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Review Article

Applications of liquid biopsy in lung cancer-diagnosis, prognosis prediction, and disease monitoring

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Abstract: Lung cancer is the most common cancer in both the developing and developed countries, which has an unoptimistic outcome. As a result of high rates in mortality and considerable difficulties in treatment, early detection of lung cancer is thought as one of the potential solutions for this stigma. Tissue biopsy has been widely used for cancer diagnosis, but the invasive nature limits their application, especially when repeated biopsies are needed. Liquid biopsy, a minimally invasive procedure aiming to primarily analyze circulating tumor cells (CTCs) and/or circulating tumor DNA (ctDNA) for diagnosing and profiling cancer, has gained interest from oncologists and basic researchers. A great number of achievements in the field of liquid biopsy has been developed, thus liquid biopsy is more feasible in clinical practice than before. More importantly, liquid biopsy is being used, in addition to the diagnosis of lung cancer, to predict prognosis according to genetic alterations and monitor disease based on signature molecular markers. In this review, we briefly summarize techniques in liquid biopsy and focus on its applications in disease diagnosis, prognosis prediction, and condition monitoring of lung cancer.

Keywords: Lung cancer, liquid biopsy, prognosis, treatment responses

Introduction

Lung cancer is the most common cancer in the developing and developed worlds, and the leading cause of cancer-related death. According to the report of GLOBOCAN 2012 [1, 2], it has been estimated that there are 1.8 million (12.9% of all cancer cases) new cases of lung cancer, and nearly 1.6 million deaths related to lung cancer (19.4% of all death cases) in the single year of 2012. The 5-year survival rate of lung cancer patients is between 4 and 17%, depending on different stages and regions [3]. These data indicate a cold fact that lung cancer heavily affects the population health. The major reasons for the poor prognosis of lung cancer are a large fraction of patients are in advanced stages when diagnosed and many of them are lack of therapeutic responses [3]. Symptoms of lung cancer are usually covert and unspecific, which allows few patients to seek medical attention due to symptoms. Currently, the only screening method in effect for lung cancer is low-dose computed tomography [4] recommended by the US Preventive Services Task Force [5], which also requires traditional tissue biopsy to confirm the characteristics of nodules observed by CT scan.

Compared to tissue biopsy, liquid biopsy has many advancements. Liquid biopsy is a collection of methods that are used to enrich, detect, and analyze circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) in cancer patients [6] (**Figure 1**; **Tables 1** and **2**). CTCs migrate from solid tumors and enter the bloodstream due to tumor invasion and shedding, or in response to a mechanical stress such as surgery. It is generally accepted that CTCs are a group of heterogeneous tumor cells derived from primary and/or metastatic tumors. CTCs, presenting at a low level in the circulation, are difficult to isolate and distinguish from the peripheral blood. Current technologies to iso-

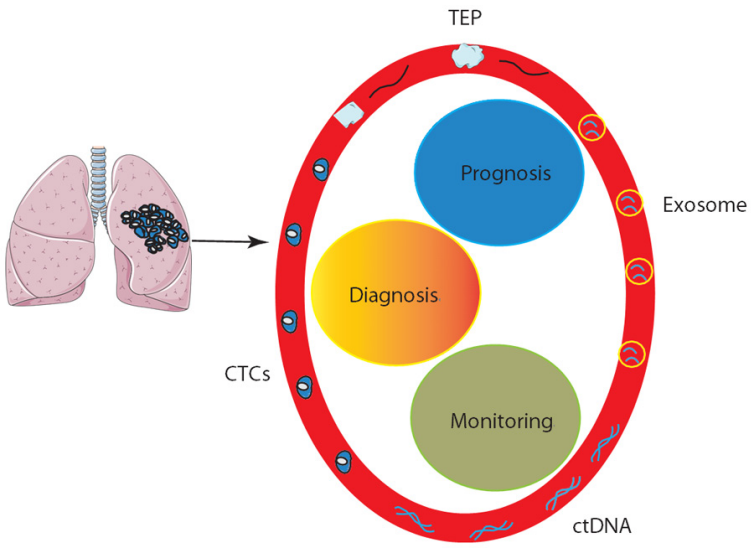

Figure 1. Overview of liquid biopsy in lung cancer. Circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), exosomes, and tumor educated platelets (TEP) can be detected from the blood stream and utilized for diagnosis, prognosis prediction, and disease monitoring of patients with lung cancer.

