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Circulating Cell-Free Tumor Deoxyribonucleic Acid Analysis as a Tool for the Diagnosis and Monitoring of Pediatric Solid Tumors

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ABSTRACT

The utility of cell-free tumor deoxyribonucleic acid analysis is currently being evaluated in a wide range of clinical studies. The validity of cell-free tumor deoxyribonucleic acid analysis methods used for screening and detecting malignant diseases, monitoring the effectiveness of treatment and disease progression, and identifying potential relapse is tested. Molecular technologies used for cell-free tumor deoxyribonucleic acid analysis include targeted polymerase chain reaction assays and next-generation sequencing approaches along with newly introduced epigenetic analysis methods such as methylation-specific polymerase chain reaction. The aim of this review was to compare the methods, pitfalls, and advantages of tests developed for the analysis of cell-free tumor deoxyribonucleic acid in the diagnosis and treatment of pediatric solid tumors. For this purpose, the PubMed database was searched for articles published in the last 10 years, in English, that investigated a human cohort aged 0 to 18 years. A total of 272 references were analyzed. A total of 33 studies were included in the review. Cell-free tumor deoxyribonucleic acid analysis has emerged as a promising novel approach that could potentially bring a significant improvement in the field of pediatric oncology, but the implementation of cell-free tumor deoxyribonucleic acid in clinical practice is largely hindered by the lack of standardized methods for processing and analysis.

Keywords: Children, circulating cell-free tumor DNA, diagnostic, solid tumor

INTRODUCTION

Cancer is the primary cause of disease-related mortality in pediatric patients.¹ Most common childhood malignant tumors originate from hematopoietic and brain tissue followed by sarcomas and specific pediatric entities such as retinoblastoma, neuroblastoma, and nephroblastoma.² The majority of childhood malignant solid tumor tissues consist of small round blue cells; therefore, molecular analysis of biopsied tissue is necessary for definitive diagnosis.^{3,4} Although tissue biopsy still remains a gold standard for tumor diagnosis, liquid biopsy has emerged as a valuable diagnostic tool in oncology.^{5,6} Liquid biopsy signifies the detection and analysis of tumor-specific biomarkers, such as circulating tumor cells, cell-free circulating nucleic acids (cell-free tumor deoxyribonucleic acid [ctDNA], cell-free tumor ribonucleic acid [ctRNA], and micro ribonucleic acid [miRNA]), macromolecular structures such as nucleosomes and exosomes, proteins, and metabolites isolated from various liquid mediums (blood, urine, saliva, and cerebrospinal fluid).⁷⁻⁹ It allows for minimally invasive tissue collection and lessens the need for invasive surgical procedures under general anesthesia in order to obtain tumor tissue samples.^{6,7}

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Cell-free deoxyribonucleic acid (cfDNA) represents approximately 120–220 bp long fragments of double-stranded DNA found in blood or other body fluids.¹⁰ It was first discovered in 1948 by Mendel and Metais.^{9,11} Owing to the recent findings on isolation and molecular analysis technologies, cfDNA has emerged as a complementary assay for cancer tissue genomic profiling.^{4,9,11} The utility of analyzing ctDNA as a fraction of cfDNA released in the bloodstream through apoptosis and necrosis of cancer cells is currently evaluated in a broad range of clinical studies. The validity of ctDNA analysis methods used to screen for and detect malignant disease, monitor treatment efficiency, and disease progression as well as recognize potential relapse is being investigated.^{2,5,7}

Malignant tumors are genetically and phenotypically heterogeneous. Consequently, a sample of tissue obtained by biopsy reflects only the genetic landscape of that portion of the tumor. On the contrary, liquid biopsy constitutes the entire tumor mass including novel subclones and metastases, represents tumor heterogeneity, and eliminates the risk of sampling bias.^{2,7,12} Quick and non-invasive sample collection as well as a half-life of just 16–90 minutes allows for longitudinal analysis with high temporal resolution and real-time assessment of responsiveness to therapy.^{2,5}

The cellular origin and tumor biology together with the genomic landscape differ drastically between adult and pediatric malignant tumors. As a result of limited exposure to mutagenic factors, recurrent somatic point mutations and short indels are rare, chromosomal abnormalities such as copy number variations and formation of fusion genes by translocations being the most frequently observed genetic alterations in pediatric population.^{2,7,9} Molecular technologies used for ctDNA analysis include targeted PCR assays and next-generation sequencing approaches along with newly introduced epigenetic analysis methods such as methylation-specific PCR.^{5,7}

The translation of cancer liquid biopsy into precision medicine practice requires an exhaustive assessment of the validity and clinical utility of mentioned technologies. In this review, we aim to compare the methods, pitfalls, and benefits of the assays developed for the analysis of ctDNA in the diagnosis and treatment of pediatric solid tumors.

MATERIALS AND METHODS

On 3 August 2022, we searched the PubMed database. The search was performed using a combination of the keywords or their equivalents, including: “cell-free tumor DNA,” “liquid biopsy,” “solid tumor,” “neuroblastoma,” “retinoblastoma,” “central nervous system tumor,” “medulloblastoma,” “glioma,” “sarcoma,” “Ewing,” “rhabdomyosarcoma,” “osteosarcoma,” “chondrosarcoma,” “Wilms tumor,” “kidney tumor,” and “pediatric.” The search was limited to articles published in the last 10 years, in the English language, investigating the human cohort aged 0–18 years. Overall, 272 references were analyzed. Researchers reviewed titles and abstracts and then screened full-text reports of selected articles. Finally, a total of 33 studies were included in the review (Table 1).

