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Review Article

Detection and application of circulating tumor cell and circulating tumor DNA in the non-small cell lung cancer

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Abstract: Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related death in both men and women. The ability of cancer cells to break-off from the primary tumor and spread to distant organs is the main cause of death of cancer patients. The detection of circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) is a considerable part of liquid biopsy, which contributes to the diagnosis, treatment and prognosis, and especially to identify the targetable mutations of NSCLC. This review is to discuss the detection and application of CTC and ctDNA in the diagnosis, prognostic evaluation and guiding targeted therapy of NSCLC.

Keywords: Non-small cell lung cancer (NSCLC), circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), liquid biopsy, targeted therapy

Introduction

Lung cancer, with 80-85% being non-small cell lung cancer (NSCLC), is the leading cause of cancer-related death in both men and women [1-3]. More than one-third of all newly diagnosed lung cancers occurred in China, making it a large social and economic burden. According to the annual report on the status of cancer in China, in total, 651,053 patients were newly diagnosed with lung cancer in 2011, including 441,364 men and 209,689 women [4-6].

The ability of cancer cells to break-off from the primary tumor and spread to distant organs is the main cause of death in cancer patients. The detection of circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) serving as liquid biopsies is novel developed technology [7]. Quantification of genetic mutations using CTCs and ctDNA may provide a noninvasive means for early cancer detection and disease monitoring. Compared with tissue biopsy, liquid biopsies are far less invasive and more repeatable [7-9]. Furthermore, because of the high degree of heterogeneity among tumor cells in different regions, liquid biopsy can provide more comprehensive tumor mutation analysis results [10]. CTCs and ctDNA as the cornerstones of liquid biopsy diagnostics have their own advantages and can complement each other. CTCs analysis can provide the information of cancer DNA, RNA and protein, whereas ctDNA can only provide genetic abnormality information. These information can help clinicians to identify drug resistance mutations, minimum residual disease after treatment, treatment progress. Although the CTCs have more information available, ctDNA is easier to save and can be used for a longer time [7, 9]. In the development of personalized medicine, CTCs and ctDNA play a crucial part in the diagnosis, treatment and prognosis of non-small cell lung cancer [11-13].

Detection of CTCs and ctDNA in NSCLC

Detection of CTCs for NSCLC

CTCs are tumor cells shedding from either primary tumors or its metastases that circulate in the peripheral blood [14]. These cells can be divided into single CTCs and CTC clusters, with the latter being defined as groups of tumor cells (exceed two or three cells, varied among previous researches) that march in the bloodstream

