Trends in Cancer

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REVIEWS

Review

Improving Cancer Detection and Treatment with Liquid Biopsies and ptDNA

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Liquid biopsy, or the capacity to noninvasively isolate and analyze plasma tumor DNA (ptDNA) using blood samples, represents an important tool for modern oncology that enables increasingly safe, personalized, and robust cancer diagnosis and treatment. Here, we review advances in the development and implementation of liquid biopsy approaches, and we focus on the capacity of liquid biopsy to noninvasively detect oncological disease and enhance early detection strategies. In addition to noting the distinctions between mutation-targeted and mutation-agnostic approaches, we discuss the potential for genomic analysis and longitudinal testing to identify somatic lesions early and to guide intervention at more manageable disease stages.

Highlights

Liquid biopsy approaches are becoming increasingly effective at detecting and monitoring signatures of oncological disease in a noninvasive fashion.

Recent technological developments have greatly increased the sensitivity with which rare cancer-specific mutations can be identified from the blood.

Mutation-targeted approaches that probe for an individual's specific mutations have the highest sensitivity but also require prior biopsy and/or personalized assay development.

Mutation-agnostic approaches, particularly those reliant on extreme-depth massively parallel sequencing, allow for the identification of mutations common to subsets of cancers and represent the best opportunity to actualize the promise of liquid biopsy.

Liquid Biopsy of ptDNA for the Treatment of Cancer

Liquid biopsy, or the analysis of a liquid specimen that is often obtained in a noninvasive or minimally invasive manner, has emerged as a potentially useful way to personalize and improve the detection and treatment of cancer. Cell-free DNA (cfDNA), which is released from cells by apoptosis, necrosis, or active secretion (Figure 1), is often sampled by liquid biopsy from body fluids such as blood [1–5], urine [5–8], saliva [5,9], and cerebrospinal fluid [5,10–15] (Figure 2 and Box 1). There has been considerable focus on the use of blood for the identification of potential tumor biomarkers, including circulating tumor cells [4,16], extracellular tumor-derived vesicles [16,17], and the cfDNA that is released from cancer cells [5,15,18,19]. Since the term cfDNA refers to cell-free DNA found in a multitude of body fluids, and most liquid biopsies aim to isolate cfDNA specifically from blood plasma, the acronym pDNA would appear to be a more specific term for the cfDNA detected in blood plasma (Figure 2). Genetic and epigenetic analyses suggest that the majority of pDNA in both healthy and diseased individuals originates from hematopoietic cells (in particular from white blood cells), and that cancer patients have elevated levels of pDNA deriving from tumor tissue [5,20–22]. In individuals with cancer, the subset of pDNA that derives from tumor cells is often referred to as plasma tumor DNA (ptDNA) [23] (Figure 2). While circulating tumor DNA, or ctDNA, is another frequent term used for tumor-derived pDNA [24–28], we prefer the more specific term ptDNA, since ctDNA can also refer to the tumor-derived cfDNA that is found in any body fluid (Figure 2).

Due to its short half-life and tendency to carry unique and cancer-associated mutations, ptDNA is an attractive tumor biomarker that facilitates the highly specific assessment of tumor dynamics, disease burden, and genomic alterations [27,29]. This specificity is particularly important given the current dearth of highly specific tumor biomarkers [27,30,31]. However, the very low concentration of ptDNA compared with background normal DNA, especially during early stages of cancer, presents a technical challenge to the use of ptDNA as a clinical tool. Nonetheless, increasingly sensitive and scalable methods for the detection of ptDNA are

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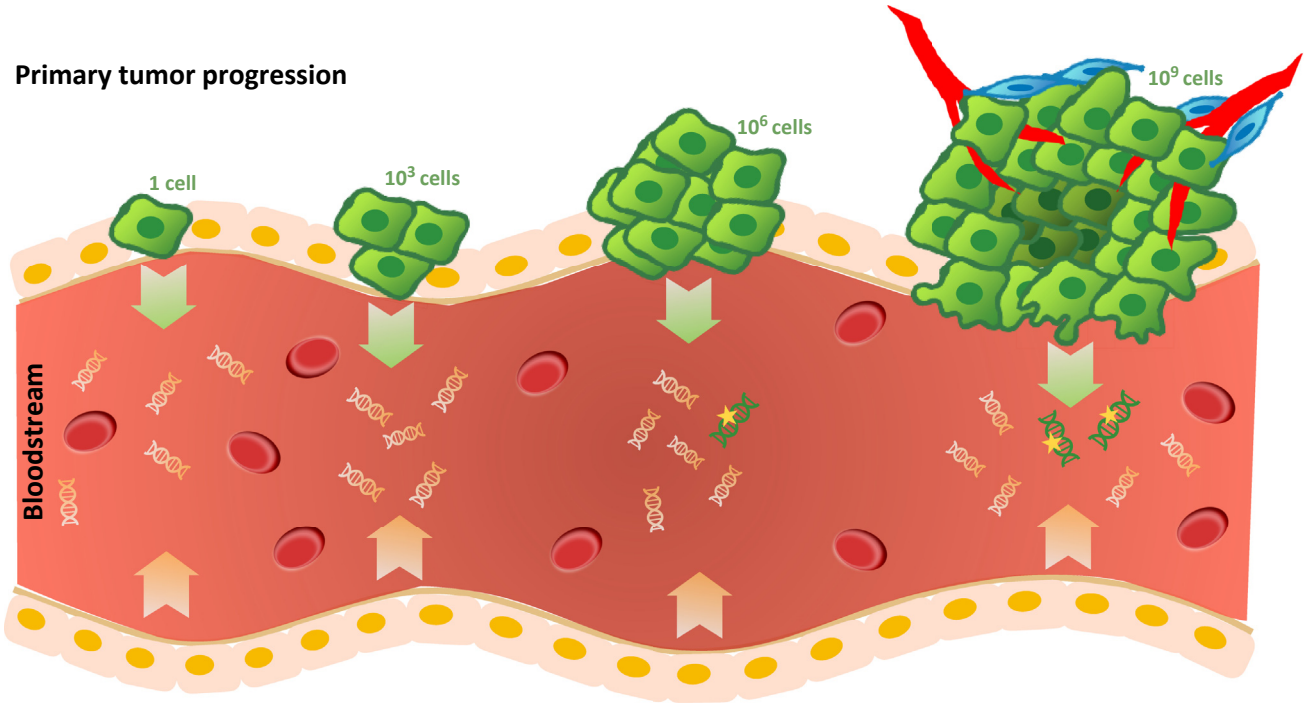

