Liquid biopsies in pediatric oncology: opportunities and obstacles

CURRENT OPINION

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Purpose of review
Liquid biopsies have emerged as a noninvasive alternative to tissue biopsy with potential applications during all stages of pediatric oncology care. The purpose of this review is to provide a survey of pediatric cell-free DNA (cfDNA) studies, illustrate their potential applications in pediatric oncology, and to discuss technological challenges and approaches to overcome these hurdles.

Recent findings
Recent literature has demonstrated liquid biopsies' ability to inform treatment selection at diagnosis, monitor clonal evolution during treatment, sensitively detect minimum residual disease following local control, and provide sensitive posttherapy surveillance. Advantages include reduced procedural anesthesia, molecular profiling unbiased by tissue heterogeneity, and ability to track clonal evolution. Challenges to wider implementation in pediatric oncology, however, include blood volume restrictions and relatively low mutational burden in childhood cancers. Multiomic approaches address challenges presented by low-mutational burden, and novel bioinformatic analyses allow a single assay to yield increasing amounts of information, reducing blood volume requirements.

Summary
Liquid biopsies hold tremendous promise in pediatric oncology, enabling noninvasive serial surveillance with adaptive care. Already integrated into adult care, recent advances in technologies and bioinformatics have improved applicability to the pediatric cancer landscape.

Keywords
biomarkers, cell-free DNA, circulating DNA, liquid biopsy

INTRODUCTION

First identified in 1948, cell-free DNA (cfDNA) has become a promising circulating biomarker in oncology [1,2]. Developments in liquid biopsy, the capture of cfDNA, circulating proteins, and circulating tumor cells (CTCs), have led to adoption of technologies across stages of patient management including screening and diagnosis, molecular prognostication, tracking treatment response, detection of minimal residual disease (MRD), monitoring clonal evolution, and posttherapy surveillance [3–7]. The US Food and Drug Administration approved the first digital drop PCR (ddPCR) liquid biopsy tests in 2016, and two next generation sequencing (NGS) liquid biopsy panels in 2020 as companion diagnostics for associated targeted therapies [8–10]. As of the time of publication, no liquid biopsy tests have been Food and Drug Administration (FDA)-approved in pediatrics.

Liquid biopsies are of particular interest for pediatric oncology as they are noninvasive, avoiding procedural sedation, and enabling serial sampling. Furthermore, liquid biopsies mitigate diagnostic challenges of tumor heterogeneity and accessibility by capturing genetic material shed throughout the body. This promise, however, comes with unique challenges that have limited wider implementation in pediatrics. Pediatric and adult cancers have differing genomic properties; pediatric cancers have low mutational burden [11–14] with few recurrent hotspots [15■■,16–20]. Adult cancers feature more point mutations [single nucleotide

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KEY POINTS

- Liquid biopsies noninvasively capture cfDNA and circulating biomarkers shed from cancerous and healthy cells.

- Liquid biopsies enable accurate molecular diagnostics and prognostication, tracking treatment response, detection of MRD, monitoring clonal evolution, and posttherapy surveillance.

- Multiomic liquid biopsies hold promise for the overcoming the low-mutational burden challenge in pediatric cancers.

variants (SNV)] and insertion/deletion errors (indels), whereas pediatric cancers are characterized by chromosomal structural variations including copy number alterations (CNA), translocations, and fusion genes [12,14,15▪▪,21–23]. This review highlights studies that illustrate the potential applications of cfDNA in pediatric cancers. We discuss technological challenges and emerging approaches to overcome these hurdles.

