Available online at www.sciencedirect.com

ScienceDirect

Chronic Diseases and Translational Medicine 2 (2016) 223–230

www.keaipublishing.com/en/journals/cdtm/
www.cdatm.org

Perspective

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

Jun-Liang Lu, Zhi-Yong Liang*

Department of Pathology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China

Received 20 September 2016
Available online 22 December 2016

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.

Keywords: Circulating free DNA; Characterization; Extraction; Validation; Interpretation

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.[1] 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.

* Corresponding author.
E-mail address: liangzhiyong1220@yahoo.com (Z.-Y. Liang).
Peer review under responsibility of Chinese Medical Association.

Production and Hosting by Elsevier on behalf of KeAi

http://dx.doi.org/10.1016/j.cdtm.2016.12.001

Intriguingly, although clinical trials indicating the efficacy of gefitinib toward non-small cell lung cancer were published in 2003,[2] the capability of the EGFR mutation for predicting responsiveness to gefitinib was not revealed until 2005.[3,4] 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.[5] The scope of the term "precision medicine" largely overlaps with the older term "personalized medicine".[6] 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."[7,8]

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.[9] 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,[10,11] and viable cells (which actively secrete DNA).[12] 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.[13−19] 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.[20] However, concerns regarding the validity of cfDNA-based assays persist. Such concerns have been directly expressed by experts worldwide,[21,22] and the fact that there are currently very few Food and Drug Administration (FDA)-approved plasma/cfDNA-based molecular genetic assays[23] 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[24] and others,[25−27] 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%.[19,28] 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.[19] 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.[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.[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,[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.[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.[28] Apart from genetic aberrations, some epigenetic features, such as methylation, can also be used to discriminate ctDNA from total cfDNA.[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.[41] Taking advantage of this characteristic, it may be possible to differentiate ctDNA from genomic DNA.[42−44] Jiang et al[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.[45] However, the instability of circulating RNA drastically limits its application.[46] Schep et al[47] overcame 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[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.[49,50] 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).[51] 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.[52] 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 vein of the primary tumor could reduce the degree of degradation 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.[53] 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).[54] However, it was also shown that when preserved at 4°C, the K_2EDTA tube was as good as the Streck tube.[55]

A review by Bronkhorst et al[56] 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.[57]

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.[58] These results were consistent with the results of two earlier studies, one of which included a modified chloroform-phenol separation and isopropanol precipitation method.[59,60] For laboratories that handle batched specimens, automation might be considered.[60,61] 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.[62] Another issue worth noting is that many researchers have adopted quantitative PCR (qPCR)-based methods to evaluate the quality and quantity of extracted cfDNA,[59,62] 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.[63] 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.[64] Attempts to optimize the pre-analytical procedures have been made to reduce DNA loss and improve cfDNA yield,[65–67] and other advancements in biomaterials could also improve cfDNA extraction.[68]

A highly innovative plasma processing approach is not to extract the DNA at all. Umetani et al[69] were the first to publish data on the clinical application of direct PCR without DNA extraction. A study by Breitbach et al[70] 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. Regretfully, 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,[71] options for pre-validated, FDA-approved assays remain limited.[23] 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[72] and ISO17025,[73] 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.[71,74,75] 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.[75] 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.[71] More examples on the validation of various types of molecular genetic tests can be found in the references.[76,77]

However, there is currently no guidance on cfDNA extraction procedure validation.[78] 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.[9] 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.[79−81] 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[82] 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.