DISCUSSION

Sarcomas

Ewing Sarcoma. Ewing sarcoma is an aggressive tumor of bones and surrounding soft tissue. It mainly affects children and adolescents—15 years being the median age of diagnosis, but it is also seen in adults. At the time of diagnosis, around 25% of patients have already developed metastases and show a 5-year survival of only 10%–30%.¹³ Even if the disease is detected in the localized stage, 25%–30% of patients' multimodal therapy regimes still show inadequate results.¹⁴ Histologically, Ewing sarcoma is identified by small round blue cells. Phenotypic and genetic characterization by immunohistochemical staining and molecular methods such as fluorescence in situ hybridization is necessary for definitive diagnosis.¹³ Although mutationally silent tumor, reciprocal translocation of EWSR1 gene and a member of the erythroblast transformation-specific family is pathognomonic for Ewing sarcoma; therefore, its identification nowadays presents a standard criterion in diagnostic evaluation. Indeed, this type of rearrangement and consequential fusion gene formation is the driver of the initiation and continuance of this sarcoma phenotype. It is stable in all tumor cells throughout the disease progression and is never lost in new subclones, making it an ideal biomarker for early detection and longitudinal assessment of therapy results.^{13,15}

Krumbholz et al¹³ investigated the utility of this fusion sequence as a plasma marker in pediatric patients suffering from Ewing sarcoma. Due to the fact that the fusion gene of interest results from chromosomal rearrangement, a nucleotide sequence of EWSR1-FLI1 or EWSR1-ERG genes is patient-specific and it is necessary to identify it from biopsy material in pursuance of manufacturing specific probe sets spanning the chromosomal breakpoint in order to detect them and quantify ctDNA using droplet digital polymerase chain reaction (ddPCR). The above-mentioned was done on a sample of 20 patients with Ewing sarcoma previously confirmed by histology and FISH. As blood samples were collected at the time of diagnosis and during the course of multimodal treatment, they observed a consistent correlation between tumor burden and ctDNA copy number. A fast reduction of ctDNA was observed after the onset of chemotherapy and/or tumor resection. Three patients whose ctDNA was still detectable after the last course of chemotherapy later on presented with disease relapse. Also, using the xenograft mouse model, they demonstrated the technical feasibility and prospect of ddPCR while dealing with small sample volumes of just 100 µL which is crucial in pediatric oncology.

Shulman et al¹⁴ and Shah et al¹⁶ used next-generation sequencing-based methods for ctDNA detection in Ewing sarcoma patients. These methods do not require prior knowledge of patient-specific intronic breakpoint of the EWS-FLI1 fusion gene and therefore can be utilized in cases when tissue biopsy is not feasible. They enable the detection of copy number alterations that direct prognosis and therapeutic decision-making. Still, they suffer from low sensitivity and higher cost; hence, precise indications for their use should be met.^{15,16} Using this method, ctDNA was detected in 44.0% of newly diagnosed patients with localized disease and 69.2% of patients with metastases. Detectable ctDNA was associated with inferior

Table 1. Studies Included in the Review

Study	Country	Number of Participants	Liquid Sample	Molecular Method	Molecular Feature of Interest	Tumor Entity
Shah et al, 2021 ¹⁶	USA	17	Plasma	CAPP-Seq targeted gene panel	CNAs	Ewing sarcoma, osteosarcoma, rhabdomyosarcoma, synovial sarcoma
Tombolan et al, 2022 ¹⁸	Italy	17	Plasma	ddPCR	SNVs	Rhabdomyosarcoma
Tombolan et al, 2018 ¹⁹	Italy	1	Plasma	ddPCR	BRAF V600E	Rhabdomyosarcoma
Lyskjær et al, 2022 ¹⁷	England	72	Plasma	ddPCR	Methylation signatures	Osteosarcoma
Shulman et al, 2018 ¹⁴	USA	94+72	Plasma	NGS	CNAs, SNVs	Ewing sarcoma, osteosarcoma
Krumbholz et al, 2016 ¹³	Germany	20	Plasma	ddPCR	EWSR1	Ewing sarcoma
Peneder et al, 2021 ¹⁵	International	148	Plasma	WGS	Fragmentation pattern	Ewing sarcoma, osteosarcoma, rhabdomyosarcoma, synovial sarcoma
Jiménez et al, 2021 ²⁶	France	19	Plasma	High-deep NGS	RB1	Retinoblastoma
Kothari et al, 2020 ²⁰	USA	10	Plasma	NGS	RB1	Retinoblastoma
Poliski et al, 2020 ²¹	USA	50	AH	Shallow WGS	SCNAs	Retinoblastoma
Xu et al, 2020 ²⁴	USA	46	AH	Shallow WGS	SCNAs (6p amplification)	Retinoblastoma
Berry et al, 2020 ²⁵	USA	17	AH, plasma	Shallow WGS	SCNAs	Retinoblastoma
Berry et al, 2018 ²³	USA	26	AH	Shallow WGS	SCNAs, MYCN	Retinoblastoma
Berry et al, 2017 ²²	USA	3	AH	Shallow WGS	CNVs, RB1	Retinoblastoma
Sun et al, 2021 ²⁸	China	58	CSF, plasma	NGS/WES	KMT2D, KMT2C, SMARCA4, BCOR, TP53, PTCH1, EP300, NF1, SETD2, MED12, SPEN	Medulloblastoma
Li et al, 2020 ²⁹	USA	4+8 healthy controls	CSF	WGBS, CMS-IP-Seq	Methylation and hydroxymethylation signatures	Medulloblastoma
Bruzek et al, 2020 ³¹	USA	12+6 healthy controls	CSF	Nanopore sequencing	HISTH3B K27M, H3F3A K27M	pHGG
Li et al, 2021 ³²	USA	10	CSF	ddPCR	H3.3K27M	Diffuse midline glioma
Stallard et al, 2018 ³³	USA	4	CSF	ddPCR	H3F3A K27M	pHGG, Diffuse midline glioma
Mueller et al, 2019 ³⁵	USA	17	Plasma	ddPCR	HISTH3B K27M, H3F3A K27M	Diffuse midline glioma
Cantor et al, 2022 ³⁴	USA	28	CSF, plasma	ddPCR	H3F3A K27M	Diffuse midline glioma
Pagès et al, 2022 ³⁰	USA	258	CSF, plasma, urine	ULP-WGS deep sequencing	SCNAs, SNVs	Over 13 types of CNS tumors
Applebaum et al, 2020 ⁴³	USA	53+34 healthy controls	Plasma	Nano-hmC-Seal technology	5-hmC	Neuroblastoma
Su et al, 2020 ³⁶	China	116	Plasma	qPCR	cfDNA concentration	Neuroblastoma
Su et al, 2019 ³⁷	China	58	Plasma	qPCR	cfDNA concentration	Neuroblastoma
Ishihara et al, 2019 ³⁸	Japan	80	Plasma	qRT-PCR	MYCN	Neuroblastoma
Combarret et al, 2015 ⁴²	France	114	Plasma	ddPCR	ALK	Neuroblastoma
Chicard et al, 2016 ⁴⁰	France	70	Plasma	MIP array	Genomic copy number profiling	Neuroblastoma
Van Roy et al, 2017 ³⁹	Belgium	37	Plasma	Shallow WGS	Genomic copy number profiling	Neuroblastoma
Yagyu et al, 2016 ⁴¹	Japan/USA	151	Plasma	qRT-PCR	MYCN	Neuroblastoma
Miguez et al, 2020 ⁴⁵	Brazil	6	Plasma, urine	Deep amplicon sequencing	CNAs and SNVs	WT
Jiménez et al, 2019 ⁴⁴	France	18	Plasma	WGS	CNAs and SNVs	WT, CCSK, RCC
Ueno-Yokohata et al, 2018 ⁴⁶	Japan	3+1 healthy control	Plasma	PCR	BCOR	CCSK