CELL-FREE DNA OVERVIEW

cfDNA are ∼120–220 bp long fragments [24] of double-stranded DNA found in plasma, cerebral spinal fluid (CSF), saliva, pleural fluid, ascites, stool, aqueous humor, and urine [25–28]. cfDNA molecules are released from healthy and malignant cells through apoptosis, necrosis, and secretion, then cleared from circulation with a half-life of several minutes to 2.5 h [29,30]. cfDNA's rapid clearance and dynamic changes make it an ideal biomarker for 'real-time' analyses compared with classic biomarkers like alpha-fetoprotein (AFP) [31].

cfDNA originating from tumors (circulating tumor DNA; ctDNA) are shorter than normal cfDNA (∼90 to 150 bp) [32,33,34▪]. The portion of overall cfDNA constituted of ctDNA varies with cancer type, tumor location, tumor burden, and metastases. In low-burden and early disease, ctDNA fraction is minute [35,36]. The detection of ctDNA, therefore, requires ultrasensitive methods that detect somatic variations. Thus, a broad understanding of the available technologies and evaluation of these considerations is necessary when selecting an approach for analyzing cfDNA.

TECHNOLOGIES

PCR

PCR specifically amplify targeted cfDNA templates. To improve sensitivity and amplify a minute quantity of ctDNA, ddPCR technology subdivides PCR reactions into numerous nano-liter droplets, and can detect variant allele fractions (VAF) as low as 0.001% [7,37]. Although ddPCR attains high sensitivities, it has limitations. First, it requires a priori knowledge of disease-specific or patient-specific mutations [37], thereby missing any de novo mutations. Similarly, genome-wide surveys of translocations, indels, and CNA are limited, given the focused nature of the assay.

Next generation sequencing

NGS methods do not require prior knowledge of mutations but have worse limits of detection than ddPCR and increased cost. The primary parameters affecting cost are breadth and depth of sequencing. Breadth is the proportion of the genome that is sequenced. NGS breadth varies from a panel of genes of interest (e.g. CAPPseq [38]), to regions of the genome [e.g. whole exome sequencing (WES)], to the entire genome [whole genome sequencing (WGS)]. This gives NGS the capacity to detect both recurrent hotspot mutations and previously unknown or uncommon variants.

Depth refers to the average number of times a base pair is sequenced. Shallow WGS [e.g. 0.1–2x ultra-low pass WGS (ULP-WGS)] accurately detects CNAs [39] at low cost but has poor sensitivity for specific somatic variants. As depth increases, SNVs, indels, and translocations may be identified. However, as assay cost increases, deep-sequencing panels typically restrict breadth, focusing on smaller genomic regions and missing abnormalities outside of those regions.