Figure 1. Clonal Expansion of a Solid Tumor and DNA Release into the Blood. As a tumor develops and clonally expands its cell number, fragments of tumor DNA (ptDNA; green) begin to enter the blood stream to join the normal DNA (white) that exists at baseline in the blood of healthy individuals. Together, the ptDNA and normal DNA make up the total DNA found in the plasma (pDNA). As the tumor increases in size, additional copies of tumor-derived DNA enter the bloodstream, although this correlation appears to differ across tumor type and stage [27]. These tumor DNA fragments can be identified by canonical mutations (gold stars) that underpin oncogenic features. Note: tumor size/cell number is representative of overall trends in tumor progression as opposed to the expected number of cells in a tumor when tumor-derived DNA is first being appreciably released.

emerging. Here we review the use of ptDNA for detecting tumor burden, assessing relapse risk, dynamically monitoring response to treatment, and informing personalized therapy. We highlight the challenges of using ptDNA to detect early-stage cancer and discuss the potential of *in silico* analytical approaches and longitudinal surveillance to improve the use of ptDNA.

Using ptDNA to Assess Relapse Risk and Monitor Tumor Dynamics in Advanced Cancers

PCR approaches have shown that patients with advanced colorectal cancers (CRCs) have mutations in the adenomatous polyposis coli (APC) gene in 8% of total APC blood DNA fragments [32]. In ovarian cancer mouse models, ptDNA strongly correlates with tumor weight/burden and can potentially be used as a marker of response to therapy [33]. Mutant ptDNA could also be used to track tumor dynamics in response to treatment. In CRC patients who had complete surgical resection, the levels of mutant ptDNA dropped markedly, whereas patients who had partial resection showed only moderate decreases [29]. Remarkably, patients with detectable ptDNA levels at their first follow-up visit relapsed (15 of 16 patients), whereas patients with no detectable ptDNA did not relapse (four of four patients) [29]. Similarly, ptDNA decreased in patients receiving chemotherapy (three of 11 patients) and rose immediately after discontinuation of chemotherapy (six of 11 patients) [29]. Ultimately, ptDNA can potentially be used to successfully identify residual disease that is undetectable by imaging after local resection and could track patient tumor responses to treatment.

(A)

Acronym	Full term	Description
cfDNA	Cell-free DNA	Free DNA (i.e. not within cells) found in any of a number of bodily fluids
ctDNA	Circulating tumor DNA	Cell-free DNA in bodily fluids that derives from tumor cells
pDNA	Plasma DNA	Free DNA (i.e. not within cells) found specifically in the blood plasma
ptDNA	Plasma tumor DNA	Plasma DNA that derives from tumor cells

(B)

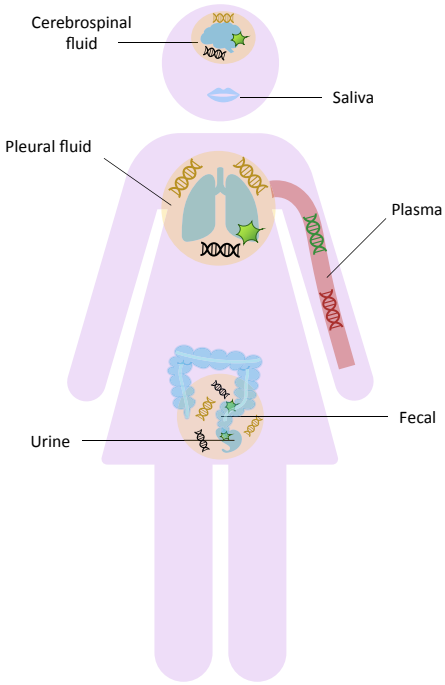

Figure 2. Acronyms Describing Free DNA in Body Fluids. Acronyms and terms are presented in (A) and representative locations of frequently analyzed body fluids are illustrated in (B). Green hexagons represent tumors.

