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Review Article

Advances in Liquid Biopsy

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Abstract

Cancer is a lethal disease and ranks as the world's second most prevalent cause of death. So far, tissue-based biopsy is conducted routinely to detect and monitor the progression of cancer. However, the traditional technique is deemed too invasive and cannot be used repeatedly. On the other hand, liquid biopsy, also known as blood-sample test, has recently surged and has been proved to be more and more promising. Using cell-free DNA (cfDNA) and circulating tumor DNA (ctDNA), liquid biopsy can effectively profile the genetic landscape of cancer and can detect the presence of cancer in patients at very early stages due to its high sensitivity. Liquid biopsy is not only capable of monitoring the risk or potential of tumorigenesis, but also capable of predicting and tracking metastasis and relapse. Therefore, liquid biopsy is believed to revolutionize cancer detection, prognosis, and personalized medicine treatment. In this review, the concept, history, recent advances of liquid biopsy, and existing commercial companies' most commonly used techniques were summarized; the advantages and disadvantages and the broad applications of liquid biopsy in various fields including cancer were discussed.

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Introduction

Cancer is a lethal disease; with more than 1.68 million of estimated new cases and more than 595,000 of estimated deaths in 2016 [1], it is the second leading cause of death in the United States after heart disease. Heterogeneous both at the cellular and molecular levels, cancers are a variety of diseases that involve accelerated cell growth and can invade other parts of the body, a process known as metastasis [2]. Because cancer patients harbor unique mutations in genes, effective cancer detection and treatment have to become more personalized and are, therefore, more challenging to accomplish [3], [4]. Tissue biopsy, a procedure that involves removing a piece of tissue for further laboratory analysis, is the conventional way to determine tumor progression but is criticized as being too invasive [5]. On the other hand, liquid biopsy, or blood sample tests conducted to identify tumor cells, is deemed non-invasive [6]. Therefore, scientists and researchers now consider liquid biopsy more promising in cancer early detection. While tissue biopsy is invasive, has unsatisfactory cancer early detection, and has limited evaluation of prognosis, liquid biopsy can detect the mutations of the original tumor, is highly sensitive and specific, and can determine tumor prognosis and progression [7].

The desire to use non-invasive techniques to detect diseases has driven scientists and researchers for centuries. In 1869, Australian pathologist John Ashworth discovered the presence of circulating tumor cells, or CTCs, in the blood that could be used to study tumor biology [8]. In 1948, another type of identification was discovered: circulating tumor DNA, or ctDNA [9]. It was not until 1977 that ctDNA was demonstrated to be linked to cancer [10]. Following a series of technological advancements, a sequencing approach using mutation-specific primers to facilitate the polymerase chain reaction, or PCR, was developed in the 1990s [11], [12]. Studies implementing this method allowed scientists to discover specific mutations in ctDNA [13]. Besides cancer, other areas of study using ctDNA include prenatal diagnostics, like determining fetal aneuploidy, as well as organ transplants [14], [13].

Extensive advancements in technology have allowed for the development of new approaches and methods in liquid biopsy. Besides the PCR-based sequencing technique, massively parallel sequencing, or MPS, and target and whole genome/exome sequencing have become popular for their generalized and comprehensive approach [13]. Improvements in technology have also driven the cost of sequencing down. Just a couple years ago, sequencing an entire genome would have taken millions of dollars; however, Illumina's recently proposed sequencing method called NovaSeq aims to charge only $100 per genome and may be completed in an hour [15]. The advancements made have also led to the accumulation of knowledge about somatic mutations in DNA of diseases, such as cancer. For example, the Catalogue of Somatic Mutations in Cancer, or COSMIC, is one of the most comprehensive databases depositing the somatic mutations in human cancer [16]. Circulating tumor DNA (ctDNA) is produced by dead tumor cells and flows into the bloodstream [17]. Based on the knowledge obtained, the ctDNA in cancer patients' plasma have led to the detection of somatic mutations in DNA, and some actionable mutations in cancer genes have been identified using liquid biopsy [18]. CAPP-Seq, a recently developed method by Stanford University and acquired by Roche, evaluates ctDNA to profile lung cancer and would reduce costs and increase sensitivity [19]. Currently, many commercial start-ups have been established focusing on further developing this field. Racing to develop liquid biopsies, more than 40 commercial companies are currently working on furthering this promising field: to name a few, Guardant Health, Grail, and Foundation Medicine. In addition to

