Drug Discovery: Recent Progress and the Future

Recent Advances in Liquid Biopsy in Precision Oncology Research

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Liquid biopsy is a minimally invasive test for cancer genetic status based on circulating tumor DNA (ctDNA), circulating tumor cells, or other tumor-derived materials in blood plasma. Although the minimal invasiveness and time resolution are attractive features of liquid biopsy, the limited amount of ctDNA in plasma poses problems. Recent developments in digital PCR and next-generation sequencing (NGS)-based technology have improved the accuracy of liquid biopsy. In particular, molecular barcoding technology in NGS-based methods, i.e., tagging of molecular barcodes to cell-free DNA before amplification, reduces technical errors by validating the consensus of sequences originating from a single molecule, leading to marked improvement of the accuracy and detection limit. However, substitutions caused by DNA damage and somatic mutations originating from normal cells are still obstacles to the sensitive detection of mutations on ctDNA. Since there have been only a few clinical applications, a deeper understanding of ctDNA biology and more advanced analytical technology are needed for the practical application of liquid biopsy.

Key words liquid biopsy; circulating tumor DNA; minimally invasive tumor genotyping; molecular barcoding technology; ultrarare mutation detection

1. INTRODUCTION

Liquid biopsy is a promising minimally invasive genetic test examining circulating tumor DNA (ctDNA) or circulating tumor cells (CTCs) detected in the blood. Although the presence of ctDNA was first described more than 30 years ago,1) intensive R&D on it began only recently in the 2010s.

In this review, we introduce current progress in analytical technology for liquid biopsy, mainly focusing on the applications of next-generation sequencing (NGS). In addition, we review the challenges and difficulties in the clinical application of liquid biopsy as suggested by the results of recent clinical studies.

2. BIOLOGY OF CT DNA

Both active and passive mechanisms were proposed as the origin of cell-free DNA (cfDNA). In the passive mechanism, cfDNA is released into the blood via apoptosis and necrosis. Phagocytosis of apoptotic/necrotic cells by phagosomes and macrophages is associated with the turnover of cfDNA. In the active mechanism, tumor cells secrete cytoplasmic fragmented DNA actively to communicate with distant tissue through exosomes.2) The turnover of fetal cfDNA in the maternal blood was first estimated, and then the half-life after birth was estimated to be an average 16.3 min.3) The half-life of ctDNA after surgical resection was estimated to be 114 min.4) The level of cfDNA was estimated to be 3-fold higher in cancer patients compared with that in healthy individuals.4) Furthermore, ctDNA levels in cancer patients appear to show a wide variety of ranges.5) The detection rate of ctDNA differs with cancer stage, and it was found in 47% of stage 1, 55% of stage 2, 69% of stage 3, and 82% of stage 4 patients in one study.6) Diehl et al. reported that the ctDNA level correlates with tumor burden.7) The fragment size of cfDNA is 170–200 base pairs (bp), reflecting the structure of nucleosomes, because nuclease digests genomic DNA during apoptosis. The fragment size profile differs between cfDNA from normal cells and ctDNA from tumor cells.7) Shorter fragments (<100 bp) are frequently observed in ctDNA from cancer patients.8) The majority of the cfDNA from normal cells is released after apoptosis, while ctDNA can be also released by necrosis.9) Necrosis-based fragmentation appears to create shorter DNA fragments, leading to smaller-sized ctDNA compared with cfDNA from normal cells. Short ctDNA fragments are more frequently observed in patients with metastatic disease than in those with early-stage cancer.10)

3. METHODOLOGIES FOR LIQUID BIOPSY

3.1. Digital PCR The concept of digital PCR was first described in the report by Vogelstein and Kinzler,11) in which RAS mutations were quantified in 384 separate wells in a microplate. The digital PCR assay is performed with one DNA fragment molecule per well. The end-point PCR assay results in a 0 (wild type) or 1 (mutant) sequence, and the mutant allele frequencies are calculated by dividing the number of mutant-containing wells by the number of total wells based on the absolute quantitation of each allele. Because real-time PCR and related techniques amplify a mixture of mutated and normal alleles, the detection of rare mutations may be affected by the vast number of normal alleles present. In contrast, because digital PCR amplifies both types of alleles separately, it is suitable for the accurate, sensitive detection of
rare mutations. Using droplet digital PCR (Bio-Rad, Hercules, CA, U.S.A.), 20000 droplets were used for an assay in which the rational detection limit is 0.005% (1/20000). Digital PCR is useful when the number of target mutations is small. Examples include detection of the KRAS mutation in pancreatic cancer patients, because most of them have the KRAS G12X mutation. Digital PCR is also used to monitor patients for recurrence by assay of single mutations preexamined in tissue biopsies.