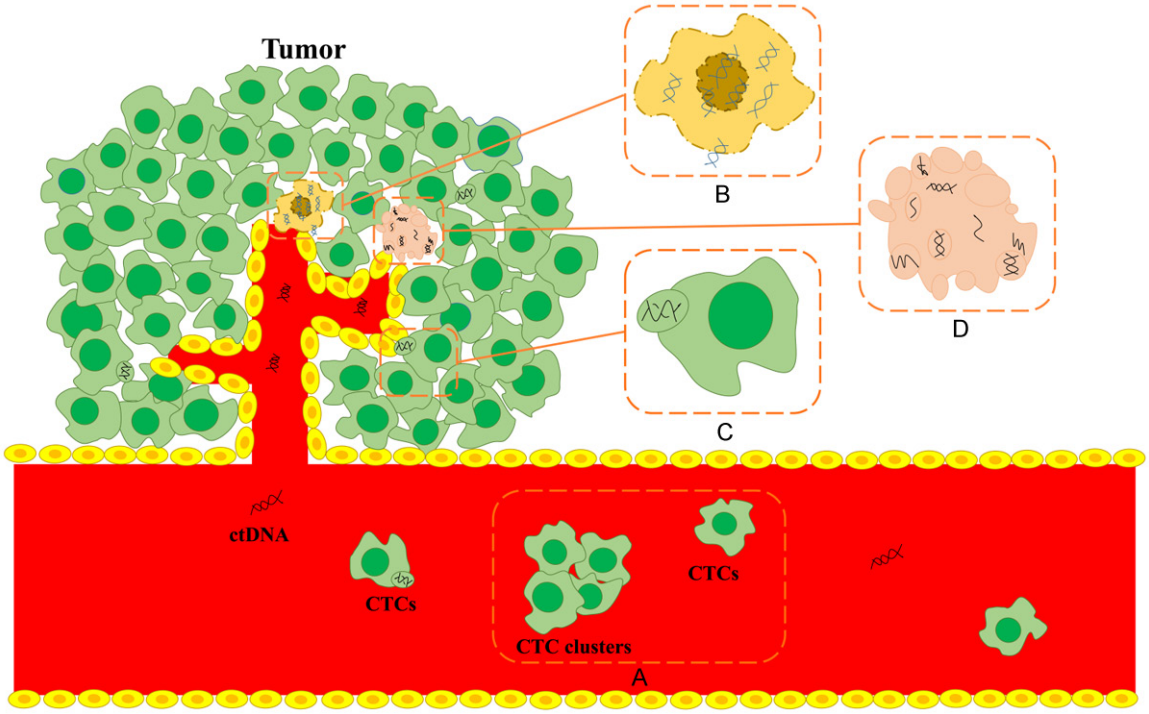

Figure 1. Formation mechanism of CTCs and ctDNA. A: Circulating tumor cells (CTCs) are shed from primary tumors or its metastases; CTC clusters can also be generated by single CTCs aggregation or proliferation; B: Circulating tumor DNA (ctDNA) is released by necrotic cells; C: Active cells (including CTC) actively secrete DNA; D: CtDNA is released by apoptotic cells.

[15] (**Figure 1A**). In the early stage of tumor formation, tumor cells can be extensively shed into the circulatory system [14]. The CTCs can be detected in the blood of patients with only primary tumor and no metastasis, and the correlation between CTCs count and cancer prognosis is observed [16]. Clinical trials and clinical data have shown that CTCs can still be detected in cancer patients after their primary tumors have been removed for several months or even years, indicating that tumor cells can be re-disseminated from the metastases into the blood [9, 17].

Currently, the CellSearch system (Janssen Diagnostics Company, USA) is the only FDA-approved assay for CTCs detection. Since the CellSearch system detects CTCs based on their expression of epithelial biomarkers which are likely to be lost in CTCs due to epithelial-mesenchymal transition (EMT) during circulating, the detection rate for CTCs is limited [18]. Many other strategies are proposed and developed. Herringbone-chip or "HB-Chip" creates an enhanced platform for CTCs insulation, which increases the capture rate of CTCs [19]. A microcavity array (MCA) system has been developed by Hosokawa M et al. and was demonstrated to have higher detection sensitivity for NSCLC than the CellSearch system [20]. An approach named isolation by size of epithelial tumor cell (ISET) can capture all the circulating non-haematological cells (CNHCs) and characterize as the CTCs among them [21]. Also many flow cytometry and chip based systems have been developed, including FISHMAN-R, On-chip Sort (On-Chip Biotechnologies) and microfluidic chips [22-24].

Detection of ctDNA for NSCLC

The cell-free DNA (cfDNA) level in the blood increases due to various pathological processes. Both benign and malignant lesions may release their DNA into the blood through apoptosis and necrosis, forming cfDNA (**Figure 1B** and **1D**). Although cfDNA may be elevated in a variety of pathological processes, cancer patients have a greater increase than those patients with benign lesions [25]. Higher concentrations of cfDNA often were measured in the plasma of cancer patients, referring as cir-

culating tumor DNA (ctDNA). Another view about the origin of cfDNA is that cells actively secrete DNA, which has nothing to do with apoptosis or necrosis (**Figure 1C**). Since all cells release cfDNA, the cfDNA in the blood of cancer patients may originate from the cancer cells or other non-cancerous cells [26]. Some scholars believe the two mechanisms mentioned above are both the main sources of cfDNA, and they are characterized as passive and active respectively [27].

