7 Circulating Cell-Free DNA for Molecular Diagnostics and Therapeutic Monitoring

Natasha B. Hunter, Julia A. Beaver, and Ben Ho Park

Introduction

The presence of circulating cell-free DNA (ccfDNA) in the human blood was originally discovered in 1948 by Mandel and Métais, a full 32 years before Frederick Sanger would win the Nobel prize for DNA sequencing [1]. At the time, the discovery was of purely biochemical interest, with no clinical utility. But with technological advances making molecular studies feasible and affordable in recent decades, interest in the potential medical applications of ccfDNA has resurfaced. Initially, scientists explored uses in maternal–fetal medicine and oncology, with more recent efforts broadening to include other disease processes such as sepsis, myocardial infarction, stroke, and diabetes [2–5].

This free-floating or "naked" DNA in the blood appears to derive from cells in both healthy and diseased states. Its origin is not fully understood and could involve multiple mechanisms. Some portion of ccfDNA is likely shed into the circulation by macrophage release of necrotic or apoptotic cellular debris [6], or some cells may actively secrete ccfDNA into the circulation [7].

Circulating cell-free DNA has been found in most human fluids: whole blood, serum, plasma, urine, and cerebral spinal fluid, with fragments ranging from 70 to 1200 base pairs in length [8–12]. Its half-life is short, on the order of 15 min to a few hours; it is quickly cleared by the kidney and liver [10, 13–15]. Patients with metastatic cancer, trauma, myocardial infarction, and sepsis display higher concentrations of overall ccfDNA than normal controls [9, 16–20], perhaps because all these processes involve high cell turnover.

Researchers are investigating numerous potential applications for ccfDNA-based assays in various disease states, but few have been approved by national regulatory bodies, and no standard testing platform exists. It should be mentioned that this review discusses free DNA in plasma, with an emphasis on oncologic applications; this is distinct from circulating tumor cells (CTCs), which are also being investigated as cancer biomarkers.

Applications in Maternal–Fetal Medicine

In the late 1970s, fetal cells were first discovered in the maternal circulation, and subsequent work demonstrated that small amounts of fetal ccfDNA was also present in maternal blood [21–23]. The prospect of noninvasive prenatal testing (NIPD) using maternal–fetal ccfDNA fueled interest in the field. Fetus-derived ccfDNA was determined to be likely placental in origin and shorter than maternal DNA (with fetal at <300 base pairs) [24]. The fetal fraction of ccfDNA accounts for approximately 10% of the total ccfDNA, although studies differ slightly, and the percentage may rise as gestation progresses [25–27].

The fetal ccfDNA in maternal circulation not only represents a small portion of the total, it is also haplotypically identical to the mother, so paternally inherited characteristics have been comparatively easier to decipher. For example, investigators in the 1990s were already demonstrating fetal rhesus D genotyping and fetal sex assessment by PCR for Y chromosome sequences [22, 28]. Later Fan et al. used direct shotgun sequencing followed by chromosome mapping to establish over- or underrepresentation of chromosomes in maternal plasma ccfDNA, thereby identifying potential aneuploidy (i.e., an abnormal number of chromosomes, most commonly trisomies 21, 18, or 13) [29]. Numerous other investigators have employed next-generation massively parallel sequencing (MPS) to detect aneuploidy, many in large-scale studies [30–32]. Indeed, NIPD using ccfDNA has already entered clinical practice: in 2015, the American College of Maternal Fetal Medicine released guidelines on cell-free DNA screening for fetal aneuploidy, cautiously endorsing ccfDNA testing for the most common trisomies and for sex chromosome analysis [33].

N. B. Hunter · J. A. Beaver · B. H. Park (✉)
The Sidney Kimmel Comprehensive, Cancer Center at Johns Hopkins, Baltimore, MD, USA
e-mail: bpark2@jhmi.edu

G. J. Netto, K. L. Kaul (eds.), *Genomic Applications in Pathology*, https://doi.org/10.1007/978-3-319-96830-8_7

Determining the maternally inherited portion of the fetal genome initially presented a difficult problem, as it required detection of genomic information that is identical to its background. Lo and colleagues were able to develop a method called "relative haplotype dosage analysis" (RHDO), which used highly accurate quantification to devise a solution; that is, if 10 percent of ccfDNA is fetal, then there should be a 5% overrepresentation of the maternal haplotype in the total ccfDNA [34]. Further studies are ongoing to detect monogenic diseases, which present similarly tough detection problems, with autosomal recessive conditions posing a particular challenge [32].

Applications in Cancer Diagnostics

Using radioimmunoassays, Leon et al. in 1977 first demonstrated higher levels of circulating ccfDNA in cancer patients compared to healthy individuals [35]. The range of ccfDNA concentrations in patients with malignancies varied substantially, however—between 0 and >1000 ng/ml, while normal subjects exhibited ccfDNA concentrations between 0 and 100 ng/ml [14, 36–38]. Given this overlap in ccfDNA concentrations in normal and cancer patients, it seemed unlikely that the total quantity of ccfDNA would prove a reliable diagnostic tool. This was confirmed in 2016 with the first large prospective study showing ccfDNA levels in NSCLC could not predict disease recurrence [39].

The promise of ccfDNA in cancer applications, therefore, depends on distinguishing a small population of DNA shed by the tumor from a larger population of normal patient DNA. In this regard, the task bears similarity to fetal DNA detection, with sometimes only a few small changes distinguishing the proverbial needle from the haystack of normal host genetic material. Indeed, studies have demonstrated that a patient with a solid tumor comprising 3×10^{10} cells, tumor DNA will make up about 3.3% of the already small amount of ccfDNA found in that patient's bloodstream daily [9].

The changes that signal the presence of plasma tumor DNA, or ptDNA, include mutations, epigenetic alterations, amplifications, and rearrangements resulting from translocations and deletions or insertions. Multiple groups have demonstrated that the size of ptDNA molecules is smaller than that of plasma DNA derived from normal cells and typically ranges from 70 to 200 bp [8, 9, 40]. Some mutations and rearrangements in tumor suppressor genes or oncogenes drive the development and progression of a given cancer; others are so-called "passenger" mutations, i.e., genetic alterations that probably result from tumor genetic instability but carry no functional consequence. All these mutations represent somatic changes (i.e., not present in the patient's germline DNA and therefore not heritable—but also not present in the patient's normal tissue) and thus potential cancer markers [41].

The implications of all of this are tantalizing: the short half-life of ptDNA lends itself to providing a snapshot of tumor burden and response to therapies. The ability to use a blood sample to perform a "liquid biopsy" offers a noninvasive, real-time assessment of both qualitative (molecular tumor genotype) and quantitative (burden of disease) aspects of a patient's tumor, providing a potentially more sensitive analysis than radiography or even surgery. Moreover, ptDNA could be a more specific biomarker than others in use clinically today, as it represents direct evidence of tumor, not just an associated nonspecific proxy of disease.