Digital droplet PCR (ddPCR) increased analytical sensitivity and enabled the detection of one mutant DNA molecule per 100 000 normal DNA molecules [34]. This suggested that ptDNA was a far more sensitive biomarker than circulating tumor cells, and that ptDNA can more accurately capture a tumor's mutational profile [16]. Using targeted amplification followed by

Box 1. A Brief History of Cell-Free Tumor DNA

As early as 1977, Leon *et al.* [63] recognized the value of cell-free tumor DNA in blood, and reported significantly higher levels of DNA in the serum of patients with metastatic disease. Despite limited methods, the signal was strong enough to see a reduction in circulating DNA in response to treatment, a general correlation between DNA reduction and improved clinical condition, and differences in these signals across tumor type. However, the authors struggled to understand the identification of seemingly normal DNA in 50% of cancer patients, and concluded that their test may have limited diagnostic value. This foreshadowed the persistent challenges with achieving adequate diagnostic sensitivity that researchers have faced when using liquid biopsy. Nonetheless, the potential was recognized for better detection methods to improve the use of ptDNA, and this laid important groundwork for future studies.

However, despite this potential, and the subsequent investigation by this group into circulating DNA in cancer patients [64], the next two decades saw a focus on the analysis of circulating tumor cells and non-nucleic acid biomolecules as candidate tumor biomarkers. Little progress was made with circulating DNA until the 1990s, when the successful isolation and analysis of cell-free fetal DNA from the blood of pregnant mothers seemed to reinvigorate the study of circulating tumor DNA [1,65]. Improvements in methodology and the optimization of powerful molecular and genetic tools, such as PCR and DNA sequencing, facilitated increases in analytical sensitivity that propelled cell-free tumor DNA research forward [2,66]. By 1999, digital PCR methods that utilized the dilution and subsequent single molecule amplification of ptDNA were enabling the detection of approximately four mutant alleles among 100 total alleles [66] (Box 2). Within a few years, significant improvements in isolation techniques had enabled researchers to detect tumor DNA molecules as rare as one per 10 000 normal DNA molecules [32]. These technical advances, in conjunction with the onset of next-generation sequencing and the enormous amount of tumor mutation data it generated, set the stage for the increased clinical application of ptDNA.

Box 2. PCR-Based Methods for Detecting ptDNA

As next-generation sequencing facilitated the identification of characteristic cancer (driving) mutations, it became clear that somatic driver mutations found in tumor samples were highly specific and could be utilized to track disease [29,67]. Since DNA arising from solid tumors can be released into the blood (see Figure 3 in main text), DNA extracted from blood plasma can be interrogated for tumor specific mutations using a variety of technical approaches. The general framework involves the dilution of pDNA to a level where every two aliquots has an average of one DNA molecule (i.e., a single genomic fragment) [66]. The genomic area flanking identified mutation(s) is then amplified from the isolated pDNA molecules with quantitative PCR-based techniques, which allows for the calculation of total DNA concentration per volume of plasma. Probes specific for a patient's identified mutation(s) can then be designed and used to identify mutated DNA among the amplified molecules. These probes are often fluorescent, and facilitate the quantitation of mutant DNA relative to wild-type DNA. Finally, this fluorescence signal can be used along with the calculated total DNA to report an estimate of the number of mutant DNA fragments per sample. This approach utilizes *a priori* knowledge of a patient's specific tumor mutations to evaluate pDNA in a personalized way. Applying this approach to the plasma of patients collected after tumor resection, or to the plasma of patients receiving chemical therapies, can enable the assessment of treatment-associated changes in ptDNA with potentially predictive value. However, since ptDNA molecules derived from cancers are often exceedingly rare compared with normal circulating DNA molecules [32], analytical sensitivity thresholds can be a limiting factor. Knowing this, many investigators have focused on accurately quantifying ptDNA in advanced stage cancers, which are associated with elevated amounts of tumor DNA.

next-generation sequencing, a novel approach called tagged amplicon deep sequencing (TAm-Seq), enabled entire genes of interest to be sequenced and scrutinized for genetic alterations without *a priori* knowledge of a tumor's driver mutations [24]. This also allowed for the identification of mutations not found in primary tumors and potentially underpinning acquired resistance. While this method has been clinically limited by high read depth requirements and an analytical sensitivity requiring allele frequencies of at least 2%, it has facilitated the use of numerous mutations in parallel to accurately monitor disease dynamics [24]. The use of multiple mutations in parallel has also been used by others [25] and can greatly reduce the likelihood of false negatives due to sampling noise.

Further advances were made by using both digital PCR, which requires targeting personalized mutations from patient-derived tumor specimens, and the mutation-nonspecific TAm-Seq platform to show that metastatic breast cancer can be effectively tracked with ptDNA [25]. This study showed that ptDNA could signal progressive disease an average of 5 months earlier than imaging in a majority of patients [25]. Others also showed that a targeted approach with digital PCR could be used to detect the acquisition of gene amplifications in metastatic breast cancers [35]. Focusing on *HER2* gene amplifications, which can be acquired or lost over the course of disease and can be successfully targeted by therapeutic antibodies, this study highlighted the potential of noninvasive assays to guide hormone-based treatments in an increasingly personalized manner [35]. Exome-focused analysis of ptDNA was used to complement traditional invasive approaches in identifying drug resistance–mediating mutations in advanced cancers [26] (Figure 3). This study included two cases for which blood samples were collected along with traditional biopsies, which allowed full representation of the tumor genomes in the blood plasma [26] (Figure 3).

