Assessment of circulating tumor DNA in pediatric solid tumors: The promise of liquid biopsies

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Abstract

Circulating tumor DNA (ctDNA) can be detected in the blood and body fluids of patients using ultra-sensitive technologies which have the potential to improve cancer diagnosis, risk stratification, non-invasive tumor profiling, and tracking of treatment response and disease recurrence. As we begin to apply "liquid biopsy" strategies in children with cancer, it is important to tailor our efforts to the unique genomic features of these tumors and address the technical and logistical challenges of integrating biomarker testing. This article reviews the literature demonstrating the feasibility of applying liquid biopsy to pediatric solid malignancies and suggests new directions for future studies.

Keywords

Circulating tumor DNA; pediatric cancer; next generation sequencing; digital PCR; liquid biopsy

Introduction

As the field of precision oncology grows, the analysis of circulating tumor DNA (ctDNA) holds incredible potential for advancing cancer treatment. After cell-free DNA was first identified in the peripheral blood in 1948, ctDNA was described in the late 1970s and has since been quantified and characterized across a range of cancers.[1–6] Potential applications of ctDNA are far-reaching, including cancer screening and diagnosis, non-invasive tumor profiling and identification of targetable somatic variants, pretreatment risk-stratification, tracking of treatment response, detection of minimal residual disease, and surveillance for relapse.[7–9] For children with cancer, these applications could reduce exposure to anesthesia and radiation by decreasing reliance on surgical biopsies and serial imaging for diagnosis, staging, and disease monitoring. Furthermore, the opportunity to analyze serial liquid biopsy

samples could vastly increase our understanding of tumor evolution and genomic patterns of treatment resistance in children with relapsed cancer.

Analysis of ctDNA in pediatric malignancies presents unique methodologic and clinical challenges, requiring tailored approaches in the liquid biopsy field. Much of the early success of ctDNA evaluation in adult malignancies focused on the identification of highly-recurrent hotspot mutations in oncogenes such as *EGFR* or *KRAS*, including polymerase chain reaction (PCR)-based assays that have gained regulatory approval in the United States and Europe.[4–6,10–20] Such recurrent single nucleotide variants (SNVs) are rare in pediatric malignancies. Instead, pediatric tumors are more commonly characterized by recurrent copy-number changes and translocations.[21–27] Detecting these structural variants requires customized DNA profiling approaches that differ from assays optimized for detecting somatic SNVs. Clinical implementation and validation of these custom assays is also challenging due to the rarity and diversity of pediatric cancers.[28]

Beyond the studies examining ctDNA in solid tumors, there is a growing body of literature evaluating ctDNA in adult patients with hematologic malignancies.[29–36] While ctDNA has been evaluated in the peripheral blood of children with leukemia, these assays lack the sensitivity of modern minimal residual disease assays (MRD).[37,38] In CNS tumors, detection of ctDNA in the peripheral blood is possible, but sensitivity appears to be a challenge in these patients.[4,39–41] Instead, recent efforts have focused on detection of tumor DNA in the CSF.[41,42] Given the paucity of data from studies of ctDNA in children with hematologic and CNS malignancies, our review will focus on current technologies being applied to interrogate ctDNA in children with non-CNS solid tumors. The goals of this paper are to familiarize pediatric oncologists with the principles and techniques used for ctDNA work and to highlight unique aspects of applying liquid biopsy approaches to pediatric cancer care and research.

Detection, quantification, and characterization of ctDNA in children with solid tumors

Cell-free DNA is composed of short fragments (~135-170 base pairs) of double-stranded DNA found in the non-cellular fraction of the blood. Cell-free DNA originates from multiple cellular sources in the body, including normal cells, injured tissues, fetal cells, and cancer cells and has a half-life in the circulation of less than 2 hours.[43–52] In patients with cancer, ctDNA accounts for a portion of the cell-free DNA present in the circulation (Fig. 1). Circulating tumor DNA can be detected and quantified by measuring the presence of somatic events, including SNVs, insertion/deletions (indels), copy-number changes, translocations, and methylation patterns that differentiate tumor DNA from cell-free DNA originating from normal cells.[53–55] One recent article demonstrated that methylation patterns in cell-free DNA can be used to detect the presence of ctDNA and correlate ctDNA methylation with specific cancer histologies.[56] However, this strategy has yet to be applied to pediatric solid tumors. The two most common methods for detecting somatic variants in any DNA sample are PCR and next-generation sequencing (NGS)(Fig. 1). Both approaches can be applied to the detection of ctDNA but differ in how well they can be optimized for

classes of somatic variants and clinical scenarios (Fig. 2). There are numerous technology-specific and context-specific considerations that must be evaluated when choosing an approach for the detection of ctDNA. We have summarized what we consider to be the most important and commonly encountered considerations in Table 1.[57–63]

PCR-based assays

Many approaches to ctDNA analysis rely on PCR, a technology used to detect or quantify a segment of DNA by sequence-specific amplification (Table 1). In the late 1990s, it was shown that ctDNA microsatellites could be detected by PCR in patients with head and neck cancer.[64] Since then, many studies have focused on using PCR to detect highly recurrent hotspot mutations in genes such as *TP53*, *EGFR* or *BRAF* (Fig. 2A). PCR can also be adapted to the detection of structural variants which are more common in pediatric malignancies, such as copy-number alterations (i.e. *MYCN* amplifications) and translocations (i.e. *EWSR1/FLI1*) (Figs. 2B and 2C). These assays rely on the development of mutation-specific primers and/or probes which are often employed in the setting of digital PCR (dPCR) and can attain a sensitivity to detect variants that are present in as little as 0.005% of a DNA sample.[4,19,20,65–68] dPCR partitions DNA into a large number of PCR reactions, allowing for quantification, improved sensitivity, and some degree of multiplexing.[69]

