Review Article

Latest development of liquid biopsy

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Abstract: Liquid biopsy provides the opportunity of detecting and analyzing cancer in various body fluids. In peripheral blood, apart from circulating cell free DNA, circulating cancer cells and other tumor-associated compounds such as extracellular vesicles are also emerging candidates for detection. Compared to conventional tissue or cytology samples, liquid biopsy is non-invasive, safe, and easy to repeat. In view of tumor heterogeneity, it is also suggested that circulating cell free DNA may be more representative of the whole tumor cells population than a biopsy or cytology sample. In addition to assisting in the initial diagnosis, liquid biopsy can also be tailored for disease monitoring, detecting resistance mutation, tumor recurrence, and perhaps for screening in the future. The accuracy of this test is greatly facilitated by the advances of molecular techniques, from PCR-based methods, DNA sequencing, Digital PCR, to the more state-of-the-art next generation sequencing technologies. Despite the tremendous potential of liquid biopsy, there are limitations and not all clinical relevant cancer biomarkers can be detected in liquid biopsy at the present moment. The clinical utility of many of the tests derived from liquid biopsy required further investigations and clinical validation. This review provides an overview of the concept of liquid biopsy, its clinical applications, and discuss the multifaceted advances in this field.

Keywords: Liquid biopsy; lung cancer; personalized medicine

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Introduction

Biopsy is obtaining tissue samples from a lesion for detecting a pathological process. Traditionally, biopsy could be classified as needle biopsy, incisional and excisional biopsy. Taking tissue biopsy is thus generally an invasive procedure. Aspiration cytology is a less invasive procedure to obtain cytology samples from the pathological lesion. With the advance of personalized cancer therapy, samples obtained from the cancer tissues are many a times not only used for diagnostic purposes, but also for detecting predictive biomarkers. Thus, there is increasing demand to obtain adequate sample for detection of the ever expanding repertoire of biomarkers. Furthermore, when patients develop resistance to targeting therapy, obtaining additional cancer samples, preferably from the resistance clone, is critical to delineate the resistance mechanism in guiding appropriate treatment. However, obtaining tissue or cytology sample may often require invasive procedures or may be practically impossible.

The advance in liquid biopsy allows an alternative and attractive non-invasive procedure to obtain biomaterials from cancers for diagnosis. Liquid biopsy would be defined as obtaining circulating cancer cells, tumor-derived cell free DNA (cfDNA) or other compounds in body fluids. The body fluids not only include peripheral blood, but also others, like urine, cerebrospinal fluid, or effusion fluids. However, it is most commonly refer to peripheral blood,

Table 1 Clinical applications of detection of different cancer-derived molecules or cells in liquid biopsy

Cancer-derived molecules/cells	Clinical applications	Reference
Circulating tumor cells (CTCs)	Cancer monitoring by quantification of CTC	(7,8)
Tumor-derived cell-free DNA	Screening of gastric and colorectal cancers by detection of methylated cell-free DNA in serum	(9,10)
	Screening of EBV-associated cancers	(11-13)
	Detection of actionable mutations in tumor-derived cell-free DNA for treatment selection	(14-22)
	Tracking progression and clonal evolution of cancers	(23-26)
Tumor-derived cell-free miRNA	Plasma or serum miRNA for detection of cancers	(27,28)
Extracellular vesicles	Detection of tumor-derived extracellular vesicles by surface markers in serum of colorectal cancer patients	(29)

and the current review is focused and limited to this scope.

Although the application of liquid biopsy into clinical use in cancer patients is relatively recent, the concept of its utility in cancer diagnosis has been there for many years. Elevated DNA levels were detected by radioimmunoassay in cancer patients in 1977 (1). A 1983 study determined that elevated DNA levels coupled with high circulating carcinoembryonic antigen (CEA) may be useful to diagnose gastrointestinal tract cancer (2). In this review, we discuss the current understanding and latest advances of this diagnostic method.