Whereas nearly all early studies on the use of ptDNA in cancer treatment had dealt with a single tumor type, Bettegowda *et al*. [27] set out to examine over 15 cancer types from 640 patients. Since certain tumor samples can be difficult to obtain due to clinical and/or logistic limitations [36], and others are obtained through needle-based biopsies that provide insufficient material for genetic analysis [37], understanding the potential of liquid biopsy across tumor type is paramount. The authors focused on estimating the percentages of patients with detectable mutant DNA across different tumor types, how the amounts of detected mutant DNA differed between and within tumor types, how this detection capacity varied based on disease

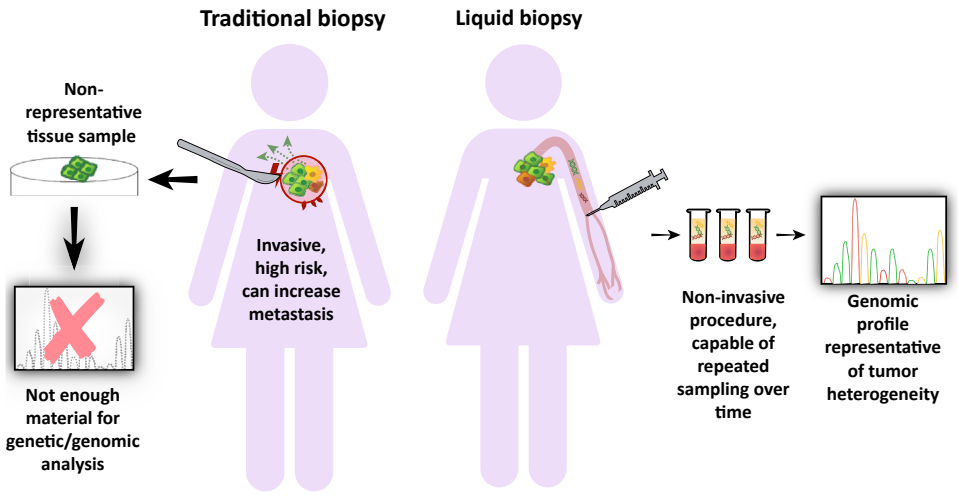

Figure 3. Traditional Biopsy versus Liquid Biopsy. Tumor biopsies are an essential component of cancer care. Traditional tumor biopsies frequently require invasive procedures, and can be limited by the patient's poor health, the location of the tumor, tumor heterogeneity, and insufficient tumor tissue. Compared with traditional biopsy, liquid biopsy offers a noninvasive option for detecting and monitoring oncological disease. By extracting tumor DNA from the blood plasma, tissue injury and complications associated with traditional biopsy are eliminated. A more complete survey of the heterogenous genomic landscape of a tumor may also be obtained, and the process is easily repeatable over time in order to perform longitudinal and replicative analysis. As a result of the short half life of tumor DNA in the blood (half-life on the scale of minutes to hours), liquid biopsy is a much more dynamic approach, and can facilitate the nearly real-time monitoring of disease dynamics in response to surgical intervention and treatment. Liquid biopsy also avoids the issues of insufficient genomic material and pathogenic disruption of the local tumor environment that are associated with traditional biopsy. Finally, while sensitivity thresholds are not high enough yet to allow for the use of liquid biopsy in the *de novo* detection of cancer, the ease of access to the blood and the nearly nonexistent morbidity of this approach suggest that routine cancer surveillance using liquid biopsy is possible.

advancement, and how mutant DNA amount varied by disease stage. The somatic mutations identified in each of the tumors of 410 patients with advanced cancers were found to be highly specific for cancer cells, and a specificity of 99.2% for *KRAS* mutations was identified in a distinct cohort of 206 metastatic CRC patients. The diagnostic sensitivity in this context was also impressive (>87%,) and was higher than most conventional biomarkers [27,30]. Furthermore, high levels of ptDNA prognosticated a poor 2-year survival, with implications for disease tracking and clinical planning, and there was significant variation across and within tumor type in the number of mutant molecules per 5 ml of plasma. While some tumor types, such as gliomas, showed both low mutant molecule amounts and a low percentage of patients with detectable ptDNA levels, numerous other tumors showed moderate to high levels of ptDNA in spite of a low number of patients with these tumors having detectable ptDNA. Some of this variation in detection is likely due to differences in the average disease stage at the time of detection across tumor type, as well as differences in the amounts of tumor DNA released into the bloodstream by different tumor types at similar stages (Figure 1). These sensitivity issues may also stem from aspects of genetic heterogeneity that we have not yet adequately characterized, and that differ across tumor type (reviewed by Martincorena *et al*. [38]).