As an example, one can envision testing postsurgical patients for residual micrometastatic disease to assess the need for adjuvant treatment, possibly preventing the administration of unnecessary toxic systemic therapies. Such a validated technique could guide the substitution of various therapies (e.g., chemotherapy vs. hormonal vs. biologic) in the adjuvant or metastatic setting by triggering a change when personalized DNA markers do not respond to therapy or when new markers arise. Real-time assessment of the molecular profile of a tumor could drive rapid and mutation-enriched clinical trial enrollment and create new surrogate endpoints, expediting drug approval. The sections that follow will discuss current and developing clinical applications, along with anticipated directions and possible roadblocks.

The Rapid Advance of Genomic Technologies

As researchers hail ptDNA as a new frontier in cancer biomarkers, it is easy to forget that we have been performing blood-based molecular genetic testing in hematologic malignancies for decades. The Philadelphia chromosome that defines chronic myelocytic leukemia (CML) was identified in 1959 using chromosomal electron microscopy; its transcript was later characterized by the quantitative real-time PCR (qPCR) methods developed by Heid et al. [42]. This standardized assay has enabled real-time monitoring of disease burden and response to treatment using peripheral blood or bone marrow samples [43].

Because leukemias are by definition cancers of the blood, abundance of circulating leukemic (i.e., tumor) cells in the peripheral blood and bone marrow facilitates easier detection of fusion transcripts. Modern assays also identify other commonly occurring mutations, e.g., tandem repeats of the FLT3 gene. The majority of hematologic malignancies display a limited array of driver mutations, and many of these define the disease and establish the diagnosis. In contrast, the ability to identify the corresponding circulating tumor cells (CTCs) for most solid malignancies has been hindered by

low sensitivity (though newer capture methods have shown promise) [44]. And while cell-free tumor DNA in solid malignancies is more easily detected than CTCs, the rarity of predictable recurrent somatic rearrangements in these diseases has complicated assay development.

Over recent decades, new technologies have emerged that allow faster, cheaper gene exploration as well as highly sensitive detection of known genes, placing clinical applications for ptDNA in solid tumors within ever-easier reach. Simple PCR entered common practice in the late 1980s, and dramatically advanced the ability to detect genetic changes. In the 1990s, several labs independently developed variations on a new, highly sensitive PCR method by which individual strands of DNA were amplified separately, generating a binary result for each molecule and allowing both detection and quantification [45]; Kinzler and Vogelstein coined the term "digital" PCR for this technique in their 1999 paper describing sample partitioning in 384-well microplates [46]. The same group in 2005 developed an emulsion-based digital PCR (ddPCR) method called BEAMing (for Beads, Emulsion, Amplification, and Magnetics, outlining the steps involved); using this semiautomated technique, the group was able to identify patients with point mutations in mutant APC molecules in both early-stage and metastatic colorectal cancer patients [9].

In the mid-2000s, high-throughput DNA sequencing, termed next-generation sequencing (NGS), transformed genomics by enabling DNA processing on an order of magnitude larger than prior Sanger methods could accomplish in the same timeframe [47]. NGS technologies advanced rapidly, and new platforms along with fierce competition within the industry have driven costs down exponentially. Sequencing delivers not only detection and quantification of known mutations as digital PCR provides but also allows for identification of new mutations and alterations. This has dramatically accelerated the discovery of patterns of mutation and tumor evolution in cancer research. The newest methods also boast high sensitivity; e.g., a platform called TAM-seq described by Forshew et al. can detect mutations with allele frequencies as low as 2 percent in the ptDNA of patients with advanced cancer [48]. A technique called SafeSeqs employs a "barcoding" method to tag DNA strands before amplifying them, producing tagged "families" of clones that must be 95% identical to be called as a true variant, thereby reducing error. Duplex sequencing also employs tags, marking double-stranded DNA before PCR amplification and then establishing single-stranded consensus sequences that are compared with their complementary strands to yield a "duplex consensus sequence"; this has greatly improved accuracy, allowing detection of one mutation among 10^7 bases [49, 50].

Despite these refinements, NGS still lags behind PCR in sensitivity and lacks the speed and cost-efficiency of PCR for applications where discovery of new mutations is not an issue. Therefore, researchers developing ptDNA assays must weigh depth against breadth of DNA sequencing, as well as time constraints and cost. To elaborate, all sequencing involves some error; increasing the number of times one "reads" each string of bases reduces this error. The number of reads is the "depth" of sequencing. One can sequence larger portions of the genome at a lower depth and gain a broader range of information, or "breadth" of sequencing, but with less certainty as to its accuracy. In other words, knowing exactly which mutation to expect, e.g., a KRAS G12 V mutation in pancreatic cancer, allows one to deploy a sensitive, fast, and cheap test (PCR) for this specific alteration, with the understanding that if other mutations exist, they will not be detected. Conversely, one can design a broader, more expensive and usually slower assay using NGS to look for a variety of mutations, with the understanding that sensitivity for each of these may be lower.

These questions are particularly relevant in ptDNA, because of the small amounts of DNA in circulation. Leary and colleagues demonstrated a 0.61- to 1.97-fold copy number increase in the plasma of cancer patients compared to normalized controls, but the assay succeeded only when the percentage of ptDNA compared to ccfDNA was at least 0.75%, at which point it carried a sensitivity of >90% and specificity of >99% [51]. This study illustrates the critical consideration of depth of sequencing, given the low concentration of ptDNA at baseline, especially in the setting of early-stage cancers or minimal residual disease.

Without large amounts of DNA available for testing, one must balance breadth and depth when choosing an assay. For this reason, many studies use targeted NGS (focused on frequently mutated genes, e.g., TP53) to find alterations in more plentiful DNA from tumor tissue and then employ PCR to detect those mutations in plasma, which allows more sensitive, rapid, and inexpensive serial monitoring. This method, however, obviously requires the availability of tumor tissue, which is frequently scant or difficult to obtain. Moreover, initial biopsies may not be reflective of the tumor at the point of ptDNA assay, as the cancer cells may have developed new mutations.

The appropriate depth of coverage to achieve an adequate sensitivity for ptDNA studies therefore depends on the use indication. For assessing minimal residual disease in early-stage cancer, we would suggest depths of coverage in the 10,000- to 100,000-fold range to achieve a reliable sensitivity of 0.01% to 0.02% allelic frequency. This is best achieved with digital PCR and/or barcoding amplicon sequencing. For metastatic disease, finding such rare clonal populations may not be needed, so using NGS in the 1000- to-10,000-fold coverage range is likely adequate to obtain a 1% to 0.1% allelic frequency sensitivity.

