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Commentary

COMMENTARY

Circulating Tumor DNA Assays in Clinical Cancer Research

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Abstract

The importance of circulating free DNA (cfDNA) in cancer clinical research was recognized in 1994 when a mutated *RAS* gene fragment was detected in a patient's blood sample. Up to 1% of the total circulating DNA in patients with cancer is circulating tumor DNA (ctDNA) that originates from tumor cells. As ctDNA is rapidly cleared from the blood stream and can be obtained by minimally invasive methods, it can be used as a dynamic cancer biomarker for cancer early detection, diagnosis, and treatment monitoring. Despite the potential for clinical use, few ctDNA assays have been cleared or approved by the US Food and Drug Administration. As tools for clinical and translational research, current ctDNA assays face some challenges, and more research is needed to advance use of these assays. On September 29–30, 2016, the Division of Cancer Treatment and Diagnosis at the National Cancer Institute convened a workshop entitled "Circulating Tumor DNA Assays in Clinical Cancer Research" to garner input from industry experts, academia, and government research and regulatory agencies to understand and promote the translation of ctDNA assays to clinical research, with potential to advance to use in clinical practice. This Commentary presents the topics of the workshop covered in the presentations and points made in the discussions that followed: 1) background on ctDNA, 2) potential clinical utility of ctDNA assays, 3) assay technology, 4) assay clinical and analytical validation, and 5) industry perspectives. Additional relevant information that has come to light since the workshop has been included.

Circulating cell-free DNA (cfDNA) is DNA shed from normal or tumor cells, by apoptotic or necrotic processes, into the bloodstream (1,2). cfDNA was originally reported in cancer patients in 1977, but its importance as a tumor biomarker was not recognized until 1994, when a mutated *RAS* gene fragment was detected in a patient's blood sample, attracting the interest of the research community (3,4). Patients with cancer have been reported to have higher levels of cfDNA than healthy individuals. However, the concentration of cfDNA in cancer patients, which ranges from 0 ng/mL to 1000 ng/mL, overlaps with the cfDNA concentrations of 0–100 ng/mL in healthy individuals (1,3), and this impedes the use of cfDNA concentration alone for cancer screening or diagnosis (3). In addition, high concentrations of cfDNA are not specific to cancer, and the concentration varies considerably in the plasma/serum of cancer patients and individuals with nonmalignant disease or inflammatory states and trauma, reflecting a pathological or physiological condition (1,3).

Circulating tumor DNA (ctDNA) is a subset of cfDNA representing up to 1% of the total cfDNA (2). ctDNA can be identified and differentiated from cfDNA by fragments size and/or variations in the genetic abnormalities compared with normal cells (1). In general, ctDNA fragments are shorter than the somatic DNA fragments in plasma, but the profiles of fragments of ctDNA have been found to vary across histologic tumor types and tumor burden, or stages of cancer. For example, in breast cancer, short fragments of ctDNA are more frequently found in patients with metastatic disease compared with patients with early-stage disease (5). Fragments of cfDNA of approximately 10 000 bp originating from necrotic cancers are found in the bloodstream, in contrast with fragments that range from 180 bp to 1000 bp originating from apoptotic tumors (1,2,5). Another way to distinguish ctDNA from cfDNA is by the presence of genetic variations found in cancer, as these genetic variations can be identified using genomic technologies such as digital polymerase chain reaction (PCR) or next-generation sequencing (NGS).

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Because ctDNA is rapidly cleared from the blood stream, it may be used as a dynamic biomarker for tumor biology, and fluctuations in the concentration of ctDNA may assist in monitoring the success of cancer treatment. For example, in stage II colorectal cancer, the recurrence rate is about 20% and chemotherapy recommendations may be needed for the small group of patients with recurrence, if they can be identified. A study in 230 patients (6) using the genetic fingerprint of the patient's own colon cancer showed that the ctDNA could be detected four to 10 weeks after surgery in 8.7% of 178 patients who did not receive adjuvant chemotherapy. Among these patients, 78.6% had radiological recurrence, whereas only 9.8% of patients without evidence of circulating tumor DNA recurred (6). For patients with metastatic cancer, resistance mutations have been detected in plasma before evidence of radiologic progression, and selection of patients for treatment with drugs specific to inhibition of certain mutations has also been detected in this way (7–9).