5-hmC, 5-hydroxymethylcytosine; AH, aqueous humor; CAPP-Seq, cancer personalized profiling by deep sequencing; CCSK, clear cell sarcoma of the kidney; cfDNA, cell-free DNA; CMS-IP-Seq, anti-cytosine-5-methylene sulfonate immunoprecipitation sequencing; CNS, central nervous system; CNV, copy number variation; CSF, cerebrospinal fluid; ddPCR, droplet digital polymerase chain reaction; MIP, molecular inversion probe; NGS, next-generation sequencing; pHGG, pediatric high-grade glioma; qPCR, quantitative polymerase chain reaction; qRT-PCR, real-time quantitative polymerase chain reaction; RCC, renal cell carcinoma; SCNA, somatic copy number alteration; SNV, single nucleotide variant; ULP-WGS, ultra-low-pass whole genome sequencing; USA, United States of America; WGBS, whole genome bisulfite sequencing; WGS, whole genome sequencing; WT, Wilms tumor.

outcomes. Along with standard EWSR-FLI1 and EWSR-ERG, a novel EWSR1-CSMD2 fusion gene was identified. Moreover, mutations in TP53 and STAG2 genes were detected in a limited set of Ewing sarcoma patients.¹⁴

CAnCER Personalized Profiling by deep-Sequencing (CAPP-Seq) ctDNA assay covering 25 intronic regions from 11 genes identified 4/6 canonic EWSR1 translocations with a median asymmetry factor of 12.9%. It detected previously not described EWSR1-PKNOX2 and EWSR1-CBLN4 translocations at asymmetry factors of 16.3% and 0.31%, respectively. Copy number alterations in chromosomes 8 and 12 were identified in addition to 1q gain that may bear a negative prognostic significance in Ewing sarcoma patients.¹⁶

A study by Peneder et al⁵ demonstrated the possibility of distinguishing ctDNA in the cfDNA samples independent of any genetic aberration leveraging fragmentation patterns of cfDNA. They noticed that Ewing sarcoma-derived DNA tends to be more fragmented than DNA originating from other sources. They used this discovery for fragment-size filtering of cfDNA in order to increase the sensitivity of detecting Ewing-specific copy number alterations (CNAs). Observed fragmentation patterns are considered to be an aftereffect of epigenetic changes such as histone H3K27 methylation and not CNAs. These results illustrate the potential distinction between patients suffering from Ewing sarcoma and healthy individuals without the knowledge of tumors' genetic characteristics.

Osteosarcoma. Osteosarcoma is the most common primary bone malignancy in the pediatric population. It is poorly differentiated and in spite of multimodal therapy approaches, the mortality rates still reach as high as 30%-40%. In contrast to Ewing sarcoma and rhabdomyosarcoma, it is not characterized by a canonical fusion gene. Genetically, it shows complex translocations and copy number alterations, 8q gain being the most common one.^{14,17}

Using the next-generation sequencing technology, 56.9% of patients with localized tumor had detectable ctDNA at the time of diagnosis. The site of the tumor influenced the rate of detection—ctDNA was detectable in 71% of patients with femoral primary tumor compared to 46.3% with other primary tumor sites. The median fraction of ctDNA in total cfDNA was 11%, and the median quantity of ctDNA was 4.5 ng/mL. The association between ctDNA levels and event-free and overall survival rates were not proven. Potentially prognostic genetic feature—8q gain that harbors MYC gene was detectable in 74.5% ctDNA-positive patients. Their 3-year event-free survival was lower than in patients without 8q gain—60% compared to 80.9%.¹⁴

The above-mentioned CAPP-Seq ctDNA assay detected TP53 translocations in 3/3 patients with known aberration at a median AF of 1.2%. The ATRX intronic translocations that are normally present in 14% of osteosarcoma tumors were not identified in any of the patients in the study. Copy number alterations were detected throughout the genome including 8q gain—suggestive of possible AT7519 targeted treatment.¹⁶

The analysis of ctDNA methylation patterns arises as a new approach in osteosarcoma diagnosis. It presents a valuable biomarker as methylation of CpG islands occurs as an early

event in tumor tissue formation and remains constant through tumor evolution. Using 2 duplexed ddPCR assays containing methylation markers cg02169391, cg22082800, cg25680486, and cg26100986, 40% of patients were evaluated as ctDNA osteosarcoma positive after setting a minimum of 2 markers as a limit of detection. A higher rate of detection was observed among patients with metastasized versus patients with localized disease—63% compared with 32%. The detection of pretreatment ctDNA was associated with inferior survival outcomes but not with tumor size. False-positive ctDNA results were observed in 2.6% of healthy patients.¹⁷

Rhabdomyosarcoma. Rhabdomyosarcoma is an aggressive malignant tumor of striated muscle origin. It derives from mesenchymal tissue that failed to complete myogenic differentiation but retained expression of desmin and myogenin. Despite improvement in therapy, it is still characterized by a high mortality rate in children and adolescents—up to 70% over 3 years in patients with metastases at the time of diagnosis. The most common histological subtypes of rhabdomyosarcoma are embryonal (60% of cases), alveolar (30%), and botryoid (10%).^{18,19}

Tombolan et al¹⁸ obtained multiple plasma samples from 17 pediatric patients during the course of treatment and quantified both circulating tumor cells and cfDNA at different points of disease states. They found a positive correlation between the number of CTCs and cfDNA levels. After processing tumor tissue obtained by biopsy by whole exome sequencing and identifying somatic alterations (MAP3K4, FES, FGFR4, MCTP1, TEK, STAG2), they also managed to detect them in cfDNA samples using ddPCR technology. They demonstrated the potential use of these tracking biomarkers for the evaluation of disease evolution and prediction of relapse.¹⁸