Traditional biopsy is limited by the amount of the tissue that can be sampled, which in turn is limited by the size of instrument used and the areas of sampling that can be achieved. Counterintuitively, a fine needle aspiration biopsy may be able to yield more diagnostic materials than a thick bore core needle because the fine needle can enter the suspicious mass in different angles, such that more parts of the lesion can be sampled. Apart from the invasiveness of these procedures, intra-tumoral heterogeneity is always a potential issue on the accuracy of a tissue biopsy (3-6). Any parts of the tumor can in theory shed materials into the bloodstream, be they nucleic acid, protein, secretory vesicles (such as exosomes), or tumor cells (*Table 1*). Thus, it is suggested that liquid biopsy may provide an even more representative sampling of the biomaterials from cancer (30).

Liquid biopsy in detecting tumor-derived circulating cfDNA—diagnostic potential

It was hypothesized that circulating cell-free DNA (cfDNA) is produced by tumor cell apoptosis or necrosis (31). The concept of detecting tumor-derived DNA in peripheral blood is not entirely new. For examples, the detection of circulating gene promotor methylation in serum of gastric and colorectal cancer patients has been reported (9,10). In addition, tumor-associated virus could also be a useful biomarker for cancer detection. For example, detecting circulating Epstein-Barr virus (EBV) DNA in blood has been established as a tumor marker for nasopharyngeal carcinoma (NPC) (11). In the context of lung cancer, similar to NPC, high level of circulating EBV DNA could be detected in lymphoepithelial-like carcinoma of lung, a rare lung cancer subtype (12,13). These suggest that tracing the circulating cell-free viral DNA may be useful in cancer screening and detecting cancer recurrence.

With the advances of cancer targeting therapy, the current interest is focusing on detecting critical gene mutations, be they driver mutation, resistance mutation or mutations that may be potentially actionable. While liquid biopsy has an advantage of being safe and easy to collect, the level of tumor-derived DNA is usually very low. In this regard, the major hurdle in liquid biopsy lies not in sampling but in the sample analysis. To differentiate circulating tumor DNA from DNA derived from normal cells, sensitive methods are required to detect cancer-specific genetic aberrations (*Table 2*).

Allele-specific PCR [also known as amplification refractory mutation system (ARMS)], digital PCR or quantitative PCR are examples (37). These methods require the mutations to be well characterized, so that allele-specific primers (as in ARMS) or probes (as in digital PCR) can be designed specifically for the mutation at the specific locus. There were several attempts to detect mutations in cfDNA on various cancer types, such as *KRAS* (e.g., G12V, G12C, G12V, G13D) and *BRAF* (V600E) in colon cancer (23,38-40), or *PIK3CA* (E545K, H1047R, H1047L) in breast cancer (14). However, most of the developments

Table 2 Detection platforms for tumor-derived cfDNA in liquid biopsy

Platforms	Advantage	Disadvantage	References
Single gene platform			
Real-time PCR	Fast turn-around-time	Fairly sensitive. Difficult to detect <1% mutation level	(14-16,32,33)
	Streamlined workflow on validated platform	Only detect limited targets per assay	
Digital PCR	Highly sensitive	In house-developed assay needs full validation	(17,18)
	Low running cost	Only detect limited targets per assay	
Panel-based platform			
Next-generation sequencing	Cost-effective. Screen of mutations in a panel of genes in a single assay	Long turn-around-time due to complex procedure	(34-36)
	Highly sensitive	Need to develop special data analysis pipeline for variant calling	
		High running cost	

were research-based and extensive validation was needed before putting them into clinical use. The first FDA approval on cfDNA-based liquid biopsy only occurred relatively recently, in June 2016 (32). This allele-specific PCR test was developed by Roche (Cobas cfEGFR Test) for detection of 42 *EGFR* mutations in peripheral blood for non-small cell lung cancer (NSCLC). *EGFR* mutation is the first actionable mutation discovered in NSCLC, in particular adenocarcinoma (ADC) that can be targeted by small molecule inhibitors. The prevalence of *EGFR* mutation varies among ethical and geographic group and being much higher in Asian population, it accounts for up to 40% to 60% in Lung ADC in Asian countries (33). Comparing to tissue genotyping result, the specificity for this blood *EGFR* test ranges from 82% to 96%, and the sensitivity ranges from 60.7% to 76% (15,16). However, the sensitivity in detecting T790M is lower (15,16). The reason for this lower sensitivity in detecting T790M mutation is not entirely clear. However, it may be attributed to that only a subpopulation of cancer cells harbors this resistance mutation (41).