late CTCs focus on the development of high-efficiency methods to capture pure tumor cell populations across many stages and types of tumor cells [7]. After tumor cell death, DNA released into the blood is referred to as cell-free tumor DNA (cfDNA), among which ctDNA is a component. Circulating DNA levels tend to be higher in patients with metastatic cancers compared to healthy individuals and non-metastatic patients [8]. Levels of cfDNA in the blood are positively correlated with the severity of cancer. The significance of CTCs and ctDNA in predicting prognosis has been demonstrated in some tumors, as CTCs and ctDNA can be used to detect drug-resistant mutations, mainly of which are acquired. ctDNA contains tumor-specific mutations that can be used to determine mutation conditions in tumor DNA. To be succinct, we summarize techniques used in liquid biopsy in **Tables 1** and **2**, and concentrate the values of these methods in disease diagnosis, prognosis prediction, and treatment monitoring.

Applications of liquid biopsy in diagnosis of lung cancer

Liquid biopsy can be used to diagnose lung cancer in patients with high risks. One of advantages of liquid biopsy, compared to tissue biopsy, is that liquid biopsy can be performed repeatedly, especially when a confirmed diagnosis fails to be initially achieved. Ilie et al reported that CTCs were detected in patients with COPD patients when lung cancer was suspected [9]. In this study, patients with COPD were screened annually by low-dose spiral CT, and approximately 3% of these patients (5 of 168 COPD patients) had positive CTCs but normal CT results. The annual monitor of these five patients revealed lung nodules 1 to 4 years after the initial detection of CTCs, leading to surgical resection and histological diagnosis of early lung cancer. More importantly, CTCs failed to be detected in control individuals regardless of smoking or not. These indicate CTCs, at least combined with CT scans, might serve to detect early lung cancer.

cfDNA and circulating miRNA profiles have been tested in values of diagnosing lung cancer. A study involving 60 non-small cell lung cancer (NSCLC) patients and 40 COPD individuals analyzed the diagnostic values of cfDNA (both the short and long fragment) [10], which indicated that levels of cfDNA have an accuracy rate of 92.1% (the long fragment) and 83.6% (the short fragment) in lung cancer diagnosis, respectively. Levels of plasma miRNA expression can be used to predict lung cancer. Wozniak et al [11] analyzed plasma miRNA profiles in patients with stage I-IIIA lung cancer with the comparison with the control individuals, which detected 24 miRNA patterns different between the two groups. In another study [12], levels of miR944 and miR3662 were reported to be at least a fourfold increase in NSCLC patients with respect to healthy control. Moreover, in addition to the overall sensitivity (86%) and specificity (97%), a combination of four miRNAs (miR21, miR126, miR210, and miR485-5p) in the plasma is more sensitive in adenocarcinoma than squamous cell carcinoma (91% v.s. 82%) [13]. These data raise the possibility that circulating miRNA profiles can be combined with CT scans to identify lung can-

Table 1. Methods utilized for analyzing CTCs and ctDNA

Methods		Mechanisms	Applications	Available products or markers
CTC isolation	Positive selection	Selection of epithelial adhesion molecule (EpCAM) positive cells [64, 65]	Magnetic beads, columns, cartridges, magnetic nanoparticles (MNPs) [66, 67], nanoclusters [68], immunomagnetic nanospheres (IMNs) [69, 70], iron oxide magnetic particles [71], lipid microbubbles [72], and microchips [64]	CellSearch® system [73]
	Negative selection	Selection of CD45 negative cells [74, 75]		
	Physical isolation	Selection by the density [76], size [77, 78], and deformability [79, 80] by centrifugation [81], microporous filters [82], microfluidic technology [83], and adhesion-based methods [84]	The microfluid platform [85].	
CTC analysis	Nucleic acid analysis		PCR, qRT-PCR, sequencing, array-comparative genomic hybridization [86]	KRAS, PIK3CA,and APC [86]
	Cell count	To count cell numbers and those that express certain markers.	Cytology, immunocytochemistry, flow cytometry	
ctDNA analysis		To measure levels of ctDNA, and analyze sequences.	Real time PCR [88], droplet digital PCR (ddPCR) [89]; whole genome sequencing (WGS) [90], whole exome sequencing(WES) [91] and targeted region sequencing(TRS) [90, 91]	Loss of heterozygosity, gene amplification, cancer-related viral sequences, hypermethylation in promoters, single-nucleotide mutations