mutations, RNA and DNA methylation patterns have also been detected using ctDNA [20]. The applications of liquid biopsy are not limited to diseases. With the advent of machine learning, scientists aim to combine both sequencing depth and genomic coverage breadth to accurately categorize people into normal or disease-associated [21].

CTCs and ctDNA

The discovery of circulating tumor cells, or CTCs, in the 1800s brought about a deeper understanding of cancer and tumor biomarkers [8]. Clusters of these cells usually enter the bloodstream via epithelial-to-mesenchymal transition, or EMT, and are considered to be one of the most likely reasons for metastasis, or spreading of the tumor [22]. A study revealed that a lower level of CTCs, less than 5 per 7.5 mL of blood, correlated to better overall survivability of about 8 months in 177 breast cancer patients [23]. Another study concluded that how well a particular drug treatment performed corresponded to certain changes in the CTCs' pathways, such as androgen receptor reactivation and amplification in prostate cancer: about 99% of the CTCs disappeared after 3 months of initiation of androgen deprivation therapy [24]. Therefore, for many years, scientists and researchers used CTCs and the amount of CTCs in a patient's blood as a tumor biomarker and way to determine the effectiveness of treatment methods.

Although CTCs have allowed scientists and researchers to understand the cancer better, there are still many problems that occur and improvements that can be made, especially involving tumor heterogeneity, surface markers, and detection methods. For example, the gene expression patterns varied between CTCs from the same patient and between different patients' CTCs, which indicates that the clinical value of CTCs is yet to be determined because of its high variability [25]. In addition, because CTCs undergo EMT, they do not express epithelial phenotypes and are harder to detect since CTC detection relies on epithelial markers [26], [27]. Because of this, CTC detection needs to be improved to include biomarkers that will not be influenced by EMT [28]. Moreover, some CTCs can adopt endothelial cell behaviors, which would allow them easier access to the bloodstream [8]. Therefore, current methods can only detect a subset of the CTCs. Another challenge in CTC detection lies with the techniques used; many methods involve permeating the cells, which reduces their viability and prevents further characterization to occur [26].

Besides CTCs, circulating tumor DNA, or ctDNA, is also a promising tumor biomarker, one that has become the more preferred over CTCs. As tumor cells die or undergo apoptosis, many of these cells release ctDNA into the bloodstream; another source of ctDNA is determined to be CTCs [18]. Cell-free DNA, or cfDNA, is double-stranded and highly fragmented, most of which are 150 bbp in length. Therefore, the detection of cfDNA/ctDNA is easier. Because, unlike CTCs, ctDNA is directly exposed in the blood, it does not need to undergo cell isolation, making it more accessible. ctDNA is characterized by its single-nucleotide mutations, methylation changes, and cancer-derived viral sequences [7]. Currently, several methods have been developed to detect cfDNA, including BEAMing, dPCR, next-generation sequencing [29], and so on. Numerous companies have emerged with their own technologies and gene panels.

The wide applications of ctDNA include cancer early detection; identification of tumorigenesis, detection of metastasis and cancer relapse; as well as selection of treatment methods and drugs. For cancer early detection, one study found that serum from cancer patients were able to induce in vitro normal cell tumorigenesis but did not occur when the serum was deprived of DNA [30], [31]. Another study discovered that ctDNA levels differed in different stages of diseases like