References

1. Goldman JM. Chronic myeloid leukemia: a historical perspective. *Semin Hematol*. 2010;47:302–311.
2. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) [corrected]. *J Clin Oncol*. 2003;21:2237–2246.
3. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2005;352:786–792.
4. Fukuoka M, Wu YL, Thongprasert S, et al. Biomarker analyses and final overall survival results from a phase III, randomized, open-label, first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients with advanced non-small-cell lung cancer in Asia (IPASS). *J Clin Oncol*. 2011;29:2866–2874.
5. Collins FS, Varmus H. A new initiative on precision medicine. *N Engl J Med*. 2015;372:793–795.
6. Katsnelson A. Momentum grows to make ‘personalized’ medicine more ‘precise’. *Nat Med*. 2013;19:249.
7. Emmert-Streib F, Tuomisto L, Yli-Harja O. The need for formally defining “Modern Medicine” by means of experimental design. *Front Genet*. 2016;7:60.
8. National Research Council (US) Committee on A Framework for Developing a New Taxonomy of Disease. *Toward Precision Medicine: Building a Knowledge Network for Biomedical Research and a New Taxonomy of Disease*. Washington, DC: National Academies Press (US); 2011.
9. Dietz S, Schirmer U, Mercé C, et al. Low input whole-exome sequencing to determine the representation of the tumor exome in circulating DNA of non-small cell lung cancer patients. *PLoS One*. 2016;11:e0161012.
10. Jahr S, Hentze H, Englisch S, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res*. 2001;61:1659–1665.
11. Zhivotosky B, Orrenius S. Assessment of apoptosis and necrosis by DNA fragmentation and morphological criteria. In: Bonifacino JS, ed. *Current Protocol in Cell Biology*. Vol 12. Hoboken, NJ: John Wiley & Sons, Inc; 2001: chap 18.
12. Stroun M, Lyautey J, Lederrey C, Olson-Sand A, Anker P. About the possible origin and mechanism of circulating DNA apoptosis and active DNA release. *Clin Chim Acta*. 2001;313:139–142.
13. Lee JY, Qing X, Xiumin W, et al. Longitudinal monitoring of EGFR mutations in plasma predicts outcomes of NSCLC patients treated with EGFR TKIs: Korean Lung Cancer Consortium (KLCC-12-02). *Oncotarget*. 2016;7:6984–6993.
14. Spindler KL, Pallisgaard N, Andersen RF, Brandslund I, Jakobsen A. Circulating free DNA as biomarker and source for mutation detection in metastatic colorectal cancer. *PLoS One*. 2015;10:e0108247.
15. Goto K, Ichinose Y, Ohe Y, et al. Epidermal growth factor receptor mutation status in circulating free DNA in serum: from IPASS, a phase III study of gefitinib or carboplatin/paclitaxel in non-small cell lung cancer. *J Thorac Oncol*. 2012;7:115–121.
16. Brychta N, Krahn T, von AO. Detection of KRAS mutations in circulating tumor DNA by digital PCR in early stages of pancreatic cancer. *Clin Chem*. 2016;62:1482–1491.
17. Chen KZ, Lou F, Yang F, et al. Circulating tumor DNA detection in early-stage non-small cell lung cancer patients by targeted sequencing. *Sci Rep*. 2016;6:31985.
18. Jovelet C, Ileana E, Le DMC, et al. Circulating cell-free tumor DNA analysis of 50 genes by next-generation sequencing in the prospective MOSCATO trial. *Clin Cancer Res*. 2016;22:2960–2968.
19. Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. *Nat Med*. 2008;14:985–990.
20. Wu YL, Wang CL, Sun Y, et al. Liquid biopsy: specification with precision. *J Evid Based Med*. 2016;16:193–198.
21. Hegemann M, Stenzl A, Bedke J, Chi KN, Black PC, Todenhöfer T. Liquid biopsy: ready to guide therapy in advanced prostate cancer. *BJU Int*. 2016;118:855–863.
22. Hofman P, Popper HH. Pathologists and liquid biopsies: to be or not to be. *Virchows Arch*. 2016;469:601–609.
23. List of Cleared or Approved Companion Diagnostic Devices (In Vitro and Imaging Tools). http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm301431.htm. Updated October 28, 2016. Accessed December 1, 2016.
24. Qin Z, Ljubimov VA, Zhou C, Tong Y, Liang J. Cell-free circulating tumor DNA in cancer. *Chin J Cancer*. 2016;35:36.
25. Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer*. 2011;11:426–437.
26. Elshimali YI, Khaddour H, Sarkissyan M, Wu Y, Vadgama JV. The clinical utilization of circulating cell free DNA (CCFDNA)