3.2. NGS-Based Deep Sequencing and Noise Reduction by Molecular Barcoding Technology

Massive parallel deep sequencing is the most feasible current approach to liquid biopsy. An advantage of the NGS approach is its ability to determine sequences, in contrast to digital PCR that can detect only single mutations. For example, because mutations in TP53 are scattered throughout almost the entire domain of the protein, NGS is indispensable and the application of digital PCR is difficult.

The NGS-based panel assay, i.e., assay of mutations in multiple genes, is a very powerful tool to detect tumor mutations. However, the scope for error in NGS is problematic in rare mutation detection, especially in ctDNA analysis requiring high sensitivity (for example ca. 0.1% sensitivity) due to the relatively high sequencing error rate or PCR errors introduced during library construction. To overcome this problem, noise reduction by molecular barcoding has been established. In this approach, random nucleotide sequences (degenerate mix nucleotides, e.g., N12) are ligated to the DNA before PCR amplification. After PCR amplification, a consensus sequence for the amplified DNA molecules with single-molecule origin having the same molecular barcode is obtained. PCR errors and sequencing errors can be removed through this procedure, because such errors distribute randomly among the sequences analyzed, while the true mutation is assumed to locate consistently at the same position.

Three major types of assay for molecular barcoding have been established: 1) the amplicon-based assay, 2) one-sided gene-specific amplification, and 3) hybrid capture-based approach (Fig. 1). In the amplicon-based assay, classical PCR amplification by forward and reverse gene-specific primers is performed. To introduce a molecular barcode, overhang PCR with a molecular-barcoded primer is performed in the first several cycles. The advantage of this assay is its high accuracy for target amplification due to the two sides of gene-specific primers. The second approach is based on PCR amplification of the target region by the one-sided gene-specific primer and universal adapter primer. This assay has an advantage in the detection of gene fusion or structural rearrangements, because one-sided gene-specific amplification can analyze unknown chimeric fragments joined to a gene-specific region. In the hybrid-capture approach, molecular-barcoded adapter ligation is followed by amplification with a universal primer on the adapter. Target panel regions are enriched with complement RNA capture probes. This type of assay has long been utilized in exome sequencing.

Two methods deserve specific attention. Duplex sequencing attaches molecular barcodes to two strands of DNA separately and can distinguish substitutions appearing in both strands and those in only one strand. Because the damage is introduced into a single strand, those appearing in both strands of DNA are regarded as mutations. Kukita et al. reported that errors in the barcode itself are problematic for the accurate
estimation of molecular count. In the assay system called the non-overlapping integrated read sequencing system (NOIR-SS), such erroneous molecular barcodes are removed, and the system can estimate the absolute number of molecules accurately.  

3.3. Other Problems in Mutation Detection  
Many studies have found that the genotyping results of tissue biopsies differ from those of liquid biopsies. Intratumor heterogeneity indicates that local assessment of tissue can cause misinterpretations. On the other hand, liquid biopsies may reflect mutations from multiple regions or the entire body.  
Perkins et al. examined the concordance between tissue biopsy and liquid biopsy. The concordance rates for metastatic cancer patients and for patients with primary tumors were 83.3 and 78.3%, respectively.  

There are two major problems in the accurate detection of rare mutations other than PCR errors and sequencing errors. DNA damage is the first. Long incubation times during the hybrid capture-based assay were reported to cause oxidative damage to DNA and deamination resulting in the G > A/C > T substitution. However, with improvements in assay systems and analysis pipelines, CAPP-seq, a capture-based method, achieved a detection limit of 0.02%, which is comparable to the results of site-specific digital PCR.  

