Liquid Biopsy to Characterize Cell-Free DNA in Cancer Detection and Monitoring

Nguyen Ngoc Tran

Department of Computational Biomedicine, Vingroup Big Data Institute, Hanoi, Vietnam

Email: v.trannn3@vintech.net.vn

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Abstract: Liquid biopsy, a concept introduced approximately a decade ago, refers to noninvasive approaches that have become the focus of biomedical research. In clinical oncology and research, the term liquid biopsy is used in a broad sense as the sampling and analysis of analytes including cell-free DNA from various accessible biological fluids for diagnosis, prognosis and prediction of a therapeutic response. This technology has the potential to be used in tracking the genomic evolution of tumors over time. It may also have therapeutic implications in terms of its ability to detect actionable events or resistant subclonal populations while avoiding the need to conduct repeated biopsies. This paper briefly reviews the major advances in liquid biopsy assay technologies and discusses the types of cancers that most likely benefit from early detection.

Keywords: *Cell-free DNA, liquid biopsy, cancer detection.*

I. Introduction

Cancer has a significant impact on public health worldwide. One approach to lower its burden is through cancer screening and early detection. It is well established that patients have a higher cure rate and 5-year survival if diagnosed at early stages [1].

Tissue biopsy is the most widely-used tool for cancer detection, staging, and prognosis, but sometimes tumor tissues can be challenging to acquire. Moreover, it is impractical to use tissue biopsy for cancer screening and early diagnosis when the tumors are non-existent. Currently, several methods have been proved useful for cancer prevention such as mammography, Pap test and colonoscopy [2]. However, these screening methods have limited sensitivity and specificity, as well as limited applicability to certain cancer types. In order to perform large-scale cancer screening among healthy individuals, a more general and cost-effective approach is crucial.

In recent years, the rapid development of next-generation sequencing (NGS) technologies has led to a significant reduction in sequencing cost with improved accuracy. In the area of liquid biopsy, NGS has been applied to sequence circulating analytes. Analytes in the blood include cell-free DNA (cfDNA), which in cancer patients contains circulating tumor DNA (ctDNA), circulating extracellular vesicles (*e.g.*, exosomes, proteins, and metabolites) [3]. Collectively, these analytes can provide information about features of primary tumors or metastases for screening and early diagnosis of cancer (Figure 1).

In addition to the information about genomic mutations and copy number alterations that is usually obtained from cfDNA, liquid biopsies are increasingly being used to generate information about the transcriptome, the epigenome, the proteome, and the metabolome. Among all analytes, cfDNA has the potential to revolutionize detection and monitoring of tumors. However, the methods reported to date have been limited by modest sensitivity, applicability to only a minority of patients, and the need for patient-specific optimization. To overcome these limitations, several new strategies have been developed for the analysis of cfDNA.

II. Cancer Types that Benefit from Early Detection

1. Pancreatic Cancer

Pancreatic cancer patients will most likely benefit from early detection by liquid biopsy. It is estimated that in 2019, about $57,000$ people will be diagnosed with pancreatic cancer, which has fewer than 9% of patients surviving 5 years after diagnosis [4]. With no biomarkers for early detection, the deadly nature of pancreatic cancer is largely the result of late onset of symptoms when the cancer has already metastasized. Chemotherapy for pancreatic cancer involves conventional cytotoxic drug combinations but only extends life by clinically unimpressive margin [5].

The prevalence of pancreatic cancer is low in asymptomatic adults. Thus, an effective screening test for pancreatic cancer would have to be highly specific to achieve a