in blood of cancer patients. *Int J Mol Sci*. 2013;14: 18925—18958.
27. Levy B, Hu ZI, Cordova KN, Close S, Lee K, Becker D. Clinical utility of liquid diagnostic platforms in non-small cell lung cancer. *Oncologist*. 2016;21:1121—1130.
28. Newman AM, Bratman SV, To J, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. *Nat Med*. 2014;20:548—554.
29. Sidow A, Spies N. Concepts in solid tumor evolution. *Trends Genet*. 2015;31:208—214.
30. Swanton C, McGranahan N, Starrett GJ, Harris RS. APOBEC enzymes: mutagenic fuel for cancer evolution and heterogeneity. *Cancer Discov*. 2015;5:704—712.
31. Bozic I, Gerold JM, Nowak MA. Quantifying clonal and subclonal passenger mutations in cancer evolution. *PLoS Comput Biol*. 2016;12:e1004731.
32. Bunz F. *Principles of Cancer Genetics*. 2nd ed. Dordrecht, Netherlands: Springer; 2016.
33. Gonzalez-Rivera M, Picornell AC, Alvarez EL, Martin M. A cross-sectional comparison of druggable mutations in primary tumors, metastatic tissue, circulating tumor cells, and cell-free circulating DNA in patients with metastatic breast cancer: the MIRROR study protocol. *JMIR Res Protoc*. 2016;5:e167.
34. López-Knowles E, Gao Q, Cheang MC, et al. Heterogeneity in global gene expression profiles between biopsy specimens taken peri-surgically from primary ER-positive breast carcinomas. *Breast Cancer Res*. 2016;18:39.
35. Russo M, Siravegna G, Blaszkowsky LS, et al. Tumor heterogeneity and lesion-specific response to targeted therapy in colorectal cancer. *Cancer Discov*. 2016;6:147—153.
36. Pleasance ED, Cheetham RK, Stephens PJ, et al. A comprehensive catalogue of somatic mutations from a human cancer genome. *Nature*. 2010;463:191—196.
37. Warton K, Mahon KL, Samimi G. Methylated circulating tumor DNA in blood: power in cancer prognosis and response. *Endocr Relat Cancer*. 2016;23:R157—R171.
38. Lehmann-Werman R, Neiman D, Zemmour H, et al. Identification of tissue-specific cell death using methylation patterns of circulating DNA. *Proc Natl Acad Sci U S A*. 2016;113:E1826—E1834.
39. Garrigou S, Perkins G, Garlan F, et al. A study of hypermethylated circulating tumor DNA as a universal colorectal cancer biomarker. *Clin Chem*. 2016;62:1129—1139.
40. Tost J. DNA methylation signatures in circulating cell-free DNA for the monitoring of at-risk populations progressing to lung cancer. *EBioMedicine*. 2015;2:798—799.
41. Underhill HR, Kitzman JO, Hellwig S, et al. Fragment length of circulating tumor DNA. *PLoS Genet*. 2016;12:e1006162.
42. Jiang P, Chan CW, Chan KC, et al. Lengthening and shortening of plasma DNA in hepatocellular carcinoma patients. *Proc Natl Acad Sci U S A*. 2015;112:E1317—E1325.
43. Mouliere F, Rosenfeld N. Circulating tumor-derived DNA is shorter than somatic DNA in plasma. *Proc Natl Acad Sci U S A*. 2015;112:3178—3179.
44. Mouliere F, Robert B, Arnau PE, et al. High fragmentation characterizes tumour-derived circulating DNA. *PLoS One*. 2011;6:e23418.
45. Rapin N, Bagger FO, Jendholm J, et al. Comparing cancer vs normal gene expression profiles identifies new disease entities and common transcriptional programs in AML patients. *Blood*. 2014;123:894—904.
