Circulating free DNA in the era of precision oncology: Pre- and post-analytical concerns

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Abstract

Cancer treatment has entered the era of precision medicine, where knowledge of a patient's genetic profile is used to facilitate early diagnosis, drug selection, prognosis, prediction of drug responsiveness, the onset of secondary resistance, and relapse. Circulating free DNA (cfDNA) has emerged as an ideal source of genetic information for cancer patients, and numerous studies have explored its validity in various clinical applications. However, clinical implementation of cfDNA-based tests has been slow. In this review, we addressed some of the pre- and post-analytical issues regarding cfDNA tests. First, we summarized the characteristics of cfDNA and reviewed the methods used to identify tumor-derived cfDNA from the pool of total cfDNA. Second, we described the procedures used to extract cfDNA, which have a great impact on representativeness and yield. Finally, we discussed our thoughts on the validation of cfDNA-based tests and the reporting of test results amid drastic limitations.

Introduction

Over the past several decades, efforts have been made to craft standards-of-care for patients with various types of cancer. Numerous guidelines have been published based on large case-controlled trials. However, regardless of these efforts, non-responsiveness to treatment and overt adverse effects have occurred, indicating that there are some flaws in this “one-size-fits-all” strategy. Precision medicine, which takes into account each cancer patient's genetic profile, is believed to be the key to overcoming these flaws. The earliest identification of molecular alterations in malignancies dates back to the 1960s, when a t(9, 22) translocation was found in chronic myeloid leukemia patients. In 1998, imatinib, a tyrosine kinase inhibitor, was found to be a very efficient treatment for these patients. The success of imatinib shed light on the path toward targeted therapy, and many successors have followed. Gefitinib, an agent directed against the tyrosine kinase domain of epithelial growth factor receptor (EGFR), is one such successful example.
Intriguingly, although clinical trials indicating the efficacy of gefitinib toward non-small cell lung cancer were published in 2003, the capability of the EGFR mutation for predicting responsiveness to gefitinib was not revealed until 2005. With the introduction of agents targeting only the “abnormal” pathways in neoplasms, the need to obtain information about the associated molecular changes and tailor treatment regimens has never been so important. Since U.S. President Obama announced his precision medicine initiative program in last year’s State of the Union Address (www.whitehouse.gov/precisionmedicine), the concept has won ever increasing acceptance by medical professionals as well as the general population. The scope of the term “precision medicine” largely overlaps with the older term “personalized medicine.” Precision medicine means to “classify individuals into subpopulations that differ in their susceptibility to a particular disease (cancer in the context of the present review), in the biology and/or prognosis of those diseases they may develop, or in their response to a specific treatment.”

The growing field of precision medicine is imposing an urgent need to be able to determine patients’ genetic status with cheaper, faster, and more accurate methods. Thanks to the ever-increasing penetrance of technologies like amplification refractory mutation system-polymerase chain reaction (ARMS-PCR), next-generation sequencing (NGS), and digital polymerase chain reaction (PCR), we are now able to obtain the genetic and epigenetic profiles of cancer patients with very limited amounts of low-quality nucleic acid using specimens from small biopsies or cytological examinations. For patients who are too sick to undergo a biopsy and those from whom a mutational profile over time is desired, it is now possible to analyze circulating free DNA (cfDNA) using the techniques mentioned above. CfDNAs represent the DNA fragments that are present in the blood stream. It is generally accepted that the DNA fragments in peripheral blood originate from three sources: apoptotic cells, necrotic cells, and viable cells (which actively secrete DNA). Analyzing cfDNA from a cancer patient allows determination of the genetic changes that occurred in the tumor and may provide clues for individualized management of the patient.