Similarly, like Ewing sarcoma, rhabdomyosarcoma is also characterized by canonic fusion genes PAX3-FOXO1 and PAX7-FOXO1 which are associated with dismal prognosis. CAPP-Seq approach managed to detect PAX3-FOXO1 gene in 3 patients with a median AF of 10.7%. The analysis of ctDNA of a patient suffering from alveolar rhabdomyosarcoma revealed a rare PAX3-NCOA1 translocation which is also associated with a worse prognosis. This approach managed to predict clinical relapse by recognizing new positivity for PAX3-NCOA1 fusion gene in the plasma of an RMS patient in remission.¹⁶

Retinoblastoma

Retinoblastoma is the most common intraocular malignancy in the pediatric population with an annual incidence of 10-14 cases per million.²⁰ The initiating factor for tumor genesis is the biallelic inactivation of RB1 tumor suppressor gene. In the hereditary form of disease, which presented in 40% of patients, 1 mutant allele originates from germline cells while the other mutation is somatic and occurs in the retinal cell. In the case of a non-hereditary tumor, both alleles are inactivated due to somatic mutations. Secondary genomic events such as chromosomal rearrangements—in particular, recurrent somatic copy number alterations contribute to disease progression.²⁰⁻²² Retinoblastoma belongs to a group of rare cancers in which biopsy is contraindicated due to the risk of tumor seeding. In current clinical practice, the prognosis is based on the

International Intraocular Retinoblastoma Classification that takes into consideration solely clinical parameters. Hereof, liquid biopsy rises as the appropriate method for molecular profiling.^{20,23} A sampling of aqueous humor is a minimally invasive procedure most often performed at the time of intravitreal chemotherapy application. Safety protocol for minimizing the risk of seeding recommends initial paracentesis in order to reduce intraocular pressure and prevent the reflux of malignant cells into the injection site.²²

Leading world research on the use of aqueous humor as a medium for ctDNA extraction in retinoblastoma is performed at the Children's Hospital Los Angeles. Since 2014, they performed a series of studies. The standard procedure includes a collection of 0.1 mL of aqueous humor (AH) and the analysis of extracted ctDNA by shallow whole-genome sequencing. First, they demonstrated that chromosome copy number alterations represented in ctDNA extracted from AH mainly correlated with tumor DNA (concordance ranged from 84.3% to 100%). Some incongruous CNAs were explained by the presumable existence of multiple tumor subclones that were not represented in the biopsied sample of the tumor after enucleation.^{22,23} The most commonly observed alterations included a gain of 1q, 2p, 6p, loss of 13q, 16q, and MYCN amplification. The presence of somatic copy number alterations (SCNAs) was predictive of eye salvage. Retinoblastoma-specific SCNAs were shown to be present in 92% of enucleated versus 38% of salvaged eyes. Gain of 6p alone, as the most common SCNA-containing driver oncogenes DEK and E2F3, was also foretelling the need for enucleation as it was present in 77% of enucleated compared with 25% of salvaged eyes. The presence of 1q gain demonstrated a marginal predictive effect. There is a possible benefit of a longitudinal evaluation of SCNA levels in AH in the future as they are proven to be in correlation with real-time clinical response to therapy.²³ Contrary to popular belief that hereditary disease occurs among younger children in comparison to non-hereditary form, studies showed no statistically significant difference in median age at diagnosis. Instead, age at the time of diagnosis was positively associated with the number of present SCNAs regardless of germline RB1 status. Genomic instability increases with the age of patients as SCNAs are already present in the retinoma stage of the tumor and accumulate during a time before malignant alteration occurs.^{21,24}

Even though the risk while performing AH sampling is minimal, it is still an invasive procedure and carries the danger of infection, trauma, and tumor seeding. Therefore, a blood-based non-invasive source of ctDNA is being explored. The study by Berry et al²⁵ used the whole-genome sequencing method in order to compare the sensitivity of detecting SCNAs in AH and plasma. They demonstrated significant superiority of AH as a medium—while they managed to identify 11/20 tumor-associated chromosomal abnormalities in AH, they did not detect any in plasma (0/20). The quantity of ctDNA in plasma is limited because of the blood-ocular barrier and is directly related to tumor burden. Therefore, AH is still the preferable liquid source of ctDNA in retinoblastoma. Another study investigated the possibilities of using plasma for identifying the RB1 tumor suppressor gene in non-hereditary retinoblastoma patients. The median cfDNA concentration at the time of diagnosis was noticeably higher than in complete remission (119 ng/mL in

comparison to 27 ng/mL). Of known RB1 somatic mutations, 77.8% were detected by a targeted exon capture for a depth of coverage of 30,000X. Still, this technology is limited to the detection of point mutations and short indels.²⁶ In cases where RB1 mutation is not known, it is possible that these tumors harbor a different type of gene abnormality or that they do not have any RB1 alteration but MYCN amplification that was shown as sufficient malignant alteration promotor in a minority of retinoblastoma cases.^{20,26} In addition, novel methods for the identification of unknown RB1 mutations in plasma and determination of inheritance in cases where tumor tissue is not available are being investigated.²⁰

Central Nervous System Tumors

Tumors of the central nervous system are the most common solid tumors in the pediatric population. Due to the specific anatomical location, tissue biopsy often cannot be safely performed and liquid biopsy provides an alternative approach for the molecular characterization of malignant tissue.²⁷ The largest amount of ctDNA is detected in cerebrospinal fluid, especially with tumor adjacent to the cerebrospinal fluid (CSF) reservoir.^{28,29} In fact, even the proximity of the sampling site to the tumor affects the concentration of ctDNA. Significantly lower concentrations are found in plasma as an effect of the blood-brain barrier.^{29,30}

