Liquid biopsy for pediatric diffuse midline glioma: a review of circulating tumor DNA and cerebrospinal fluid tumor DNA

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Diffuse midline glioma (DMG) is a highly malignant childhood tumor with an exceedingly poor prognosis and limited treatment options. The majority of these tumors harbor somatic mutations in genes encoding histone variants. These recurrent mutations correlate with treatment response and are forming the basis for molecularly guided clinical trials. The ability to detect these mutations, either in circulating tumor DNA (ctDNA) or cerebrospinal fluid tumor DNA (CSF-tDNA), may enable noninvasive molecular profiling and earlier prediction of treatment response. Here, the authors review ctDNA and CSF-tDNA detection methods, detail recent studies that have explored detection of ctDNA and CSF-tDNA in patients with DMG, and discuss the implications of liquid biopsies for patients with DMG.

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DIFFUSE midline gliomas (DMGs) are highly malignant childhood tumors with poor clinical outcomes. The anatomical location of these lesions, the majority of which are found in the brainstem, often precludes attempts at resection (Fig. 1). Thus, treatment often consists of fractionated radiation therapy (RT).[7] Several chemotherapy regimens have been trialed with minimal benefit achieved.[52] Given the paucity of treatment options for these patients, molecularly informed targeted therapies are being actively investigated.

DMGs are characterized by recurrent somatic mutations in the genes encoding histone H3 variants. Mutations in *H3F3A*, encoding H3.3, and in *HIST1H3B*, encoding H3.1, are found in the majority of pediatric DMG, with the substitution of lysine 27 to methionine (K27M) in H3.3 or H3.1 reported in up to 80% of DMG.[47,56] Tumors with H3 mutations are more aggressive and demonstrate worse responses to treatment, allowing molecular profiling to inform treatment planning and patient counseling.[3] Several recent studies of pediatric brain tumors have demonstrated

the feasibility of molecular profiling of biopsy tissue in order to guide treatment decisions.[8,23,24,42,44] Patients with DMG were more likely to have molecular features that changed management decisions than patients with other tumor types.[30] In light of these studies, consensus guidelines were recently released, recommending tumor next-generation sequencing (NGS) of cancer-related genes for pediatric high-grade glioma patients in addition to immunohistochemistry (IHC) of H3K27M for DMG.[30] Increasing the availability of tumor tissue for molecular profiling is a key step forward in enabling targeted therapies for DMG patients. Historically, offering surgical biopsies to these patients was controversial given an uncertain risk-benefit tradeoff. However, with possible targeted therapies and a prospective multicenter study demonstrating the safety of neurosurgical biopsy for DMG,[16] the historical paradigm has begun to shift.

Alternatives to neurosurgical biopsy for DMG patients are beginning to emerge. Specifically, the sampling of cerebrospinal fluid (CSF) and peripheral blood to detect
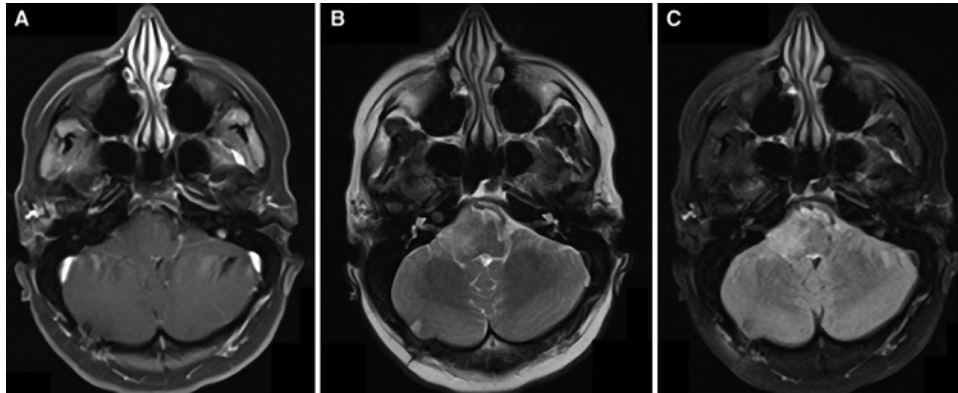
FIG. 1. Axial MR images obtained in a patient with a newly diagnosed brainstem glioma: T1-weighted postcontrast (**A**), T2-weighted (**B**), and FLAIR (**C**).

tumor mutations offers diagnostic and prognostic leverage without the risks associated with a neurosurgical procedure. We review these recent developments in "liquid biopsy" for DMG, providing an overview of circulating tumor DNA (ctDNA) and CSF tumor DNA (CSF-tDNA) and methods of detecting these molecules. We further detail the recent studies that have demonstrated the feasibility of detecting ctDNA and CSF-tDNA in patients with DMG and provide a framework for incorporating these assays into the clinical care of DMG.