Lung cancer represents one oncological setting within which ptDNA-based liquid biopsy is already in widespread use and where actionable mutations (such as those in the EGFR gene) can be reliably identified in the clinic using liquid biopsy. For example, plasma-based genotyping of non-small cell lung cancers (NSCLCs), via the cobas EGFR Mutation Test v2 (Roche

Molecular Systems, Branchburg, NJ, USA), has recently received FDA approval for the diagnosis of specific EGFR mutations (exon 19 deletions, L858R substitution) that can help to determine overall prognosis and eligibility of NSCLC patients for a variety of tyrosine kinase inhibitor (TKI)-based therapies [39]. This is the first FDA-approved ptDNA cancer diagnostic test and foreshadows the potential for ptDNA to survey a tumor's genetic landscape noninvasively in order to guide cancer treatments. This FDA approval has been based on results from a number of recent studies and clinical trials, such as the IGNITE [40,41], ASSESS [40], EURTAC [42], and NCT01203917 trials and their follow-up studies [39,43], which showed that informative EGFR mutations could be detected reliably enough with ptDNA to serve as an alternative to tissue-based genotyping. Furthermore, the cobas EGFR Mutation Test v2 can identify up to 42 EGFR mutations, and many of these (like the T790M mutation) represent good candidates for upcoming FDA approval. Recently published retrospective and prospective studies have demonstrated the equivalence in outcomes for patients with both common EGFR-activating mutations and the EGFR T790M resistance mutation that received a third-line EGFR TKI, regardless of whether these mutations were identified using DNA extracted from tumor tissue or blood [44,45]. While these studies support the efficacy of liquid biopsy–based methods in genotyping NSCLCs, the modest diagnostic sensitivity of these approaches (~70%) has led to the continued use of both traditional biopsy and tissue-based genotyping as diagnostic tools, whenever clinically feasible, for individuals whose blood-based genotyping is negative [39]. However, the turnaround time for blood-based genotyping can be much faster than traditional approaches, and repeated biopsies over the course of treatment are much more feasible using liquid biopsy [36,39,45,46] (Figure 3).

ptDNA in Early-Stage Cancers

Given the limitations of current therapeutics in the treatment of advanced cancers, and the efficacy of surgery in earlier stage cancers when tumors are locally confined, successful use of ptDNA in the context of early-stage disease is likely to bring the most clinical benefit. However, since ptDNA levels correlate with tumor stage across tumor types [27,47] (Figure 1), the use of ptDNA in early-stage cancers has been challenging due to low ptDNA levels that impede sensitivity in a rate-limiting fashion. Nonetheless, in their seminal study of ptDNA across tumor type, Bettegowda *et al*. detected cancerous disease in slightly more than half of early-stage patients [27]. Close to 50% of stage I patients had detectable ptDNA levels, while over two-thirds of stage III patients had detectable ptDNA [27]. These results suggested that ptDNA assays might serve as a viable monitoring approach in high-risk individuals (e.g., BRCA gene carriers) who would benefit greatly from early detection and for whom repeated biopsy or imaging is not feasible (Figures 1 and 4). Similarly, ptDNA may provide an alternative option for disease detection or mutational profiling in patients for whom traditional biopsy is contra-indicated, or for patients whose tumor types tend to release more ptDNA.

In a recent study on the use of ptDNA in early-stage breast cancer, Beaver *et al*. [23] used Sanger sequencing and newer ddPCR approaches to search for mutations at hotspots in the *PIK3CA* gene. In addition to confirming the enhanced capacity of ddPCR to identify mutations compared with Sanger sequencing, they showed that a small number of common cancer mutations were found in almost 50% of early-stage breast cancer tumors [23]. This demonstrated that ptDNA assays could be developed around common mutation subsets that are profiled simultaneously without the need to first identify mutations in a biopsied tumor specimen. Furthermore, when searching for ptDNA using the identified *PIK3CA* mutations, they reported a specificity of 100%, a sensitivity of 93.3%, and an accuracy of 96.7% [23]. Lastly, the authors reported the ptDNA mutational status of patients before and after surgery in the context of the histopathology and general presentation of these patients, with half of the patients with