In contrast to liquid biopsies, tumor tissue is more difficult to obtain, especially in metastatic disease, and tumor biopsy is beset with sampling bias and may potentially miss critical information about tumor heterogeneity (1,2,10). Bardelli's group at the University of Torino (Italy), in one the first prospective analyses in metastatic colorectal cancer, indicated that plasma ctDNA profiling improved detection of RAS mutation (compared with paired tissue biopsies) and showed that liquid biopsies are more comprehensive than tissue biopsies to evaluate tumor heterogeneity (11). In addition, resampling would be greatly facilitated with liquid vs tumor biopsy. However, despite potential for clinical use, ctDNA assays face some challenges for adoption into clinical practice. For example, there is the need to establish a better correlation of ctDNA abnormalities detected in the blood with those detected in solid tumor biopsies and with clinical outcomes. Some mutations detected in ctDNA do not match with mutations detected in the primary tumor DNA. This could be in part due to the fact that apoptotic/necrotic cells are one source of ctDNA and variants in these cells may differ from living cells within a tumor. Another explanation could be attributed in part to limitations in accuracy of current technologies to detect specific mutations in very low copy numbers of ctDNA among large amounts of wild-type cfDNA (12). Another factor to consider could be that tumor cells releasing ctDNA may be genetically different from most tumor cells in primary tumors due to tumor heterogeneity as ctDNA represents a mixture of DNA fragments released into the blood (1,13).

On September 29–30, 2016, the Division of Cancer Treatment and Diagnosis at the National Cancer Institute convened a workshop entitled "Circulating Tumor DNA Assays in Clinical Cancer Research. This Commentary presents the topics of the workshop that were covered in the presentations and points made in the discussions that followed: 1) background on ctDNA, 2) potential clinical utility of ctDNA assays, 3) assay technology, 4) assay clinical and analytical validation, and 5) industry perspectives. Additional relevant information that has come to light since the workshop has been included.

ctDNA Clinical Utility

Clinical utility is defined by the availability of effective interventions and a demonstration that the use of the test results provides a favorable benefit-to-risk ratio for the patient.

Clinical Applications of cfDNA Genotyping for Cancer Care

Genotyping cfDNA is a rapid and convenient option that can offer insights into the heterogeneity of cancer evolution, can potentially detect emergence of resistance, and can allow noninvasive monitoring of disease. However, as pointed out in the workshop by Dr. Geoffrey Oxnard (Dana-Farber Cancer Institute), the number of variants identified by genotyping has increased considerably in the last decade, creating challenges for interpretation of the clinically actionable variants and for delivering meaningful data in a timely fashion to guide patient care. These challenges required development of new technologies such as digital droplet PCR (ddPCR) and NGS. In ddPCR, the DNA is PCR-amplified using fluorescent nucleotides, and the fluorescence is measured in an automated droplet flow cytometer. Each droplet is individually scored based on fluorescence intensity for the mutant or wild-type allele of a target locus (9). In a prospective validation study in 180 patients with non–small cell lung cancer (NSCLC), plasma ddPCR showed an overall sensitivity of 64%–82% for detection of known tumor for EGFR 19 del, 74% for L858R, and 77% for T790M, but lower sensitivity for KRAS at 64% (14). The rate of detection increases with increased tumor burden. Plasma ddPCR exhibited a positive predictive value (PPV) of 100% for EGFR 19 del, 100% for L858R, and 100% for KRAS, but lower PPV for T790M at 79% (14). A clinical trial is underway that is using ddPCR to evaluate use of plasma ctDNA for *EGFR* mutation to rapidly initiate erlotinib as a therapy in metastatic NSCLC (NCT02770014) (15). Limitations of ddPCR include detection of only known genotypes, inability to detect rearrangements, and difficulty in multiplexing to detect more than a few genetic variants. On the other hand, NGS of plasma DNA, when focused on a small gene panel, can detect a range of genomic alterations in NSCLC including *ALK*, *ROS1*, and *RET* rearrangements, *HER2* insertions, and *MET* amplification, with 100% specificity, whereas sensitivity is equivalent to that of ddPCR (16).