Sensitivity of ptDNA and Concordance with Tissue

Two related questions arise when considering ptDNA as a reliable proxy for disease presence or burden: sensitivity of detection in the plasma and concordance between the mutations found in plasma and tumor tissue. Prior to NGS technologies becoming commonplace, investigators typically employed PCR to identify common mutations in tumor biopsies and then looked for these same mutations in plasma; in this case, the sensitivity of detection is defined by its concordance with tissue biopsy. But as sequencing technology has advanced, along with the field's understanding of tumor evolution and heterogeneity, tissue mutations may be proving less useful as a gold standard, with de novo sequencing of plasma providing a complementary and potentially broader look at the tumor mutational landscape.

Some of the earliest ptDNA assays employed PCR to analyze microsatellite instability and loss of heterozygosity (LOH) in plasma of breast cancer patients [41] and detected KRAS mutations in pancreatic cancer [52]. Other groups examined mutant KRAS in a primary tumor and identified corresponding KRAS mutations in the plasma, with higher sensitivity than in prior assays [53, 54]. These and studies like them provided an exciting proof of concept, spurring interest in ptDNA as a biomarker.

From the beginning, studies reported varying sensitivity. Mutations in TP53 were found in 42.9% of the plasma DNA from patients harboring TP53 mutations in their tumor [55]. Other studies reported 100% concordance [10]. Some of these discrepancies could be explained by differences in study design—e.g., retrospective vs. prospective trials. Lab techniques also differed—e.g., improperly collected or inadequately spun blood can result in a high fraction of white blood cell DNA diluting the plasma sample. Issues of ptDNA dilution by total ccfDNA may have complicated the detection of ptDNA in several studies [14, 56–58]. Also, the raw number of genome equivalents sampled by the investigators is a measure of DNA yield, which correlates with sensitivity; studies vary in the amount of plasma collected, which affects results [59].

Generally, metastatic disease has been consistently easier to detect in ptDNA, likely owing to bulkier disease shedding larger amounts DNA into the circulation. For example, Diehl and colleagues found 100% concordance between APC mutations in the plasma and solid tumor specimens of six metastatic colorectal cancer patients [9], but the group's subsequent analysis of early-stage colorectal cancer patients with proven APC mutations detected mutant APC DNA in only 63% of corresponding plasma samples. Similarly, Bettegowda and colleagues looked at multiple tumor types and were able to detect ptDNA in 75% of patients with advanced disease, but less than 50% in some tumor types with localized tumors (this also suggested varying ptDNA levels by tumor type, still an issue under investigation) [60]. However, this study used varying analytes (serum tumor DNA and ptDNA), as well as multiple methods for mutation detection (NGS and digital PCR), so it is unclear how much information from this study can be extrapolated into newer studies using higher-quality analytes and uniform detection platforms.

Sensitivity continues to improve for early-stage disease and in settings (e.g., postsurgical) where detection of micrometastatic disease is key. Technological advances are improving sensitivity, e.g., a 2016 study assay of KRAS in pancreatic cancer patients undergoing resection could detect down to a mutation prevalence of 0.01–0.1%, corresponding to 1 mutant copy per 1000–10,000 wild-type copies [61]. Investigators have also recognized that given lower concentrations of ptDNA in early-stage patients, increasing the number of genome equivalents sampled in early-stage cancer patients should also increase assay sensitivity [35].

As investigators worked to establish concordance between plasma and tissue in various tumor types, many noted issues arising when using archival specimens to assess mutational status in patients with metastatic disease [62, 63]. For example, our lab evaluated "hotspot" PIK3CA mutations (a gene commonly mutated in breast and other cancers) in metastatic breast cancer patients from 49 archival matched tumor and plasma samples for exon 9 (E542K and E545K) and 20 (H1047R) mutations. We found 100% concordance between the specific PIK3CA mutation in each tumor and its matched plasma sample. However, a subsequent prospective study by our group identified only 70% concordant PIK3CA mutations between tumor tissue and peripheral blood. This disparate result could be explained by tumor heterogeneity and clonal evolution: the prospective study used archived primary cancer tissues from any source and blood drawn at the time of study enrollment, while in the retrospective trial, blood and tissue samples were acquired concurrently [64]. Notably, in the prospective study, discordant results were only seen when tissue and blood were collected greater than 3 years apart.

Numerous studies have now characterized tumor heterogeneity in ptDNA; for example, its concordance with tissue mutations was confirmed recently by de Mattos Arruda and colleagues, who tracked mutations in both plasma and serial biopsies (primary tumor and metastatic disease) in a single breast cancer patient. They showed that high-depth massively parallel sequencing (MPS) could capture all mutations in the primary tumor and in liver metastases, indicating that from a mutation standpoint, ptDNA could represent an alternative to biopsy in the metastatic setting, if performed in a timely fashion [65]. Rothe and colleagues reported more discordant results in 17 patients with metastatic breast cancer, in which concurrent plasma and tissue samples showed 4 of

17 with different mutations identified (with the rest being concordant), indicating that ptDNA may in some cases provide complementary information to metastatic biopsy [66].

Regardless of these differences, one distinct advantage ptDNA offers is availability; we recently conducted a feasibility study, Individualized Mutational Analysis Guides Efforts (IMAGE), attempting to acquire metastatic biopsies and ptDNA for evaluation by our molecular tumor board within a 28-day period; the study was halted owing to inability to acquire tumor tissue in the requisite time frame, but plasma studies proved quite amenable to acquisition and evaluation in a clinically actionable interval [67].

Differing mutational and genomic signatures between primary and metastatic disease sites complicates validation efforts. The majority of cancers are thought to arise through the accumulation of 3 to 8 "driver" mutations [68]. In theory, these mutations are the initial foundation or "trunk" of the cancer's evolutionary tree. As cancer cells progress and metastasize to different sites in the body, they acquire additional driver mutations that are then unique to those subpopulations. These "branch/leaf" mutations can continue to evolve, especially when selective pressures such as chemotherapy and other therapies are applied.

While ptDNA may in some cases offer a more complete profile of a tumor's mutational profile than tissue biopsy, this picture is also perhaps more error-prone owing to smaller amounts of DNA, which often undergoes additional cycles of PCR amplification prior to sequencing. Tumor heterogeneity also makes results harder to interpret; e.g., if a mutation displays a low clonal allele fraction within a solid tumor, wild-type sequences shed from other tumor and normal cells may reduce the relative fraction of ptDNA for that mutation and may fail to reflect the overall tumor burden [52, 69, 70]. The use of multiple somatic alterations as markers can mitigate some of these concerns. Investigators have also considered the possibility of using stool, urine, and increased volumes of plasma to improve the sensitivity of detecting rare mutations within ptDNA [71, 72].

Residual Disease, Recurrence, and Tumor Dynamics

Given the complexities described above, the story of how ptDNA will fit into cancer detection, monitoring of response to therapies, and clinical decision making continues to evolve.