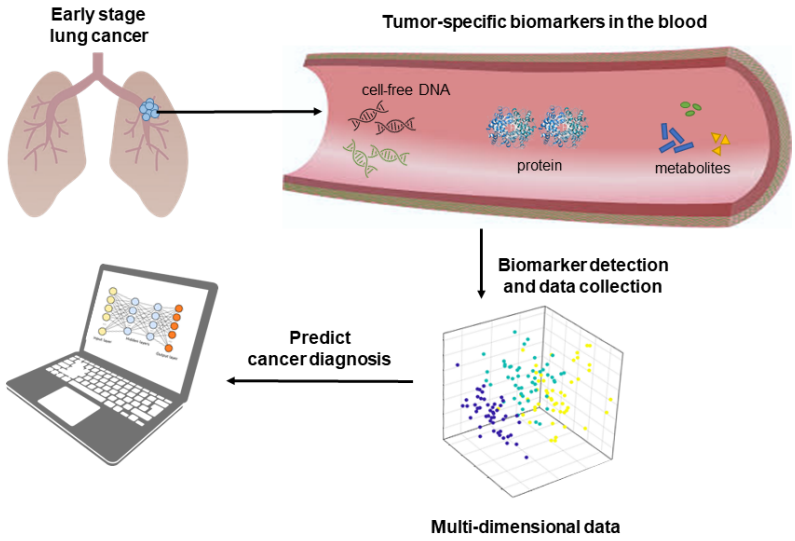

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Figure 1. Liquid biopsies track individual's plasma for biomarkers such as DNA alterations, proteins, and metabolites released by tumor cells. Once these biomarkers are analyzed, the data are used to train machine-learning algorithms to distinguish between cancerous and noncancerous blood samples.

reasonable positive predictive value and to minimize the incorrect identification of healthy individuals. Currently used circulating biomarkers for pancreatic cancer, such as CA19-9, lack adequate sensitivity and specificity for early diagnosis when used alone. Additional biomarkers, including CA125 and LAMC2 have been investigated *in silico* as combinatory diagnostic strategies to CA19-9 with varying degrees of success [6]. The diagnosis and molecular analysis of pancreatic cancer may involve fine-needle aspiration or core needle biopsy. Yet due to the location of the pancreas and expense, repeated biopsies for longitudinal analysis are generally avoided [7]. Therefore, using tissue biopsies to longitudinally monitor pancreatic cancer is infeasible, whereas liquid biopsies provide a new opportunity for molecular profiling of the genetic landscapes of pancreatic cancer throughout disease progression.

The study of cfDNA in pancreatic cancer started in the 1980s, when a high level of cfDNA (> 100 ng/ml) was detected in 90% of pancreatic cancer patients [8]. Later on, KRAS mutations in the cfDNA of plasma from three pancreatic ductal adenocarcinoma (PDAC) patients were detected by PCR [9]. More recently, several groups have attempted to validate the clinical utility of mutation detection in the cfDNA of pancreatic cancer patients. For instance, Kinugasa *et al.* [10], detected KRAS mutations in 62.5% of serum samples from pancreatic cancer patients and found that these mutations were correlated with worse overall survival (OS). By ddPCR, Sausen *et al.* [11] detected KRAS mutations in the plasma cfDNA of 43% patients with early-stage pancreatic cancer. Moreover, using matched plasma samples before and after operations from 59 pancreatic cancer patients, Lee *et al.* [12] was able to detect KRAS mutations from 38 out of 42 (90.5%) patients. The ctDNA detected prior to operation was correlated with worse median recurrence free survival (RFS), 10.3 months versus not reached and after operation was 5.4 months versus 17.1 months. These data suggest that cfDNA is a promising prognostic biomarker for early diagnosis of pancreatic cancer.

2. Lung Cancer

Lung cancer is the most common cancer worldwide, accounting for 2.1 million new cases and 1.8 million deaths in 2018 [13]. More than half of people with lung cancer die within one year of being diagnosed [14]. When the cancer is detected at a localized stage (I–II), the 5-year survival rate is about 56% [4]. However, only 16% of lung cancer cases are diagnosed at an early stage. For advanced and metastatic tumors (stage IV), the 5-year survival rate is only 5% [15]. Therefore, it is imperative to identify diagnostic methods for early detection of lung cancer, enabling a timely treatment plan while potentially reducing healthcare costs [16].

One of the earliest and best examples is the use of cfDNA testing to identify the emergence of the epidermal

growth factor receptor (EGFR) T790M gatekeeper mutation after EGFR inhibitor therapy in *EGFR* mutant non–small-cell lung cancer. EGFR tyrosine kinase domain mutation status was strongly associated with the responsiveness to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI). Several studies have shown that *EGFR* mutations were detectable in serum samples obtained from patients with NSCLC. The *EGFR* mutation status in serum detected by different methods was correlated statistically with responsiveness to, and the progression-free survival of EGFR-TKI treatment [17, 18]. Indeed, the cobas *EGFR* Mutation Test can identify T790M and other *EGFR* driver mutations in plasma and has been approved by the Food and Drug Administration as a companion diagnosis for choosing specific *EGFR* therapies [19].