One-fifth of children diagnosed with intracranial malignancy suffer from medulloblastoma.²⁸ Based on molecular features, medulloblastoma can be classified into 4 subgroups (WNT, SHH, Group 3, and Group 4).^{28,29} Sun et al²⁸ detected cfDNA in 15/58 CSF samples of medulloblastoma patients. Its presence was a positive correlation with the recurrence of disease, occurrence of metastases, and tumor progression. Furthermore, tumor-specific mutations such as KMT2D, KMT2C, SMARCA4, BCOR, TP53, PTCH1, EP300, and NF1 were detected in CSF and plasma samples using the next-generation sequencing platform with a 500-gene panel. The alterations were identified in the plasma of patients previously treated with radiotherapy and chemotherapy as they damage the blood-brain barrier. Shared alterations between tumor tissue and CSF were detected in cases with a narrow time interval between obtaining tissue and liquid biopsy samples. Medulloblastoma nuclear DNA undergoes distinctive epigenetic alterations during the course of the disease and ctDNA methylomes and hydroxymethylomes faithfully reflect the tumor epigenetic landscape. Using whole-genome bisulfite sequencing and anti-cytosine-5-methylenesulfonate immunoprecipitation sequencing, Li et al²⁹ demonstrated the capability of utilizing epigenetic signatures found in ctDNA isolated from CSF for the detection of tumor as well as monitoring the disease progression and response to treatment.

Pediatric high-grade glioma in an aggressive primary tumor of central nervous system with less than 10% of pediatric patients surviving 2 years.³¹ A subtype of pediatric high-grade glioma (pHGG) is a diffuse midline glioma formerly known as diffuse intrinsic pontine glioma. Over 50% of pHGGs and over 80% of diffuse midline gliomas (DMGs) harbor a mutation in histone H3 encoding genes H3.3A (H3F3A) or H3C2 (HIST1H3B) which leads to lysine-27-to-methionine substitution (H3K27M). This mutation is associated with more aggressive disease and a dissatisfactory response to treatment.³²⁻³⁴ The most commonly

used method for its detection in ctDNA is ddPCR.³²⁻³⁵ A significant increase in ctDNA 3-5 days after radiotherapy suggests the potential of this method for longitudinal evaluation of therapy effectiveness.³³ In support of that, speaks the achievement of Mueller et al³⁵ who correctly identified 85% H3K27M mutation-positive plasma samples at the time of diagnosis and 100% during the course of oncology treatment. In addition, this technology demonstrates the potential for predicting clinical progression 1-3 months before any radiological changes by an increase of ctDNA VAF of at least 25% from the baseline. Accordingly, it is able to distinguish pseudo-progression and pseudo-response. Identification of histone mutations shows higher sensitivity using CSF compared to plasma samples (85.4% and 96.5%).³⁴ The most important conditions for translation and clinical implementation are high sensitivity, specificity, and reproducibility of prospected methodology demonstrated in a recent study conducted across 3 relevant institutions and 2 ddPCR assays.³² The pHGGs also express alterations in ATRX, TP53, ACVR1, BRAF, EGFR, and PIK3CA genes. These recurrent hotspot mutations were successfully detected by Nanopore electronic sequencing technology in ctDNA from 127 CSF samples with 85% sensitivity and 100% specificity demonstrating the feasibility of using low input samples of just 0.1 fmol of DNA. On top of that, the method imposes as an incredibly time-saving option with final results available in just 12 hours.³¹

Neuroblastoma

Neuroblastoma originates from neural crest precursor cells that underwent a defective sympathetic neuronal differentiation. It is the most common childhood extracranial solid malignancy characterized by a wide range of clinical behavior varying from maturation to benign form and spontaneous regression to an aggressive form with a long-term survival based on risk stratification of 30%-90%.³⁶⁻³⁸ Therefore, risk stratification at the time of diagnosis is essential.

Nowadays, cfDNA analysis is being evaluated as a prospective tool for neuroblastoma (NB) disease monitoring. It is shown that when solely the concentration of cfDNA in plasma is used to assess the response during early chemotherapy in patients suffering from neuroblastoma, a statistically significant difference between patients in partial remission and the ones with the stable disease is noticed after the fourth chemotherapy cycle (8.0 ng/mL in comparison to 18.0 ng/mL).³⁷ Su et al³⁶ monitored cfDNA concentration in patients with high-risk neuroblastoma during maintenance treatment with intention of predicting possible relapse of the disease. The concentration of cfDNA was quantified every 3 months and was noticed to be significantly higher at the time of measurement that indicated the relapse than throughout the maintenance treatment (the median of 29.34 ng/mL in comparison to 10.32 ng/mL). These findings could be leveraged to non-invasively distinguish patients with adequate and dissatisfactory responses to treatment and predict disease recurrence using only venous blood sampling.

The genomic copy number profile of the tumor tissue carries a high prognostic value in neuroblastoma patients.³⁹ Recurrent segmental chromosomal alterations, such as 17q gain, 1p deletion, and 11q deletion, are associated with dismal prognosis whereas numerical chromosomal alterations offer optimistic projections. The rate of recurrent somatic mutations is low, the

most commonly evaluated being MYCN and anaplastic lymphoma kinase (ALK).^{39,40}

The amplification of MYCN oncogene present in 20%-25% of patients is associated with an aggressive form of the disease.⁴⁰ It is assessed by MYCN/N-acetyl glucosamine kinase ratio. The NAGK (2p12.3) gene is used as a reference because of its position close to the centromere of the same chromosome on which MYCN (2p24) is located.³⁸ In comparison to the biopsied primary tumor tissue evaluation by Interphase FISH (I-FISH) and Southern blotting, ctDNA analysis by real-time quantitative polymerase chain reaction (PCR) and the ratio of MYCN and NAGK gene dosage (M/N ratio) detected MYCN gene amplification with a sensitivity of 86%-100% and specificity of 95%-100%. While lebara et al³⁸ correctly classified all the samples, Yagyu et al⁴¹ observed lower sensitivity of just 67% in stage 1 and stage 2 neuroblastoma cases. The MYCN amplification (MNA) status is proven as a valuable negatively associated prognostic factor in infantile NB while the statistically significant difference in survival between MNA-positive and MNA-negative patients over 18 months of age was not observed.⁴¹

Activating mutation of ALK tyrosine kinase receptor became the center of interest after the development of targeted therapy with crizotinib. The mutation of ALK is present in 8%-10% of neuroblastoma patients at the time of diagnosis. To date, more than 50 different ALK mutations have been described. Mutations in hotspots F1174L in exon 23 (positions 3520 T > C and 3522 C > A) and R1275Q in exon 25 (position 3824 G > A) account for 70% of all ALK mutations. Using ddPCR, they have been detected in 21.62% of patients included in a study by Combaret et al⁴² with a sensitivity of 100%, 85%, and 92%, and specificity of 100%, 91%, and 98%, respectively.