Capturing Tumor Heterogeneity and Clonal Evolution Using ctDNA Analysis

Dr. Muhammed Murtaza (Translational Genomic Research Institute, Mayo Clinic) in his talk discussed the identification of cancer mutations in ctDNA at low allele frequency (2%) and high specificity (>97%) using tagged-amplicon deep sequencing (Tam-Seq). Using this method, he identified tumor suppressor gene *TP53* and *EGFR* mutations not found in the original ovarian tissue biopsy in circulating DNA from advanced ovarian cancer patients (17). Dr. Murtaza followed six patients with advanced cancer for one to two years and showed that ctDNA analysis, using exome sequencing, could complement biopsy for identifying mutations associated with acquired drug resistance in advanced cancers (7). In a proof-of-principle study, a patient with metastatic estrogen receptor–positive (ER+), human epidermal growth factor receptor 2–positive (HER2+) breast cancer who received two lines of targeted therapy was followed for three years in a demonstration that the simplicity of ctDNA assays allows the sampling and assessment of multifocal clonal evolution over time (18). The mutations in the patient's plasma DNA reflected the clonal hierarchy inferred from the sequencing of tumor biopsies. Subclone-specific mutations in ctDNA in serial samples correlated with varying treatment responses in different metastatic sites (19). Comparison of biopsy and plasma

samples in individual patients with metastatic breast cancer demonstrated that ctDNA provides a dynamic sampling of somatic alterations that reflects the size and activity of distinct tumor subclones. Analysis of ctDNA mutations also mirrors the clonal hierarchy determined by multiregional tumor sequencing and tracks the variability of treatment responses by different metastases (18). More extensive studies beyond proof-of-principle demonstrations are required to rigorously evaluate this potential.

Deep Sequencing of ctDNA for Cancer Detection and Monitoring

Dr. Maximilian Diehn (Stanford University) discussed an NGS-based approach of using Cancer Personalized Profiling by Deep Sequencing (CAPP-Seq) for analysis of ctDNA developed by his group at Stanford. This technology achieves both an ultra-low detection limit and broad patient coverage in that the assay is applicable to many tumor types at stage II and above at a reasonable cost (20). This method uses labeled DNA oligonucleotides (selectors) that target known DNA sequences mutated in a cancer of interest. The methodology was further refined by utilizing integrated digital error suppression (iDES), an approach that eliminates most technical artifacts observed in cfDNA sequencing (21). When iDES was applied to pretreatment plasma from a 30-patient NSCLC cancer cohort, ctDNA was detectable in 93% of patients. ctDNA was also detectable in 73% of pre- and post-treatment plasma samples, with a specificity of 100%. With these refinements, CAPP-Seq can achieve equivalent or better sensitivity than ddPCR (four in 105 cfDNA molecules) and can test a much wider number of loci on the genome (21).

Utility of Monitoring ctDNA in Metastatic Melanoma Disease Surveillance

Melanoma is a highly curable disease when treated at early stage (22,23). Serum lactate dehydrogenase (LDH) concentration is the only current serologic marker for melanoma, and though used in staging and in monitoring advanced cases, its sensitivity and specificity are low. Dr. David Polsky (NYU School of Medicine) in his talk discussed a clinical study using ddPCR with amplification of melanoma mutation hotspots in *BRAF* and *NRAS*, using the QX100 ddPCR system (Bio-Rad). In this study, he demonstrated that ctDNA has a higher sensitivity than LDH (82% and 40%, respectively) to evaluate disease progression. ctDNA was also elevated in 83% of cases of new brain metastases compared with LDH, which was elevated in 50% of cases of new brain metastases. This study showed the potential for ctDNA as a biomarker for monitoring melanoma (24).

Diagnosing and Treatment Monitoring in Esophageal Adenocarcinoma Using ctDNA

There are promising data showing that ctDNA is detectable in 100% of metastatic cases of gastroesophageal cancer (25), but one issue is the small (often fragmented) amount of DNA in plasma (~10–50 ng/mL of cfDNA), which translates to only about 1–10 copies/mL. Dr. Tony Godfrey (Boston University) focused his talk on his studies on esophageal adenocarcinoma (EAC) using a modified version of the SafeSeq-S sequencing methodology (26), called Simple, Multiplexed, PCR-based barcoding of DNA for Sensitive mutation detection using Sequencing (SiMSen-Seq). SiMSen-Seq uses primers with barcodes in an internal hairpin design, and this modification protects the barcode sequence during PCR-based NGS library preparation for more efficient identification of true mutations from polymerase-induced errors (27). This method enables flexible multiplexing with a background consensus error rate below 0.1%. Dr. Godfrey found that greater sensitivity of mutation detection can be achieved with shorter amplicons of less than 80 bp. The strengths of the SiMSen-Seq methodology are that it requires less than 5 ng of DNA, allows rapid and easy library preparation, is useful for coverage of less than 1000 bp, is easily customizable for individual diagnostic needs, and the cost per sample is low.