For the past decade, numerous studies have been conducted to explore the feasibility of using cfDNA as an analyte for molecular genetics in cancer patients. Very recently, the Chinese Society of Clinical Oncology and Chinese Society of Lung Cancer jointly released expert consensus on liquid biopsy, marking the recognition and acceptance of liquid biopsy, including cfDNA, by clinical experts in a developing country. However, concerns regarding the validity of cfDNA-based assays persist. Such concerns have been directly expressed by experts worldwide, and the fact that there are currently very few Food and Drug Administration (FDA)-approved plasma/cfDNA-based molecular genetic assays may, to some extent, indicate the cautiousness of healthcare authorities.

Since the methodologies for and clinical applications of cfDNA have been well summarized in earlier reviews by Qin et al and others, we have refrained from duplicating their works here. Alternatively, we focused on some unsolved issues that exist before and after cfDNA analysis to clarify the limitations and trace the source of general concerns regarding cfDNA-based assays. These issues include (1) analytical methods and the importance of characterization, (2) extraction methods and the bias they introduce, and (3) issues related to the validation of cfDNA-based assays.

Characterization of cfDNA

Given that the major use of cfDNA-based assays is to obtain knowledge about the genetic changes in tumor cells, when cfDNA from a patient is analyzed, the goal is to analyze the DNA fragments derived from tumor cells, i.e., the circulating tumor DNA (ctDNA). Unfortunately, the fraction of ctDNA within the total cfDNA in cancer patients is rather low, ranging from 0 (undetectable) to 11.7%. The mixture of tumorous and non-tumorous DNA present in the blood causes problems for clinical analysis of cfDNA. It also places extremely high sensitivity and specificity requirements on the methodologies used to analyze cfDNA because of the need to differentiate genetic aberrations from the large fraction of DNA from normal cells. However, there is currently no convenient, economical way to quantify the ctDNA fraction within the pool of cfDNA, which further limits the clinical applications of cfDNA-based assays. This issue is discussed later in the present review.

Early attempts to quantify the ctDNA fraction relied on the identification of tumor-specific genetic aberrations. The most successful example was the study by Diehl et al. By expanding a panel containing APC, TP53, PIK3CA, and KRAS in resected colorectal cancer specimens, they obtained baseline somatic mutation profiles for the enrolled patients. In follow-up, plasma from these patients was subjected to mutational
analyses using the digital PCR-based beads, emulsions, amplification, and magnetics (BEAMing) technique. Defining the ctDNAs as those containing at least one of the mutations identified in the corresponding tumor tissue, they managed to calculate the fraction and copy number of ctDNA in each sample. However, one must be cautious when selecting appropriate molecular fingerprints used for the identification of ctDNA, given the evolutionary growth model of solid tumors.\textsuperscript{29,30} Theoretically, founder mutations or those that occur in the founder cell population in the primary tumor, while they may not provide selective advantages, should be given priority, as these aberrations tend to be present throughout the microevolution of the tumor and thus persist in circulating blood.\textsuperscript{31–33} While substantial evidence has pointed to the potential of this strategy, it is subjected to certain limitations. First is the need for a thorough knowledge of the genetic or epigenetic profile of the primary tumor, which may not be accessible for inoperable tumors. As biopsies usually introduce biased information due to intratumor heterogeneity,\textsuperscript{34,35} they should not be used to develop the baseline mutational profile of a cancer patient. Another major problem is that the genetic aberrations eligible as ctDNA markers may be difficult to find.\textsuperscript{36} Thanks to the advancements in massively parallel sequencing techniques, it is now possible to screen over 500 regions in more than 130 candidate genes at the same time to plot the baseline mutational profile of an individual, thus improving ctDNA identification.\textsuperscript{28} Apart from genetic aberrations, some epigenetic features, such as methylation, can also be used to discriminate ctDNA from total cfDNA.\textsuperscript{37–40}