KRAS is an important molecule in the downstream signaling network of EGFR. The mutation rate of *KRAS* is about 15% to 30% in NSCLC. However, the occurrences of *KRAS* and *EGFR* mutations are often mutually exclusive and patients with *KRAS* mutations are found to be nonresponsive to EGFR-TKIs [20–22]. It has been determined that lung cancer with *KRAS* mutation may be resistant to EGFR-TKI and that *KRAS* mutation in plasma DNA correlates with the mutation status in the matched tumor tissues of patients with NSCLC [23, 24]. However, at present, no mature technology exists for early cancer detection. Such an approach would require a highly sensitive method in order to detect trace amounts of cfDNA released by preneoplastic lesions. It would also require high specificity to minimize false positive results in the large unaffected population undergoing screening. Also, even though we have made considerable progress in finding therapeutic biomarkers, less progress has been made to identify patients who are likely to have relapse after surgical resection.

One key study has shown that the postoperative detection of tumor-specific mutations in cfDNA can predict residual disease and tumor relapse in lung cancer [25]. This approach has the potential to become a critical tool in the postoperative management of the care of patients with cancer and is currently being tested in prospective clinical trials that will assess the usefulness of residual postoperative cfDNA detection to guide adjuvant chemotherapy.

3. Liver Cancer

Liver cancer ranks the fourth leading cause of cancer-related death worldwide. There are more than 841,000 patients diagnosed with liver cancer globally with the five-year survival rate of 18% [26]. Currently, surgical resection or liver transplantation is the primary therapy for liver cancer patients. Unfortunately, the majority of patients at the time of first liver cancer diagnosis, have already reached an advanced cancer stage, and less than 30% of the patients are eligible for surgical intervention [27]. The fact that the only clinically accepted molecular assay for detection of liver cancer is serum alpha-fetoprotein levels makes it critical to find a novel method to detect early liver cancer [27].

In liver cancer, the molecular pathogenesis is tremendously complex and heterogeneous. Thus, analysis of therapeutic targets and drug resistance-conferring gene mutations from ctDNA released into the circulation contributes to a better understanding and clinical management of drug resistance in cancer patients. Studies have found that cfDNA concentration in serum or plasma of liver cancer patients is 3-4 times higher than in patients with chronic hepatitis and is up to 20 times higher than in healthy individuals [28, 29]. Ng *et al.* demonstrated that a number of somatic variants can be reliably detected in plasma but can only be found by interrogation in the biopsy counterparts, supporting the notion that genetic analysis of a single diagnostic biopsy may not be representative of the disease [30].

In a small number of liver cancer patients, ultra-deep sequencing of DNA from plasma was able to detect variants on commonly mutated genes in HCC including *TERT*, *JAK1*, *CTNNB1*, *BRAF* and *TP53* [31]. Another study in advanced liver cancer demonstrated that serial assessment of alterations in cancer-related driver genes using cfDNA NGS can reveal genomic changes with time. The concordance levels between genomic alterations found in plasma and tissue were high for the three most commonly altered genes, *TP53*, *CTNNB1*, and *ARID1A*, indicating that liquid biopsy is relatively accurate compared to traditional biopsy in monitoring of advanced liver cancer patients [32]. Cai *et al.* demonstrated that both single nucleotide variants (SNVs) and copy number variants (CNVs) of cfDNA found in plasma samples can quantifiably reflect the tumor size of liver cancer patients and may be prognostic [33]. However, even though SNVs are a more sensitive measurement than CNVs, they failed to detect tumor burden when the tumor fraction of cfDNA is relatively low [33].

III. Advances in the Detection of cfDNA

Recent research has positioned the detection of genetic alterations in the cfDNA at the frontline of cancer management. In this part of the article, we discuss contemporary NGS approaches to cfDNA analysis (Table I). Here, we explain the technical and analytical challenges to low-frequency mutation detection in the context of early cancer detection. An overview of the effects of biological noise on the detection of low frequency mutations in plasma cfDNA is provided.