cfDNA Assay Technology

Electric Field Induced Release and Measurement–Based Liquid Biopsy Technology

Dr. David Wong (UCLA) discussed the utility of Electric Field–Induced Release and Measurement (EFIRM) technology in the detection of *EGFR* mutations in saliva and plasma for monitoring treatment response on NSCLC cancer patients (28,29). The EFIRM technology works with noninvasive samples and rapid turnaround time (<30 minutes). The technology is sensitive (95%), requires only 40 μL sample volume collected non- or minimally invasively, and can be multiplexed. In addition, the EFIRM device is suitable for use in low-resource settings and can transmit data wirelessly to a primary care provider. The method uses an electric field to release nucleic acids trapped in exosomes and other extracellular vesicles with phospholipid bilayers that are captured on a microarray using specific probes for capture and detection (30).

Nanotechnology and Microfluidics for Analysis of DNA Methylation

Increased DNA methylation can contribute progression of carcinogenesis, for example, by silencing tumor suppressor genes. These epigenetic changes can occur before somatic mutations are detectable. Measuring promoter methylation may be promising for early cancer diagnosis; however, conventional methylation-specific PCR-based methods are not sensitive enough to detect DNA methylation in body fluids (nested PCR or digital PCR is required). Dr. Jeff Wang (Johns Hopkins University) in his talk presented a DNA methylation sensor based on an inorganic/organic hybrid FRET nanosensor linked to streptavidin-quantum dot (QD-FRET) with a low level of background fluorescence not seen with conventional organic FRET probes (31,32).

Dr. Wang also discussed another method for ultrasensitive assessment of locus-specific epigenetic heterogeneity in a liquid biopsy called Discrimination of Rare EpiAlleles by Melt (DREAMing). DREAMing uses precise melt curve analysis to distinguish and enumerate individual copies of epiallelic variants at single-CpG-site resolution in liquid biopsies, even with variant fractions as low as 0.005% (33). The method can identify a methylated variant in the presence of 10 000 excess unmethylated alleles and requires only 15 pg of cfDNA or tumor DNA.

Dr. Wang is currently optimizing a streamlined microfluidics-based approach that fully integrates DNA extraction, bisulfite conversion, and PCR detection to allow high-throughput detection and analysis of DNA methylation variants at a point-of-care setting.

Patch-PCR: A Targeted Sequencing Approach to Quantify Breast Cancer ctDNA

Dr. Katherine Varley (University of Utah) presented Patch PCR. Patch PCR was introduced in 2008 as a sequencing method for detection of somatic mutations in tumors (34–36). However, this technology has not yet been translated to clinical use. In 2016, Dr. Varley received a grant from the National Cancer Institute to translate Patch PCR for ctDNA testing for breast cancer recurrence (37), which will hopefully lead to rapid translation of the Patch PCR ctDNA assay from small-scale academic projects to a robust clinical-grade Clinical Laboratory Improvement Amendments laboratory test. Dr. Varley adapted this technology to create a small Patch PCR panel that can be effective in large portion of breast cancer patients. For example, 75% of breast cancer patients can be identified by mutations among four specific genes (PIK3CA, TP53, GATA3, and CDH1); therefore, increasing the panel size will not necessarily improve its efficacy. In addition, Dr. Varley proposes to include in the Patch PCR panel hotspot mutations that could provide information about tumor resistance to therapy; for example, hotspot mutations in ESR1 and LYN inhibitory SH2 domain that confer resistance to hormone therapy. There are many opportunities to study ctDNA analysis in clinical trials to improve clinical management of breast cancer patients, impacting a large population. As a clinically relevant example, each year in the United States, 39 million mammograms and 1.6 million biopsies are performed, and approximately 246 000 new cases of invasive breast cancer are diagnosed, implying that mammography identifies many false-positive cases (38).

Clinical and Analytical Validation of ctDNA Assays

Challenges for the Validation of ctDNA Assays for Use in Clinical Trials

Dr. Christopher Gocke (Johns Hopkins University) in his talk described challenges for the validation of ctDNA assays including the development of standardized protocols, development of purpose-driven (fit-for-purpose) tests, and selection of appropriate analytes.

A prime example of the challenge in this area is the lack of consistent, reproducible, and reliable protocols to isolate DNA from blood using blood collection tubes. As none of the published protocols have been compared directly, there is little evidence indicating whether one protocol is better than another and how the DNA isolated from each method differ in yield or other variables. More information about the importance of cfDNA stabilization and extraction is presented in the Supplementary Material (available online).