pancreatic, breast, and colorectal cancer [18]. In addition to tumorigenesis, it has been discovered that ctDNA is associated with distant metastasis. For example, a significant increase in ctDNA levels was correlated to metastasis of colorectal cancer [32], and distant metastasis resulted from the transfer of ctDNA from tumor to normal cells through apoptosis [31]. ctDNA analysis can also be applied to detection of relapse. Because ctDNA has a relatively short half-life compared to other protein-based biomarkers, monitoring a patient's ctDNA levels more accurately reflects the real-time situation of the patient [7], and the varying levels of ctDNA can be used to identify relapsing and non-relapsing patients [20]. Since tumors constantly evolve, treatment resistance has become a major issue. Studies have shown that through analyzing ctDNA, mutations in markers of therapy resistance can be detected. For example, some KRAS gene mutations that are markers of therapy resistance were detected while analyzing ctDNA in lung cancer [33], and another study using ctDNA discovered mutations that prevented the drug from binding with its targets [29]. Therefore, ctDNA can also be used to help clinicians choose the most effective drugs for each patient. The promising potential of ctDNA analysis in clinical applications has led to the establishment of many commercial companies and a funding of $1 billion for Grail's liquid biopsy tests among large populations [34].

Commercial Companies

Liquid biopsy is a promising direction for cancer early detection and has potential applications in therapy and diagnosis. Therefore, many commercial companies have developed their own products for liquid biopsy tests for use in research or clinical trials. Three of these nationwide companies that are located in Silicon Valley are Grail, Guardant Health, and Natera. Table 1 summarizes the oncology product and method for each company.

TABLE 1. Commercial companies developing liquid biopsy tests

Company (Year)	Product (Year)	Method	References
Biocartis (2009)	Idylla (2014)	Fully automated, real-time PCR based molecular diagnostics system, 30 biomarkers	[35]
Chronix Biomedical (1997)	Second Opinion (2016)	Next generation sequencing – the technique that has successfully mapped the whole human genome	[36]
Cynvenio (2008)	ClearID (2015)	Targeting and sequencing of genes known to be somatically altered in cancer	[37]
Exosome Diagnostics (2008)	ExoDx (2016)	Detection of mutations in certain genes from exosomes extracted from urine and blood	[38]
Foundation Medicine (2010)	FoundationACT (2016)	Blood-based ctDNA assay identifying base substitutions, insertions and deletions, copy number variations, and rearrangements/gene fusions, 62 cancer-associated genes	[39]
Genomic Health (2000)	Oncotype SEQ (2016)	Next-generation sequencing to identify and assess actionable genomic alterations, 17 genes	[40]
GRAIL (2016)	---	High-intensity sequencing to surface clinically actionable insights from vast amounts of tumor genome data	[41]
Guardant Health (2013)	Guardant360 (2014)	Digital sequencing to identify actionable somatic alterations across all solid tumor sites, 73 genes	[42]
Inivata (2014)	InVision (2016)	Tagged-amplicon sequencing (TAm-Seq™) method, which allows amplification and deep sequencing of genomic regions spanning thousands of bases from individual copies of fragmented DNA, 34 genes	[43]

MDxHealth (2003)	ConfirmMDx (2015)	Detection of a field effect or halo associated with the presence of cancer at the DNA Level	[44]
Myriad Genetics (1991)	myRisk (2013)	Representation of the next generation of hereditary cancer risk testing, 28 genes	[45]
Natera (2004)	Constellation (software) (2015)	Next generation sequencing in oncology and non-invasive prenatal testing (NIPT)	[46]
NeoGenomics Laboratories (2002)	NeoLAB (2015)	Targeted tumor profiles and single gene assays for solid tumors and hematologic diseases	[47]
OncoCyte (2014)	PanC-Dx (2015)	Comparison of gene expression patterns exhibited by cancer tissues that get picked up in blood or urine	[48]
Personal Genome Diagnostics (2010)	PlasmaSELECT (2016)	Illumina next-generation sequencing to identify mutations and translocations, 64 genes	[49]
Resolution Bioscience (2012)	ctDX-Lung (2015)	Next-generation sequencing of SNPs, insertion and deletions, copy number variations (CNVs) / amplifications, and fusions / translocations, 21 genes	[50]

