PD-L1 expression is directly related to the EMT of CTCs. Furthermore, PD-L1 expression as well as EMT of CTCs are negative predictors of survival in NSCLC patients.[45] Increased expression of multiple immune checkpoints that lead to immune tolerance is associated with EMT. Upregulation of PD-L1 in tumor tissues can evade immune surveillance by inhibiting immune cell activation.[92]

In addition to anti-PD-1/PD-L1 treatment, adoptive natural killer (NK) cell transfer proves to be a formidable cancer immunotherapy tool for the treatment of various cancers. Lin et al.[93] measured the peripheral blood CTC values from 31 patients with advanced NSCLC. Samples were collected 1 day before NK cell treatment and again at 7 and 30 days after NK cell treatment. CTC counts decreased significantly across the time points (counting result: 18.11 ± 5.813, 15.13 ± 5.984, and 10.32 ± 5.623, respectively). NK cell treatment provides a method for the treatment of NSCLC, and CTCs have been used as a biomarker to evaluate the efficacy of NK cell treatment[93] (Table 2).

Table 3. CTC counts are related to OS, PFS, DFS, and TFS

Authors (year)	Isolation method	Staging	Population	Cutoff values	Outcome	Reference
Tengteng Wei et al. (2019)	nano-enrichment	Tis, I, II, III, IV NSCLC	73	CTC numbers > 5	PFS was 7.2 months	Wei et al.[52]
				CTC numbers ≤ 5	PFS was 11.3 months	
Angela Alama et al. (2019)	ScreenCell CYTO (ScreenCell)	advanced NSCLC	89	CTC numbers ≥ 2	OS was 6.2 months	Alama et al.[91]
				CTC numbers ≤ 2	OS was 8.8 months	
Jingsi Dong et al. (2019)	CanPatrol	I–III NSCLC	114	CTC numbers ≥ 15	postoperative DFS was 15.3 months	Dong et al.[103]
				CTC numbers < 15	postoperative DFS was 24.7 months	
Bing Tong et al. (2018)	combines subtraction enrichment, leukocyte common antigen (CD45) immunostaining, and fluorescence *in situ* hybridization	IIIb or IV NSCLC	43	CTC numbers ≥ 8	PFS was 8.5 months, OS was 17.7 months	Tong et al.[105]
				CTC numbers < 8	PFS was 11.6 months, OS was 21.0 months	
Yunsong Li et al. (2017)	FAMCell System	I–IIIA NSCLC	23	PPB-CTC density > 5	TFS was 22.0 months, OS was 27.0 months	Li et al.[106]
				PPB-CTC density ≤ 5	TFS was >60 months, OS was >60 months	
				IPVB-CTC density > 25	TFS was 25.0 months, OS was 30.0 months	
				IPVB-CTC density ≤ 25	TFS was >60.0 months, OS was >60.0 months	
Jia Zhou et al. (2017)	CellSearch	III or IV NSCLC	59	CTC numbers ≥ 2	PFS was 4.3 months	Zhou et al.[107]
				CTC numbers < 2	PFS was 6.2 months	
Lindsay et al. (2017)	CellSearch	IIIb–IV NSCLC	125	CTC numbers ≥ 5	PFS was 4.9 months, OS was 6.6 months	Lindsay et al.[108]
				CTC numbers < 5	PFS was 5.8 months, OS was 12.9 months	
Yuanling Qi et al. (2016)	CellSearch	IIIA, IIIB, or IV NSCLC	100	CTC numbers ≥ 5	PFS 2.5 months, OS was 3.9 months	Qi and Wang[109]
				CTC numbers < 5	PFS was 6.3 months, OS was 8.3 months	
Matthew G. Krebs et al. (2011)	CellSearch	III or IV NSCLC	101	CTC numbers ≥ 5	PFS was 2.4 months, OS was 4.3 months	Krebs et al.[54]
				CTC numbers < 5	PFS was 6.8 months, OS was 8.1 months	
Laura Muinelo-Romay et al. (2014)	CellSearch	IIIB or IV NSCLC	43	CTC numbers ≥ 5	PFS was 4.1 months, OS was 4.6 months	Muinelo-Romay et al.[55]
				CTC numbers < 5	PFS was 7.6 months, OS was 10.7 months	

CTCs, circulating tumor cells; NSCLC, non-small cell lung cancer; PFS, progression-free survival; OS, overall survival; DFS, disease-free survival; TFS, tumor-free survival.