CtDNA detection technology has went through many development periods, from the conventional karyotype analysis to the various molecular cytogenetic methods such as spectral karyotyping, chromosome-based comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH), and to the modern technologies including microarray-based CGH and single nucleotide polymorphism (SNP) analysis and next-generation sequencing (NGS) [28]. Currently, detecting ctDNA is clinically practical. By sending ctDNA and tumor tissue of the same patients to NGS at the same time, researchers found that the concordance rate for clinically actionable DNA alteration detection in the two samples can reach 54.9% with a sensitivity of 53.2% and a specificity of 75.0% [29]. NGS has been widely used in clinics as routine for personalized therapy, and it can detect multiple mutations at the same time [30, 31]. Many PCR-based assays are also potential methods for detecting genotype mutations in plasma, such as microfluidics digital PCR and droplet digital PCR (ddPCR) which have all been applied to the detection of EGFR mutations in NSCLC [32, 33].

CTCs and ctDNA can be used as the diagnostic biomarkers of NSCLC

CTCs and ctDNA are emerging as noninvasive multifunctional biomarkers in liquid biopsy. Lung cancer is the most common cause of cancer-related death worldwide [34], and the five-year survival rate of lung cancer varies from 4% to 17% depending on geographic and stage differences [35]. The lack of clinical symptoms in early stage is the crucial reason for this extremely low survival rate of lung cancer. In addition, physicians never practice a biopsy in the patients without any clinical symptom. Early lung cancer patients have far better prognosis versus advanced patients. For these reasons, exploring and researching biomarkers for early diagnosis of lung cancer is an inevitable trend [36, 37].

CTCs as the diagnostic biomarkers of NSCLC

Tanaka F et al. found that the quantity of CTCs in patients with lung cancer was much higher than those without malignant disease, and increased CTCs significantly associated with the progression or metastasis of lung cancer. Therefore, CTCs counts may be useful for the diagnosis and metastasis of primary lung cancer [38].

Ilie M et al. examined the presence of CTCs in 245 patients without cancer including 168 chronic obstructive pulmonary disease (COPD) patients and 77 subjects without COPD by using ISET technology, CTCs were detected in 5 COPD patients. These patients are all diagnosed with pulmonary nodules in the one to four years after the detection of CTCs, and the pathological diagnosis after surgery was confirmed as early stage lung cancer. Neither recurrence nor CTCs were found at 16 months after operation because of early diagnosis and intervention. This result preliminarily confirmed the potential of CTCs testing for the early diagnosis of lung cancer in high-risk patients [39]. Many studies have demonstrated that Folate Receptor-positive CTCs can be used as a biomarker for the early diagnosis and progress monitoring in NSCLC patients [40, 41].

ctDNA as the diagnostic biomarkers of NSCLC

Some studies have found that circulating DNA in plasma of NSCLC patients is higher than normal subjects. By sequencing specific gene regions with PCR, the proportion of tumor derived DNA in all ctDNA can be determined. Also, some studies found that NSCLC patients with lymph node metastasis or distance metastasis had higher circulating DNA levels, and those patients with higher circulating DNA levels had less overall survival time. These findings indicated that circulating DNA can act as a potential diagnostic marker for NSCLC [42-44].

At the present stage, the diagnosis and treatment of cancer is greatly depending on molecular detection of tumor-drived genes, but limited tumor tissue has made it difficult to obtain molecular detection data. The proposal to

replace tumor DNA with ctDNA for molecular phenotype determing has gradually been taken seriously. EGFR mutation status is an important biomarker for NSCLC targeted therapy. It is, therefore, helpful to have an early diagnosis about mutation type for receiving optimal targeted therapy. Studies have shown that ctDNA is capable of detecting epidermal growth factor receptor (EGFR) mutations in ctDNA of patients with NSCLC [45-47].

CTCs and ctDNA can act as prognostic biomarkers of NSCLC

Although the prognosis of NSCLC patients has been greatly improved with the development of targeted therapies, the overall prognosis of NSCLC remains poor. Blood circulation markers can be an effective indicator about the prognosis and treatment response of lung cancer patients [48, 49].