The need to define a purpose-driven or fit-for-purpose test is also a challenge. Essentially, the purpose of a test or assay must be decided before the test is defined. Even deciding between three of the broadest purposes, namely, screening vs diagnostic vs monitoring, is critical to developing the assay; each purpose demands a different test and imposes radically different requirements.

Analyte selection provides another challenge. The analyte selected must represent a true measure of a target or a process the assay will detect, measure, or monitor and must be reliably measured by the selected platform. For example, if the intention is to measure ctDNA concentration, it is implied that the type of cancer is not important. In reality, not all cancers shed cells/DNA at the same rate. The ability to monitor cancer burden or minimal residual disease will be related to the rate of shedding. Likewise, if testing for point mutations or indels, the platform characteristics and depth of coverage are critical for making correct calls on the mutation's presence. Although the science of ctDNA identification began decades ago, it will continue to require extensive development to refine its use in clinical trials. More information about general principles of analytical validation is presented in the Supplementary Material (available online).

Regulatory Aspects of ctDNA Assays as Companion Diagnostics

Dr. Reena Philip, Director of the Division of Molecular Genetics and Pathology for the US Food and Drug Administration (FDA; Center for Diagnostics and Radiologic Health), discussed the regulatory aspects of ctDNA assays using the first FDA-approved liquid biopsy test as an example. The Cobas *EGFR* Mutation Test v2, which detects the gene mutations associated with sensitivity to EGFR inhibitors in non–small cell lung cancer, has been approved as a companion diagnostic for the cancer drug Tarceva (erlotinib). The FDA reviewed many aspects of the test, including the target patient population, the specific marker(s) detected by the assay, the assay cutoff values, and the defined specimen type to ensure adequate analytical validation and clinical validation. However, even with the FDA's approval of one liquid biopsy test, there are still big challenges for ctDNA assay development. Some of the issues include limited availability of clinical specimens for analytical validation, lack of appropriate samples representative of the intended-use patient population, and lack of reference methods and standard reference materials to assess results' concordance between liquid biopsy and tissue. The FDA requires relevance to clinical outcomes, not just analytical status.

ctDNA Assays, Industry Perspective

Dr. Teresa Zhang, Personal Genome Diagnostics (PGD) Vice President, described the potential advantages of the cfDNA assay PLASMASELECT (developed by PDG), which was designed to evaluate a targeted panel of alterations in 64 well-characterized cancer genes. This panel identifies clinically actionable and functionally important sequence mutations and structural alterations across multiple cancer types, reports microsatellite instability status (MSI), and identifies sequence mutations, amplifications, and translocations. The assay could be useful in choosing treatment for patients with targetable mutations. She commented that after treatment with EGFR inhibitors, the majority of patients with lung adenocarcinoma develop resistance gate keeper mutations (eg, *EGFR* T790M), but 15% of patients develop *MET* amplification as a resistance mechanism.

Kelli Bramlett discussed the new Thermo Fisher Scientific plasma cfDNA assay, Oncomine, for rare variant detection. Sequencing is performed on the nsION-Torrent system, and analysis is performed on a Torrent suite reporter. The Oncomine cfDNA library kit requires 1–20 ng of DNA and utilizes a unique identifier (added before sample processing), which allows tracking molecules through the library preparation process and enables variants to be called at very low frequencies. The Oncomine Lung cfDNA assay covers around 169 hotspot mutations in 11 genes.

EGFR T790M mutation drives resistance in 50% of *EGFR*-mutated NSCLC patients who become resistant to a first-generation EGFR tyrosine kinase inhibitor, but as there are multiple resistance mechanisms, follow-up using only *EGFR* T790M, a more comprehensive approach, would be required for monitoring cancer progression. Therefore, a multiplex assay may be more efficient in this clinical situation. For clinical practice, the National Comprehensive Cancer Network (NCCN) guidelines recommend testing for genomic alterations in 11 somatic genomic targets in seven cancer types including NSCLC, colorectal, breast, gastric, melanoma, and gastrointestinal stromal tumor. However, testing for multiple alterations is limited by the amount of tissue available/collected. Addressing these issues, Dr. Richard Lanman (Guardant Health) discussed the Guardant 360 (G360) ctDNA assay and its use in different clinical trials. The overall accuracy of ctDNA sequencing in comparison with matched tissue DNA sequencing was 87%. The accuracy further increased to 98% when blood and tumor were collected within less than six months of each other. Somatic alteration patterns in ctDNA samples largely agree with tissue alteration patterns, except for resistance mutations. In discordant cases, assessment of clinical utility of this assay may depend on outcomes of clinical trials that use it to identify patients with eligible molecular abnormalities.