Digital PCR-based assays can serve as noninvasive surrogates to biopsies for identification of potentially targetable oncogenic hotspot mutations, tracking treatment responses, and monitoring for the development of specific treatment resistant mutations.[10,34,65–67,70–74] However, the use of PCR to detect ctDNA requires prior knowledge of the disease-specific or patient-specific mutations, including the exact location of the patient-specific DNA breakpoints in the case of oncogenic translocations.[75,76] Furthermore, while ctDNA quantification by dPCR is highly sensitive, it only measures the mono-allelic fraction of the targeted genetic region. This mono-allelic fraction may not be an accurate estimate for the total abundance of ctDNA when the targeted region is affected by copy-number alterations, especially when the magnitude of those events is heterogeneous in the tumor (Table 1).

Next-generation sequencing

Next-generation sequencing is a powerful tool for ctDNA analysis. Unlike PCR, NGS technology allows for evaluation of somatic events without previous knowledge of the abnormality of interest. These assays can be developed to sequence individual genes, selected regions of the genome, the exome, or the entire genome (Table 1). For example, recurrent mutations in driver oncogenes, such as oncogenic EGFR mutations in patients with lung cancer, can be detected and monitored by NGS while also having the capacity to identify previously unknown or uncommon variants in this gene (Fig. 2D).[10] NGS gene panels of multiple known oncogenes (i.e. *BRAF*, *EGFR*, *KRAS*, and *PIK3CA*) have been used in patients with advanced cancer to identify targetable mutations for therapy selection without the need to perform numerous tests for individual variants.[77]

Next-generation sequencing can also be utilized to detect chromosomal copy-number alterations in ctDNA, a key feature of many pediatric malignancies (Fig. 2E).[21–23,78] Leary

et al. developed an algorithm to infer presence of ctDNA and its concentration from an analysis of chromosomal copy-number variants by whole-genome sequencing (WGS) performed with an average sequencing coverage of only 8 reads per base pair (8x).[79] Economical shallow WGS, with a coverage of 0.1× and a turnaround time of 2 days, was first used to identify ctDNA in prostate cancer by detecting copy-number changes.[80] It has also been used in neuroblastoma, allowing a reliable noninvasive copy-number profiling.[81] Adalsteinsson et al. showed that ctDNA can be quantified by measuring genome-wide segmental copy-number changes from ultra-low passage WGS (ULP-WGS) using the ichorCNA algorithm.[60] We recently applied this approach to the most common non-CNS pediatric solid tumors and we demonstrated that the majority of pediatric malignant tumors shed ctDNA at detectable levels.[82,83] One advantage of this approach is that quantification of ctDNA does not require prior tumor profiling or the need for a germline sample. Therefore, ctDNA analyses can be performed in settings where access to such samples may be limited or incomplete, as is often the case in multi-institutional prospective clinical trials of rare cancers.

High-throughput NGS can also be used for translocation detection in pediatric cancer. Translocations typically occur in introns that frequently span greater than 1,000 to 100,000 bases, with unique DNA break-points seen in each patient. Given that ctDNA fragments are less than 200 base pairs in length, identifying translocation breakpoints in the cell-free DNA by PCR requires the development of patient-specific PCR assays and previous knowledge of the translocation break-point (Fig. 2C). Recently, NGS hybrid capture assays have been adapted to detect these fusions by enriching sequencing libraries for regions of the genome commonly involved in oncogenic translocations, such as those described in Ewing sarcoma and alveolar rhabdomyosarcoma (Fig. 2F).[82,84] Such an approach has allowed for detection of oncogenic translocations directly from cell-free DNA specimens without the need to profile tumor biopsy material. This technology may be readily adapted to other fusion-positive tumors not previously evaluated.

Conceptually, ultra-deep WGS of matched cell-free DNA and germline samples would allow investigators to detect and quantify ctDNA by identifying any type of somatic variant without the need for previous knowledge of the tumor genome. However, this approach is currently cost prohibitive. Therefore, NGS strategies must be selected to balance three main profiling considerations: 1) the proportion of the genome targeted for sequencing; 2) the depth of sequencing desired; and 3) the cost of sequencing each sample (Fig. 3). For example, low-passage whole-genome sequencing can identify ctDNA by detecting chromosomal copy-number events.[62,79,81–83,85] This approach efficiently identifies copy-number events in samples with a high fraction of tumor DNA (3%) at a relatively low cost but is unable to identify specific base-pair substitutions, focal copy-number events, or translocations. Conversely, very deep sequencing (i.e. $10,000 – 100,000\times$ coverage) of a small panel of genomic regions can identify SNVs, indels, and focal copy-number alterations. With the appropriate error-suppression techniques,[86] this approach can detect variants with allelic fractions of < 0.1%, but is unable to detect chromosomal copy-number events, translocations, or SNVs occurring outside the targeted regions.