Some researchers have approached the issue of identifying the ctDNA fraction within the cfDNA another way. Briefly, the peak on the cfDNA size spectrum is ~166 bp, which resembles the length of a chromatosome, indicating that most cfDNA is generated via apoptosis. An in vivo experiment showed that the average cfDNA length is reduced to ~130 bp in xenograft rats.\textsuperscript{41} Taking advantage of this characteristic, it may be possible to differentiate ctDNA from genomic DNA.\textsuperscript{42–44} Jiang et al\textsuperscript{42} adopted a massively parallel sequencing method to sequence plasma DNA from healthy individuals and patients with hepatocellular cancer (HCC). The numbers of reads mapped to corresponding loci on chromosomes 1 and 8 were calculated for healthy individuals to set a standard reference, which was then compared to that of the HCC patients. They found that in most HCC patients, the short arms of chromosomes 1 and 8 were underrepresented, while the long arms were overrepresented compared to healthy individuals, indicating the presence of copy number variation (CNV) in HCC. DNA with such CNV was deemed to originate from the tumor. The fraction of DNA from the tumor was then calculable. Intriguingly, as the proportion of tumor-derived DNA increased, the size spectrum was significantly shifted to the left, indicating that tumor-derived DNA was shorter.

The gene expression profile may also serve as an identifier for cancer.\textsuperscript{45} However, the instability of circulating RNA drastically limits its application.\textsuperscript{46} Schep et al\textsuperscript{47} overcome this issue by taking advantage of the notion that nucleosome positioning and the length of inter-nucleosome spaces could greatly affect transcriptional regulation. Snyder et al\textsuperscript{48} used nucleosome positioning and the inter-nucleosome spacing pattern as classifiers and correlated them with the expression of corresponding genes in numerous cell lines and primary tissues. Their results showed that nucleosome phasing varied in different tissues and cells and might be useful for determining the tissue of origin for cfDNA. Differentiating ctDNA from genomic DNA based on fragment size or nucleosome spacing and positioning seems to be very promising approaches, yet it will take years for these advances to be translated into methodologies that are applicable in routine cfDNA analyses. The most broadly accepted method to calculate the fraction of ctDNA within the total cfDNA is mutation-based, and there is currently no established method to enrich ctDNA.

The recent emergence of DNA direct imaging technology allows for visualization of the double helix structure and acquisition of quantification parameters.\textsuperscript{39,50} Given the changes in the spatial relationship between histones and the DNA double helix in tumor cells, direct imaging seems very promising for the characterization of tumor-derived DNA. However, just as with other new technologies, it will take a long time for this technology to be clinically valid and feasible.

**Pre-analytical variables**

Analyses of cfDNA are preceded by several steps. First, approximately 10 ml of blood is taken from the patient in a collection tube. The blood sample is then centrifuged twice, yielding approximately 4 ml of cell-free plasma, which is followed by various DNA extraction procedures. Numerous studies focusing on these pre-analytical procedures have been conducted to optimize cfDNA yield.

Cancer patients generally have higher plasma concentrations of cfDNA than healthy individuals.
However, this figure varies dramatically among groups (1 ml of plasma from healthy controls and cancer patients contains 0.3–10.3 ng and 8–798 ng of cfDNA, respectively). This variation may reflect the distinct nature of different types of cancer; however, the blood drawing procedure itself may also affect the measured concentration. A recent but very preliminary study just revealed that the timing of a blood draw (at 7:00, 12:00, and 17:00) might contribute to the difference in the cfDNA yield, although no statistical significance was observed. Currently, there is no data on whether drawing blood from different sites could affect the quality or quantity of cfDNA, and our current knowledge about how necrotic and apoptotic DNA is degraded in vivo is poor. Thus, it is difficult to tell whether sampling blood from the proximal draining edge about how necrotic and apoptotic DNA is degraded to some extent.

For the anticoagulant used in the blood collection tube, ethylenediaminetetraacetic acid (EDTA) is preferred over heparin, as the latter does not halt endonuclease activity, which may lead to cfDNA degradation. Other options include the Streck tube (Streck, Omaha, NE, USA) and CellSave preservation tube (Janssen Diagnostics, Raritan, NJ, USA). A recent study comparing the DNA preservation capability of these tubes concluded that all three showed comparable cfDNA stability within a narrow time window (6 h between blood draw and cfDNA extraction), while the more expensive Streck tube provided better cfDNA preservation over a longer time period (~48 h in their study and up to 14 days as claimed by the manufacturer). However, it was also shown that when preserved at 4°C, the K₂EDTA tube was as good as the Streck tube.