Studies performing genomic copy number profiling using both, whole-genome sequencing and molecular inversion probe assay, have demonstrated substantial spatial and temporal heterogeneity of neuroblastoma tumors. An increase in segmental chromosomal alterations over the course of the disease as well as the emergence of new somatic mutations as the result of subclonal evolution was observed.⁴⁰ Interestingly, while most alterations were observed both by tumor tissue and cfDNA analysis (e.g., MYCN, 5p gain containing IRX1/IRX2, CCDC148/PKP4 gain, ATRX deletion, AUTS2 deletion), some were only detectable by tumor tissue (CDKN2A/B, PTPRD, and 12q deletions) or cfDNA analysis (LIN28B, IGF1R, TERT). Alterations observed solely in cfDNA were detected at lower fractions than ctDNA suggesting their subclonal origin.^{39,40}

5-Hydroxymethylcytosine (5-hmC) is formed as an intermediate in the process of cytosine demethylation—epigenetic change responsible for the activation of gene expression. By comparing epigenetic profiles of patients with and without clinical evidence of neuroblastoma, Applebaum et al⁴³ identified 347 genes that showed significantly different 5-hmC aggregates. Utilizing these findings, they managed to predict metastatic burden in a cohort of 21 patients with a sensitivity of 70% and specificity of 89.5%, respectively. Moreover, the change in 5-hmC profiles over the course of the disease reflected the changes in tumor burden; hence, real-time assessment of 5-hmC status in patients suffering from neuroblastoma may be

exploited for treatment response monitoring and prediction of relapse.

Renal Tumors

Renal tumors present 5% of childhood malignancies, Wilms tumor or nephroblastoma being the most common one (accounts for approximately 90% of cases). Other types include clear cell sarcoma of the kidney, rhabdoid tumor, renal medullary carcinoma, and renal cell carcinoma.⁴⁴

Wilms tumor is embryonal cancer caused by disrupted differentiation during renal organogenesis and therefore comprises immature mesenchymal, epithelial, and blastema cells.^{45,46} The Children's Oncology Group protocol applied mostly in North America recommends primary nephrectomy and further treatment options depending on the final pathohistological and molecular diagnosis. On the contrary, the Society of Pediatric Oncology protocol, mainly used in Europe, based on clinical and radiological findings presumes that the kidney tumor is Wilms and therefore suggests the application of neo-adjuvant chemotherapy followed by nephrectomy to reduce the risk of tumor seeding.⁴⁶ Thus, 10% of patients may be misdiagnosed. A possible solution lies in the implementation of liquid biopsies into clinical practice as they enable the identification of tumor-specific copy number alterations and single nucleotide variations avoiding tissue sampling. Wilms tumor harbors a series of recurrent mutations in genes involved in cell differentiation (CTNNB1, TP53, WT1, MYCN, SIX1/2, WTX) and miRNA processing genes (DROSHA, DGCR8, DICER1, XPO5, TARBP2).⁴⁴⁻⁴⁶ The TP53 mutation is specific for an anaplastic form of Wilms tumor while SIX1 and MYCN mutations are associated with an increased risk of relapse. Loss of heterozygosity for 1p and 16q is the only molecular characteristic currently clinically used for risk stratification and is associated with inferior event-free and overall survival as well as an increased risk of relapse.^{44,47} Miguez et al⁴⁵ analyzed cfDNA isolated from plasma and urine samples of 6 female patients using deep amplicon sequencing technology. They managed to identify mutations previously detected in matched biopsied tissue samples. The WTAP and PHF5A mutations were detected both in plasma and urine (sediment and supernatant) samples, while common CTNNB1 and WTX mutations were detectable only in plasma samples. Yet, not all mutations identified in tissue biopsy material were recognized by cfDNA analysis. Although in theory, urine exemplifies an ideal completely non-invasively obtained medium for renal and genitourinary cancer monitoring, the biology of ctDNA circulation and excretion is still not sufficiently investigated. Another study also compared CNAs and single nucleotide variants (SNVs) detected by whole-genome sequencing in both kidney tumor tissue and plasma cfDNA. The CNA profiles were consistent between tumor DNA and cfDNA in 81.3% of cases. The CNAs suggestive of Wilms tumor were identified by cfDNA analysis in 57.14% of patients, the most common being 7p loss, 7q gain, loss of heterozygosity (LOH) on chromosomes 11p and 16q, 1q gain. The CTNNB1 mutation was detected in 42.9% of cfDNA samples, and MYCN, WT1, SIX1/2, DICER1, and DROSHA were also found. A mutation of TP53 was detected in a patient with an anaplastic type of WT. At least 1 SNV indicative of Wilms tumor was detected in 64.3% of cases. Not all CNAs or SNVs identified in cfDNA were present in the primary tumor DNA.

Furthermore, 23.6% of detected SNVs occurred at a minor fraction of ctDNA. This could be explained by the presence of intratumor heterogeneity.⁴⁴

Clear cell sarcoma of the kidney constitutes about 5% of pediatric renal tumors. It is impossible to distinguish it from other renal tumor types just by clinical and radiological findings. A definitive diagnosis cannot be established without pathohistological evaluation. With the advances in molecular diagnostics, internal tandem duplication of BCOR gene has been noticed as an authentic aberration in clear cell sarcoma of the kidney (CCSK). Using PCR, Ueno-Yokohata et al⁴⁸ managed to correctly identify it in ctDNA of 2 CCSK patients. A minority of CCSK tumors express other translocations such as YWHAE-NUTM2B and BCOR-CCNB3. In another study, neither BCOR internal tandem duplication identified in CCSK tumor tissue DNA nor SPL/TFE3 translocation found in tumor fragment samples of renal cell carcinoma were identifiable in cfDNA.⁴⁴

CONCLUSION

Cell-free tumor DNA analysis has emerged as a promising novel approach that could potentially bring a significant improvement in the field of pediatric oncology. Still, there are many obstacles needed to be overcome before its implementation into clinical practice. It is essential to further investigate the physiology of ctDNA release, circulation, and clearance. Moreover, taking into consideration the relatively small prevalence of studied conditions, standardization of pre-analytical procedures of sample collection in addition to harmonization of protocols between large collaborative studies are necessary in order to enable the pooling of patient cohorts and merge of data in meta-analyses.