Clinical exploration of ctDNA in pediatric solid tumors

The available data indicate that circulating tumor DNA is detectable in a range of pediatric solid tumors at diagnosis, during treatment, and at the time of relapse (Table 2). Criteria for determining how ctDNA assays should adopted into clinical practice were recently outlined in an American Society of Clinical Oncology and College of American Pathologists joint review.[8] They concluded that ctDNA assays must demonstrate 1) analytical validity, meaning the assay is able to detect a targeted variant with accuracy, reproducibility, and reliability, 2) clinical validity, meaning that the assay can divide a clinical group in to one or more cohorts with significantly different outcomes, and 3) clinical utility, that the knowledge gained from the assay significantly improves clinical care. At the time of this review, there are no ctDNA assays that have gained regulatory approval for clinical use in pediatric oncology. However, numerous opportunities exist for incorporating ctDNA assays into the care of patients with childhood cancers. Results from ongoing and future studies are needed to validate these assays and justify their use in routine clinical care.

Diagnosis and prognostication

Circulating tumor DNA may provide a means of early cancer detection or of obtaining a diagnosis in cases where viable diagnostic tumor material cannot be obtained. To date, there have been no systematic evaluations of ctDNA for the purpose of early detection or diagnosis, however a number of studies have demonstrated the feasibility of using ctDNA for diagnostic purposes. We recently demonstrated that custom hybrid-capture NGS assays as well as an ULP-WGS NGS assay can be utilized in Ewing sarcoma, rhabdomyosarcoma, neuroblastoma, osteosarcoma and Wilms tumor to identify ctDNA without prior sequencing of tumor biopsy material.[82,84] For now, it is unlikely that such technology would replace conventional biopsies given the frequent need to assess histologic and pathologic characteristics of the tumor tissue for making a definitive diagnosis and risk group classification. However, in instances where tissue cannot be obtained, analysis of ctDNA using a validated assay may ultimately provide a diagnostic alternative, particularly in diseases with pathognomonic translocations.

A number of studies have examined ctDNA for the purpose of detecting prognostic genomic features. For example, studies have demonstrated the feasibility of detecting *MYCN* amplification in the peripheral blood of patients with neuroblastoma using real-time PCR with a sensitivity of 75-100% and specificity of 100%.[87–89] Another study utilized methylation of the *DCR2* gene in serum using real-time PCR to identify patients with an inferior event-free survival in patients with and without *MYCN* amplification.[90] Proof of concept studies found a correlation between plasma dPCR and tumor genomic analysis for detection of *MYCN* amplification, *ALK* amplification, and *ALK* hotspot mutations in neuroblastoma. [72,82,91,92] NGS assays have also been utilized to detect variants associated with a worse outcome, including *TP53* mutations and *STAG2* loss in Ewing sarcoma, 8q gain in osteosarcoma, *MYCN* amplification in neuroblastoma, 1q gain in Wilms tumor, and *PAX3* gene rearrangements in alveolar rhabdomyosarcoma.[82,83]

While the prior studies identified known prognostic genetic features in ctDNA, we demonstrated that detection of ctDNA itself provided prognostic information at diagnosis in

patients with Ewing sarcoma and osteosarcoma.[83] Using a hybrid-capture NGS assay, ctDNA could be detected in over half of patients with Ewing sarcoma from a cooperative group biobank of plasma samples. In these patients, the detection of ctDNA was associated with an inferior EFS and OS. Similarly, using an ULP-WGS approach capitalizing on the complex CNVs in osteosarcoma, ctDNA could be detected in over half of all baseline plasma samples. For patients with newly diagnosed localized osteosarcoma, increasing ctDNA levels were associated with incrementally inferior EFS and OS. These assays are now undergoing clinical validation in a prospective, multicenter biomarker study.

Measurement of residual disease and disease surveillance

Multiple studies have evaluated ctDNA levels throughout treatment for the purposes of assessing response to therapy and post-treatment disease surveillance. In this setting, one would expect that a very high sensitivity may be required to detect the earliest signs of relapse, although the sensitivity needed for these assays to demonstrate clinical utility is unknown. In Ewing sarcoma, ctDNA levels by patient-specific dPCR were shown to drop early in treatment, and rise with disease recurrence, sometimes prior to radiologic detection. [76,82,93] A case report described a similar approach utilized in a patient with desmoplastic small round cell tumor.[94] In one study of patients with *MYCN*-amplified neuroblastoma, *MYCN* was detected by real-time PCR in plasma. The authors demonstrated that *MYCN* levels remained elevated in patients with sub-total resections, but returned to baseline levels in patients with complete resections.[95] Wilms tumor has a genome with few recurrent mutations, but frequently has a characteristic aberrant methylation at 11p15. As proof of concept, differentially methylated regions have been used to identify ctDNA in patients with Wilms' tumor.[96]

Highly sensitive measurements of treatment response, such as MRD testing in hematologic malignancies, are lacking in solid tumors. Instead, much reliance is placed on radiologic evaluations often carrying increased radiation exposure and cancer risk, or sedation in young children, possibly affecting neuro-developmental outcomes.[97–99] In some cases, serial radiographic measurements of tumor burden have failed to be significantly predictive of outcome.[100,101] If validated and utilized appropriately, ctDNA assays may provide a sensitive biomarker of treatment response for solid tumors and decrease reliance on serial imaging and sedation in young children.

Exploration of tumor biology and identification of targets for therapy

Increasingly, ctDNA is recognized as an avenue to explore tumor biology that may exceed what is possible with tumor tissue alone. Particularly in solid tumors, biopsies may under-represent tumor heterogeneity and evaluation of ctDNA may provider further insight into spatially diverse aspects of the tumor genome. Furthermore, serial surgical biopsies of tumors are not currently feasible, limiting our ability to assess tumor evolution in solid tumor malignancies. However, serial liquid biopsy sampling is readily implemented and may provide insights into tumor heterogeneity and its evolution in response to conventional and novel therapies. For example, sequencing of matched pre-treatment tumor samples, relapsed biopsy material and plasma samples from patients with relapsed breast cancer following chemotherapy allowed for longitudinal comparison of somatic copy-number alterations.