TABLE I
ASSAYS DEVELOPED FOR THE EARLY DETECTION OF CANCERS

Assay	**CAPP-Seq**	**TEC-Seq**	**TRACERx**	**CancerSEEK**
Technique	Deep sequencing	targeted sequencing	multiplex PCR	multiplex PCR
Application	cfDNA	ctDNA	ctDNA	ctDNA and protein
Panel size	128 genes	58 genes	61 ROI (16 genes)	Median of 18 patient specific SNVs
Technology	NimbleGen SeqCap	Agilent SureSelect	Signatera	Safe-Seq
Year published	2014	2017	2014	2018
Citation	[34]	[35]	[36]	[37]

TABLE II
ABBREVIATIONS FOR THE TERMS USED IN TABLE I

Abbreviation	**Definition**
CAPP-Seq	Cancer Personalized Profiling by deep Sequencing
ctDNA	circulating tumour DNA
ROI	Regions Of Interest
Safe-Seq	Safe-Sequencing system
SNV	Single-Nucleotide Variant
TEC-Seq	Targeted Error Correction Sequencing
TRACERx	Tracking Cancer Evolution Through Therapy

1. CancerSEEK

The recent paper by Cohen *et al.* [37] addresses the possibility of detecting solid tumors at an early stage with a combined assay for genetic alterations and protein biomarkers (Figure 1). The approach is simple and cost-effective, potentially resulting in significant time savings for diagnosis, and improved patient survival. Although solid outcome has not yet been achieved, this manuscript advances the field of early cancer detection.

In the original study, the patient cohort included 1,005 participants diagnosed with non-metastatic forms of one of eight cancer types (ovarian, liver, stomach, pancreatic, oesophageal, colorectal, lung and breast). CancerSEEK had a median sensitivity of 70% among the eight cancer types with a specificity of 99% [38]. To achieve this, the test was developed with rigorous statistical methods to ensure the accuracy. The most important attribute to the algorithm was the presence of cfDNA mutation followed by the elevation of cancer antigens (including CA-125 and HGF) [38]. Since the driver gene mutations are usually not tissue specific, the majority of localization information was derived from protein markers. The accuracy of prediction was highest for colorectal cancers and lowest for lung cancers [37].

The study design is perhaps the weakest point of the paper. First, the study analyzed the plasma of a comparatively small number of healthy controls without matched controls for inflammatory lesions that could imitate the disease process. Second, among eight chosen cancer types, the detection capability of the test was 100% for ovarian cancer but only reasonable for other common cancers, such as breast and lung cancers, which led to another main caveat of the test. The predictive value of the test which relies on the prevalence of the disease within the tested population. The prevalence of the chosen cancers in healthy individuals over age 64 is approximately 1% [37]. Thus, even if CancerSEEK could achieve a 99% sensitivity and 99% specificity, the resulting positive predictive value would be only 50% (50% of test positives would be false positive). An established challenge in early cancer detection is that patients at increased risk for cancer may also have precancerous conditions resulting in elevation of serum protein biomarkers, a factor that was not well addressed in the healthy control population of the CancerSEEK test [38].

2. CAPP-Seq

The cancer personalized profiling by deep sequencing (CAPP-Seq) method assesses plasma cfDNA for alterations in 128 genes that are recurrently mutated in NSCLC. When being assessed for disease monitoring and minimal residual disease detection, the method achieved the sensitivity of 50% for stage-I tumors, and 100% among those with stage-II to -IV tumors, with a specificity for both groups of 96%. Notably, it was able to detect a specific *TP53* hotspot variant at a median frequency of approximately 0.18% across all plasma DNA samples. When CAPP-Seq was combined with a computational error correction approach called integrated digital error suppression (iDES), the specificity in detecting EGFR hotspot variants was 100% [34].