Methylation, fragmentomics, and transcriptomics

Although previous methods identify genetic variations to characterize ctDNA, recent techniques leverage other ctDNA signatures. Pediatric cancer's low mutational burden may be better suited to alternative markers or combinatorial approaches. Methylation, for example, is a promising pediatric marker as epigenetic dysregulation is a recurrent characteristic of childhood cancers [40]. The circulating methylome can be measured in targeted cfMeDIP-seq [41] or ddMethyLight assays [42] as well as genome-wide approaches [43]. Methylation fingerprints may also define cfDNA tissues of origin [44,45]. Emerging bioinformatic techniques, such as fragmentomics and transcriptomics, leverage non-random differences in cfDNA fragments and sequence coverage for in silico enrichment of ctDNA [33,34▪,46], and infer transcriptome profiles based on chromatin availability and nucleosome footprints [47–50,51▪▪].
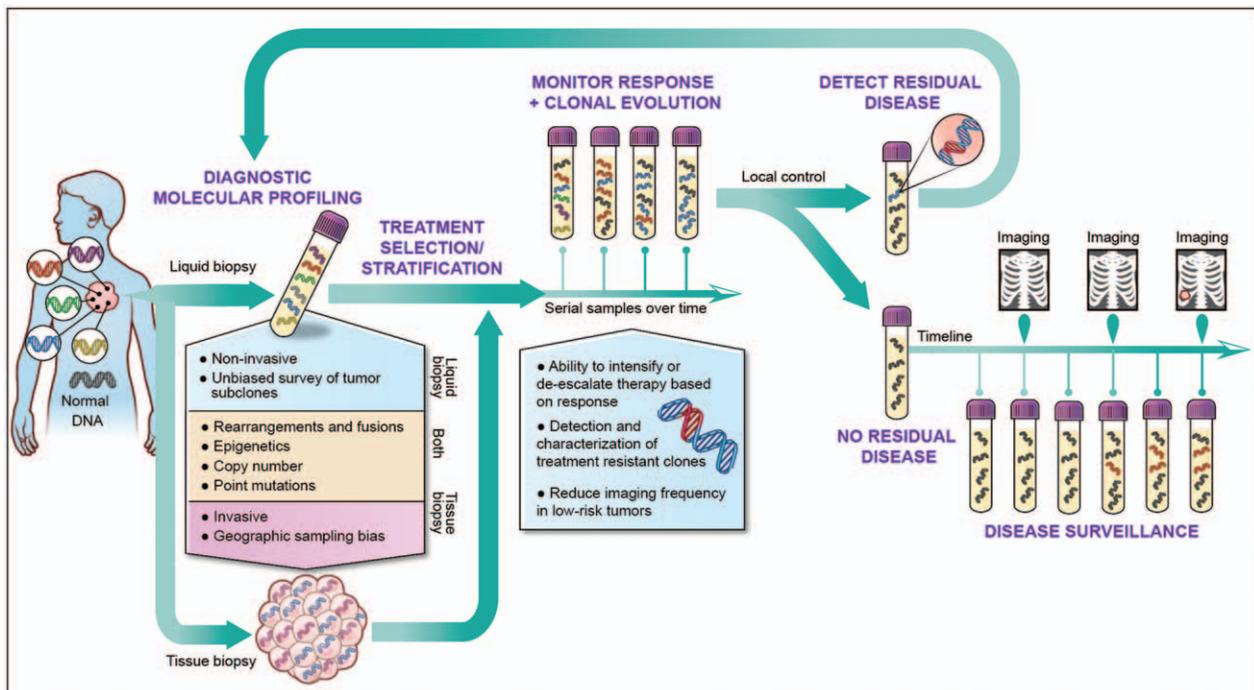
FIGURE 1. Clinical applications of liquid biopsy in pediatric oncology. Liquid biopsies capture genetic material shed throughout the body, enabling noninvasive molecular profiling without the confounding variable of tumor heterogeneity. Liquid biopsies can, therefore, inform care and treatment selection at diagnosis, monitor clonal evolution during treatment, sensitively detect minimum residual disease following local control and provide sensitive posttherapy surveillance. Multiple studies have demonstrated that liquid biopsies have the potential to detect relapsed disease before radiographically evident.

CLINICAL APPLICATIONS OF LIQUID BIOPSIES

The diversity of assays available to profile cfDNA is matched by the range of potential applications for these tools in the clinic (Fig. 1).

Molecular profiling and treatment selection

Sequencing from multiple regions of the same tumor reveals significant intratumor genetic heterogeneity [52,53]. Up to 69% of detected somatic mutations are absent from other regions of the same tumor [52], leading to potential sampling biases during biopsy. Liquid biopsy of cfDNA captures genetic materials released from multiple tissue regions and separate disease foci, mitigating sampling biases. Current adult clinical trials employ cfDNA for risk stratification and treatment selection [7,54].

Liquid biopsy could similarly be used to inform management of pediatric cancers, guiding treatment intensification and de-escalation. Intralesional and interlesional heterogeneity of *MYCN* and *ALK*, for example, is well described in neuroblastoma [55,56]. Accurate assessment of *MYCN*, *ALK*, and segmental chromosome alterations

(SCA), has prognostic implications [57–59] and is incorporated into treatment selection. Intermediate-risk neuroblastoma patients without *MYCN* amplification or specific SCA maintain excellent outcomes with treatment reduction [60]; therefore, genomic misclassification would lead to inappropriate de-escalation of treatment. Multiple studies demonstrate sensitive and tissue concordant molecular characterization of these genes and SCA in neuroblastoma using liquid biopsies [61–65]. The promise of liquid biopsy for risk stratification is further illustrated in pediatric sarcomas. In Ewing sarcoma (EWS) and osteosarcoma, the presence of detectable ctDNA alone predicts inferior outcomes. Furthermore, 8q gain in osteosarcoma cfDNA portended poorer 3-year event-free survival (EFS) (60 vs. 80.9%) [66], and EWS high-risk co-mutations of *STAG* and *TP53* [67] are detectable in cfDNA [68].