While the majority of copy-number alterations were present in primary, metastatic, and ctDNA samples, copy-number events that were specific for metastatic samples, such as *NOTCH2*, *AKT2* and *AKT3*, were also observed in the plasma samples obtained at the time of relapse.[102]

In pediatrics, tumor genomic heterogeneity and tumor evolution over time can also be characterized from the peripheral blood using ctDNA. Chicard et al. provides two illustrative examples of this approach in the context of neuroblastoma.[78,103] In the first study of 70 patients, somatic copy-number alterations found in ctDNA were highly correlated with those in matched tumor samples. Interestingly, additional alterations were found in the ctDNA of some patients that were not present in the match tumor biopsy, indicating the possibility of heterogeneity among metastatic tumors.[78] In particular, there were two alterations involving *IGF1R* and two involving *TERT*, genes thought to confer increased metastatic potential, that were identified only in the ctDNA. In the second study, a combination of whole-exome sequencing and targeted sequencing demonstrated the utility of ctDNA to understand tumor heterogeneity and tumor evolution of neuroblastoma.[63] The authors found that sub-clonal events present at diagnosis frequently evolved into clonal mutations at relapse. These studies provide the most compelling evidence thus far that ctDNA may provide an avenue for exploration of tumor biology in pediatric solid tumors.

Challenges in clinical implementation

With an array of technologies now available for detection and characterization of ctDNA in pediatric solid tumors, it is important to consider the relevance of the information that can be derived from each assay as well as the logistical limitations of each approach. For example, NGS may be able to identify pathognomonic translocations in the plasma of patients with an undiagnosed tumor, however, clinical sequencing cannot currently be performed quickly enough for diagnostic purposes. In the case of prognostication, dPCR assays may have a greater sensitivity than NGS assays in the context of a minimal residual disease testing, but these assays often first require profiling of a tumor biopsy followed by the development and validation of patient-specific assays. While there may be sufficient time to develop such patient-specific assays for use in surveillance, after completion of therapy, this workflow is unlikely to be feasible for pre-treatment or early response-based risk-stratification. Therefore, we believe that a complement of approaches are needed to meet the clinical needs that could be addressed by ctDNA and we provide one example of how such assays could be applied longitudinally to patient care (Fig. 4).

Future directions

The feasibility of detecting, quantifying, and profiling ctDNA in patients with pediatric solid tumors has now been established. A variety of technical approaches can be readily adapted to the development of ctDNA assays. Choosing the optimal approach depends on a thorough understanding of the strengths and limitations of each technology as well as the goals of each clinical scenario. This review provides a framework for making such decisions. Translating these new tools into clinically meaningful and validated assays will require a focused and coordinated effort within the pediatric oncology community. Validation of

clinically relevant biomarkers for pediatric cancer can be challenging due to the relative rarity of these diseases. Each biomarker must be studied in the context of a well-defined patient cohort treated with a unified approach as part of a prospective analysis. Such studies in pediatric solid tumors are generally performed through multi-institutional cooperative efforts. Furthermore, studies sufficiently large to provide statistical power to validate a prognostic biomarker are typically conducted over several years. Given the rarity of these opportunities, it is critical that the appropriate samples be collected on multi-institutional trials whenever possible. The collection of blood is a minimal-risk procedure and as we explore which timepoints during therapy provide the most useful information, we recommend collecting blood samples prior to therapy, frequently during planned treatment, and serially while patients remain at risk for relapse. Other considerations, such as methods to collect and store samples for central processing have largely been standardized, making these studies immediately feasible.

As a scientific research tool, ctDNA provides a new avenue for understanding aspects of tumor biology that were largely inaccessible until now. The paucity of clinically annotated, matched diagnostic and relapsed tumor biopsy samples has resulted in few opportunities to study patterns of tumor evolution and treatment resistance in pediatric solid tumors. Rigorous efforts to collect and annotate matched diagnostic and relapsed blood samples from patients on banking studies and prospective trials will yield a new source of tumor DNA. Similarly, ctDNA technologies may help us explore the prevalence of tumor heterogeneity, a challenge that was previously restricted by a lack of access to geographically distinct biopsy samples collected simultaneously from a single tumor and from multiple metastatic tumors.

Beyond these immediate applications, ctDNA assays may ultimately have the potential to change the way patients are diagnosed by generating genomic information which can be integrated with clinical and pathologic data even when tumor biopsy material is significantly limited. As novel therapies become available for use in pediatric cancers, ctDNA may facilitate the identification of targetable mutations that direct the selection of specific agents and may also allow the detection of resistance mutations for patients undergoing targeted therapy. The early detection of ctDNA may inform the use of maintenance therapies designed to keep minimal residual disease in check and could play a role in surveillance strategies for patients with cancer predisposition syndromes.

Through the inherent collaborative nature of the pediatric oncology community and the tradition of enrolling our patients in prospective clinical trials, we have a unique opportunity to rapidly validate and implement ctDNA studies into the care of our patients. With plasma samples now being appropriately collected for ctDNA studies in the Children's Oncology Group banking study (Project: EveryChild) and into the majority of recent prospective trials, we are well poised to validate the clinical and scientific potential of liquid biopsy studies in pediatric oncology.