In 2008, Diehl and colleagues correlated amounts of ptDNA with rising or falling tumor burden using BEAMing for four genes (APC, PIK3CA, TP53, and KRAS) in 18 colorectal cancer patients [10]. Recent studies demonstrate similar results using personalized assays. For example, in a 2016 comparison of 30 women with metastatic breast cancer, ptDNA displayed a greater dynamic range and better correlation with changes in tumor burden during treatment than CTCs and a greater sensitivity than the protein tumor marker CA 15–3 or CTCs (97% vs. 78% and 87%, respectively).

If ptDNA reliably reflects tumor dynamics, this suggests clinical utility at the very least in prognostication, for guiding clinical decision making, defining clinical trial populations enriched for high-risk features, or assisting in patient–physician communication. A recent study risk-stratifying breast cancer patients into ptDNA high, low, or free correlated patient status with DFS and OS [73]. Olsson and colleagues used whole genome sequencing to identify tumor-specific mutations in 20 breast cancer patients and showed that these personalized signatures discriminated between patients with and without recurrence, preceding clinical detection in 86% with an average lead time of 11 months [74]. Pietrasch et al. were recently able to develop sequencing libraries from ptDNA alone—not preceded by sequencing of tissue biopsy—in patients with various stages of pancreatic cancer and were able to correlate detection of ptDNA with poor OS [75].

Tracking multiple tumor-specific mutations over multiple time points appears to have some sensitivity in detecting minimal residual disease, also potentially guiding therapies. Tie et al. used ptDNA to detect MRD in stage II colon cancer patients, a population in which risk-stratification to determine potential benefit from adjuvant chemotherapy has proven challenging. The researchers subjected 231 operative tumor samples to MPS, revealing somatic mutations in 99.6% of cases. Patients positive for ptDNA postoperatively had a reduced recurrence-free survival (RFS) compared with ptDNA-negative patients. The authors went on to perform mutation tracking for ptDNA-positive patients who underwent chemotherapy and were able to demonstrate correlation between mutational load and radiologic recurrence [76].

A similar effort by Garcia Murillas and colleagues used MPS to detect patient-specific mutations in the tumors of 55 patients with early-stage breast cancer who received neoadjuvant therapy. Using PCR assays personalized for each tumor, they tested patient plasma collected prior to therapy and serially after completion of treatment. ptDNA detection at baseline did not predict disease-free survival, but ptDNA found in a single postsurgical sample predicted early relapse (HR 25, C-index 0.78), and mutation tracking (i.e., serial testing) improved relapse prediction. Moreover, ptDNA became detectable at a median of 7.9 months before clinical relapse, implying that the assay could potentially direct intervention prior to macroscopic recurrence, at a time point when salvage and cure might prove feasible [77].

Many hope that ptDNA may offer a more practical substitute for metastatic biopsy. Lebofsky scanned 46 genes and more than 6800 mutations from the COSMIC database using a multiplexed NGS panel on both ptDNA and tissue and was

able to detect mutations in 27 patients; however, matching ptDNA to tumor was impossible in several patients due to inadequate biopsy material [78]. Our group's IMAGE study, mentioned above, while stopped owing to similar problems with lack of biopsy tissue, did demonstrate that mutations in the patients' plasma could be assessed by NGS within 28 days and that tracking mutations was reflective of tumor burden and response to therapies [67].

Resistance Mutations and Agile Treatment Algorithms

One powerful application of ptDNA is to test for the emergence of resistance clones. Indeed, this represents the only area in which US and European regulatory bodies have already approved clinical assays; work in this field is progressing rapidly and may lead to other resistance assays entering standard practice in the near future.

In 2011, Taniguchi and colleagues demonstrated the ability to detect second-site ptDNA T790 M epidermal growth factor receptor (EGFR) mutations in lung cancer patients treated with EGFR tyrosine kinase inhibitors (TKIs); this mutation confers resistance to standard EGFR-targeted medications such as erlotinib and gefitinib and indicates a possible response to third-generation EGFR TKIs such as osimertinib [79]. This resistance mutation was observed in a significant fraction of EGFR-inhibitor naïve patients, suggesting the natural existence of a minority population of cancer cells that subsequent EGFR inhibition may select for. Conversely, Piotrowska and colleagues demonstrated that T790 M may also revert to wild type in response to third-generation therapies and that ptDNA can detect the emergence of these "new" wild-type clones [80]. Other groups have also created ptDNA assays for T790 M mutations [81, 82]; one study was able to show ptDNA emergence of a resistance clone predating clinical progression by months [83]. While most assays rely on digital PCR to detect previously characterized mutations, Chabon et al. recently developed a targeted NGS method (CAPP-Seq) to study emerging changes in ptDNA from patients receiving a third-generation TKI (rociletinib, no longer in clinical development) and were able to identify several novel alterations in EGFR, ERBB2, and most notably MET, all suspected to confer resistance to TKI therapies [84].

Such work presaged the announcement in 2016 of the first Food and Drug Administration (FDA)-approved plasma-based companion diagnostic, the cobas EGFR Mutation Test v2. The approval for the assay relied on testing of specimens from the large EURTAC trial [85] and complemented the FDA's previous approval granted in 2013 for EGFR testing in tissue as a selection for treatment with Tarceva. The test detects EGFR mutations (exon 19 deletion or exon 21 [L858R] substitution mutations) in NSCLC patients' blood samples. If these mutations are not detected in the plasma, then a tumor biopsy should be performed to better indicate who may benefit from Tarceva treatment [86].

Resistance to endocrine therapy in hormone-positive positive breast cancers may prove the next relatively low-hanging fruit for ptDNA validation. An emerging literature demonstrates that mutations in the gene encoding for estrogen receptor-alpha, ESR1, can develop in the tumors of metastatic breast cancer patients whose disease progresses on hormone therapy. Our group was able to retrospectively and prospectively detect ESR1 mutations in plasma of patients where a simultaneous biopsy proved negative for the same mutation, suggesting the emergence of a resistance clone; moreover, in some plasma samples, multiple mutations occurred at measurable allelic frequencies, indicating the presence of parallel development of resistant clonal populations [87]. Schiavon and Turner examined ptDNA from 171 women with advanced breast cancer and uncovered ESR1 mutations exclusively in patients treated with aromatase inhibitors (AIs)—who were also found to have shorter progression-free survival [88]. Other investigations of ptDNA assays for ESR1 mutations have likewise shown utility in predicting AI resistance [89, 90].

Similar studies hold hope for validation of ptDNA assays in other resistance mutations; e.g., two separate studies have reported the use of BEAMing to detect the emergence of KRAS mutations that are known to confer resistance to antibody-mediated EGFR-targeted therapies for colorectal cancer [91, 92], and one recent trial used ptDNA to detect acquired PIK3CA mutations that may contribute to cetuximab resistance in metastatic colon cancer [93]. Siravegna et al. developed a targeted NGS panel to explore colorectal tumor evolution in response to therapies, not only identifying the emergence of potentially novel resistance mutations in EGFR but also demonstrating rise and fall of KRAS mutational burden in response to targeted treatment [94]. The relatively common V600E BRAF mutation has also been detected in ptDNA in melanoma patients and showed promise in monitoring response to BRAF-directed therapy [95].