Implementation of liquid biopsy for genomic characterization of Wilms tumor could reconcile international treatment paradigms. European consortium paradigms recommend neoadjuvant chemotherapy without tissue diagnosis because of the prevalence of favorable disease and risk of metastatic seeding with biopsy or surgery. In contrast, North American algorithms recommend upfront

nephrectomy for biology-guided therapy. In small studies, *TP53* mutations were detectable in 100% of prenephrectomy urine cfDNA [69] and tumor-confirmed mutations were identified in plasma [70]. cfDNA profiles of poor prognosis *TP53* mutations [71,72] and 1p/16q alterations [70], therefore, could guide therapy without risking seeding tumor.

Treatment response and clonal evolution

Although mortality from primary pediatric cancers has decreased, intensification of treatments has led to increased treatment-related morbidity [73]. Clinical trials, therefore, have examined response-adapted protocols that reduce long-term morbidities in hematologic malignancies [74–76]. In pediatric solid tumors, response-adapted therapy is hindered by lack of serial biopsies; response markers are limited to imaging [77], and necrosis estimates on postneoadjuvant resections [78]. Liquid biopsies offer noninvasive, serial monitoring to assess tumor burden, clonal evolution, and epigenetic changes.

In a small osteosarcoma cohort, patients with more than 80% necrosis in resections had undetectable ctDNA following initiation of neoadjuvant therapy. Patients with less than 70% necrosis; however, had persistence of ctDNA throughout therapy [64]. Although larger validation studies are needed, these data suggest that ctDNA may predict prognostic postneoadjuvant percentage necrosis in osteosarcoma [78]. Similarly, dynamic changes in ctDNA have been shown to correlate with tumor burden in Wilms tumor [79], hepatoblastoma [80], retinoblastoma [81–83], EWS [51▪▪,77,84], and neuroblastoma [61,62]. Additionally, circulating epigenetic signatures approximate tumor burden and response. Data suggest that regional differences in coverage over DNase I hypersensitive sites (DHSs), a surrogate for chromatin status and epigenetic signatures [49,51▪▪], infer tissue type and can estimate tumor burden [51▪▪]. Applebaum *et al.* measured epigenetic signatures using 5-hydroxymethylcytosine (5-hmC) to predict disease burden and response in metastatic neuroblastoma. 5hmC deposition on *MYCN* predicted relapse and end of induction total 5-hmC levels are prognostic. In two patients with no clinical evidence of disease, 5hmC profiling predicted subsequent relapse [85▪].

Finally, serial cfDNA can monitor clonal evolution and identify therapy-resistant subclones. A landmark study in lung cancer reported that cfDNA is more sensitive than tissue biopsy for detection of acquired resistance to erlotinib through $EGFR^{T790M}$ mutations [86]. In pediatrics, cfDNA temporally resolved heterogeneity in progressive neuroblastoma with a mean of 22 new SNV between

diagnostic and subsequent samples, including 17 commonly acquired relapse-specific mutations [61]. Similarly, Barris *et al.* [87] identified *TP53* mutations in relapsed osteosarcoma plasma not identified in initial tumor or germline. In medulloblastoma, dynamic changes in CSF cfDNA methylation mirror tissue changes during treatment and progression [43].