Acknowledgments

Abbreviation table:

ctDNA	Circulating tumor DNA
PCR	Polymerase chain reaction
SNVs	Single nucleotide variants
MRD	minimal residual disease assays
Indels	insertion/deletions
NGS	next-generation sequencing
dPCR	digital PCR
WGS	whole-genome sequencing
ULP-WGS	ultra-low passage WGS

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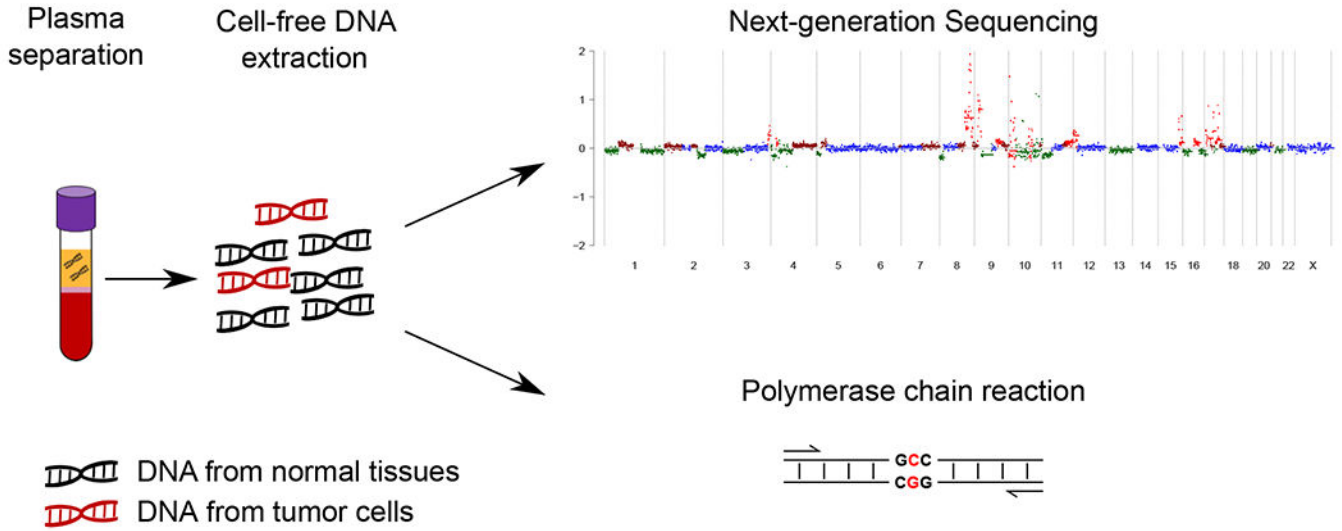
Figure 1. Overview of cell-free DNA extraction and processing.
Cartoon depicts the presence of cell-free DNA in the plasma layer of a blood sample collected in an EDTA tube (left). The extracted cell-free DNA is a mixture of DNA originating from normal tissues (black strands of DNA) and DNA originating from tumor cells (red strands of ctDNA), the latter being often a small fraction of the total DNA in the sample (middle). Identifying and quantifying ctDNA from a cell-free DNA sample requires detection of somatic variants that are present only in the tumor. This is typically done with either next-generation sequencing or polymerase chain reaction assays (right).
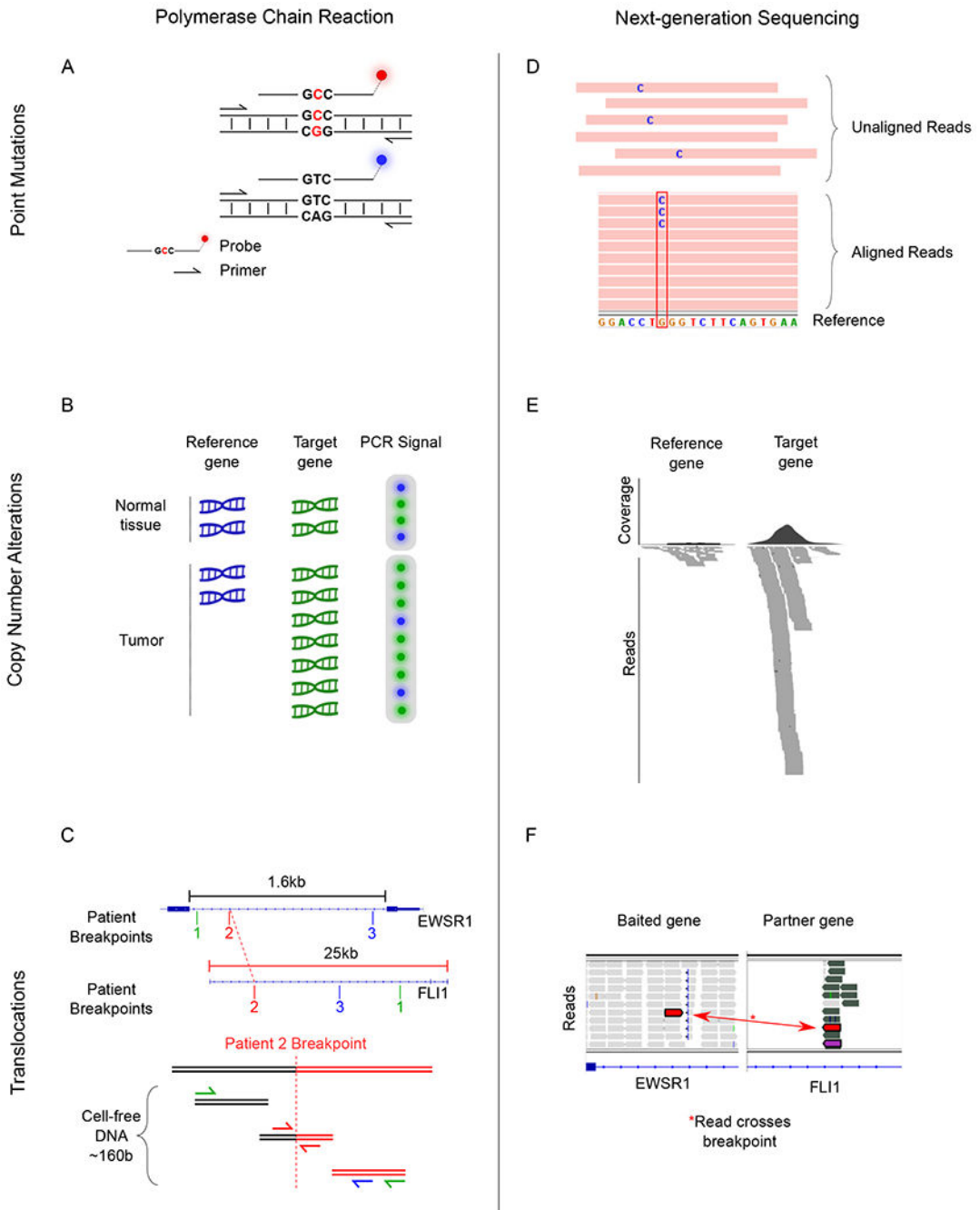
Figure 2. PCR and next-generation sequencing approaches to detecting somatic variants in cell-free DNA.