The main caveat of CAPP-Seq is that it used cfDNA levels to detect tumor burden since cfDNA can only predict residual tumor rather than the tissue of origin. This means cfDNA should be used in combination with another method such as imaging for disease burden. Note that CAPP-Seq is currently unable to acknowledge whether cfDNA rates from primary lesions and metastatic disease are released at the same. Potentially, the different rates in which tumors or clones release their DNA could cause problems with determining tumor burden and clonal evolution [34].

3. TEC-Seq

Targeted error correction sequencing or TEC-Seq was developed to allow the profiling of tiny amounts of ctDNA present in early-stage cancer using massively parallel sequencing. In brief, DNA fragments were isolated from a patient's blood, the researchers labeled each fragment with a unique barcode. These barcodes allowed the tracking of each fragment as they sequenced it to approximately 30,000x in depth. By cross-checking sequences at each position to each other and to a reference genome, the researchers could confirm whether a detected mutation was genuine or a false positive [35].

TEC-Seq detects 58 cancer-related genes encompassing 81 kb that are often altered in certain tumors and can detect tumor-specific mutations. They used the technique to analyze plasma samples from 44 healthy individuals and 200 individuals with breast, colorectal, lung, or ovarian cancers in various stages. TEC-Seq correctly identified 56%, 83%, 62%, and 71% of these individuals, respectively, identifying gene alterations for each case. TEC-Seq was sensitive enough to detect early-stage tumors. From a pool of 138 stage-I and -II cancers, TEC-Seq identified 86, or more than 60% of these diseases. Overall, the test was most successful at detecting colorectal and ovarian cancers [35].

The key for any early detection assay is to determine whether the mutations present in the blood are tumor-derived. Here, it was found that in 82% of cases, a mutation detected in the blood was also present in the tumor when analyzing matched tumor and plasma samples. In a further test, the researchers applied TEC-Seq to blood samples from 44 healthy individuals. They did not detect any alterations among any of the investigated 80,000 bases, leading to a very high specifivity [35].

TEC-Seq may also be useful for forecasting cancer relapse. The study applied the method to plasma samples from 38 individuals with colorectal cancer who had undergone removal. They found that patients with large abundant of ctDNA at diagnosis had shorter survival with less ctDNA [35].

IV. Future Perspectives

We have discussed technical and biological challenges associated with cfDNA detection in patients in the context of liquid biopsy. Liquid biopsy is increasingly being adopted for an extensive variety of applications in oncology. Unquestionably, using cfDNA detected from liquid biopsy for effective screening of early cancers as well as monitoring developed diseases represents the best hope for reducing cancer mortality.

The conceptual improvements and the practicability of the discussed assays are important milestones toward the application of early cancer detection. The ongoing development of cost effective and sensitive blood-based cancer screenings is set to revolutionize cancer management. Nevertheless, the use of these promising biomarkers requires a fundamental understanding of the mechanisms underlying liquid analytes and the resolution of important technology. Additional preclinical studies addressing the biology of liquid biopsy analytes are needed. Most existing assays have focused on a single analyte; however, sensitivity and accuracy could be improved by adopting multiparametric assays that integrate data from the multiple analytes present in a single sample. Innovative assay technology must be accompanied by novel statistical and machine learning tools that make use of high dimensional and large amounts of data. Thus, future liquid biopsy developments are expected to be multi-disciplinary. Critically, it is only after clinical validity and clinical utility have been demonstrated that liquid biopsies will reach their full potential and have the expected impact on clinical oncology and the clinical management of patients.

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Nguyen Ngoc Tran was born in Ho Chi Minh City in 1989. She received the Bachelor in Pharmacy from The University of Medicine and Pharmacy in Ho Chi Minh City, Ho Chi Minh, Vietnam in 2011. She went on to receive a Ph.D. in Cancer Biology from The University of Texas at MD Anderson Cancer Center, Houston, Texas in 2018. Her thesis work focused on studying the mechanism of skin cancer development. She spent two years working at Moffitt Cancer Center, Tampa, Florida as a Research Associate and Postdoctoral Scholar. Since 2019, she has been with Vingroup Big Data Institute, Hanoi, Vietnam, where she is currently a Research Scientist. Her main area of research interest is translational research, including identifying preventative biomarkers and developing diagnostic tests for cancer. Dr. Nguyen Ngoc Tran is a member of the American Association for Cancer Research.