Detection of minimum residual disease and posttherapy surveillance

cfDNA's short half-life [29,30] and representation of spatial heterogeneity [88] make it an ideal biomarker to detect postoperative minimum residual disease (MRD) and early recurrence. In adults, postoperative persistence of ctDNA portends a more than 80% risk of relapse in colorectal cancers [89] and urothelial carcinomas [90]. These findings suggest that ctDNA positivity postneoadjuvent therapy or surgery could inform clinical decisions regarding follow-up frequency and need for adjuvant or radiation therapy. Indeed, the addition of immunotherapy to urothelial carcinoma patients with postoperative ctDNA improved disease-free survival and overall survival [90]. Furthermore, application of cfDNA for MRD may enable earlier relapse detection. In lung cancer, recurrence of detectable ctDNA identified relapse a median of 5.2 months earlier than imaging [91]; in colon and breast cancer, cfDNA outperforms biochemical biomarkers CEA and CA 15-3 for early, sensitive detection of recurrence [92,93].

To date, there have been no large MRD pediatric studies using cfDNA; however, case series demonstrate feasibility. Hayashi *et al.* used tumor-informed EWS-ETS ddPCR to detect fusion genes in plasma from three EWS patients. Two patients had persistent postoperative ctDNA and clinically relapsed. The only ctDNA-negative patient remained in remission [84]. In osteosarcoma, three of seven patients followed with targeted NGS liquid biopsy relapsed, all of whom had recurrence of detectable ctDNA prior to radiographic relapse [87]. Circulating mutant *Rb1* becomes undetectable following enucleation of intraocular retinoblastoma [94,95] but was again detectable in regionally recurrent or metastatic relapsed disease [83]. Finally, previously detectable circulating *CTNNB1* in hepatoblastoma becomes undetectable following total resection with no histological or radiographic evidence of residual disease [80].

CHALLENGES AND FUTURE DIRECTIONS

Rapid technological advances coupled with exciting preliminary studies in pediatric histologies portend

an important future for cfDNA in pediatric oncology; however, several important considerations must be considered in this population.

Blood draw limitations

An assay's limit of detection is constrained by the number of unique sequencing reads generated. Each milliliter of blood has approximately 1000 genome equivalents [96] and sequencing to a depth greater than unique genome equivalents results in duplicated reads with no improvement in limits of detection [97**]. Theoretically, this presents a barrier in pediatrics because of weight-based limits in blood draw volume. Kahana-Edwin *et al.*, however, highlight that the percentage of total blood volume collected during weight-based draws in pediatrics is consistent with adults. Furthermore, even early-stage pediatric cancers often represent a larger tumor burden relative to patient size than adult counterparts. Taken together, pediatric assays should capture a proportional or higher ratio of ctDNA:cfDNA because of relative tumor burden in similarly staged diseases [98]. Indeed, pediatric studies in neuroblastoma [65,99], EWS [66], and hepatoblastoma [80] have demonstrated the feasibility of sensitive detection from less than 1 ml of plasma, and studies of unilateral intraocular retinoblastoma, characterized by exceptionally small tumor volumes, detect ctDNA in plasma [83,100].

Clonal hematopoiesis of indeterminate potential

An emerging challenge in liquid biopsies is false positives because of somatic mutations present in peripheral blood but not in tumor, termed clonal hematopoiesis of indeterminate potential (CHIP) [101]. A common phenomenon associated with aging, CHIP is the presence of a mutant clone in the blood without evidence of dysplasia, neoplasm, or cytopenia [102]. CHIP are attributed to survival advantages in certain mutations acquired during normal hematopoietic stem cell (HSC) divisions [103]. Studies of serial samples banked 10 years apart suggest that CHIP rarely undergo continued expansion [104] and have minimal risk of transforming into hematologic malignancy [105,106].