Latest Developments in ctDNA Cancer Research

Several recent publications have pointed toward the utility of ctDNA assays, in some cases coupled with proteomic analysis, for the detection and localization of surgically resectable cancers (39) and for depicting early-stage lung cancer evolution (40). This work demonstrates the ability of ctDNA assays to assist clinicians in the identification of tumor characteristics and tumor behavior using noninvasive methodologies.

In March 2018, a review has been published jointly by the American Society for Clinical Oncology (ASCO) and the College of American Pathologists (CAP) to summarize the evolving state of knowledge regarding the value of ctDNA assays for the care of cancer patients (41,42). The reviewers noted that some consensus is emerging as to the preferred methods for sample collection and preparation to optimize the analytic validity of ctDNA assays. The number of instances where there is sufficient evidence of clinical validity to support regulatory approval of a ctDNA test is still quite small. And even in these instances, the clinical sensitivity of the tests is modest; cases where the blood test is negative but the variant can be detected in the tumor tissue are fairly common. Although the Updated Molecular Testing Guideline for the Selection of Lung Cancer Patients for Treatment with Targeted Tyrosine Kinase Inhibitors recently issued jointly by the Association for Molecular Pathology, the College of American Pathologists, and the International Association for the Study of Lung Cancer addressed the role of ctDNA tests for this indication, the recommendations are relatively cautious (43). These guidelines recommend that cell-free plasma DNA molecular methods may be used to identify EGFR mutations in settings where tumor tissue is limited or insufficient for molecular testing, and may also be used to identify EGFR T790M mutations in patients with resistance to EGFR-targeted inhibitors, with the caveat that tissue should be tested if the blood sample is negative. But the same guidelines state that there is currently insufficient evidence to recommend the use of cfDNA for the diagnosis of primary lung adenocarcinoma. Similarly, the ASCO/CAP reviewers note that efforts to establish clinical validity for ctDNA assays in other forms of advanced cancer are ongoing but face considerable challenges, both technical and biological. For the use of ctDNA assays in other settings, such as detection of residual disease following treatment for primary cancers or detection of tumors in screening asymptomatic individuals, the evidence for clinical validity does not yet support their use outside clinical trials.

Conclusions

Analysis of ctDNA shows promising clinical value for cancer research and practice. ctDNA assays are noninvasive and feasible to track tumor behavior longitudinally, thus potentially enhancing cancer management by assessing tumor burden, detecting recurrence, monitoring early response, and identifying drug resistance. Specific genomic alterations identified in the ctDNA can be used for quantifying cancer dynamics and as a genomic tool for molecular characterization of tumors.

Despite this exciting potential, few ctDNA assays have been fully integrated into the clinical workflow. There is a need to establish a better correlation of ctDNA abnormalities detected in the blood with those found in solid tumor biopsies and with clinical outcomes. There are approximately 100 clinical trials registered at ClinicalTrials.gov in which the main focus is to evaluate ctDNA as a biomarker, and an additional 100 trials in which ctDNA is a secondary outcome measurement. Many of these trials (about 67%) were opened to accrual in 2016 (the earliest trial registered started in 2005), indicating the growing interest of the community to validate ctDNA assays. Results should begin to be available within the next five years. Although ctDNA assays are beginning to be approved and used for a small panel of molecular alterations, to date, ctDNA assays that measure as large a number of genomic alterations accurately are not available, thus theoretically preventing a ctDNA assay being used for molecular screening for a large trial such as NCI-MATCH. Currently, early diagnosis of cancer using ctDNA assays represents a formidable challenge, not only for detecting minute concentrations of aberrant DNA while minimizing false-positive results, but for pinpointing from which type of tumor (and which location) the ctDNA may originate, absent imaging evidence of a tumor mass. Early detection was not a focus of this workshop but does represent an opportunity and challenge for ctDNA assessment. A study on ctDNA from 1059 asymptomatic high-risk cancer individuals using a nine-gene, 96-mutation panel found a large false-positive rate, limiting the value of the ctDNA test for early detection (44). Other review articles on ctDNA also suggest that realization of the potential of ctDNA for early detection of cancer requires further advancements in sample processing, nucleic acid extraction and sequencing technologies addressing artifacts, improving sensitivity and specificity of the entire analysis pipeline, identification of possible combinations of cancer-specific mutations, and defining potential quantitative thresholds to avoid overdiagnosis (45).