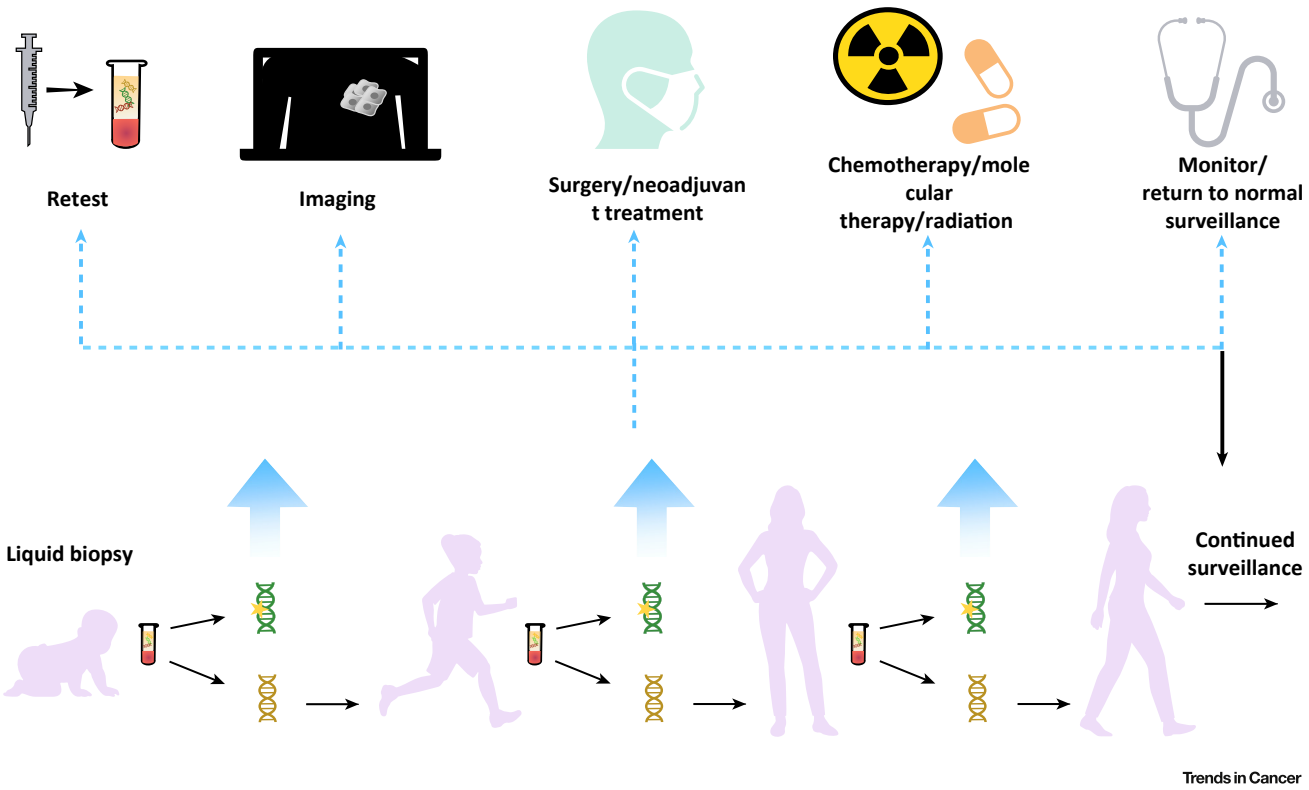

Figure 4. Hypothetical Longitudinal Patient Surveillance and Detection of Early Cancer Biomarkers Using Liquid Biopsies. A theoretical approach to a liquid biopsy-based cancer detection blood test is depicted. From birth, and at established intervals, an individual would receive a blood test to evaluate their pDNA for features representative of cancer. Those whose tests were positive, based on a compendium of cancer mutation information and associations, could be referred for more in depth clinical evaluation and monitoring. This would hopefully increase the likelihood of identifying potential cancers at their earliest and most treatable stages, and might help to significantly reduce cancer mortality rates. Individuals with cancer who achieve remission might similarly be monitored by liquid biopsy-based approaches, and might eventually re-enter the standard surveillance paradigm.

detectable ptDNA pre-surgery having detectable ptDNA post-surgery despite showing no other signs of residual disease [23]. These data suggest that ptDNA can be used to help stratify patients as more or less likely to benefit from morbid adjuvant treatments that only benefit a minority of patients [23,48].

While many studies have continued to demonstrate the specificity of ptDNA as a broad cancer biomarker, some studies have focused on identifying clinical niches where ptDNA can offer predictive value. In seminal work on the ability of ptDNA to predict relapse in early-stage disease, Garcia-Murillas *et al*. [28] reported that the identification of ptDNA after neoadjuvant treatment and surgery accurately predicted relapse in early-stage breast cancer. This prediction could be made accurately with only a single post-surgical plasma sample and was more sensitive with serially collected plasma samples (Figure 3), which could signal disease progression a median of 7.9 months before clinical relapse [28]. The authors also showed that targeted capture and sequencing of ptDNA could characterize the genetic landscape of minimally residual disease and that this predicted the mutational profile of the metastatic relapse better than sequencing of the primary tumor sample [28] (Figure 3). In another encouraging study, patients with stage II CRC that did not receive adjuvant chemotherapy and had detectable ptDNA levels post-surgery relapsed ~80% of the time by a median follow up of 27 months [49]. Contrastingly, similar patients without detectable ptDNA post-surgery

relapsed <10% of the time [49]. A similar association between ptDNA levels and recurrence risk was seen in stage II CRC patients receiving adjuvant chemotherapy treatment [49]. A recent study evaluating the use of liquid biopsy in localized lung cancer identified ptDNA in the first post-treatment blood sample in 94% of patients who had recurrence, and all stage I patients tested had detectable ptDNA [50]. This post-treatment ptDNA was identifiable in over 70% of patients a median of 5.2 months before radiographic identification of disease progression and was further used to identify mutation profiles that predicted favorable responses to targeted therapies. Scherer *et al*. could similarly detect ptDNA in the post-treatment plasma of >70% (8/11) of lymphoma patients who ultimately had recurrence, and ptDNA was not found in any of the 10 patients that were disease-free for 24 months, or 54 healthy patients [51]. In addition to noninvasively and successfully profiling the genomic heterogeneity and clonal evolution of these lymphoma tumors, Scherer *et al*. could predict clinically relevant histologic cancer transformations and progressions using genomic features ascertained exclusively with noninvasive liquid biopsy.