CHIP are classically defined as having a VAF of at least 2% [102–105,107]; however, this threshold is based on previous limits of detection using WES [105]. Recent studies using error suppression algorithms suggest that CHIP accumulation likely begins in fetal development. 18.2% of sequenced cord blood samples harbor low frequency (VAF 0.002–0.006) somatic mutations [108] and, during normal hematopoiesis, individuals gain one mutation in HSCs per decade [103,109]. It is hypothesized however, that these mutations do not confer survival advantages in youth [110] and, consequently, VAF of CHIP typically remains less than 0.5% until age 50 years [107,111]. The VAF of CHIP in pediatrics, therefore, remains undetectable by many liquid biopsy assays [7,39]. As technologies continue to push limits of detection in an effort to identify early-stage cancer, low-VAF CHIP may become a relevant source of false-positives in pediatric oncology. Multiomic or combinatorial imaging-cfDNA assays may mitigate this.

CHIP frequency increases in adults who received chemotherapy [105,112,113] and radiation therapy [105,113]. These CHIP are enriched for *TP53* mutations [113], the most common somatically mutated gene in pediatric cancer [13], presenting a potential source of false positives in posttherapy surveillance and MRD detection. The only study examining CHIP in pediatric cancer survivors, however, showed no increases using a 14-gene NGS panel [110]. Larger, longitudinal studies including broader panels or WGS with error suppression are necessary for validation.

Low mutational burden

With early-stage disease, increasing the number of targeted mutations improves probability of detection [5]. Pediatric cancers, however, have approximately one-fourteenth of the mutation burden of adult cancers [13], with as few as one SNV per exome [12], limiting targeted panels' utility in early-stage detection.