46. Tsui NB, Ng EK, Lo YM. Stability of endogenous and added RNA in blood specimens, serum, and plasma. *Clin Chem*. 2002;48:1647—1653.
47. Schep AN, Buenrostro JD, Denny SK, Schwartz K, Sherlock G, Greenleaf WJ. Structured nucleosome fingerprints enable high-resolution mapping of chromatin architecture within regulatory regions. *Genome Res*. 2015;25:1757—1770.
48. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA comprises an in vivo nucleosome footprint that informs its tissues-of-origin. *Cell*. 2016;164:57—68.
49. Marini M, Falqui A, Moretti M, et al. The structure of DNA by direct imaging. *Sci Adv*. 2015;1:e1500734.
50. Gentile F, Moretti M, Limongi T, et al. Direct imaging of DNA fibers: the visage of double helix. *Nano Lett*. 2012;12: 6453—6458.
51. Rykova EY, Morozkin ES, Ponomaryova AA, et al. Cell-free and cell-bound circulating nucleic acid complexes: mechanisms of generation, concentration and content. *Expert Opin Biol Ther*. 2012;12(suppl 1):S141—S153.
52. Korabecna M, Horinek A, Bila N, Opatrna S. Circadian rhythmicity and clearance of cell-free DNA in human plasma. In: Gahan BP, ed. *Circulating Nucleic Acids in Plasma and Serum: Proceedings of the 6th International Conference on Circulating Nucleic Acids in Plasma and Serum Held on 9—11 November 2009 in Hong Kong*. Dordrecht, Netherlands: Springer; 2011:195—198.
53. Lam NY, Rainer TH, Chiu RW, Lo YM. EDTA is a better anticoagulant than heparin or citrate for delayed blood processing for plasma DNA analysis. *Clin Chem*. 2004;50: 256—257.
54. Kang Q, Henry NL, Paoletti C, et al. Comparative analysis of circulating tumor DNA stability in K3EDTA, streck, and cell save blood collection tubes. *Clin Biochem*. 2016;49:1354—1360.
55. Barrett AN, Zimmermann BG, Wang D, Holloway A, Chitty LS. Implementing prenatal diagnosis based on cell-free fetal DNA: accurate identification of factors affecting fetal DNA yield. *PLoS One*. 2011;6:e25202.
56. Bronkhorst AJ, Aucamp J, Pretorius PJ. Cell-free DNA: preanalytical variables. *Clin Chim Acta*. 2015;450:243—253.
57. Bronkhorst AJ, Aucamp J, Pretorius PJ. Adjustments to the preanalytical phase of quantitative cell-free DNA analysis. *Data Brief*. 2016;6:326—329.
58. Malentacchi F, Pizzamiglio S, Verderio P, et al. Influence of storage conditions and extraction methods on the quantity and quality of circulating cell-free DNA (ccfDNA): the SPIDIA-DNAplas External Quality Assessment experience. *Clin Chem Lab Med*. 2015;53:1935—1942.
59. Yuan H, Zhu ZZ, Lu Y, et al. A modified extraction method of circulating free DNA for epidermal growth factor receptor mutation analysis. *Yonsei Med J*. 2012;53:132—137.
60. Fleischhacker M, Schmidt B, Weickmann S, et al. Methods for isolation of cell-free plasma DNA strongly affect DNA yield. *Clin Chim Acta*. 2011;412:2085—2088.
61. Wolf A, Beller K, Groemminger S, Hofmann W, Sachse M, Fassunke J. Purification of circulating cell-free DNA from plasma and urine using the automated large-volume extraction on the QIAsymphony® SP Instrument. *Adv Exp Med Biol*. 2016;924:179—185.
62. Page K, Guttery DS, Zahra N, et al. Influence of plasma processing on recovery and analysis of circulating nucleic acids. *PLoS One*. 2013;8:e77963.