Recent work by Abbosh *et al*. also utilized ptDNA in early disease contexts to both assess ptDNA levels in early-stage patients and determine the clonal and subclonal evolution of tumors during relapse and progression [52]. The authors found at least one single nucleotide mutation in ~60% (58/96) of patients with early-stage NSCLC, as well as significant differences in ptDNA detection rates based on a histological subtype that correlated with necrosis and other tumor features associated with ptDNA release. Furthermore, ptDNA was found in 13 of 14 NSCLC patients that relapsed, whereas only one of 10 patients that did not relapse by at least 688 days had detectable ptDNA. This ptDNA in relapsed patients was found a median of 70 days before radiographic confirmation, with almost 50% of patients demonstrating ptDNA-based lead time of greater than 150 days. Treatment response and resistance was also represented by ptDNA mutation profiling, and subclonal mutation frequency changes predicted which malignant subclones dominated relapse and progression.

Specificity Challenges of ptDNA Assays

In spite of the strong body of evidence supporting ptDNA as a highly specific biomarker, several studies have challenged this notion by identifying cancer-associated mutations in the blood plasma of healthy individuals [53,54]. While these may represent early mutational events during tumorigenesis, and carriers of these characteristic mutations seem to have elevated cancer risks, significant numbers of patients seem to progress without any manifestation of oncological disease. For example, Fernandez-Cuesta *et al*. [54] reported that 11.4% of 123 healthy patients were positive for mutations in the canonical tumor suppressor gene *TP53* and were able to replicate these results in an independent cohort of 102 noncancerous controls. The finding of purportedly cancer-causing mutations in apparently healthy individuals poses a clinical diagnostic challenge to otherwise highly specific mutation-targeted ptDNA assays. This may represent a significant obstacle to the widespread adoption of ptDNA as a foundational biomarker in initial cancer detection. On the other hand, if the majority of cancer mutations found in the pDNA of healthy individuals represent the DNA of cells that are becoming clinically malignant, then the routine surveillance of pDNA for cancer mutations may help to identify and monitor at-risk individuals in a preventative fashion (Figure 4).

Improving ptDNA Detection with *In Silico* Analytical Methods

Due to increased computational capacity, availability of massively parallel sequencing technologies, and high-throughput bioinformatic methods, liquid biopsy approaches are now notably more powerful. For instance, Newman *et al*. [55,56] recently developed a pivotal

approach to liquid biopsy that targets a compilation of recurrently mutated regions in a particular cancer of interest. Once a 'selector' has been designed for a particular cancer type, a patient's tumor DNA is targeted by this selector to capture these recurrently mutated regions. These regions are then deep sequenced to depths of ~10 000 × and analyzed for cancerous mutations. This approach allowed for the detection of multiple types of mutations and was able to identify mutations in >95% of NSCLC tumors. The approach, called cancer personalized profiling by deep sequencing (CAPP-Seq), was highly specific for mutant alleles with a frequency of ≥0.02%, and was able to identify 100% of stage II–IV disease cases and 50% of stage I disease cases [55]. While the identification of 50% of stage I patients may seem suboptimal, these results are significantly better than those achieved by other methods [27,54]. In lung cancer in particular, where biopsies are difficult and hazardous to obtain [36,46], resulting in small amounts of genetic material [37], and therapies are of limited efficacy, the ability of CAPP-Seq to utilize very small amounts of tumor DNA to identify disease in early-stage patients is very promising (Figure 3). The authors also applied CAPP-Seq to ptDNA to differentiate between radiographically ambiguous results, which could suggest when a radiographic signal was an artifact of radiation treatment or when negative radiographic results were missing minimally residual disease [55]. Furthermore, when CAPP-Seq was used in a separate study to analyze ptDNA for mutations underlying acquired resistance in NSCLC, multiple apparent resistance mutations were found in 46% of patients who had previously received first-line targeted inhibitor treatment, and other point mutations and amplifications were differentially associated with different inhibitor-based therapies [57]. These results speak to the ubiquity and clinical relevance of tumor heterogeneity and support the use of CAPP-Seq-based liquid biopsy to dynamically identify mutations to guide treatment.

In a follow-up study, Newman *et al*. [56] were able to further enhance their CAPP-Seq approach with the addition of an *in silico*–based integrated digital error suppression (iDES) technique. By recognizing that the background noise they experienced was not random, they utilized datasets to mathematically identify and account for stereotypical errors, significantly increasing the sensitivity, specificity, and accuracy of CAPP-Seq [56]. Notably, their computational method alone improved analytical sensitivity by about threefold and represents the kinds of improvements than can be made by the appropriate use of *in silico* techniques. When this computational approach was combined with a strategic molecular barcoding enhancement, the two compounded one another and led to an ~15-fold improvement in analytical sensitivity. Ultimately, the iDES CAPP-Seq method enabled the biopsy-free detection of EGFR kinase domain mutations with nearly 100% specificity and 92% sensitivity at the variant level. At the patient level, sensitivity for ptDNA was over 90% and specificity was over 95% [56]. While the sample size of early-stage patients was small, ptDNA was identified in three of three pretreatment plasma samples from early-stage patients [56]. Furthermore, a more recent study employing iDES CAPP-Seq identified tumorous DNA from pretreatment blood samples in seven of seven stage IB cancers, and were also able to effectively identify and track minimal residual disease [50].