Methods

We restricted the scope of this review to the detection of cell-free DNA (cfDNA) molecules in the blood or CSF. Other liquid biopsy approaches (e.g., cell-free RNA, circulating tumor cells, circulating proteins) are well described in a recent review by Bonner and Bornhorst et al.[2] We searched the PubMed/MEDLINE electronic database and the references of relevant articles for English-language articles published up to August 1, 2019. To identify ctDNA publications related to DMG, the following search phrase was employed: "(DMG OR DIPG OR "diffuse glioma" OR "diffuse intrinsic pontine glioma" OR "diffuse midline glioma") AND (cfDNA OR "cell free" OR "cell-free" OR ctDNA OR "circulating DNA" OR "liquid biopsy" OR "plasma DNA" OR "circulating tumor DNA" OR "circulating tumour DNA")." To identify CSF-tDNA publications related to DMG, the above Boolean phrase was modified to "(DMG OR DIPG OR "diffuse glioma" OR "diffuse intrinsic pontine glioma" OR "diffuse midline glioma") AND ("cerebrospinal fluid" OR CSF)." Articles describing results of ctDNA or CSF-tDNA assays in the blood or CSF of DMG patients were retained (Fig. 2).

Results

Overview of ctDNA and CSF-tDNA

Cell-free DNA is composed of nuclear DNA released into the peripheral blood by dying cells and circulates as short fragments between 170 and 340 bp in length.[17,51] Circulating tumor DNA, the fraction of cfDNA that originates from the tumor, holds promise as an emerging cancer bio-

marker for both the genotyping of tumors and the detection of disease during or after therapy.[1,17,19,36] Particularly when assessing serially collected samples, the noninvasive nature of ctDNA acquisition offers considerable advantages over biopsies and allows for robust monitoring of tumor response to therapy.[5,17,54] Furthermore, ctDNA summarizes the entirety of the tumor, reducing bias associated with spatial heterogeneity and metastatic disease.[54] Applications of ctDNA have been explored in a number of cancers, notably lymphoma, lung cancer, and colorectal cancer; however, there have been relatively fewer studies exploring ctDNA in the context of central nervous system (CNS) tumors.

Detection of ctDNA from CNS malignancies remains difficult. The blood-brain barrier remains a formidable obstacle isolating the CNS from the peripheral circulation. Furthermore, as ctDNA quantitation relies on the accuracy of somatic variant identification to interrogate the circulating tumor burden, the relatively low mutational burden in CNS tumors complicates ctDNA assessment. This conundrum is even more pronounced in pediatric brain tumors, which have been shown to carry approximately 10-fold fewer coding mutations.[15] In these patients, tumor-derived DNA fragments spanning a somatic mutation are exceedingly rare, likening the problem of tumor detection to "finding a needle in a haystack."