A review by Bronkhorst et al concluded that each procedure before analysis, from blood draw to DNA extraction, affects the final yield of cfDNA. The factors considered included centrifugation, storage temperature, thawing temperature, and extracting reagents used. Those interested in this topic may refer to their review. Another dataset reporting in vitro experimental results on the impact of each of these factors using cancer cell culture medium as a highly reproducible model has also been published by the same group.

From a technical perspective, current cfDNA extraction methodologies can be classified into three categories: phase isolation, silicon membrane-based spin column, and magnetic bead-based isolation. In the standardization and improvement of generic pre-analytical tools and procedures for in vitro diagnostics (SPIDIA) external quality control program conducted in late 2015, when other pre-analytical variables were controlled, it was shown that precipitation-based methods yielded a higher DNA integrity index than spin column-based methods. These results were consistent with the results of two earlier studies, one of which included a modified chloroform-phenol separation and isopropanol precipitation method. For laboratories that handle batched specimens, automation might be considered. One thing to pay attention to is that both spin columns and magnetic beads show selective recovery for DNA fragments of a certain size. For example, the QIAamp blood mini kit (Cat. No: 51104; Qiagen, Hilden, Germany) preferentially enriches DNA >200 bp; thus it is inferior to the QIAamp Circulating Nucleic Acid Kit (Cat. No: 55114; Qiagen, Hilden, Germany) for the extraction of cfDNA, given that the peak size of cfDNA is usually 166–200 bp. Another issue worth noting is that many researchers have adopted quantitative PCR (qPCR)-based methods to evaluate the quality and quantity of extracted cfDNA, which is a standard practice and they could not be blamed for that. However, these methods risk introducing bias when evaluating the effectiveness of an extraction method, since qPCR primers preferentially amplify longer, more intact DNA sequences. Thus, qPCR is prone to overestimation because it preferentially recovers longer DNA fragments. Comparison of qPCR and capillary electrophoresis analyses for cfDNA yield and integrity showed higher reproducibility for the latter technique. The latest technique enables extraction from droplet amounts of plasma, which is sufficient for amplicon-based NGS, suggesting the future possibility of profiling one’s own genes at home. Attempts to optimize the pre-analytical procedures have been made to reduce DNA loss and improve cfDNA yield, and other advancements in biomaterials could also improve cfDNA extraction.

A highly innovative plasma processing approach is not to extract the DNA at all. Umetani et al were the first to publish data on the clinical application of direct PCR without DNA extraction. A study by Breitbach et al not only provided further evidence for the feasibility of direct PCR without the need for DNA extraction from plasma but also showed that the concentration of cfDNA in unpurified plasma was 2.79 and 1.14 times of that in the eluate from the QIAamp blood mini kit and phenol-chloroform-isoamyl extraction, respectively. Regrettfully, the authors did not present the fragment spectrum of DNA molecules obtained from unpurified plasma. For ordinary downstream
analyses, such as real-time PCR and NGS, phase isolation, spin column, and magnetic bead-based extraction remain the first-line choices as they can be used to concentrate cfDNA from plasma to meet the input DNA concentration requirement. However, in the future, a time-saving, bias-free non-extraction protocol is likely to gain popularity with the on-going implementation of highly sensitive digital PCR-based assays.