A) Image depicts the ability to detect DNA with single-nucleotide variants (top) from wild-type DNA (bottom) by utilizing sequence-specific fluorescent PCR probes. In this approach, primers are specific to the sequence flanking the region of interest while each fluorescent probe has a sequence complementary to either the mutated strand (red) or the wild-type strand (blue). DNA is depicted by two horizontal lines connected by small vertical lines with the three central base pairs indicating the sequence of interest. Red letters indicated mutated single-nucleotide variant. B) dPCR can be used to identify copy-number alterations by

comparing the number of PCR reactions from the target gene to the number of PCR reactions from a reference gene. Top, DNA originating from normal tissue contains two copies of the reference gene (blue DNA) and two copies of the target gene (green DNA) and results in two droplets containing reference gene PCR product (blue circle) and two droplets containing the target gene product (green circles). Bottom, DNA originating from tumor has an amplification of the target gene (green DNA) resulting in more droplets with PCR product from the target gene (green circles) compared to the number of droplets with reference gene (blue circle). C) The *EWSR1/FLI1* translocation breakpoints occur at intronic regions of each gene and are patient specific. Top, intronic regions of *EWSR1* and *FLI1* with patient-specific breakpoints indicated for hypothetical patients 1 through 3. Bottom, rearranged DNA from patient 2 is shown with the breakpoint indicated by a vertical dotted red line. Short fragments of cell-free DNA from patient 2 align to the same genomic region as the translocation. Only patient-specific primers designed for patient 2 (red) can successfully result in a PCR product. PCR primers are depicted as arrows and the color is specific to primers designed to amplify the matched patient-specific translocation D) Top, next-generation sequencing reads are generated from cell-free DNA. Bottom, reads are then aligned to the reference genome. Mutated DNA has a sequence mismatch at the site of the somatic single-nucleotide variant indicated by the letter "C" in blue. E) Average coverage and aligned sequencing reads for a reference gene (left) and the target gene (right). Amplification of the target gene results in many more sequencing reads compared to the reference gene. F) Using a hybrid-capture sequencing panel designed to enrich sequencing reads for the intronic region of *EWSR1*, DNA translocations are identified as reads that map on one side to the *EWSR1* intron and on the other side map to the *FLI1* intron. Two red rectangles represent the two sides of a single sequencing read.
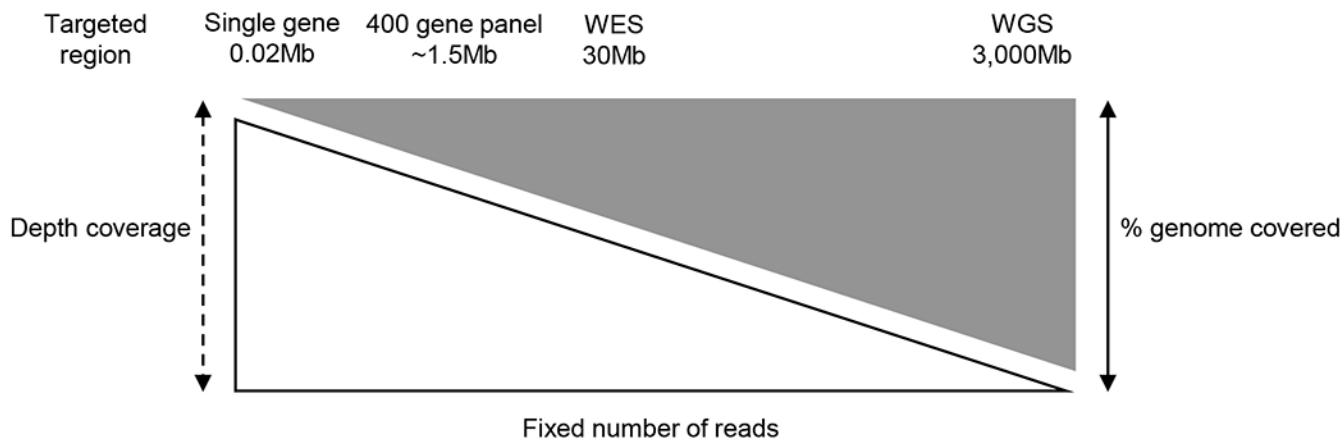
Figure 3. Relationship between sequencing depth and DNA coverage for next-generation sequencing.