These results achieved by iDES CAPP-Seq are very encouraging and represent sensitivity levels that may facilitate the widespread and reliable use of liquid biopsy for both early-stage cancer detection and advanced disease monitoring (Figure 4). Recent work by Phallen *et al*. [58] builds on elements of this approach and offers an interesting alternative for ptDNA-based cancer detection using liquid biopsy. By extending the selected regions to include additional genes and somatic hotspot positions and by calibrating this set of captured regions to apply equally well across different cancer types, the authors were able to detect somatic mutations highly specific for cancer in 71%, 59%, 59%, and 68% of early-stage colorectal, breast, lung,

and ovarian cancer patients, respectively [58]. This approach, termed targeted error-correction sequencing, or (TEC-Seq), strategically prepares and barcodes pDNA, sequences samples to extreme depths (>30 000 × coverage), requires mutations to be seen numerous times across identical reads and distinct read pairs, and applies robust filtering criterion to achieve an error rate of $<3.3 \times 10^{-7}$ false-positive mutation calls per base [58]. While such high-depth sequencing is currently prohibitively expensive, and the definition of this error rate is somewhat different than other methods such as iDES CAPP-Seq (false positive rate vs. technical error rate), this approach enabled the authors to detect no false-positive bases among the 80 930 bases analyzed in error-assessing dilution assays [56,58]. Furthermore, and similar to iDES CAPP-Seq, the TEC-Seq approach aims to reduce false positives by using DNA from matched timepoint blood cells, and was able to identify genomic alterations indicative of clonal hematopoiesis in 16% of the 44 healthy individuals they assessed. This suggests the possible use of such an approach to monitor healthy individuals for the clonal expansion of cellular populations. While the detection rates of early-stage disease by these newer methods are still lower than what would ideally underpin a reliable diagnostic test, they represent forward progress towards the use of ptDNA and liquid biopsy for the detection of early-stage cancers.

Concluding Remarks

While the findings reviewed thus far demonstrate significant fulfillment of the promise of liquid biopsy to noninvasively and reliably identify cancer patients and guide treatment, the identification of ptDNA in the earliest stages of tumor development has been limited. Numerous questions remain regarding how to improve the detection of ptDNA in early-stage cancers, and whether such detection can ultimately be used for cancer surveillance and diagnosis (see Outstanding Questions). One potential way to enhance the sensitivity of ptDNA methods is to identify and employ genomic metrics that vary with tumor progression. While independent mutations require exquisite precision and sensitivity to detect, genome-wide measures are summed across billions of base pairs and may reflect aggregated signal that is undetectable in isolation. When calculated from deeply-sequenced genomes produced by advanced sequencing technologies, such metrics can increase the power to detect otherwise hidden ptDNA signals. This is likely why the inclusion of non-canonical mutations (i.e., private mutations) in commonly mutated regions has proven important to the detection of cancerous disease by modern ptDNA-based analyses [50,58]. While certain variations may require cost-prohibitive ultra-deep sequencing to detect the signal from low-abundance tumor DNA, sequencing costs continue to drop exponentially, and such an approach may ultimately be feasible. An example of one measure that may reflect the onset of cancer is the change over time in the proportion of rare variants found in an individual's plasma DNA. Based on the population genetic concept that rapidly expanding populations exhibit an elevated level of rare variants as they accumulate novel genetic mutations [59,60], one might expect changes in the proportion of rare variants found in pDNA as tumors clonally expand and release their DNA into the blood. Genome-wide measures could potentially also be calibrated around the amount of structural variation found in pDNA or the epigenetic profiles of pDNA.

As the amount of genomic data from tumor samples continues to grow, it should be possible to develop optimal recurrent mutational profiles from all previously analyzed cancers [61,62]. As emphasized and demonstrated by Newman *et al*. and Phallen *et al*. [55,56,58], changing focus from patient specific mutations to cancer specific assays can be extremely powerful. Mutations identified in this way can be prognostic and can signal that a patient's disease course is likely to resemble that of patients with similar mutational profiles, regardless of tumor tissue type. Furthermore, as outlined in the study by Ciriello *et al*. [61], the increasing characterization of cancer mutational signatures can potentially facilitate the redefinition of tumor classification

Outstanding Questions

Given the low levels of ptDNA during initial cancer development and the limited analytical sensitivity of current approaches, how can the detection of ptDNA in patients with early-stage cancers be improved?

How can we use genome-wide measures that are summed across billions of base pairs, like rare variation levels or shifts in copy number variation, to aggregate signal that is undetectable in isolation and indicate the development of cancers?

Can we develop optimal recurrent mutational profiles from previously analyzed cancers to underpin cancer targeting assays that do not need *a priori* knowledge of a patient's specific mutations?

Will ptDNA-based liquid biopsy enable surveillance of the general population for DNA mutations that signal the development of cancerous disease?

from a tissue-based system to a mutation-based one. By combining newer deep-sequencing techniques that avoid the need to target a patient's specific mutations with the potential of genome-wide analysis, surveillance of the general population for DNA-based cancer signals may become a reality. As outlined in Figure 4, one might imagine a standard blood test that is performed longitudinally, and which focuses on changes in pDNA signals over time that correlate with cancer risk. Liquid biopsy has already shown the potential to significantly impact the treatment of cancer in the clinic, and by leveraging features of ptDNA further, clinicians and cancer genomicists can hopefully use liquid biopsy for the noninvasive *de novo* detection of cancer.

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