The use of CSF-tDNA circumvents this dilemma by reducing the size of the proverbial haystack. The CNS, while not as immune-privileged as once believed, is home to far fewer myeloid, epithelial, and endothelial cells than the rest of the body. By reducing the mass of cfDNA originating from myeloid, epithelial, and endothelial derivatives, which constitute more than 95% of peripheral blood cfDNA in healthy individuals, the fraction of CSF-tDNA that is tumor derived can be greatly enriched.[41] Early CSF-tDNA studies demonstrated improved sensitivity compared to peripheral blood ctDNA and recapitulated tumor response to treatment using quantitation of the CSF tumor burden.[10,33,41,55] More recent investigations profiling WHO grade II–IV gliomas using CSF cfDNA obtained through lumbar puncture comprehensively characterized somatic alterations associated with the pretreatment sample and uncovered temporal evolution of the tumor genome during therapy in a subset of the cohort.[31]
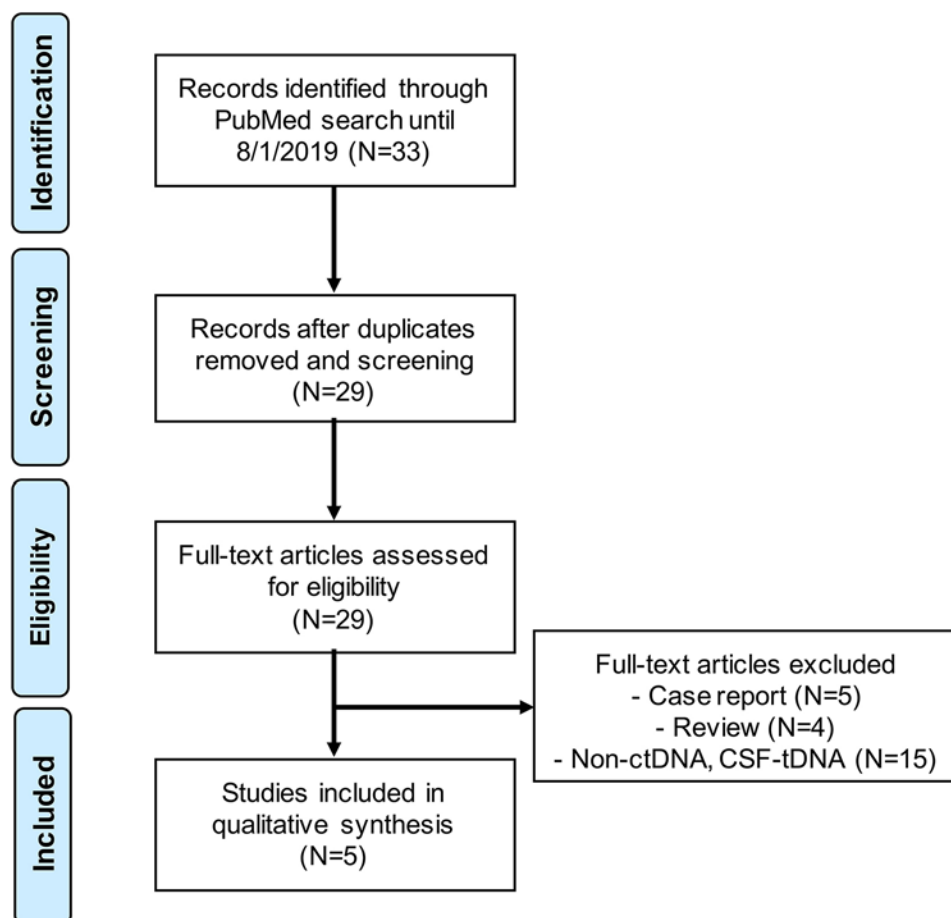
FIG. 2. PRISMA flowchart illustrating literature review strategy, including included and excluded articles. Excluded full-length papers were review articles (n = 4) or case reports (n = 5) or did not describe results of ctDNA or CSF-tDNA assays in DMG (n = 15).

Methods of ctDNA and CSF-tDNA Detection

As with the genotyping of nuclear DNA, ctDNA and CSF-tDNA can be profiled to assess the mutational landscape, structural alterations, and tumor burden. Multiple high-throughput sequencing approaches have evolved to allow for accurate and scalable interrogation of nucleotide content, including digital polymerase chain reaction (PCR),[11–13,53] amplicon sequencing,[14,22] hybrid capture sequencing,[36,37] whole-exome sequencing (WES),[35] and whole-genome sequencing (WGS).[18,26] An overview, including advantages and disadvantages of these methods, is provided in Table 1.

Digital PCR harnesses in vitro PCR to quantitate target sequences within an input mass of DNA fragments by detecting the presence of fluorescence in individual partitions of the original sample after amplification.[43,53] Methods that generate nanoliter-scale water-oil emulsions called "droplets" prior to amplification can be grouped under the category of "droplet digital PCR" (ddPCR), a process that has markedly increased the scalability of digital PCR.[43] In contrast, "NGS" refers to a broad range of highly scalable approaches built off the principles of Sanger sequencing. Fundamentally, these can be broken down into hybridization-based, amplicon-based, and whole-genome approach-

es. Sequencing scope can range from a few hundred base pairs to the entirety of the genome.[27] Hybridization-based approaches utilize hybridization probes aligning to a predefined set of genomic coordinates to isolate DNA fragments spanning regions of interest.[20,38] An alternative approach, amplicon sequencing, interrogates amplicons generated from PCR using paired probes flanking regions of interest.[46] These approaches are further summarized in Table 1.