The graph assumes that a fixed number of sequencing reads are generated for each sequencing strategy. As the targeted region increases (left to right), the depth of coverage decreases. These changes in depth can be overcome by increasing the number of sequencing reads generated, but that also results in a significant increase in cost.
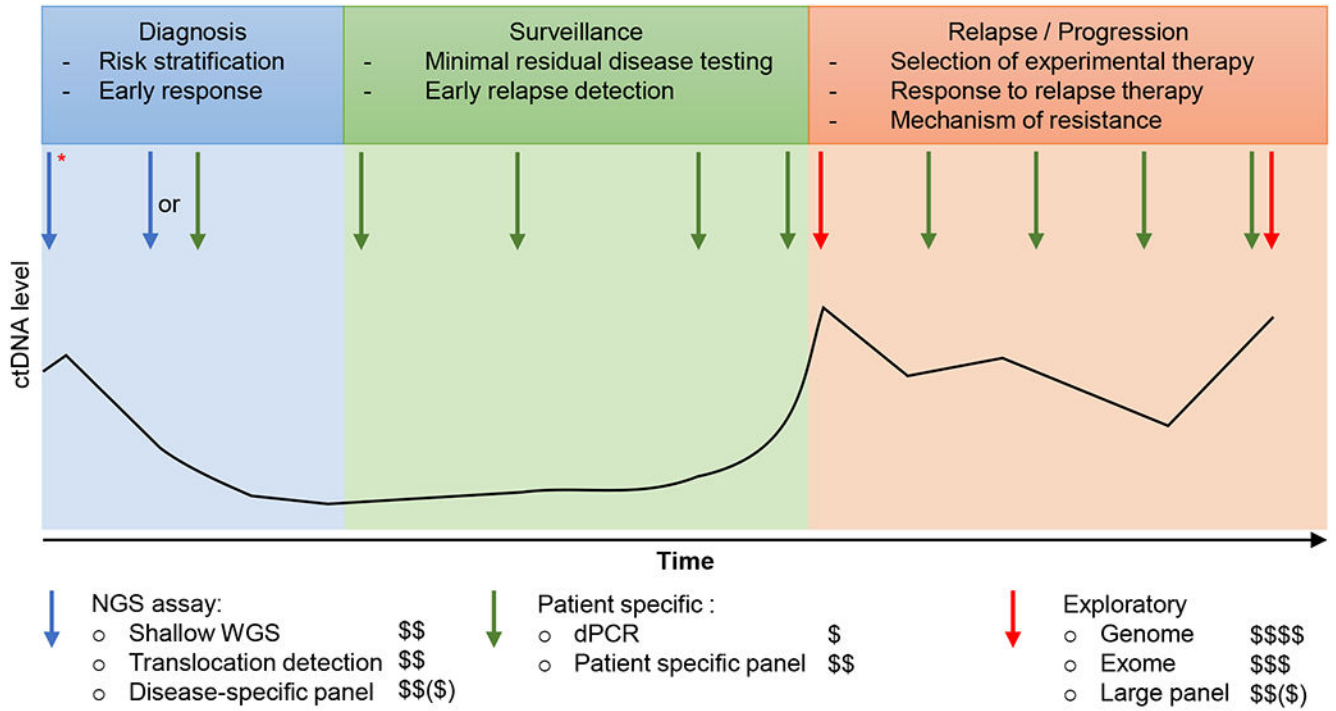
Figure 4. Potential workflow for clinical application of different ctDNA assays.
This graph depicts one potential strategy to combine complementary technologies to detect, quantify, and profile ctDNA throughout the course of a patient's care. At diagnosis and early in therapy, focused NGS assays can be used to detect and quantify ctDNA without requiring existing genomic data from the tumor (blue arrows). Highly-sensitive patient-specific assays can be developed for use later in therapy to detect minimal residual disease and for surveillance (green arrows). Broad genomic profiling can performed on ctDNA at relapse or progression (red arrows) and compared to broad profiling of the initial diagnostic sample (red asterisk) to identify patterns of tumor evolution, treatment resistance, and identify new targetable variants for clinical trial enrollment. Dollar signs indicate relative cost of each approach. The black line indicates ctDNA levels in the patient throughout the course of treatment.

TABLE 1.

Features of PCR and NGS.

Listed are common features and limitations of PCR and NGS that should be considered when choosing a strategy for ctDNA assay development.