CTCs AS PROGNOSTIC BIOMARKERS IN LUNG CANCER

CTCs are promising as a biomarker for monitoring prognosis and recurrence in NSCLC patients. In a TRACERx study, pulmonary venous CTCs (PV-CTCs) were detected by CellSearch in 48% of 100 patients. PV-CTCs from surgical resection of NSCLC represent subclones that influence recurrence. PV-CTCs, genomic profiling, and enumeration have the potential to be prominent as early predictors of NSCLC relapse.[98] In patients, longitudinal monitoring of CTCs in blood circulation is more meaningful than radiological evidence to indicate disease recurrence.[99] The molecular characterization of CTCs can provide useful messages based on counts. The CTCs of patients with different metastatic potential have specific gene characteristics with different expression profiles that can be applied to identify high risk of recurrence in patients with early-stage NSCLC.[100] Liu et al.[101] used a CanPatrol platform and RNA *in situ* hybridization technology to analyze the baseline counts of CTCs, EMT classification, and galactocerebrosidase (GALC) expression in 47 patients with NSCLC. Epithelial CTCs were detected in 55.4% of patients, and mesenchymal and hybrid CTCs were detected in 61.7% and 78.7% of patients, respectively. Patients with higher EMT-CTC levels usually have distant metastasis and poor therapeutic efficacy.[101] GALC is positive in 80.6% of patient CTCs and is closely related to the number of tumors, distant metastases, and therapy results.[101] It was found that CTCs in which the expression of PD-L1 was positive were significantly related to adverse outcomes.[102,103] In addition, high expression of octamer-binding transcription factor 4 (*OCT4*) tends to occur in advanced patients and in patients with distant metastases[104] (Table 3).

CONCLUSIONS

The discovery of CTCs dramatically changes the traditional medical model. CTCs allow for early disease detection, prognosis, recurrence monitoring, and personalized cancer treatment. However, a highly sensitive EpCAM-independent CTC capture platform to isolate CTCs and subsequently perform a comprehensive molecular analysis of a pure population of sufficient numbers of tumor cells has not yet been developed. In the meantime, it would be helpful to establish a permanent CTC cell line to be used for screening of anti-metastatic drugs and analysis of the mechanism of lung cancer metastasis. In recent years, many researchers have applied single-cell sequencing technology to the study of CTCs.[110] Single-cell sequencing of CTCs can obtain the pattern of whole-genome copy number changes in CTCs of cancer patients, identify cancer subtypes, and further reveal the molecular mechanisms of tumor metastasis and clone origin.[111,112] In addition, single-cell sequencing of CTCs has the ability to identify point mutations in oncogenes, reveal dynamic changes in gene mutations in patients undergoing cancer treatment, and predict drug resistance and phenotypic transformation, ultimately providing a truly personalized approach to medicine.[113] Liquid biopsy and noninvasive analysis will no doubt continue to play an important part in the overall assessment and treatment of patients with NSCLC. In addition, this approach encourages continued research into biomarkers to guide the diagnosis and treatment of NSCLC. With additional research, CTCs can be expected to become as indispensable to cancer diagnosis and treatment as routine imaging evaluations.

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AUTHOR CONTRIBUTIONS

W.G., J.H., and J.X. designed the study. H.Q. and Y.Z. drafted the manuscript. W.G., J.H., and J.X. revised the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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