Table 2. Comparisons between CTCs and ctDNA

	CTCs	ctDNA
Concentration in blood	Rare	Rich
Mechanism of release into the blood	Unclear	Unclear
Quantification	Yes	Difficult to do
Detection specificity	Higher than ctDNA	Lower than CTCs
Mutation detection	Yes	Yes
Predict prognosis	Yes	Yes
Monitor disease status	Yes	Yes
Predict treatment response	Yes	Yes
Predict relapse	Yes	Yes
Predict drug resistance	Yes	Yes
Detect molecular markers	Yes	Yes
Detection in CSF	Difficult to do	Yes
Indication of tumor burden and kinetics	Difficult to do	Yes
In vitro culture	Yes	No

cer patients, which has been tested in small cohorts [14]. In the future, values of liquid biopsy in diagnosis of lung cancer, especially during the screening for early lung cancer in the high-risk population, requires well-designed prospective studies involving large cohorts.

Prognosis prediction of CTCs in lung cancer

Values of CTCs in predicting prognosis in lung cancer patients have been appreciated by clinical oncologists (**Table 3**).

Baseline CTC counts

Accurately predicting the prognosis of cancer patients using CTCs has been achieved in several types of tumors. Baseline CTC counts are an important prognostic factor, as patients with CTCs at baseline indicates a poor prognosis. Dedicated studies demonstrating significant correlations between CTC counts and prognosis have been observed in many tumor types, including small cell lung cancer (SCLC) [15] and NSCLC [16]. In a study, the correlation of CTC counts and prognosis in 51 patients with newly diagnosed SCLC was analyzed, where baseline CTCs were detected in 40 patients with a median of 4 CTCs [15]. At baseline, after treatment, and at relapse, patients with a CTC count < 9 (the favorable population), or conversion from a CTC count > 9 (the unfavorable population) to a favorable CTC-count, had better survival rates than those with a CTC count > 9 [15, 17]. Similarly, in NSCLC patients, detection of CTCs at baseline may predict a poorer prognosis when compared to patients without detectable CTCs at baseline [17]. The presence of CTCs in metastatic NSCLC patients and SCLC is associated with poor outcome [18, 19]. Krebs and colleagues [18] also reported that the progression-free survival (PFS) was 6.8 months in patients bearing fewer than 5 CTCs and 2.4 months in those having more than 5 CTCs before chemotherapy (the overall survival: 8.1 v.s. 4.3 months). Similar results were observed in lung cancer patients with radiotherapy [20] and surgery [21].

As disease progress, the positive detection rate of CTCs increases. Thus, CTCs may be used as a factor in staging advanced diseases. A study showed that the positive rates and the range of CTC counts at baseline were 13% and 0-1 among patients with stage IIIA, 20% and 0-16 among patients with stage IIIB, 38% and 0-4 among patients with stage IV, and 60% and 0-67 among naive NSCLC patients, respectively [17].

These results are from relatively small cohorts, but suggest important values of CTCs in disease staging and prognosis prediction. It is also imperative to compare the values of CTCs and the current TNM system in selected lung cancer patients to emphasize CTCs' significance.

Apoptotic CTCs

Apoptotic CTCs are also an independent prognostic factor, as the presence of CTCs with apoptotic nuclear morphology appears to indicate a poorer prognosis [22]. Positive detection of CTC apoptosis was associated with a worse prognosis in SCLC patients with a shorter PFS and OS [23], indicating the importance of CTC apoptosis in predicting the outcome. It is worth noting that the biological functions of apoptotic CTCs have not been elucidated, which warrants investigations in the future.

Table 3. Values of liquid biopsy in lung cancer diagnosis, prognosis prediction, and disease monitoring

	Diagnosis	Prognosis	Disease monitoring
CTCs	Lung cancer patients bear more CTCs	High number indicates poor prognosis	Higher number indicates high risks for recurrence
			KRAS and P53 reemerging indicates recurrence
Apoptotic CTCs		Positive results indicate poor prognosis	
PD-1 on CTCs		Positive results indicate poor prognosis	
ctDNA	SNV detected in early NSCLC patients	High concentration indicates poor prognosis	Determination of mutation patterns for targeted therapy decision making
			High concentration during follow-up predicts recurrence
Exosome-derived miRNA	Potential for early cancer detection		
Secretome	Differential diagnosis of pleural effusion		
TEP-derived mRNA	mRNA profiles for diagnosis	Detection of KRAS mutation, EGFR mutation, MET overexpression	