	PCR	Next Generation Sequencing			
		Gene Panel[1]	Translocation Panel[1]	Shallow Sequencing (WIGS)	Deep Sequencing (WES/WGS)
Detectable variants	SNV/indel, translocation, CNA	SNV/indel, translocation, CNA	Translocation	CNA (Mb scale)	SNV/indel, CNA, Translocation (WGS)
Mutation-specific assay	Yes	No[2]	No[3]	No	No
Patient-specific assay	Yes (Translocation and non-recurrent variants)	No	No	No	No
Patient germline required	Variant dependent[4]	Variant dependent[4]	No	No	Yes
ctDNA quantification	ctDNA estimation complicated by CNA	Measuring multiple variants offsets effects of CNA	ctDNA estimation complicated by CNA	Multiple CNA improve ctDNA estimation	Measuring multiple variants offsets effects of CNA
Time to result	Day(s)	Week(s)	Week(s)	Week(s)	Week(s)
Relative cost	$	$$($)	$$	$$	$$$$
Sensitivity	Up to 0.005% of tumor fraction	Sensitivity increases with coverage[5]	Sensitivity increases with coverage	~3% (Adalsteinsson et al.)	Sensitivity increases with coverage[5]
Detects novel variants	No	Novel variants in targeted gene(s)	Unknown translocation partners	Yes	Yes

[1] - Gene and translocation panels can be combined because they use the same hybrid capture technology.

[2] - Assays are not mutation specific but selected genes are typically restricted to those known to be mutated in the cohort of interest.

[3] - Assays are not breakpoint specific but are enriched for intronic regions typically involved in the translocations of interest.

[4] - For variants that can be observed in the germline (e.g. TP53), either germline control must be used or prior knowledge of germline status is required to interpret results.

[5] - There is a risk of confusing sequencing artifact for low-allelic SNV/indels events. Error suppression strategies need to be incorporated when targeting these events by NGS.

SNV, single nucleotide variation; indel, small insertion or deletion; CNA, copy-number alterations; Mb, megabase.

TABLE 2:

Clinical evaluation of ctDNA in children with cancer.

First Author	Year of publication	Disease(s) evaluated	Number of patients	Key finding
Combaret[87]	2002	Neuroblastoma	102 patients 72 controls	*MYCN* amplification is detectable in ctDNA with high sensitivity and specificity using rtPCR.
Gotoh[89]	2005	Neuroblastoma	87 patients	*MYCN* amplification is detectable in ctDNA with high sensitivity and specificity using rtPCR.
Yagyu[90]	2008	Neuroblastoma	86 patients	Methylated-*DCR2* gene is detected using rtPCR in ctDNA is associated with inferior outcomes.
Combaret[88]	2009	Neuroblastoma	267 patients	*MYCN* amplification is detectable in ctDNA with high sensitivity and specificity using rtPCR, especially in patients with Stage 3 or 4 disease.
Combaret[104]	2011	Neuroblastoma	142 patients 16 controls	17q gain is variably detectable in ctDNA using rtPCR.
Yagyu[105]	2011	Neuroblastoma	24 patients	11q loss is detectable in ctDNA with high sensitivity and specificity via rapid analysis of microsatellites using polymorphic markers.
Charlton[96]	2014	Wilms tumor	120 patients	ctDNA can be identified through methylome analysis and may be useful for disease monitoring.
Combaret[91]	2015	Neuroblastoma	114 patients	*ALK* mutations are found in ctDNA using ddPCR with high sensitivity and specificity.
Chicard[78]	2016	Neuroblastoma	70 patients	ctDNA copy-number profiling using NGS is feasible and shows high concordance with the tumor genomic profile.
Ferreira[94]	2016	DSRCT	1 patient	Patient-specific ddPCR probes can be created from tumor-specific breakpoint sequencing.
Hayashi[93]	2016	Ewing sarcoma	3 patients	Patient-specific ddPCR probes can be used to detect tumor-specific breakpoint DNA fragments with high sensitivity.
Krumbholz[76]	2016	Ewing sarcoma	20 patients	ddPCR detects patient-specific *EWSR1* fusion sequences with high sensitivity and correlates with tumor volume and disease status.
Chicard[63]	2017	Neuroblastoma	19 patients	Using WES and deep target sequencing can identify tumor heterogeneity and evolution of treatment resistant clones.
Lodrini[72]	2017	Neuroblastoma	10 patients	ddPCR can be used to accurately discriminate *ALK* and *MYCN* copy-number changes.
Shukla[84]	2017	Ewing sarcoma, DSRCT	Ewing sarcoma (11), DSRCT (6)	*EWSR1* fusions are detectable using ddPCR and NGS approaches, and NGS identifies *TP53* and *STAG2* in patients with Ewing sarcoma.
Barris[106]	2018	Osteosarcoma	10 patients	Targeted NGS can be used to identify somatic aberrations in osteosarcoma tissue and ctDNA at diagnosis and during treatment, with the capacity to

First Author	Year of publication	Disease(s) evaluated	Number of patients	Key finding
				detect new alterations in serial ctDNA samples.
Klega[82]	2018	Osteosarcoma, neuroblastoma, Ewing sarcoma, alveolar rhabdomyosarcoma, and WT	Ewing sarcoma (11), osteosarcoma (10), neuroblastoma (10), WT (8), alveolar rhabdomyosarcoma (7)	Changes in ctDNA levels correlate with treatment response and disease-specific genomic biomarkers are identifiable in ctDNA.
Shulman[83]	2018	Ewing sarcoma, osteosarcoma	Ewing sarcoma (94), osteosarcoma (72)	Hybrid capture (Ewing sarcoma) and ultra-low pass WGS (osteosarcoma) identify ctDNA in ~50% of banked samples from diagnosis and detection/quantification of ctDNA is associated with inferior outcome. NGS identifies genomic features (*TP53* mutation and *STAG2* loss in Ewing sarcoma and 8q gain in osteosarcoma).

DSRCT, Desmoplastic small round cell tumor; WT, Wilms tumor; rtPCR, real-time PCR; dPCR, droplet digital PCR