The lack of consistent protocols for ctDNA isolation and the lack of correlation of the results of ctDNA assays with results of other ctDNA or tumor assays may delay the integration of ctDNA assays into the clinic. Efforts at uniform and/or fit-for-purpose collection, handling and storage techniques, and confirmation of findings of a ctDNA assay by another ctDNA technique may be helpful in this regard and could be incorporated into future clinical trials using targeted treatments. Instead of confirmation with an orthogonal assay, or along with such confirmation, the development and use of standard reference

materials will aid in the ability to compare ctDNA assays and choose the best assay for the purpose.

Novel assays that may be useful in clinical practice or clinical trials are being developed. The increasing availability of improved methods and strategies with higher sensitivity and specificity for ctDNA analysis is expected to allow detection of smaller amounts of ctDNA, as well as broader panels of genomic alterations, thus enabling extraction of more information from limited samples for the study of both early- and later-stage cancers.

Notes

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References

1. Howell JA, Khan SA, Knapp S, Thursz MR, Sharma R. The clinical role of circulating free tumor DNA in gastrointestinal malignancy. *Transl Res*. 2017;183:137–154.
2. Huang WL, et al. Liquid biopsy genotyping in lung cancer: Ready for clinical utility? *Oncotarget*. 2017;8(11):18590–18608.
3. Schwarzenbach H, Hoon DSB, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer*. 2011;11(6):426–437.
4. Yong E. Cancer biomarkers: Written in blood. *Nat News Comment*. 2014;511(7511):3.
5. Mouliere F, Rosenfeld N. Circulating tumor-derived DNA is shorter than somatic DNA in plasma. *Proc Natl Acad Sci U S A*. 2015;112(11):3178–3179.
6. Tie J, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. *Sci Transl Med*. 2016;8(346):346ra92.
7. Murtaza M, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature*. 2013;497(7447):108–112.
8. Remon J, et al. Osimertinib benefit in EGFR-mutant NSCLC patients with T790M-mutation detected by circulating tumour DNA. *Ann Oncol*. 2017;28(4):784–790.
9. Oxnard GR, et al. Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. *Clin Cancer Res*. 2014;20(6):1698–1705.
10. Diaz LA Jr, Bardelli A. Liquid biopsies: Genotyping circulating tumor DNA. *J Clin Oncol*. 2014. 32(6):579–586.
11. Siena S, et al. Dynamic molecular analysis and clinical correlates of tumor evolution within a phase II trial of panitumumab-based therapy in metastatic colorectal cancer. *Ann Oncol*. 2018;29(1):119–126.
12. Arriola E, et al. Comparison of plasma ctDNA and tissue/cytology-based techniques for the detection of EGFR mutation status in advanced NSCLC: Spanish data subset from ASSESS. *Clin Transl Oncol*. In press.
13. Toor OM, et al. Correlation of somatic genomic alterations between tissue genomics and ctDNA employing next generation sequencing: Analysis of lung and gastrointestinal cancers. *Mol Cancer Ther*. In press.
14. Sacher AG, et al. Prospective validation of rapid plasma genotyping for the detection of EGFR and KRAS mutations in advanced lung cancer. *JAMA Oncol*. 2016;2(8):1014–1022.
15. Oxnard G. Rapid plasma genotyping for early initiation of erlotinib in EGFR mutant lung cancer NCT02770014. 2016. https://clinicaltrials.gov/ct2/show/NCT02770014. Accessed May 30, 2018.
16. Paweletz CP, et al. Bias-corrected targeted next-generation sequencing for rapid, multiplexed detection of actionable alterations in cell-free DNA from advanced lung cancer patients. *Clin Cancer Res*. 2016;22(4):915–922.
17. Forshew T, et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. *Sci Transl Med*. 2012;4(136):136ra68.
18. Murtaza M, et al. Multifocal clonal evolution characterized using circulating tumour DNA in a case of metastatic breast cancer. *Nat Commun*. 2015;6:8760.
19. Kim JY, et al. Circulating tumor DNA shows variable clonal response of breast cancer during neoadjuvant chemotherapy. *Oncotarget*. 2017;8(49):86423–86434.
20. Newman AM, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. *Nat Med*. 2014;20(5):548–554.