Trials like these, as well as the emerging acceptance of ptDNA assays for resistance mutations, increasingly bring into focus the prospect of anticipatory therapy change, wherein a clinician substitutes a new treatment when the resistance mutation is detected, before clinical or radiological progression. Whether this would actually extend or improve the lives of patients with metastatic cancer remains an open question; it is a testable hypothesis currently being addressed in clinical trials.

ptDNA in Cancer Screening

A "liquid biopsy" for cancer may conjure a vision where simple blood screening tests can take the place of painful or invasive procedures such as mammography, colonoscopy, and diagnostic biopsy. However, the sparsity of DNA in early detectable disease, as well as the lack of tissue specificity for the most common cancer mutations, likely relegates these hopes to the future. Moreover, knowing that an individual has a mutation in ptDNA is not an absolute indication that this person has a malignancy. Finally, for a screening test to be clinically useful, one must demonstrate that the test reduces overall mortality from the cancer and preferably mortality in general. Such studies will take decades to prove or disprove, though several efforts continue to explore the use of ptDNA for primary screening. For example, Cohen et al. recently demonstrated a 64% sensitivity for early-stage, surgically resectable pancreatic cancer when combining KRAS detection in ptDNA with four protein biomarkers (CEA, CA19–9, HGF, and OPN) [96].

Challenges in Oncologic Applications

Despite the promise that ptDNA holds in a variety of oncologic settings outlined above, the field faces numerous challenges and complexities.

Many studies published to date are retrospective, using plasma gathered as part of clinical trials with a different primary focus. While this research is illuminating, it should be viewed as hypothesis-generating; as always, large, multicenter prospective clinical trials will provide the evidence of real-world clinical benefit. A few such trials are underway; for example, one large French study aims to determine whether patients with ESR1 mutations in ptDNA would benefit from an early switch from AI/palbociclib to fulvestrant/palbociclib (clinicaltrial.gov #NCT03079011).

While the FDA has approved one ptDNA assay as described above, overall the field lacks standardization. Publications describe a wide variety of methods to obtain and process plasma, which, as discussed, may explain some of the disparities in results reported. Moreover, in tissue vs. ptDNA studies, even ground truth has yet to be established, given that ptDNA could reasonably be expected to contain mutations not present in tissue; indeed, at least one rapid autopsy study has addressed this problem by comparing total postmortem metastatic mutational burden to ptDNA present in blood at the time of death [97].

Meanwhile, a host of companies and platforms compete for research dollars in ptDNA processing, all with varying proprietary methods; many are excellent, but again, none are uniformly received as a standard. As methods for detecting and sequencing genetic material encompass an increasing number of platforms and techniques, it will be important to understand strengths and pitfalls of available technology. Moreover, NGS assays must become increasingly nimble to accommodate the need for rapid, inexpensive serial monitoring of ptDNA if clinicians are to respond quickly to changes in mutational burden. Sensitivity must also be improved if we hope to move beyond our dependence on tissue for mutation discovery and advance to direct mutation detection from ptDNA alone.

One emerging issue complicating the study of ptDNA involves the blood dyscrasias that develop in many older patients. When sequencing genetic material from either tumor or plasma, a substantial proportion of DNA can originate from healthy tissue or from white blood cells mixed with plasma or tissue samples; mutations, therefore, are assumed to originate from the tumor under investigation—perhaps in some cases erroneously. A recent case study relates an account of a patient whose sequenced solid tumor revealed a JAK2 mutation, which was initially assumed to represent an unusual but actionable mutation from the solid tumor in question; further inquiry revealed the patient's known history of polycythemia vera. From this, it was further demonstrated that the tumor sample contained enough blood to display a detectable allelic fraction of mutant JAK2 [98].

In the above case, investigators were able to discern the source of the patient's JAK2 mutation because the mutation is associated with polycythemia vera, and the patient had been diagnosed with the disease. But an expanding set of literature describes somatic mutations of unknown significance developing in the blood of older persons [99]. This so-called clonal hematopoiesis of indeterminate potential, or CHIP, threatens to further complicate the task of distinguishing actual solid tumor-related mutations from these poorly characterized hematologic mutations.

This issue is arising in both plasma and tissue samples. The more that a tumor sample exhibits a hemorrhagic component, the more one risks CHIP mutations entering the mix if they are present. Regarding plasma, although double spun plasma can remove the majority of cellular contaminants, the very nature of CHIP suggests that mutations from these cells are also found in ccfDNA, again complicating the mutational profile of ptDNA leading to false-positive mutations. As NGS technologies increase their sensitivity, ever smaller mutant allele fractions may achieve detectability, further compounding the complexity of ptDNA analysis. With awareness and careful analysis of mutational profiles, confusion regarding CHIP mutations can be minimized; other technologies if validated, such as methylation haplotyping, could theoretically help distinguish origin tissue type further mitigating these concerns [100].

Future Directions for ccfDNA

Looking ahead, more potential applications of circulating genetic material appear on the horizon. For example, researchers are exploring the use of alternative circulating nucleic acids to detect residual disease and profile mutations in cancer and other disease states. Specifically, messenger RNA (mRNA) also appears to circulate in human serum, although its cellular origin is less clear than that of ccfDNA. Circulating cell-free mRNA can be detected using microarray technologies or reverse transcription qPCR [101], with various potential applications. For example, circulating mRNA may predict graft rejection in transplant patients using fractions of donor-specific ccfDNA and circulating mRNA [102]. It may also have applications in early diagnosis of diabetic retinopathy and neuropathy: by screening for organ-specific mRNA in the plasma, investigators envision identifying these diabetic complications sooner, with the hope that early intervention could improve outcomes [5, 103]. Finally, microRNAs (miRNAs)—noncoding RNA species that regulate gene expression—have been described in the serum of cancer patients with B cell lymphoma in several large studies, and serum miRNA levels have also been shown to correlate with solid tumor metastases [104, 105].

The ratio of long to short DNA fragments (DNA integrity) is also under investigation as a possible biomarker of tumor presence and tumor burden, which could be broadly applicable for many cancer subtypes and could also improve sensitivities of current assays [106]. In addition, studies examining epigenetic alterations in the plasma of cancer patients, specifically detection of promoter hypermethylation by methylation-specific PCR, have been performed in various cancer subtypes and hold significant promise as another biomarker of cancer burden [107–109].