CTCs as prognostic biomarkers of NSCLC

As a minimally invasive test, CTCs can be conveniently acquired for many times, thus can help patients to customize individual treatment plans, monitor treatment efficacy and observe patients' prognosis [51]. Krebs et al. conducted a single-center prospective study, detecting CTCs in untreated patients with late stage NSCLC. CTCs number detected in stage IV patients is significantly higher than in stage III patients. Moreover, the CTCs counts are positively related to overall survival (OS), suggesting CTCs can act as a novel prognostic factor of NSCLC [52]. Spiliotaki et al. have also demonstrated that monitoring the proliferation and apoptosis in CTCs can be a useful tool for long-term follow-up of cancer patients [53].

Multivariate analyses showed that the number of CTCs is significantly related to shorter disease-free survival (DFS) and progression-free survival (PFS), indicating CTCs can be an independent prognostic factor and an efficacy predictor for lung cancer [54, 55]. CTC clusters are more invasive than single CTCs, and patients who can be detected with clustered CTCs have a worse prognosis than patients with single CTCs only [56, 57].

ctDNA as prognostic biomarkers of NSCLC

A study in India demonstrated that the EGFR mutation status detected in tissue biopsies and ctDNA were highly consistent, ctDNA not only can be used as an important biomarker for cancer prognosis, but also it could be used for early diagnosis and treatment response assessment [58]. The concentration of ctDNA has a strong positive relation with cancer prognosis, with ctDNA level increasing when cancer progresses and declining after effective treatment [13]. Molecular tests are commonly used as companion diagnostics to help adjust treatment regimens and improve prognosis. The ctDNA can also be used to dynamically monitor EGFR mutations to improve patient care [59].

Another research conducted a prospective trial to measure ctDNA and tumor volume with PET-CT in advanced NSCLC patients and tracked their prognosis. Results revealed that ctDNA level has high correlation with OS but has no correlation with tumor burden [60]. Sirera et al. tracked the EGFR mutation status in plasma ctDNA in 45 NSCLC patients who are receiving EGFR targeted therapy. They found that EGFR mutation status in ctDNA changed in 26 cases, and the T790M negative to positive alteration group has a shorter PFS [61]. Another large-scale research also proved that high ctDNA level is an independent poor prognostic biomarker for advanced NSCLC, and it may be useful for improving the prognosis [62].

Detection of CTC and ctDNA can be used to guide the targeted therapy of NSCLC

In recent years, more and more attention has been paid to precision medicine, and cancer treatment is also more inclined to molecular targeted therapy which can improve the prognosis of cancer patients and enhance PFS and overall survival (OS) [63, 64]. The key to targeted therapy is to find targetable activating mutations. Tissue biopsy is gradually replaced by liquid biopsy for mutation detection. Detectable genetic alterations of NSCLC patients include not only common mutations such as EGFR, ALK, and ROS-1 but rare driver mutations like MET amplification, RET rearrangement, and HER-2 insertion. These mutations are the widely used therapeutic target to treat patients with NSCLC [65]. Some assessments based on the results of targeted therapy for patients with NSCLC suggest that CTC and ctDNA analysis are effective means for identifying genotyping of tumors, especially if tissue biopsy is not feasible [66-68]. They are also important non-in-

Table 1. Compare the differences between circulating tumor cell (CTCs) and circulating tumor DNA (ctDNA)

Comparison	CTCs	ctDNA
Definition	Tumor cells are released into the blood from primary tumors and/or metastatic sites [9]	High concentration of cell-free DNA in tumor patients [24]
Common detection technology	CellSearch method (Janssen Diagnostics Company, USA) [17]/FISHMAN-R/On-chip Sort [20, 21]	Conventional approach (karyotype analysis) Molecular cytogenetic methods (spectral karyotyping, CGH and FISH) Modern technologies (microarray-based CGH, SNP, NGS) [26] PCR-based methods (microfluidics digital PCR, ddPCR [30, 31]
Information type providing by analysis	DNA, RNA and protein [7, 9]	DNA [7, 9]
As diagnostic biomarkers	CTC counts contributes to the judgment of benign and malignant lung diseases [36, 37]	Prone to the diagnosis of molecular subtypes of lung cancer [40-45]
As prognostic biomarkers	Assess the change of patient's condition by monitoring the proliferation and apoptosis changes of CTCs [51] The number of CTCs is significantly related to DFS and PFS [52, 53]	CtDNA A can be used as an independent prognostic marker [13, 56, 58] Different mutation status of the same subtype has a different prognosis [57]
Roles of CTC and ctDNA in targeted therapy	Baseline CTC counts can be used to predict and monitor the efficacy of targeted therapies [69]	Monitor mutation status to guide the selection of targeted drugs [65-67]

vasive tools for assessing early treatment response and monitoring mutation status in real time [69-71]. And ctDNA is more sensitive than CTC in predicting and monitoring the treatment effectiveness for mutation harboring lung cancer [72].