Data Reproducibility: The data that support the findings of this study are available upon request from the corresponding author.

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REFERENCES

- Berlanga P, Pierron G, Lacroix L, et al. The European MAPPYACTS trial: precision medicine program in pediatric and adolescent patients with recurrent malignancies. *Cancer Discov.* 2022;12(5):1266-1281. [CrossRef]
- Andersson D, Fagman H, Dalin MG, Ståhlberg A. Circulating cell-free tumor DNA analysis in pediatric cancers. *Mol Aspects Med.* 2020;72:100819. [CrossRef]

3. Kurihara S, Ueda Y, Onitake Y, et al. Circulating free DNA as non-invasive diagnostic biomarker for childhood solid tumors. *J Pediatr Surg.* 2015;50(12):2094-2097. [\[CrossRef\]](#)
4. van Paemel R, de Koker A, Vandeputte C, et al. Minimally invasive classification of paediatric solid tumours using reduced representation bisulphite sequencing of cell-free DNA: a proof-of-principle study. *Epigenetics.* 2020;16(2):1-13.
5. Peneder P, Stütz AM, Surdez D, et al. Multimodal analysis of cell-free DNA whole-genome sequencing for pediatric cancers with low mutational burden. *Nat Commun.* 2021;12(1):3230. [\[CrossRef\]](#)
6. Galardi A, Colletti M, di Paolo V, et al. Exosomal MiRNAs in pediatric cancers. *Int J Mol Sci.* 2019;20(18):4600. [\[CrossRef\]](#)
7. Weiser DA, West-Szymanski DC, Frait E, et al. Progress toward liquid biopsies in pediatric solid tumors. *Cancer Metastasis Rev.* 2019;38(4):553-571. [\[CrossRef\]](#)
8. Kahana-Edwin S, Cain LE, Karpelowsky J. Roadmap to liquid biopsy biobanking from pediatric cancers-challenges and opportunities. *Biopreserv Biobank.* 2021;19(2):124-129. [\[CrossRef\]](#)
9. Vellichirammal NN, Chaturvedi NK, Joshi SS, Coulter DW, Guda C. Fusion genes as biomarkers in pediatric cancers: a review of the current state and applicability in diagnostics and personalized therapy. *Cancer Lett.* 2021;499:24-38. [\[CrossRef\]](#)
10. Sundby RT, Pan A, Shern JF. Liquid biopsies in pediatric oncology: opportunities and obstacles. *Curr Opin Pediatr.* 2022;34(1):39-47. [\[CrossRef\]](#)
11. van Paemel R, Vlug R, de Preter K, et al. The pitfalls and promise of liquid biopsies for diagnosing and treating solid tumors in children: a review. *Eur J Pediatr.* 2020;179(2):191-202. [\[CrossRef\]](#)
12. George SL, Izquierdo E, Campbell J, et al. A tailored molecular profiling programme for children with cancer to identify clinically actionable genetic alterations. *Eur J Cancer.* 2019;121:224-235. [\[CrossRef\]](#)
13. Krumbholz M, Hellberg J, Steif B, et al. Genomic EWSR1 fusion sequence as highly sensitive and dynamic plasma tumor marker in Ewing sarcoma. *Clin Cancer Res.* 2016;22(17):4356-4365. [\[CrossRef\]](#)
14. Shulman DS, Klega K, Imamovic-Tuco A, et al. Detection of circulating tumour DNA is associated with inferior outcomes in Ewing sarcoma and osteosarcoma: a report from the Children's Oncology Group. *Br J Cancer.* 2018;119(5):615-621. [\[CrossRef\]](#)
15. Bodlak A, Chang K, Channel J, et al. Circulating plasma tumor DNA is superior to plasma tumor RNA detection in Ewing sarcoma patients: ptDNA and pRNA in Ewing sarcoma. *J Mol Diagn.* 2021;23(7):872-881. [\[CrossRef\]](#)
16. Shah AT, Azad TD, Breese MR, et al. A comprehensive circulating tumor DNA assay for detection of translocation and copy-number changes in pediatric sarcomas. *Mol Cancer Ther.* 2021;20(10):2016-2025. [\[CrossRef\]](#)
17. Lyskjær I, Kara N, de Noon S, et al. Osteosarcoma: novel prognostic biomarkers using circulating and cell-free tumour DNA. *Eur J Cancer.* 2022;168:1-11. [\[CrossRef\]](#)
18. Tombolan L, Rossi E, Binatti A, et al. Clinical significance of circulating tumor cells and cell-free DNA in pediatric rhabdomyosarcoma. *Mol Oncol.* 2022;16(10):2071-2085. [\[CrossRef\]](#)
19. Tombolan L, Zin A, Bisogno G. Cell-free DNA in pediatric rhabdomyosarcoma: potential and challenges. *Methods Mol Biol.* 2019;1909:165-175. [\[CrossRef\]](#)
20. Kothari P, Marass F, Yang JL, et al. Cell-free DNA profiling in retinoblastoma patients with advanced intraocular disease: an MSKCC experience. *Cancer Med.* 2020;9(17):6093-6101. [\[CrossRef\]](#)
21. Polski A, Xu L, Prabakar RK, et al. Variability in retinoblastoma genome stability is driven by age and not heritability. *Genes Chromosomes Cancer.* 2020;59(10):584-590. [\[CrossRef\]](#)
22. Berry JL, Xu L, Murphree AL, et al. Potential of aqueous humor as a surrogate tumor biopsy for retinoblastoma. *JAMA Ophthalmol.* 2017;135(11):1221-1230. [\[CrossRef\]](#)
23. Berry JL, Xu L, Kooi I, et al. Genomic cfDNA analysis of aqueous humor in retinoblastoma predicts eye salvage: the surrogate tumor biopsy for retinoblastoma. *Mol Cancer Res.* 2018;16(11):1701-1712. [\[CrossRef\]](#)
24. Xu L, Polski A, Prabakar RK, et al. Chromosome 6p amplification in aqueous humor cell-free DNA is a prognostic biomarker for retinoblastoma ocular survival. *Mol Cancer Res.* 2020;18(8):1166-1175. [\[CrossRef\]](#)
25. Berry JL, Xu L, Polski A, et al. Aqueous humor is superior to blood as a liquid biopsy for retinoblastoma. *Ophthalmology.* 2020;127(4):552-554. [\[CrossRef\]](#)
26. Jiménez I, Frouin É, Chicard M, et al. Molecular diagnosis of retinoblastoma by circulating tumor DNA analysis. *Eur J Cancer.* 2021;154:277-287. [\[CrossRef\]](#)
27. Tang K, Gardner S, Snuderl M. The role of liquid biopsies in pediatric brain tumors. *J Neuropathol Exp Neurol.* 2020;79(9):934-940. [\[CrossRef\]](#)
28. Sun Y, Li M, Ren S, et al. Exploring genetic alterations in circulating tumor DNA from cerebrospinal fluid of pediatric medulloblastoma. *Sci Rep.* 2021;11(1):5638. [\[CrossRef\]](#)
29. Li J, Zhao S, Lee M, et al. Reliable tumor detection by whole-genome methylation sequencing of cell-free DNA in cerebrospinal fluid of pediatric medulloblastoma. *Sci Adv.* 2020;6(42):eabb5427. [\[CrossRef\]](#)
30. Pagès MDS, Rotem D, Gydush G, et al. Liquid biopsy detection of genomic alterations in pediatric brain tumors from cell-free DNA in peripheral blood, CSF, and urine. *Neuro Oncol.* 2022;24(8):1352-1363. [\[CrossRef\]](#)
31. Bruzek AK, Ravi K, Muruganand A, et al. Electronic DNA analysis of CSF cell-free tumor DNA to quantify multi-gene molecular response in pediatric high-grade glioma. *Clin Cancer Res.* 2020;26(23):6266-6276. [\[CrossRef\]](#)
32. Li D, Bonner ER, Wierzbicki K, et al. Standardization of the liquid biopsy for pediatric diffuse midline glioma using ddPCR. *Sci Rep.* 2021;11(1):5098. [\[CrossRef\]](#)
33. Stallard S, Savelieff MG, Wierzbicki K, et al. CSF H3K36A K27M circulating tumor DNA copy number quantifies tumor growth and in vitro treatment response. *Acta Neuropathol Commun.* 2018;6(1):80. [\[CrossRef\]](#)
34. Cantor E, Wierzbicki K, Tarapore RS, et al. Serial H3K27M cell-free tumor DNA (cf-tDNA) tracking predicts ONC201 treatment response and progression in diffuse midline glioma. *Neuro Oncol.* 2022;24(8):1366-1374. [\[CrossRef\]](#)
35. Mueller S, Jain P, Liang WS, et al. A pilot precision medicine trial for children with diffuse intrinsic pontine glioma-PNOC003: a report from the Pacific Pediatric Neuro-Oncology Consortium. *Int J Cancer.* 2019;145(7):1889-1901. [\[CrossRef\]](#)
36. Su Y, Wang L, Jiang C, et al. Increased plasma concentration of cell-free DNA precedes disease recurrence in children with high-risk neuroblastoma. *BMC Cancer.* 2020;20(1):102. [\[CrossRef\]](#)
37. Su Y, Wang L, Wang X, et al. Dynamic alterations of plasma cell free DNA in response to chemotherapy in children with neuroblastoma. *Cancer Med.* 2019;8(4):1558-1566. [\[CrossRef\]](#)
38. Iehara T, Yagyu S, Gotoh T, et al. A prospective evaluation of liquid biopsy for detecting MYCN amplification in neuroblastoma patients. *Jpn J Clin Oncol.* 2019;49(8):743-748. [\[CrossRef\]](#)
39. Van Roy N, van der Linden M, Menten B, et al. Shallow whole genome sequencing on circulating cell-free DNA allows reliable noninvasive copy-number profiling in neuroblastoma patients. *Clin Cancer Res.* 2017;23(20):6305-6314. [\[CrossRef\]](#)
40. Chicard M, Boyault S, Colmet Daage LC, et al. Genomic copy number profiling using circulating free tumor DNA highlights heterogeneity in neuroblastoma. *Clin Cancer Res.* 2016;22(22):5564-5573. [\[CrossRef\]](#)
41. Yagyu S, Iehara T, Tanaka S, et al. Serum-based quantification of MYCN gene amplification in young patients with neuroblastoma:

- potential utility as a surrogate biomarker for neuroblastoma. *PLoS One*. 2016;11(8):e0161039. [\[CrossRef\]](#)
42. Combaret V, Iacono I, Bellini A, et al. Detection of tumor ALK status in neuroblastoma patients using peripheral blood. *Cancer Med*. 2015;4(4):540-550. [\[CrossRef\]](#)
 43. Applebaum MA, Barr EK, Karpus J, et al. 5-hydroxymethylcytosine profiles in circulating cell-free DNA associate with disease burden in children with neuroblastoma. *Clin Cancer Res*. 2020;26(6):1309-1317. [\[CrossRef\]](#)
 44. Jiménez I, Chicard M, Colmet-Daage L, et al. Circulating tumor DNA analysis enables molecular characterization of pediatric renal tumors at diagnosis. *Int J Cancer*. 2019;144(1):68-79. [\[CrossRef\]](#)
 45. Miguez ACK, Barros BDF, de Souza JES, et al. Assessment of somatic mutations in urine and plasma of Wilms tumor patients. *Cancer Med*. 2020;9(16):5948-5959. [\[CrossRef\]](#)
 46. Charlton J, Pavasovic V, Pritchard-Jones K. Biomarkers to detect Wilms tumors in pediatric patients: where are we now? *Future Oncol*. 2015;11(15):2221-2234. [\[CrossRef\]](#)
 47. Gadd S, Huff V, Skol AD, et al. Genetic changes associated with relapse in favorable histology Wilms tumor: a Children's Oncology Group AREN03B2 study. *Cell Rep Med*. 2022;3(6):100644. [\[CrossRef\]](#)
 48. Ueno-Yokohata H, Okita H, Nakasato K, et al. Preoperative diagnosis of clear cell sarcoma of the kidney by detection of BCOR internal tandem duplication in circulating tumor DNA. *Genes Chromosomes Cancer*. 2018;57(10):525-529. [\[CrossRef\]](#)