In addition to cancer and maternal–fetal medicine, other potential uses for ccfDNA in medical diagnostics are under exploration. For example, circulating bacterial DNA fragments can diagnose a causative bacterial organism in culture-negative but clinically septic patients [110]. Cell-free DNA may be helpful in risk-stratifying trauma and burn patients, whose levels of ccfDNA may correlate with severity of injury, outcomes, and length of hospital stay [111–113]. Investigators have looked into whether ccfDNA levels can predict sepsis [114, 115] and whether increasing levels correspond with worsening myocardial damage and/or cardiac outcomes in acute coronary syndromes [116]. Others have explored correlations between classic cardiac ischemic markers such as troponin and creatinine kinase with increasing levels of ccfDNA [3] and the prognostic value of ccfDNA in stroke patients [117]. No doubt as the field matures, more applications in various areas of clinical medicine will emerge.

Conclusions

Researchers in the still-nascent ccfDNA field have made huge strides in developing ccfDNA applications in cancer and other disease states, yet far more remains to be explored. Like other areas of human research, we face the exciting but challenging task of interfacing with the private sector, governmental regulatory bodies, academic institutions, and others, to understand and select rapidly developing technology and platforms. We struggle with the technical problems of detecting a small percentage of variant molecules in a sea of wild-type DNA, all in a setting where currently neither an industry standard nor a uniformly agreed-upon platform exists. As tests increasingly require both high complexity and fast turnaround to be clinically actionable, we will need to increase speed and efficiency. Finally, in order to validate these promising assays, we must prove not only their feasibility but also their clinical utility in the real world. In short, we must work to establish the best techniques to quantify, detect, and monitor ccfDNA and develop appropriate criteria for ccfDNA surveillance through prospective clinical trials. Through standardization and improved detection, this emerging technology promises to produce rapid, noninvasive, sensitive assays that will allow clinicians and patients to make better decisions and improve clinical outcomes.

References

1. Mandel P, Metais P. Not AvailableC R Seances Soc Biol Fil. 1948;142(3–4):241–3.
2. Lam NY, et al. Plasma DNA as a prognostic marker for stroke patients with negative neuroimaging within the first 24 h of symptom onset. Resuscitation. 2006;68(1):71–8.
3. Antonatos D, et al. Cell-free DNA levels as a prognostic marker in acute myocardial infarction. Ann N Y Acad Sci. 2006;1075:278–81.
4. Saukkonen K, et al. Association of cell-free plasma DNA with hospital mortality and organ dysfunction in intensive care unit patients. Intensive Care Med. 2007;33(9):1624–7.
5. Sandhu HS, et al. Measurement of circulating neuron-specific enolase mRNA in diabetes mellitus. Ann N Y Acad Sci. 2008;1137:258–63.
6. Choi JJ, Reich CF 3rd, Pisetsky DS. The role of macrophages in the in vitro generation of extracellular DNA from apoptotic and necrotic cells. Immunology. 2005;115(1):55–62.
7. Stroun M, et al. The origin and mechanism of circulating DNA. Ann N Y Acad Sci. 2000;906:161–8.
8. Jahr 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(4):1659–65.
9. Diehl F, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. Proc Natl Acad Sci U S A. 2005;102(45):16368–73.
10. Diehl F, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med. 2008;14(9):985–90.
11. De Mattos-Arruda L, et al. Cerebrospinal fluid-derived circulating tumour DNA better represents the genomic alterations of brain tumours than plasma. Nat Commun. 2015;6:8839.

12. Pan W, et al. Brain tumor mutations detected in cerebral spinal fluid. Clin Chem. 2015;61(3):514–22.
13. Lo YM, et al. Rapid clearance of fetal DNA from maternal plasma. Am J Hum Genet. 1999;64(1):218–24.
14. Fleischhacker M, Schmidt B. Circulating nucleic acids (CNAs) and cancer--a survey. Biochim Biophys Acta. 2007;1775(1):181–232.
15. Emlen W, Mannik M. Effect of DNA size and strandedness on the in vivo clearance and organ localization of DNA. Clin Exp Immunol. 1984;56(1):185–92.
16. Chang CP, et al. Elevated cell-free serum DNA detected in patients with myocardial infarction. Clin Chim Acta. 2003;327(1–2):95–101.
17. Wimberger P, et al. Impact of platinum-based chemotherapy on circulating nucleic acid levels, protease activities in blood and disseminated tumor cells in bone marrow of ovarian cancer patients. Int J Cancer. 2011;128(11):2572–80.
18. Lo YM, et al. Plasma DNA as a prognostic marker in trauma patients. Clin Chem. 2000;46(3):319–23.
19. Chiu TW, et al. Plasma cell-free DNA as an indicator of severity of injury in burn patients. Clin Chem Lab Med. 2006;44(1):13–7.
20. Rhodes A, et al. Plasma DNA concentration as a predictor of mortality and sepsis in critically ill patients. Crit Care. 2006;10(2):R60.
21. Herzenberg LA, et al. Fetal cells in the blood of pregnant women: detection and enrichment by fluorescence-activated cell sorting. Proc Natl Acad Sci U S A. 1979;76(3):1453–5.
22. Lo YM, et al. Presence of fetal DNA in maternal plasma and serum. Lancet. 1997;350(9076):485–7.
23. Lo YM, Chiu RW. Prenatal diagnosis: progress through plasma nucleic acids. Nat Rev Genet. 2007;8(1):71–7.
24. Li Y, et al. Cell-free DNA in maternal plasma: is it all a question of size? Ann N Y Acad Sci. 2006;1075:81–7.
25. Lo YM, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet. 1998;62(4):768–75.
26. Lun FM, et al. Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. Clin Chem. 2008;54(10):1664–72.
27. Chiu RW, et al. Non-invasive prenatal assessment of trisomy 21 by multiplexed maternal plasma DNA sequencing: large scale validity study. BMJ. 2011;342:c7401.
28. Lo YM. Fetal RhD genotyping from maternal plasma. Ann Med. 1999;31(5):308–12.
29. Fan HC, et al. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. Proc Natl Acad Sci U S A. 2008;105(42):16266–71.
30. Chiu RW, et al. Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. Proc Natl Acad Sci U S A. 2008;105(51):20458–63.
31. Chiu RW, Lo YM. Clinical applications of maternal plasma fetal DNA analysis: translating the fruits of 15 years of research. Clin Chem Lab Med. 2013;51(1):197–204.
32. Liao GJ, Gronowski AM, Zhao Z. Non-invasive prenatal testing using cell-free fetal DNA in maternal circulation. Clin Chim Acta. 2014;428:44–50.
33. (ACOG), A.C.o.O.a.G., Cell-free DNA Screening for Fetal Aneuploidy. Society for Maternal-Fetal Medicine, Committee on Genetics. 2015.
34. Lo YM, et al. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. Sci Transl Med. 2010;2(61):61ra91.
35. Leon SA, et al. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res. 1977;37(3):646–50.
36. Allen D, et al. Role of cell-free plasma DNA as a diagnostic marker for prostate cancer. Ann N Y Acad Sci. 2004;1022:76–80.
37. Chun FK, et al. Circulating tumour-associated plasma DNA represents an independent and informative predictor of prostate cancer. BJU Int. 2006;98(3):544–8.
38. Schwarzenbach H, et al. Detection and monitoring of cell-free DNA in blood of patients with colorectal cancer. Ann N Y Acad Sci. 2008;1137:190–6.
39. Li BT, et al. A prospective study of total plasma cell-free DNA as a predictive biomarker for response to systemic therapy in patients with advanced non-small-cell lung cancers. Ann Oncol. 2016;27(1):154–9.
40. Giacona MB, et al. Cell-free DNA in human blood plasma: length measurements in patients with pancreatic cancer and healthy controls. Pancreas. 1998;17(1):89–97.
41. Chen X, et al. Detecting tumor-related alterations in plasma or serum DNA of patients diagnosed with breast cancer. Clin Cancer Res. 1999;5(9):2297–303.
42. Heid CA, et al. Real time quantitative PCR. Genome Res. 1996;6(10):986–94.
43. Branford S. Chronic myeloid leukemia: molecular monitoring in clinical practice. Hematology Am Soc Hematol Educ Program. 2007:376–83.
44. Yu M, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science. 2013;339(6119):580–4.
45. Morley AA. Digital PCR: a brief history. Biomol Detect Quantif. 2014;1(1):1–2.
46. Vogelstein B, Kinzler KW. Digital PCR. Proc Natl Acad Sci U S A. 1999;96(16):9236–41.
47. Goodwin S, McPherson JD, McCombie WR. Coming of age: ten years of next-generation sequencing technologies. Nat Rev Genet. 2016;17(6):333–51.
48. 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.
49. Kinde I, et al. Detection and quantification of rare mutations with massively parallel sequencing. Proc Natl Acad Sci U S A. 2011;108(23):9530–5.
50. Kennedy SR, et al. Detecting ultralow-frequency mutations by duplex sequencing. Nat Protoc. 2014;9(11):2586–606.
51. Leary RJ, et al. Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. Sci Transl Med. 2012;4(162):162ra154.
52. Castells A, et al. K-ras mutations in DNA extracted from the plasma of patients with pancreatic carcinoma: diagnostic utility and prognostic significance. J Clin Oncol. 1999;17(2):578–84.
53. Kopreski MS, et al. Somatic mutation screening: identification of individuals harboring K-ras mutations with the use of plasma DNA. J Natl Cancer Inst. 2000;92(11):918–23.
54. Dianxu F, et al. A prospective study of detection of pancreatic carcinoma by combined plasma K-ras mutations and serum CA19-9 analysis. Pancreas. 2002;25(4):336–41.
55. Garcia JM, et al. Extracellular tumor DNA in plasma and overall survival in breast cancer patients. Genes Chromosomes Cancer. 2006;45(7):692–701.
56. Boddy JL, et al. Prospective study of quantitation of plasma DNA levels in the diagnosis of malignant versus benign prostate disease. Clin Cancer Res. 2005;11(4):1394–9.
57. Schwarzenbach H, et al. Comparative evaluation of cell-free tumor DNA in blood and disseminated tumor cells in bone marrow of patients with primary breast cancer. Breast Cancer Res. 2009;11(5):R71.
58. Schwarzenbach H, et al. Cell-free tumor DNA in blood plasma as a marker for circulating tumor cells in prostate cancer. Clin Cancer Res. 2009;15(3):1032–8.
59. Toro PV, et al. Comparison of cell stabilizing blood collection tubes for circulating plasma tumor DNA. Clin Biochem. 2015;48:993.