Analysis of ctDNA revealed the frequent appearance of somatic mutations from normal cells. False-positive plasma genotyping due to clonal hematopoiesis has been recently reported as an obstacle to the accurate detection of ctDNA.  

The study found that somatic mutations originating from hematopoietic stem cells and clonal proliferation accumulate in normal blood cells. Older people tend to have a wide variety of mutations and high mutation rates. Mutations on the exonic region of cancer-related genes also can be wrongly derived from somatic mutations of hematopoietic stem cells, causing false-positive plasma genotyping of cancer. Matched-pair analysis of ctDNA and normal hematopoietic cells, and removal of such noncancer substitutions, might enable more robust detection of tumor-derived ctDNA, although this doubles the analytical cost. A method to overcome this problem is filtering of such false-positive substitutions using databases in the public domain such as the COSMIC database. Substitutions not present or with few entries are regarded as mutations not derived from tumor tissues.  

The loss of mutation alleles during the assay process is also problematic, especially for the detection of rare mutations.  
It is not possible to recover the total initial DNA material in the final analysis in most assays due to technical problems such as dead volume, splitting of tubes, and loss through purification. Such loss of DNA may cause fluctuations in mutation/ wild-type allele ratios based on the statistical probability of hypergeometric distribution. This effect is not negligible in rare mutation detection (0.1–0.01%). The development of highly efficient assays or ctDNA purification/enrichment methods would improve the yield of analysis.  

4. TARGET ANALYTEs OF LIquid BIoPSY  
Genetic profiling of ctDNA in the blood is the main target of liquid biopsy. In addition to ctDNA, CTCs are also an attractive resource for genetic analysis. An advantage of CTCs compared with ctDNA is the availability of mRNA in viable CTCs. However, the number of CTCs is considered to be very low (1 in 10⁹ cells), indicating the difficulty of isolation and gene expression analysis. Most recent studies have reported that the detection accuracy (sensitivity) of CTCs is much lower than that of ctDNA.  
A study of KRAS mutation detection revealed that the sensitivity of cancer detection by ctDNA and CTC analysis was 96 and 52%, respectively. In metastatic cancer patients, the sensitivity of ctDNA detection was 87.2%.  
Furthermore, analysis of cancer patients positive for both ctDNA and CTCs revealed that their blood contained 50-fold more mutant DNA fragments in ctDNA than in CTCs per 5-mL blood sample. Thus, liquid biopsy based on ctDNA is currently the first choice for the development of diagnostic testing due to its high sensitivity and specificity.  
Methylation profiling of ctDNA is another alternative target of liquid biopsy. An examination of 30 cancer patients revealed that methylation of the RASSFIA, CALCA, and EP300 promoters detected cancer accurately with 90% sensitivity and 86.7% specificity.  
Mahon et al. reported that methylated GSTP1-free DNA can be used as a prognostic marker of prostate cancer. In a study of colon cancer, blood-based detection with THBD promoter methylation showed 71% sensitivity and 80% specificity.  
The concept of tumor-educated platelets has been proposed recently. Tumor-derived RNA is considered to accumulate in the platelets of cancer patients. The detection of fusion genes such as ALK, ROS1, and RET oncogenes in the blood is challenging. DNA-based analysis of fusion genes is difficult due to the randomness of fusion breakpoints. RNA-based analysis using tumor-educated platelets may improve the detection accuracy of fusion genes, because fusion breakpoints on mRNA are located in much smaller regions (i.e., limited to exon regions) compared with those on DNA.  

5. CLINICAL APPLICATIONS OF LIQUID BIoPSY  
5.1. Therapeutic Selection of Molecular-Targeted Drugs  
The selection of patients to receive molecular-targeted drugs, so-called companion diagnostics, is often based on genotyping for target genes. A representative example is the screening of epidermal growth factor receptor (EGFR) mutations for EGFR tyrosine kinase inhibitor (EGFR-TKI). The sole liquid biopsy system approved by the US Food and Drug Administration and Japanese Pharmaceuticals and Medical Devices Agency is the Cobas EGFR Mutation Test (Hoffman-La Roche, Basel, Switzerland) using plasma DNA. First, the test for T790M, a resistant mutation, and then that for activating mutations was approved. The main driving force for liquid biopsy to detect the EGFR mutation was the approval of osimertinib, a third-generation EGFR-TKI, based on a study by Mok et al. Osimertinib was originally approved for the treatment of T790M-positive non-small cell lung cancer after frontline EGFR-TKI treatment. Thus, the therapy requires a T790M mutation test in a rebiopsy. Because rebiopsy is more invasive, its replacement with a noninvasive procedure such as liquid biopsy was advantageous. After the approval of osimertinib as a firstline treatment based on a study by Soria et al., the mutation test with plasma DNA became less important and its utility is now controversial.  
For companion diagnostics, so-called panel tests, i.e., simultaneous mutation tests of multiple genes using NGS, are an
emerging, highly anticipated diagnostic approach. Currently, this type of test employs tumor tissues, but several companies are developing liquid biopsy versions.