63. Sang F, Ren J. Comparisons between capillary zone electrophoresis and real-time PCR for quantification of circulating DNA levels in human sera. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2006;838:122−128.
64. Ford A, Spurgin J, Athanasuleas J, Yeh CH. Next-generation liquid biopsy: tumor monitoring from droplet volumes of blood. *J Cancer Prev Curr Res*. 2015;3:00064.
65. Xue X, Teare MD, Holen I, Zhu YM, Woll PJ. Optimizing the yield and utility of circulating cell-free DNA from plasma and serum. *Clin Chim Acta*. 2009;404:100−104.
66. Parpart-Li ST, Bartlett B, Popoli M, et al. The effect of preservative and temperature on the analysis of circulating tumor DNA [published online ahead of print November 8, 2016]. *Clin Cancer Res*. doi:10.1158/1078-0432.CCR-16-1691.
67. Sherwood JL, Corcoran C, Brown H, Sharpe AD, Musilova M, Kohlmann A. Optimised pre-analytical methods improve KRAS mutation detection in circulating tumour DNA (ctDNA) from patients with non-small cell lung cancer (NSCLC). *PLoS One*. 2016;11:e0150197.
68. Lee H, Jeon S, Seo JS, Goh SH, Han JY, Cho Y. A novel strategy for highly efficient isolation and analysis of circulating tumor-specific cell-free DNA from lung cancer patients using a reusable conducting polymer nanostructure. *Biomaterials*. 2016;101:251−257.
69. Umetani N, Giuliano AE, Hiramatsu SH, et al. Prediction of breast tumor progression by integrity of free circulating DNA in serum. *J Clin Oncol*. 2006;24:4270−4276.
70. Breitbach S, Tug S, Helmig S, et al. Direct quantification of cell-free, circulating DNA from unpurified plasma. *PLoS One*. 2014;9:e87838.
71. Cree IA, Deans Z, Ligtenberg MJ, et al. Guidance for laboratories performing molecular pathology for cancer patients. *J Clin Pathol*. 2014;67:923−931.
72. ISO/TC 212 ISO 15189:2012. *Medical Laboratories—Requirements for Quality and Competence*. 2012.
73. ISO/CASCO ISO/IEC 17025:2005. *General Requirements for the Competence of Testing and Calibration Laboratories*. 2005.
74. Saunders N, Zambon M, Sharp I, et al. Guidance on the development and validation of diagnostic tests that depend on nucleic acid amplification and detection. *J Clin Virol*. 2013;56:260−270.
75. Mattocks CJ, Morris MA, Matthijs G, et al. A standardized framework for the validation and verification of clinical molecular genetic tests. *Eur J Hum Genet*. 2010;18:1276−1288.
76. Frampton GM, Fichtenholtz A, Otto GA, et al. Development and validation of a clinical cancer genomic profiling test based on massively parallel DNA sequencing. *Nat Biotechnol*. 2013;31:1023−1031.
77. Thierry AR, Mouliere F, El MS, et al. Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. *Nat Med*. 2014;20:430−435.
78. Devonshire AS, Whale AS, Gutteridge A, et al. Towards standardisation of cell-free DNA measurement in plasma: controls for extraction efficiency, fragment size bias and quantification. *Anal Bioanal Chem*. 2014;406:6499−6512.
79. Duan H, Lu J, Lu T, et al. Comparison of EGFR mutation status between plasma and tumor tissue in non-small cell lung cancer using the Scorpion ARMS method and the possible prognostic significance of plasma EGFR mutation status. *Int J Clin Exp Pathol*. 2015;8:13136−13145.
80. Luo J, Shen L, Zheng D. Diagnostic value of circulating free DNA for the detection of EGFR mutation status in NSCLC: a systematic review and meta-analysis. *Sci Rep*. 2014;4:6269.
81. Chae YK, Davis AA, Carneiro BA. Concordance between genomic alterations assessed by next-generation sequencing in tumor tissue or circulating cell-free DNA [published online ahead of print August 30, 2016]. *Oncotarget*. doi: 10.18632/oncotarget.11692.
82. Tannock IF, Hickman JA. Limits to personalized cancer medicine. *N Engl J Med*. 2016;375:1289−1294.

Edited by Pei-Fang Wei