60. Bettegowda C, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med. 2014;6(224):224ra24.
61. Hadano N, et al. Prognostic value of circulating tumour DNA in patients undergoing curative resection for pancreatic cancer. Br J Cancer. 2016;115(1):59–65.
62. Gerlinger M, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med. 2012;366(10):883–92.
63. Yachida S, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. Nature. 2010;467(7319):1114–7.
64. Higgins MJ, et al. Detection of tumor PIK3CA status in metastatic breast cancer using peripheral blood. Clin Cancer Res. 2012;18(12):3462–9.
65. De Mattos-Arruda L, et al. Capturing intra-tumor genetic heterogeneity by de novo mutation profiling of circulating cell-free tumor DNA: a proof-of-principle. Ann Oncol. 2014;25(9):1729–35.
66. Rothé F, Laes J-F, Lambrechts D, Smeets D, Vincent D, Maetens M, Fumagalli D, Michiels S, Stylianos D, Moerman C, Detiffe J-P, Larsimont D, Awada A, Piccart M, Sotiriou C, Ignatiadis M. Plasma circulating tumor DNA as an alternative to metastatic biopsies for mutational analysis in breast cancer. Ann Oncol. 2014;25:1959.
67. Parsons HA, et al. Individualized molecular analyses guide efforts (IMAGE): a prospective study of molecular profiling of tissue and blood in metastatic triple negative breast cancer. Clin Cancer Res. 2017;23(2):379–86.
68. Tomasetti C, et al. Only three driver gene mutations are required for the development of lung and colorectal cancers. Proc Natl Acad Sci U S A. 2015;112(1):118–23.
69. Ryan BM, et al. A prospective study of circulating mutant KRAS2 in the serum of patients with colorectal neoplasia: strong prognostic indicator in postoperative follow up. Gut. 2003;52(1):101–8.
70. Wang S, et al. Potential clinical significance of a plasma-based KRAS mutation analysis in patients with advanced non-small cell lung cancer. Clin Cancer Res. 2010;16(4):1324–30.
71. Diehl F, et al. Analysis of mutations in DNA isolated from plasma and stool of colorectal cancer patients. Gastroenterology. 2008;135(2):489–98.
72. Husain H, et al. Monitoring daily dynamics of early tumor response to targeted therapy by detecting circulating tumor DNA in urine. Clin Cancer Res. 2017;23:4716.
73. Oshiro C, et al. PIK3CA mutations in serum DNA are predictive of recurrence in primary breast cancer patients. Breast Cancer Res Treat. 2015;150(2):299–307.
74. Olsson E, et al. Serial monitoring of circulating tumor DNA in patients with primary breast cancer for detection of occult metastatic disease. EMBO Mol Med. 2015;**7**(8):1034–47.
75. Pietrasz D, et al. Plasma circulating tumor DNA in pancreatic cancer patients is a prognostic marker. Clin Cancer Res. 2017;23(1):116–23.
76. Tie J, et al. Circulating tumor DNA as an early marker of therapeutic response in patients with metastatic colorectal cancer. Ann Oncol. 2015;26:1715.
77. Garcia-Murillas I, et al. Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. Sci Transl Med. 2015;7(302):302ra133.
78. Lebofsky R, et al. Circulating tumor DNA as a non-invasive substitute to metastasis biopsy for tumor genotyping and personalized medicine in a prospective trial across all tumor types. Mol Oncol. 2015;9(4):783–90.
79. Taniguchi K, et al. Quantitative detection of EGFR mutations in circulating tumor DNA derived from lung adenocarcinomas. Clin Cancer Res. 2011;17(24):7808–15.
80. Piotrowska Z, et al. Heterogeneity underlies the emergence of EGFRT790 wild-type clones following treatment of T790M-positive cancers with a third-generation EGFR inhibitor. Cancer Discov. 2015;5(7):713–22.
81. Ishii H, et al. Digital PCR analysis of plasma cell-free DNA for non-invasive detection of drug resistance mechanisms in EGFR mutant NSCLC: correlation with paired tumor samples. Oncotarget. 2015;6(31):30850–8.
82. Que D, et al. EGFR mutation status in plasma and tumor tissues in non-small cell lung cancer serves as a predictor of response to EGFR-TKI treatment. Cancer Biol Ther. 2016;17(3):320–7.
83. 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):1698–705.
84. Chabon JJ, et al. Circulating tumour DNA profiling reveals heterogeneity of EGFR inhibitor resistance mechanisms in lung cancer patients. Nat Commun. 2016;7:11815.
85. Rosell R, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol. 2012;13(3):239–46.
86. FDA, Editor. 2016. https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm504488.htm
87. Chu D, et al. ESR1 mutations in circulating plasma tumor DNA from metastatic breast cancer patients. Clin Cancer Res. 2016;22(4):993–9.
88. Schiavon G, et al. Analysis of ESR1 mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. Sci Transl Med. 2015;7(313):313ra182.
89. Fribbens C, et al. Plasma ESR1 mutations and the treatment of estrogen receptor-positive advanced breast cancer. J Clin Oncol. 2016;34:2961.
90. Wang P, et al. Sensitive detection of mono- and polyclonal ESR1 mutations in primary tumors, metastatic lesions and cell free DNA of breast cancer patients. Clin Cancer Res. 2016;22(5):1130–7.
91. Diaz LA Jr, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature. 2012;486(7404):537–40.
92. Misale S, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature. 2012;486(7404):532–6.
93. Xu JM, et al. PIK3CA mutations contribute to acquired cetuximab resistance in patients with metastatic colorectal cancer. Clin Cancer Res. 2017;23:4602.
94. Siravegna G, et al. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nat Med. 2015;21(7):795–801.
95. Shinozaki M, et al. Utility of circulating B-RAF DNA mutation in serum for monitoring melanoma patients receiving biochemotherapy. Clin Cancer Res. 2007;13(7):2068–74.
96. Cohen JD, et al. Combined circulating tumor DNA and protein biomarker-based liquid biopsy for the earlier detection of pancreatic cancers. Proc Natl Acad Sci U S A. 2017;114(38):10202–7.
97. Desmedt C, Brown DN, Szekely B, Smeets D, Szasz MA, Adnet P, Rothé F, Nagy Z, Farago Z, Tokes A, Zardavas D, Zoppoli G, Ignatiadis M, Pusztai L, Piccart M, Larsimont D, Lambrechts D, Kulka J, Sotiriou C. Unraveling breast cancer progression through geographical and temporal sequencing, in AACR 2014. San Diego; 2014.
98. Lee J, et al. A polycythemia vera JAK2 mutation masquerading as a duodenal cancer mutation. J Natl Compr Cancer Netw. 2016;14(12):1495–8.