**Issues regarding the validation and interpretation of test results**

While the potential feasibility of cfDNA as an analyte for molecular genetic testing is widely accepted, options for pre-validated, FDA-approved assays remain limited. This paradoxical situation requires laboratories to develop cfDNA-based tests, namely laboratory developed tests (LDTs). The implementation of an LDT requires independent validation in compliance with two major international standards, ISO15189 and ISO17025, in addition to local legislation. Guidance on the validation of molecular genetic tests is currently available, which serves as a reference for the development of laboratory tests. Of note, a standardized framework for the implementation of molecular genetic tests has been developed by EuroGentest, in which, the process of validating a novel assay is detailed. Guidance by the European Society of Pathology Task Force on Quality Assurance in Molecular Pathology and the Royal College of Pathologists sets a context for molecular pathology testing and describes the procedures from analysis requesting to sample preparation, analysis, and the reporting and interpretation of the test results. More examples on the validation of various types of molecular genetic tests can be found in the references.

However, there is currently no guidance on cfDNA extraction procedure validation. It is also worth noting that simply knowing the yielding and integrity of cfDNA produced by a certain extraction method is insufficient. The fragmentation profile and ctDNA fraction within the total cfDNA would be more significant. In routine molecular genetic testing using formalin fixed paraffin embedded (FFPE) specimens, a histological review of the corresponding hematoxylin and eosin (H&E) sections is required. One reason is that when the tumor fraction in that specimen is below a certain threshold, operators may either choose alternative tissue blocks, resample the patient, or enrich tumor cells by microdissection or macrodissection.

However, in the clinic, we sometimes face a situation where all three of these options are inapplicable, and we have to get by with less than 100 tumor cells within a metastatic lymph node. Under such circumstances, knowing the tumor fraction within a tissue sample prompts the molecular pathologists to pay extra attention to the druggable mutations that fall out of positive range of the routine workflow. Such information can be conveyed to the physician who determines the treatment plan for the patient. Characterizing the ctDNA fraction within cfDNA is in fact a molecular-based histological review. Knowledge of the tumor fraction within a sample will improve molecular pathologists’ confidence in the report and help provide some critical information for patient treatment.

There is currently no clear guidance on how to validate a cfDNA-based test, probably due to the lack of a consensus on the biological characteristics and representativeness of cfDNA. Thus, when designing validation experiments for a cfDNA assay, one should keep in mind the difference between analytical validation and clinical validation. That is, what we can achieve through the regular in-house validating process is the correct representation of the status of the patient’s cfDNA, rather than the clinical status of the tumor. In this regard, FFPE tissue may not serve as an appropriate gold standard for the validation of cfDNA assays, although such comparisons have been reported in early investigations on cfDNA applications. The concepts of sensitivity and specificity are also not valid in such comparisons. Because when comparing, for example, EGFR mutation status in plasma and FFPE specimens from the same patient using real-time PCR, two template pools are amplified: one representing normal and/or cancer cells that have undergone apoptosis and another representing an unexhausted combination of subclones of the primary tumor cells. Thus, cfDNA and FFPE should be regarded as two complementary genetic pools that jointly provide a map of the overall genetic profile of a cancer patient.

Barring immediate resolution of the technological limitations of cfDNA-based molecular pathology assays, the results generated from cfDNA should be reported along with ancillary information, including the type of analyte (cfDNA), scope of detection (loci that are included) and the performance characteristics of the test (capability/incapability for identifying single nucleotide substitutions/small indels/structural alterations). Most importantly, when interpreting the results, molecular pathologists need to ensure that the representativeness of ctDNA and the limitations of the
test are accurately conveyed and well understood by clinicians, so that treatment decisions can be made in a comprehensive manner, especially in the presence of negative results.

Conclusion

CfDNA is a potential source of information that conveys the genetic and epigenetic aberrations of a cancer patient to his/her physician, thus meeting the need for a convenient, non-invasive approach in the era of precision medicine. The current poor understanding of the biology of cfDNA and the lack of an approach for distinguishing ctDNA from genomic DNA within total cfDNA limit the validity of cfDNA-based assays. Thus, caution should be used when developing, validating, and reporting the result of a cfDNA-based assay.

Conflicts of interest

The authors state that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers’ bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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