To improve the sensitivity of detecting tumor-derived DNA in blood, digital PCR platform has been investigated. It could be chip-based or droplet based. The specificity for *EGFR* L858R and 19del mutation detection is up to 100% while the sensitivity ranges from 74% to 82%, depending of the mutation types (17). The sensitivity in detecting T790M is improved in this platform with a 77% sensitivity (17).

Apart from *EGFR* mutation, liquid biopsy for *BRAF* mutation is also under active investigations. This is particularly relevant to lung cancer as *BRAF* inhibitors are available for treatment and have been approved for treating metastatic non-small cell lung cancer with V600E mutation (42). *BRAF* mutation detection in the peripheral blood can be achieved by digital PCR (18).

With the advances of personalized molecular targeting therapy, one could anticipate more actionable gene mutations to become potential candidates for liquid biopsy detection. Thus, a gene panel testing becomes an attractive approach. A number of next-generation sequencing based gene panels are currently available for tissue samples (34-36). Such gene panel approach is also applicable in the setting of liquid biopsy (19). However, the sensitivity is still relatively low.

Liquid biopsy in detecting tumor-derived circulating cfDNA—disease monitoring and detecting recurrence

When a mutation can be characterized for the primary cancer, the detection of such a specific mutation for a particular patient may be utilized as disease monitoring or detecting recurrence. This personalized medicine approach of cancer patient follow-up is exemplified by several studies (24-26,37). Diehl *et al.* reported that detection of *APC*, *KRAS*, *PIK3CA*, and *TP53* mutation in cfDNA in post-operative plasma in colorectal cancer patients who had undergone surgery was highly predictive of recurrence (24). According to Garcia-Murillas *et al.*'s study, for breast cancer patients, using mutation-specific digital PCR in tracking mutation in follow-up blood samples was useful in predicting early relapse (25).

Liquid biopsy in detecting tumor-derived circulating cfDNA—implication in cancer screening

Recently, a cancer screening algorithm was developed to detect eight common cancer types, by using a panel of 16 genes, such as *EGFR*, *APC*, *KRAS*, and *TP53* (43). After an initial round of gene amplification, the samples were subjected to parallel sequencing with next generation sequencing platforms. Together with proteomics information, the algorithm was able to detect cancer with 69–98% sensitivity, with very low false negativity (7/812 subjects). The authors reasoned that the performance and the relatively low cost were major advantages of this strategy. Understandably, the relative small number of genes being tested and the overlapping spectrum of mutations that can occur in different tumors made it difficult to ascertain the causative cancer type. The authors resolved this issue by calculating the probability of each cancer type that the patient may have, by taking into account of the mutation type, patient age and sex, and other serological markers such as CEA. Although intriguing, the corollary of this strategy is that apart from molecular testing, clinical information and patient demographics are still indispensable in arriving a possible correct diagnosis.

For cancer screening, one concern is that being increasing in sensitivity, screening assays may eventually pick up mutant DNA which are derived from pre-invasive lesion, benign or indolent tumor, which may not manifest or affect an individual within his/her life span. Detecting these lesions can result in unnecessary anxiety or series of investigations for an individual. Therefore, as a screening test, it will require stringent evaluation.