99. Steensma DP, et al. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. Blood. 2015;126(1):9–16.
100. Guo S, et al. Identification of methylation haplotype blocks aids in deconvolution of heterogeneous tissue samples and tumor tissue-of-origin mapping from plasma DNA. Nat Genet. 2017;49(4):635–42.
101. O'Driscoll L, et al. Feasibility and relevance of global expression profiling of gene transcripts in serum from breast cancer patients using whole genome microarrays and quantitative RT-PCR. Cancer Genomics Proteomics. 2008;5(2):94–104.
102. Schutz E, et al. Graft-derived cell-free DNA, a noninvasive early rejection and graft damage marker in liver transplantation: a prospective, observational, multicenter cohort study. PLoS Med. 2017;14(4):e1002286.
103. Simo-Servat O, Simo R, Hernandez C. Circulating biomarkers of diabetic retinopathy: an overview based on physiopathology. J Diabetes Res. 2016;2016:5263798.
104. Bradshaw G, et al. Dysregulated MicroRNA expression profiles and potential cellular, circulating and polymorphic biomarkers in Non-Hodgkin Lymphoma. Genes (Basel). 2016;7(12):130.
105. Sapp RM, et al. Circulating microRNAs in acute and chronic exercise: more than mere biomarkers. J Appl Physiol (1985). 2017;122(3):702–17.
106. Zonta E, Nizard P, Taly V. Assessment of DNA integrity, applications for cancer research. Adv Clin Chem. 2015;70:197–246.
107. Visvanathan K, et al. Monitoring of serum DNA methylation as an early independent marker of response and survival in metastatic breast cancer: TBCRC 005 prospective biomarker study. J Clin Oncol. 2017;35(7):751–8.
108. Tang Y, et al. Promoter DNA methylation analysis reveals a combined diagnosis of CpG-based biomarker for prostate cancer. Oncotarget. 2017;8(35):58199–209.
109. Hagrass HA, Pasha HF, Ali AM. Estrogen receptor alpha (ERalpha) promoter methylation status in tumor and serum DNA in Egyptian breast cancer patients. Gene. 2014;552(1):81–6.
110. Grumaz S, et al. Next-generation sequencing diagnostics of bacteremia in septic patients. Genome Med. 2016;8(1):73.
111. Shoham Y, et al. Admission cell free DNA as a prognostic factor in burns: quantification by use of a direct rapid fluorometric technique. Biomed Res Int. 2014;2014:306580.
112. Lam NY, et al. Time course of early and late changes in plasma DNA in trauma patients. Clin Chem. 2003;49(8):1286–91.
113. Hu Q, et al. Elevated levels of plasma mitochondrial DNA are associated with clinical outcome in intra-abdominal infections caused by severe trauma. Surg Infect. 2017;18:610.
114. Long Y, et al. Diagnosis of sepsis with cell-free DNA by next-generation sequencing technology in ICU patients. Arch Med Res. 2016;47(5):365–71.
115. Hou YQ, et al. Branched DNA-based Alu quantitative assay for cell-free plasma DNA levels in patients with sepsis or systemic inflammatory response syndrome. J Crit Care. 2016;31(1):90–5.
116. Rainer TH, et al. Plasma beta-globin DNA as a prognostic marker in chest pain patients. Clin Chim Acta. 2006;368(1–2):110–3.
117. O'Connell GC, et al. Circulating extracellular DNA levels are acutely elevated in ischaemic stroke and associated with innate immune system activation. Brain Inj. 2017:1–7.