Grail is a recently established company invested by Illumina and Gates Foundation. The goal of the company is to use high-intensity sequencing to generate datasets that can be applied to cancer early detection. The main method through which the approach will develop is through analyzing the ctDNA of both cancer patients and normal individuals. Grail focuses on the breadth and depth of its technology, emphasizing its unique ability to sequence broadly across the genome and its high sensitivity to detect ctDNA signals among background noise. Implementing advanced tools in data science, Grail seeks to use machine learning, laboratory tests, and clinical trials to generate large scale gene panels that yield clinically actionable insights. Because the company's goal is to develop a comprehensive dataset, it plans on conducting laboratory and clinical tests that involve tens of thousands of individuals [41].

Guardant Health is one of the earliest commercial companies established in the liquid biopsy industry. Its product Guardant360 has already been on the market for more than 2 years and covers somatic genomic biomarkers for advanced solid tumors. The 73-gene panel identifies actionable somatic alterations using a digital sequencing method, which concentrates on improving sensitivity and specificity, on tubes of blood. Specifically, Guardant360 uses digital sequencing to identify, tag, and make copies of ctDNA. Since next-generation sequencing methods may introduce errors and false positives, the product prefers the digital engine, which reduces errors by one thousand fold and grants high level specificity to the gene panel. Guardant360 includes a professional advisory board that reviews each case after the test is applied and generates an informative report, displaying graphs indicating mutation trends, FDA-approved drugs, and current clinical trials [42].

Natera originally began as a commercial company targeting prenatal diagnosis tests but is also applying its technology to liquid biopsy for cancer detection. Its approach combines multiplexed PCR (mPCR) and next-generation sequencing [29] to detect mutations in cfDNA in early-stage cancers. In a recently published scientific paper by Natera, the company focused on determining whether both ubiquitous and heterogeneous mutations can be detected using this method in non-small cell lung cancer. Currently, Natera is collaborating with UCSF in a trial named I-SPY 2, which was launched in 2010 and has enrolled more than 1000 breast cancer patients [51]. More products targeting cancer are in development [46].

The commercial companies mentioned above have unique techniques, which they constantly keep improving upon. They seek to optimize their sequencing methods and increase sensitivity,

accuracy, and specificity of their products. In addition, they would refine their gene panels, which should make them more cost-effective, and convince insurance companies to cover the costs so that more patients can benefit from the liquid biopsy tests.

Advantages and Disadvantages

Liquid biopsy is an emerging trend in cancer detection. As an alternative to tissue biopsy, the blood test shows great promise in detecting genetic mutations in tumors. Therefore, liquid biopsy can be applied in numerous fields, including cancer early detection, disease progression, metastasis, relapse, and therapy resistance.

One of the greatest advantages of liquid biopsy lies in its ability to detect cancer at early stages. By analyzing the mutations in and level of ctDNA through liquid biopsy tests, scientists and researchers can identify a tumor at an early stage and apply treatments that will have greater success. In one particular study, researchers discovered that ctDNA was found in at least half of the patients with localized bladder, colorectal, and breast cancer. Those of which were in Stage I, 47% had detectable ctDNA levels and could be curable by surgery alone [18]. Another study by a group from Stanford University also demonstrated that 50% of Stage I patients with non-small-cell lung cancer had detectable ctDNA levels, while ctDNA was detected in 100% of Stages II-IV patients [19].

Compared to traditional tissue biopsy methods, liquid biopsy is more capable of detecting metastasis, non-invasive, and allows for sufficient follow-up tests. Contrary to tissue biopsies, liquid biopsies can detect DNA derived from distinct metastatic sites, which provides more information about the disease since one of the challenges in cancer treatment is its heterogeneity [18]. Because of liquid biopsy's non-invasiveness, tests can be performed repeatedly to follow-up with the patient, unlike for tissue biopsies. Since tissue biopsies involve removing only part of the tumor, instances may occur where there is not enough tissue left for profiling or there is insufficient quality in the tissue remaining [52]. From the patients' perspective, they may experience less pain and medical complications resulting from liquid biopsy than tissue biopsy.