21. Newman AM, et al. Integrated digital error suppression for improved detection of circulating tumor DNA. *Nat Biotechnol*. 2016;34(5):547–555.
22. Sandru A, Voinea S, Panaitescu E, Blidaru A. Survival rates of patients with metastatic malignant melanoma. *J Med Life*. 2014;7(4):572–576.
23. ACS. Survival rates for melanoma skin cancer, by stage. 2016. https://www.cancer.org/cancer/melanoma-skin-cancer/detection-diagnosis-staging/survival-rates-for-melanoma-skin-cancer-by-stage.html. Last accessed May 30, 2018.
24. Chang GA, et al. Sensitivity of plasma BRAFmutant and NRASmutant cell-free DNA assays to detect metastatic melanoma in patients with low RECIST scores and non-RECIST disease progression. *Mol Oncol*. 2016;10(1):157–165.
25. Bettegowda C, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med*. 2014;6(224):224ra24.
26. Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B. Detection and quantification of rare mutations with massively parallel sequencing. *Proc Natl Acad Sci U S A*. 2011;108(23):9530–9535.
27. Stahlberg A, et al. Simple, multiplexed, PCR-based barcoding of DNA enables sensitive mutation detection in liquid biopsies using sequencing. *Nucleic Acids Res*. 2016;44(11):e105.
28. Wei F, et al. Noninvasive saliva-based EGFR gene mutation detection in patients with lung cancer. *Am J Respir Crit Care Med*. 2014;190(10):1117–1126.
29. Pu D, et al. Evaluation of a novel saliva-based epidermal growth factor receptor mutation detection for lung cancer: A pilot study. *Thorac Cancer*. 2016;7(4):428–436.
30. Wei F, Yang J, Wong DT. Detection of exosomal biomarker by electric field-induced release and measurement (EFIRM). *Biosens Bioelectron*. 2013;44:115–121.
31. Zhang CY, Yeh HC, Kuroki MT, Wang TH. Single-quantum-dot-based DNA nanosensor. *Nat Mater*. 2005;4(11):826–831.
32. Bailey VJ, Keeley BP, Razavi CR, Griffiths E, Carraway HE, Wang TH. DNA methylation detection using MS-qFRET, a quantum dot-based nanoassay. *Methods*. 2010;52(3):237–241.
33. Pisanic TR 2nd, et al. DREAMing: A simple and ultrasensitive method for assessing intratumor epigenetic heterogeneity directly from liquid biopsies. *Nucleic Acids Res*. 2015;43(22):e154.
34. Varley KE, Mitra RD. Nested patch PCR enables highly multiplexed mutation discovery in candidate genes. *Genome Res*. 2008;18(11):1844–1850.
35. Varley KE, Mitra RD. Bisulfite patch PCR enables multiplexed sequencing of promoter methylation across cancer samples. *Genome Res*. 2010. 20(9):1279–1287.
36. Varley KE, Mitra RD. Nested patch PCR for highly multiplexed amplification of genomic loci. *Cold Spring Harb Protoc*. 2009;2009(7):pdb.prot5252.
37. NIH-RePORTER. A partnership to enable clinical ctDNA testing for breast cancer recurrence. 2016. https://projectreporter.nih.gov/project_info_description.cfm?aid=9288158&icde=38957123. Last accessed May 30, 2018.
38. Løberg M, Lousdal MT, Bretthauer M, Kalager M. Benefits and harms of mammography screening. *Breast Cancer Res*. 2015;17(1):63.
39. Cohen JD, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science*. 2018;359(6378):926–930.
40. Abbosh C, et al. Corrigendum: Phylogenetic ctDNA analysis depicts early-stage lung cancer evolution. *Nature*. 2018;554(7691):264.
41. Merker JD, et al. Circulating tumor DNA analysis in patients with cancer: American Society of Clinical Oncology and College of American Pathologists joint review. *J Clin Oncol*. In press.
42. Merker JD, et al. Circulating tumor DNA analysis in patients with cancer: American Society of Clinical Oncology and College of American Pathologists joint review. *Arch Pathol Lab Med*. In press.
43. Lindeman NI, et al. Updated molecular testing guideline for the selection of lung cancer patients for treatment with targeted tyrosine kinase inhibitors: Guideline from the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology. *J Mol Diagn*. 2018;20(2):129–159.
44. Kammesheidt A, Tonozzi TR, Lim SW, Braunstein GD. Mutation detection using plasma circulating tumor DNA (ctDNA) in a cohort of asymptomatic adults at increased risk for cancer. *Int J Mol Epidemiol Genet*. 2018;9(1):1–12.
45. Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer*. 2011;11(6):426–437.