5.2. Recurrence Prediction by Detection of Residual Cancer

Frequent monitoring of ctDNA by liquid biopsy after surgery may be helpful for early detection of recurrence based on residual cancer. In a study examining 55 breast cancer patients, after surgery the ctDNA-positive group showed a recurrence rate 4-fold higher than the ctDNA-negative group.32 After 6-month postoperative monitoring, ctDNA-positive switch patients showed a 93% recurrence rate, while ctDNA-negative patients had only a 10% recurrence rate. Spindler et al.33 reported that ctDNA detected tumor recurrence 11 months earlier than any other currently available markers or image analysis methods. Diehl et al.41 suggested that ctDNA is more useful in predicting recurrence than the widely used tumor marker CEA in postoperative settings.

Theoretically, the early detection of residual cancer by ctDNA can help to eradicate the tumors or prevent recurrence. The clinical benefit (prolongation of overall survival or improvement of quality of life) of such an approach has not yet been established, however. Hence, there is no evidence that the detection of residual tumor improves clinical outcomes with specific treatments. It is noteworthy that several randomized, prospective clinical trials examining the clinical benefits of detecting circulating tumor protein markers for this purpose failed.34,35 In the future, large prospective studies will be conducted to investigate the clinical utility of the ctDNA approach in the setting of recurrence monitoring.

5.3. Early Diagnosis of Cancer

One of the ultimate goals of liquid biopsy is early cancer diagnosis of asymptomatic individuals. The detection of fetal DNA in maternal blood and success of prenatal genotyping of aneuploidy36,37 offer the hope that the technology can be applied to early cancer detection. For the feasibility assessment of liquid biopsy, KRAS mutations in pancreatic cancer patients are a rational target, because >90% of pancreatic cancer patients have the KRAS mutation.38,39 The sensitivity of KRAS G12X detection by the NGSt-based ctDNA assay for pancreatic cancer patients was estimated to be 25–30%,21 showing performance comparable with site-specific digital PCR.40 This estimate indicates the need for improvement of the ctDNA assay as the first screening tool for the early detection of cancer. Although the utility of each single marker has some limitations, one study found that combinatorial unity and integration of multiple markers/approaches improved the detection accuracy.41 Although there is widespread enthusiasm for the utilization of liquid biopsy for this purpose, there is currently no evidence supporting the clinical validity and utility of liquid biopsy for early cancer detection.

6. CONCLUSION

The development of digital PCR and NGS technologies greatly enhanced the study of liquid biopsy with the ultimate goal of clinical application. In particular, NGS-based molecular barcoding technology has rapidly improved over the past several years. These developments make it possible to detect ultrarare ctDNA mutations located in a broad range of the genome. Many studies of liquid biopsy have recently been conducted worldwide because it is an attractive technology for minimally invasive tumor genotyping, complementing invasive tissue biopsy. However, most of these studies were retrospective, indicating that many challenges remain before the widespread use of liquid biopsy is seen in routine clinical settings. Important points are whether liquid biopsy actually provides more clinical benefits than currently established medical practice, improves patients’ QOL, or offers cost benefits from the viewpoint of health economics. To evaluate the feasibility of liquid biopsy from these viewpoints, large prospective clinical studies should be conducted in the future. The clinical validity and utility of liquid biopsy are expected to be validated in the near future.

Conflict of Interest

The authors have declared that no competing interests exist. K.K. is an advisor to DNA Chip Research Inc.

REFERENCES