Due to its high sensitivity, liquid biopsy has been regarded as a test that can predict cancer relapse, monitor real-time tumor progression, and identify therapy-resistant mutations. Since the concentration of ctDNA in circulation varies at different stages of cancer, researchers and clinicians can use the tumors' genetic makeup to monitor the real-time progression of the cancer and determine what types of therapy the patient is resistant to and what types are most effective [53]. For example, a study of 400 patients with several types of cancers demonstrated that liquid biopsy was able to identify therapy-resistant mutations that had not been detected in tissue biopsy [52]. In another study, across five years, diffuse large B-cell lymphoma patients with detectable ctDNA were more likely to experience relapse than those who did not have detectable ctDNA. 17 out of 107 had detectable ctDNA after therapy, and 15 out of these 17 experienced relapse. These assays also predicted relapse 3.5 months before CT scans detected any signs of the disease [54]. This correlates with therapy-resistance because relapse would occur when the tumor becomes resistant to the specific type of therapy offered.

Although liquid biopsy shows much promise in cancer detection, it still needs some improvements, one of which is consistency in panel design. The major concern is that many companies are currently developing their own gene panels to detect cancer. Because of this, results depending upon which gene panels were used are inconsistent and may be misleading. In addition,

some of the commercial liquid biopsy gene panels designed include an enormous number of genes, which is usually comprised of a couple hundred genes and makes the procedure less cost-efficient.

Another limitation lies in liquid biopsy tests' insurance coverage. Because commercial companies are working hard to develop liquid biopsy tests, tissue biopsy is still considered the norm, and many insurance policies do not cover the blood test costs. In addition, when conflicting results are presented by liquid biopsy and tissue biopsy, insurance policies tend to prefer the diagnoses of tissue biopsy and therefore claim that liquid biopsy is "not ready for primetime" [55].

Conclusion and Future Directions

Liquid biopsy is regarded as one of the most promising techniques in the cancer detection field in recent years. The test's unprecedented sensitivity level allows clinicians and researchers to detect cancer in patients at early stages, which provides an opportunity for early treatment. Because of liquid biopsy's non-invasive nature, follow-up examinations can be done at lower risks and repeatedly. The technique can be widely used and can be adjusted to target various states of cancer, including metastasis and relapse. In addition to cancer detection, liquid biopsy can also monitor treatment and therapy. Its ability to detect therapy-resistant mutations in patients allows clinicians and researchers to determine the most effective personalized treatment option for each individual patient.

In addition to cancer, liquid biopsy can be extended to many other types of diseases, including prenatal testing, organ transplantations, and autoimmune diseases. Non-invasive prenatal testing (NIPT) is a field that can effectively use liquid biopsy. The test analyzes blood samples that include the mother and child's cell-free DNA to detect certain types of genetic diseases that the child may carry or exhibit. Natera is well-known for conducting NIPT tests, and recently using liquid biopsy, their researchers have been able to detect fetal aneuploid abnormalities of chromosomes 13, 18, 21, and X, especially triploidy [56]. In addition to prenatal testing, cell-free DNA and liquid biopsy can also be applied to organ transplantations. A recent study demonstrated that liquid biopsy can analyze donor-derived cell-free DNA to detect the risk of rejection and monitor the health of the transplanted tissue [57].

With future improvements to the technique, liquid biopsy can be part of an annual routine examination. The expected gradual decrease in the cost of the test may provide an opportunity for liquid biopsy to become a regular screening practice. Through this, not only cancer patients but also normal individuals will have access to liquid biopsy tests. Although clinicians and researchers face certain obstacles in developing an optimal screening test, liquid biopsy is an emerging trend and promising opportunity in the field. Low-cost next-generation sequencing combined with advanced ways to process and analyze data, such as machine learning, may enable them to make breakthroughs in cancer detection and other diseases.

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