Recent guidelines for the role of testing cfDNA in lung cancer patients

The College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology published a guideline for the selection of lung cancer patients for treatment with targeted tyrosine kinase inhibitors (TKIs) (30). It recommends that physicians may use plasma cfDNA methods to identify *EGFR* mutations in lung adenocarcinoma patients when insufficient tissue is obtained. It also supports the use of plasma for *EGFR* T790M mutation test in lung adenocarcinoma patients with progression or secondary clinical resistance to *EGFR*-targeted TKIs. When the testing of *EGFR* mutation in plasma is negative, testing the tumor sample is recommended. However, there is currently insufficient evidence to support the use of liquid biopsy for the diagnosis of primary lung adenocarcinoma by the identification of *EGFR* or other mutations.

Thus, the practical usefulness of *EGFR* mutation detection in liquid biopsy is "rule in" targetable mutations when tissue sample is limited or hard to obtain. When tissue biopsy materials are insufficient for molecular testing, cfDNA-based tests for patient selection for targeting therapy are possible (20,21,30). Furthermore, when biopsy tissue is inadequate or not available, detection of resistance mutation T790M in liquid biopsy may allow the consideration of using third generation TKI (22).

Liquid biopsy is not limited to cfDNA

miRNA

Detecting other circulating tumor-derived nuclear acid is also under investigations. For example, elevated levels of several microRNAs have been described in different cancers (27,28). However, the clinical unity of these approaches remains to be further evaluated.

Extracellular vesicles

Although it was discussed at length that cfDNA is a promising tumor marker for liquid biopsy, more candidates are emerging, such as extracellular vesicles (EV) which are secreted by tumor cells into the bloodstream (44-46). These membrane bound substances can be technically challenging to detect, but recent research has indicated that their detection may be simplified by using antibody-based assays, such as CD147 and CD9 for colorectal cancer (29).

Circulating tumor cells (CTCs)

It has long been discovered that intact tumor cells were shed into the blood-stream by the main tumor bulk (47). These cells can be isolated by means of density gradient centrifugation, size centrifugation, fluorescence-assisted cell sorting, or antibody-conjugated microfluidic devices, etc. (48). The detection of the amount of CTCs has been shown to have prognostic value in patients with metastatic breast cancer (7). Based on the clinical finding, Cellsearch CTC test (Menarini Silicon Biosystems, San Diego, CA, USA) has been approved by FDA for monitoring metastatic breast cancer (8). However, the techniques of isolating CTCs are still cumbersome and not easily adapted in most laboratories. Thus, its clinical utility will require further investigations and verification.

Limitations and challenges in liquid biopsy

While promising, limitations in liquid biopsy do exist. Apart from the sensitivity issues, there are other technical challenges. For example, gene translocations are particularly difficult to detect. The fragment length of cfDNA in blood can be quite short to span translocation breakpoints and make detecting gene translocations difficult (49,50). Detecting gene copy number variation in cfDNA is limited to high copy number amplification (51). For NSCLC patients with *EGFR* mutation who developed acquired resistance to the first or second generation TKIs, T790M mutation accounts for only about half of the resistance mechanisms. Other resistance mechanism, like small cell transformation (52) may not be readily detectable from liquid biopsy.

Emerging immunotherapeutic options such as immune checkpoint blockade may require tissue assessment of PD-L1 expression for patient selection (53). This could be beyond the capability of liquid biopsy at the present moment. Further investigations in the assessment of PD-L1 RNA expression, protein expression in CTC or estimation of tumor mutation burden in liquid biopsy may shed light on predicting the response of cancer patients to immunotherapies.

Conclusions

Knowledge on tumor biology and treatment advance constantly shape our diagnostic approach in cancer patients. There is increasing demand for detecting various biomarkers in tumor samples. Coupled with the technological breakthrough, it is now feasible to detect circulating tumor-derived cfDNA, tumor-derived compounds or cancer cells in peripheral blood. It opens the possibility of non-invasive, safe and easily repeatable testing platform in detecting cancer. It may serve as an attractive alternative approach in aiding diagnosis, disease monitoring, recurrence and detection of resistance mechanism in guiding clinical management. However, there are still limitations and challenges ahead. Further investigation, technological advancement, and clinical validation are mandatory to explore the clinical utility of liquid biopsy.

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Footnote

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