One strategy to improve probability of detection is to expand assayed targets by integrating 'multiomic' features including CNA [32,34**,51**], fragmentation profiles [32–34**,46,51**], protein [114–116], and epigenetics [51**]. CNA is the most common alteration in pediatric solid tumors [13,15**,22] and may be inferred from off-target reads of targeted panels [117] or detected using ULP-WGS [34**,39, 81]. The utility of cfDNA CNA as a biomarker has been shown in neuroblastoma [61,62,65], retinoblastoma [81,82], and EWS [64], and the sensitivity of these assays can be boosted with fragmentomics [32,33,34**,46]. Our group reported that CNA combined with fragment size analysis not only detects malignancy but can accurately distinguish benign from malignant tumors in neurofibromatosis type 1 (NF1) [34**]. For small tumors, combinatorial assays with cfDNA and protein have doubled sensitivity in stage I/II pancreatic cancers and lesions measuring less than 1.5 cm [115]. Furthermore, pediatric cancers have a high incidence of mutations in
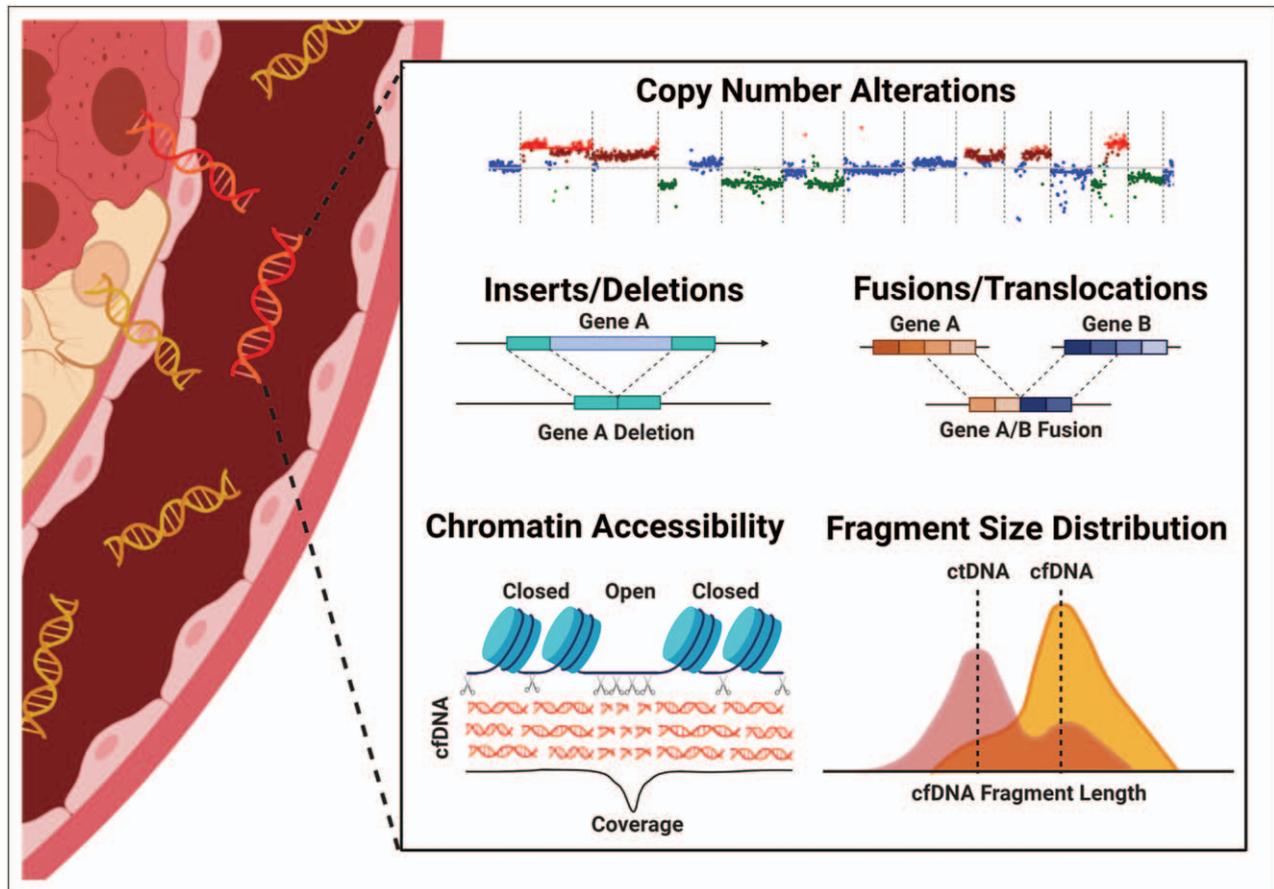
FIGURE 2. Moderate depth whole genome sequencing improve detection by enabling multiomic integration from a single assay. Recent studies have demonstrated that, with advanced bioinformatic analysis, WGS to depths of 12x–35x is sufficient for detection of copy number alterations, indels, translocations and fusions, assessment of chromatin accessibility and fragmentomics [51▪▪,97▪▪]. In addition to detecting the most common genomic alterations in childhood cancer, therefore, integration of these output also infers epigenetic and transcriptomic signatures. Combinatorial approaches, previously requiring multiple assays, enhance detection of early stage cancers.

epigenetic regulators [13,15▪▪,118]. Integrating epigenetic and genetic signatures enabled accurate classification of sarcoma types [51▪▪]. Finally, combinatorial approaches may not be multiomic but multimodality. Using a pan-cancer liquid biopsy for surveillance, Lennon *et al.* [119] increased their positive-predictive value by 45% after integrating imaging features.

CONCLUSION

Liquid biopsies have emerged as a noninvasive alternative to tissue biopsy. Despite the potential to allow serial molecular profiling while reducing invasive procedures and anesthesia events, implementation in pediatrics has been limited and liquid biopsy remains only a research tool. This is partly because of challenges presented by relatively low mutational burden. Multiomic strategies show promise for

sensitive detection of low mutational burden disease but are expensive and require more blood for parallel assays. Exciting recent studies demonstrate that bioinformatic processing of 12x–35x WGS enables characterization of copy number analysis, small indels, fusions, chromatin accessibility, and detailed fragmentomics from a single assay (Fig. 2) [51▪▪,97▪▪]. These novel approaches offer tremendous promise for childhood cancer by detecting the most common pediatric alterations in childhood cancers, incorporating multiomic input, and reducing required blood and cost.

Conflicts of interest

There are no conflicts of interest.

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