Programmed death 1 expressed on CTCs

Programmed death 1 (PD-1) is an immune checkpoint that regulates T cell-mediated responses. PD-1 and its ligand, programmed death ligand l (PD-L1), can also be used as a biomarker for prognosis of cancer patients. It has been established that lung cancer patients with high PD-L1 expression had a poor prognosis as tumor cells escaped from immune surveillance by the interaction between PD-L1 expressed by lung cancer cells and PD-1 expressed by tumor-infiltrating dendritic cells [24]. In a study that recruited 24 metastatic NSCLC patients receiving anti-PD-1 antibody Nivolumab, CTCs and surface PD-L1 on CTCs were examined by the CellSearch system. Patients who had detected CTCs and surface PD-L1 expression at baseline and 3 months after treatment had an unfavorable outcome [25]. Controversially, high expression of PD-L1 (more than 50% tumor cells expressed PD-L1) also predicted a good response upon PD-1 blockade (Pembrolizumab) in lung cancer patients [26]. The seeming difference between the above two trials [25, 26] might result from administration of two different types of antibodies, or tumors cells possibly have varied ability to leaving the tumor bed and entering the circulation because of different levels of PD-L1 expression. The further understanding activation of the PD-1-PD-L1-axis will help to unfold the underlying mechanisms.

ctDNA in diagnosis lung cancer

The cellular source of ctDNA can be both healthy and tumor cells [27], but ctDNA from different cell types have varied characteristics. ctDNA from necrotic tumor cells has various sizes, whereas ctDNA released from apoptotic healthy cells has a uniform size between 185- and 200-bp. Since the major origin of ctDNA in healthy individuals is from apoptotic cells, a preponderance of longer DNA fragments can be considered as an indication for tumors [27]. It has also been evident that tumor patients have increased levels of ctDNA compared to healthy volunteers [28, 29]. In a pilot study analyzing ctDNA profiles of lung cancer patients [30], at least two single-nucleotide variations (SNV) in ctDNA were observed in 46 out of 96 (48%) early-stage NSCLC patients, in addition to a single SNV detected in 12 cases. These valuable data indicate that profiling ctDNA can be used to screen early lung cancer.

Liquid biopsy as a means to monitor disease

CTCs to monitor disease recurrence

Wu and colleagues investigated the association between CTC counts and cancer recurrence in untreated lung cancer patients including adenocarcinoma, squamous cell carcinoma, and SCLC [31], where patients with recurrence were found to bear higher levels of CTCs at diagnosis. Reappearance of tumor-specific mutations in the circulation including KRAS and P53 indicates tumor recurrence in lung cancer patients [32]. These data highlight the importance of CTC counts for predicting recurrence. However, studies of larger scales are warranted.

ctDNA predicts treatment response

ctDNA can be used to determine mutations in lung cancer patients. A technique called “cancer personalized profiling by deep sequencing (CAPP-Seq) has been utilized in lung cancer patients [33], which has an ability to detect various types of somatic mutations. The CAPP-Seq technique also applies to ctDNA. However, as the concentration of ctDNA is low in early-stage lung cancer patients, only approximately 50% of early lung cancer patients harbor ctDNA [6]. Therefore, the value of the CAPP-Seq technique in detecting early lung cancer patients faces challenges. An increased copy number of the MET gene was reported to be determined in lung cancer patients during targeted therapy treatment [34]. It was reported that a microsatellite alteration was detected in the plasma of 71% (5 out 21) patients with small cell lung cancer [35].

According to the mutation patterns, an appropriate targeted therapy can be applied to a certain patient. Amplification of MET genes by determining the copy number in the plasma predicts treatment responses to anti-EGFR therapy in lung cancer patients [36]. The T790M mutation in the membrane receptor EGFR indicates therapeutic resistance to gefitinib and erlotinib in approximately 50% of lung cancer patients [37]. More importantly, the T790M mutation can be also observed in the

plasma of recurrent patients [38, 39], indicating the feasibility of monitoring treatment resistance by liquid biopsy. The T790M substitution increases the affinity of EGFR for ATP, therefore, inhibits the binding of the inhibitors during targeted therapy [40]. The detection of this mutation has clinical significance, as newer inhibitors (such as the third-generation inhibitor WZ4002) works effectively when the T790M mutation is evident [41]. This sheds a light that it is possible to use liquid biopsy to select a treatment regime in lung cancer patients with certain mutations.