CTC in the targeted therapy of NSCLC

The use of CTC counts in targeted therapy of NSCLC is very common. 43 cases of NSCLC patients with EGFR mutation or ALK rearrangement were classified by CTC counts. Then the prognostic analysis showed that baseline CTC counts can be used as predictive prognosis biomarkers for EGFR mutations and ALK rearrangement of NSCLC, and can better guide patients in drug therapy and monitor patient's prognosis in targeted therapy [73]. Chang et al. developed a parallel flow micro-aperture chip system for detection of CTCs and found that CTC counts in untreated patients in statically higher than treated patients with NSCLC (detailed comparison) [74].

In addition, some researchers compared the number of CTCs in patients with NSCLC and those without lung cancer finding that CTCs can only be found in NSCLC group and CTCs positive detection rate obviously rises as the pathological stage increases from I to IV. Comparing pre-treatment and post chemotherapy CTCs counts, they found that CTCs counts decreased significantly after two courses of chemotherapy. The decreasing in CTCs counts are also positively relative to the treatment efficacy [75, 76]. Other investigators have developed a microfluidic device to enrich CTCs, which not only facilitates the detection of genetic mutations in individual cells, but also dynamically monitors the genetic aberrations of patients during treatment and explores possible drug-resistant mutations in patients [77].

ctDNA in the targeted therapy of NSCLC

The detection of ctDNA can be qualitative or quantitative. Qualitative testing is mainly used to detect genetic subtypes (EGFR, ALK, KRAS, ROS1, et al.) of NSCLC patients. Therefore, liquid biopsies utilizing ctDNA testing represent a powerful approach to detect cancer genotype and monitor the development of resistance [78, 79]. Thompson's team used NGS to compare ctDNA with tissue genome sequence, demonstrating that ctDNA NGS was feasible for the detection of targeted driver and resistance mutations in NSCLC patients [80]. Particularly, for patients with unusable or inadequate tumor tissue, ctDNA detection may be the first choice for detecting drive mutations and resistance mutations (especially forthe EGFR mutation) [81, 82].

Quantitative testing, on the other hand, is used to predict the cancer response and progression and to assess efficacy and prognosis [83]. The concentration of ctDNA is related to the

response and disease progression, reflecting its ability to act as a biomarker [84]. Quantitative ctDNA detection commonly used digital PCR to quantify ctDNA mutations in plasma. The German researchers used digital PCR to quantify EGFR and KRAS mutations in circulating DNA, and monitored changes in the level of DNA mutations in the plasma of the subjects over time, and confirmed the correlation between ctDNA concentration and tumor progression [85]. Other researches also confirmed these results [86, 87] (**Table 1**).

Conclusions and futures

CTCs and ctDNA detecting are gradually developed to play roles in the diagnosis, treatment, and prognosis assessment of NSCLC. More importantly, CTCs and ctDNA are used to detect the mutation status and gene copy number of the EGFR gene in NSCLC patients which could benefit patients by providing a more convenient and dynamic monitoring for targeted therapy and improving prognosis.

With the development of liquid biopsy, CTCs and ctDNA detection have also received increasing attention in other body fluids (such as urine, cerebrospinal fluid) besides blood, and will better help diagnose and treat NSCLC and prolong the PFS of patients. In the future, further studies would be concentrated on the following aspects: (1) developing more convenient and applicable CTCs and ctDNA detection methods (2) applying CTCs and ctDNA detection in other body fluids samples (3) discovering more driver mutations using CTCs and ctDNA (4) finding more targeted drugs with higher sensitivity and specificity by tracking CTCs and ctDNA (5) further exploration of the relationship between CTCs and ctDNA detection and targeted therapy.

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Disclosure of conflict of interest

None.

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