Unbiased approaches can also be applied in liquid biopsy, where genetic mutation markers for treatment resistance can be detected in the blood of cancer patients [42]. It has been reported that the T790M mutation of EGFR was detected in the plasma of a lung cancer patient treated by gefitinib by exome sequencing [43].

ctDNA predicts risks of recurrence

Sozzi and colleagues [32] explored whether the concentration of ctDNA could be used to predict recurrence in patients with NSCLC. Patients with recurrence harbored with higher levels of ctDNA during follow-ups compared to those without regression (34 ng/ml v.s. 345 ng/ml, $P < 0.001$). More importantly, relapse-free patients showed a trend of reduction of ctDNA levels with time, indicating the dynamic changes of ctDNA has the potential to monitor disease recurrence.

Other markers with predictive values for prognosis of lung cancer patients that can be detected by liquid biopsy

Exosomes are small membrane vesicles originated from cells and are detected in almost all biological fluid, including urine, blood, ascites, and cerebrospinal fluid [44]. The size of exosomes is between 30 and 100 nm. Exosomes are composed of a phospholipid membrane, where proteins involved in membrane transport and fusion, endosomal sorting complex, and tetraspanins are also present, therefore exosomes have an important role in cell-to-cell communication via activating the expression of ligands or transferring signal molecules. Exosomes also affect cancer growth by modulating the local and systemic environment or manipulating the immune system [45]. The profiling of microRNA (miRNA) is significantly different between the primary lung cancer tissues and corresponding normal tissues, which makes miRNA a potential diagnostic marker [46]. It has been reported that exosomes are rich in genetic materials (including miRNA) and protein, and exosomes can transfer genetic information between cells [47]. In a dedicated study performed by Rabinowits and colleagues [48] indicated miRNA derived from circulating exosomes and those collected from lung cancer had a similar profile, indicating the possibility to utilizing circulating exosomes and containing miRNA profiles to detect lung cancer. Moreover, miRNA in exosomes have the capacity to switch the host to a prometastatic phenotype [49].

The secretome was first discovered in a study of proteins secreted by Bacillus subtilis [50], and this concept has been extended to the collection of proteins secreted by a cell, tissue, or organism [51], including the context of cancer [52]. Secreted proteins are involved in many processes related to cancer, such as angiogenesis, differentiation, invasion, and metastasis. Patients with NSCLC occasionally suffer the malignant pleural effusion that has been studied as a source of biomarkers for lung cancer patients [53]. However, the significance of secretome in the pleural effusion of lung cancer patients, and whether a certain protein present in the pleural effusion can be also detected in the circulation, warrants further studies.

Tumor educated platelets [46] have gained interest from researchers in oncology. Confrontation between tumor cells and platelets results in transfer of biomolecule to TEP [54]. Upon activation, platelets undergo specific splicing of pre-mRNAs [55], which leads to a unique mRNA profile that can serve as a means for cancer diagnosis [56]. A study profiling mRNA derived from TEP of cancer patients demonstrated 20 non-protein coding RNAs had altered levels with respect to those from healthy individuals [54]. Moreover, 1453 mRNAs were increased and 793 mRNAs were decreased in TEP of cancer patients comparing to healthy control, which allowed to detect KRAS mutation, EGFR mutation, and MET overexpression. Although roles of TEP and mRNAs derived TEP in lung cancer in terms of diagnosis and prediction have not been fully tested yet, it is worth to warrant a thorough trial in the near future.

Perspective

On May 23rd 2017, the US Food and Drug Administration (FDA) approved pembrolizumab, a PD-1 inhibitor, to treat unresectable or metastatic solid cancers with high microsatellite instability or mismatch repair gene deficiency in adult and pediatric patients, regardless of tumor location [49]. This is the first approved medication for cancer therapy targeting gene mutations rather than tumor sites based on previous encouraging results [57, 58]. Although the FDA has not approved a method to detect mismatch repair gene deficiency or microsatellite instability, it is reasonable to speculate that liquid biopsy can be performed to detect mismatch repair gene deficiency in ctDNA or microsatellite instability in CTCs collected from the blood of lung cancer patients to predict treatment responses upon PD-1 blockade. In other cancers like melanoma, results from mouse experiments and human trial indicated that treatment response upon PD-1 blockade relies on the composition of the microbiome in the small intestine and colon [59-62]. Bacterial 16S rRNA is used for bacterial identification and can be detected in the circulation of individuals [63], thus it is possible to use liquid biopsy methods to determine bacterial 16S rRNA to predict the presence of bacterial strains that can predict treatment response upon PD-1 blockade. In the future, investigations are required to test these speculations in lung cancer patients.

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Disclosure of conflict of interest

None.

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