More specifically, each of these approaches has advanced in numerous ways to create a diverse spectrum of options for ctDNA and CSF-tDNA profiling. The development of BEAMing (beads, emulsion, amplification, magnetics) in the early 21st century further enhanced emulsion-based ddPCR by capturing PCR products on superparamagnetic beads.[13] In contrast to emulsion digital PCR, a downstream flow cytometry step of the fluorescently tagged bead-DNA output allows for the precise determination of mutant DNA from wild-type fragments.[11–13] "NGS" is an umbrella term that spans a multitude of chemical approaches, including methods based on sequencing by synthesis and sequencing by ligation, the specifics of which are beyond the scope of this review.[48] Notably, the scope and scale of NGS is highly flexible

TABLE 1. Overview of ctDNA detection approaches

Approach	Summary	Advantages	Disadvantages	DMG Study
Digital PCR				
Emulsion PCR	Based on end-amplification detection of fluorescence in partitioned samples of the input DNA	Simple quantification of DNA of interest; high sensitivity & specificity per mutation	Requires a predefined list of mutations of interest; not optimized for genotype discovery	Huang et al., 2017; Martinez-Ricarte et al., 2018; Stallard et al., 2018; Panditharatna et al., 2018
BEAMing	Integration of superparamagnetic beads & flow cytometry to improve identification of mutant DNA templates	Additional bead capture & flow sorting improves ability to detect mutant DNA fragments compared to traditional PCR	Additional reagents required compared to traditional digital PCR; does not have the genotyping capabilities of WES/WGS	
NGS				
Hybrid capture	Enrichment of regions of interest through direct capture of DNA using probes hybridizing to predefined genomic regions	Improved coverage uniformity compared to amplicon-based sequencing	Library preparation can be lengthy; current technologies require multiplexing of samples to reduce unit costs	
Targeted	Deep sequencing of a predefined set of regions spanning alterations of interest; custom gene panels are required for isolation of DNA fragments of interest	Increased depth compared to approaches w/ broader sequencing coverage; improved recovery of rare variants	Limited hypothesis space restricts genotype discovery; increased sequencing depth can result in over-sequencing of duplicate input molecules	Pan et al., 2019
WES	Broad sequencing of the exome using a hybridization panel spanning coding regions of the genome	Spans the coding region of the genome, allowing for the genotyping of functional mutations w/o restriction; more affordable than WGS	Does not cover noncoding regions of the genome, limiting genotyping of broad alterations (e.g., whole arm copy number gains); more expensive than targeted sequencing	
Amplicon	Enrichment of regions of interest through PCR-based amplification using paired probes	Improved on-target rates compared to hybridization capture methods; sequencing library preparation may be faster than hybridization capture methods	Most effective for highly targeted sequencing (compared to WGS); limited ability to genotype genome-wide alterations	
WGS	Bulk sequencing of input DNA w/o restriction to prespecified genomic coordinates; conducted using shotgun sequencing principles	Broad hypothesis space allows for improved discovery of genome-wide aberrations	Deep sequencing can be costly compared to either WES or targeted sequencing; increased hypothesis space could increase detection of spurious variants not associated w/ pathology of interest	

BEAMing = beads, emulsion, amplification, magnetics.

for both genotype discovery and recovery. Applications of NGS include deep sequencing of select regions, which can range from hundreds of base pairs to the entirety of the exome to shallow coverage of the full genome. This flexibility allows for a broad range of applications ranging from the recovery of rare mutations in samples with low tumor content to the comprehensive profiling of point mutations, structural alterations, and copy number alterations across the genome.[48]

CSF-tDNA for DMG

Our literature review returned 4 publications that reported the results of CSF-tDNA assays in diffuse gliomas.[21,29,39,49] We describe these studies in the order in which they were published.

Huang et al. analyzed archival CSF from 11 patients with pediatric brain tumors, including 6 patients with DMG (diffuse intrinsic pontine glioma [DIPG], n = 4; thalamic anaplastic astrocytoma, n = 2).[21] CSF was collected (volume range 0.4–2 ml) either by placement of a CSF diversion device (ventricular shunt, external ventricular drain, or indwelling CSF reservoir, 2/11) or by accessing an existing CSF diversion device (9/11 [82%]). cfDNA concentrations isolated from the CSF of the DMG patients ranged from 10 to 280 ng/ml. Two independent techniques to detect H3 mutations in the CSF were employed: Sanger sequencing of *H3F3A* and *HIST1H3B*, and nested PCR with primers specific to *H3F3A* c.83A > T transversion (H3.3K27M). The authors reported sufficient DNA (accounting for quality and quantity) for analysis of 5/6 DMG samples by nested PCR and 3/6 samples by Sanger sequencing. The PCR strategy resulted in detection of the *H3F3A* mutation in 4/5 samples, while Sanger sequencing detected *H3F3A* mutations in 2/3 samples. Concordance of CSF-tDNA mutation status was confirmed by IHC or Sanger sequencing of matched tumor biopsy DNA in 3/3 DMG patients with available tissue.[21]

Martínez-Ricarte et al. investigated detection of CSF-tDNA in a series of 20 diffuse gliomas, including 3 cases with DMG (pediatric, n = 2).[29] Two milliliters of CSF were collected from these patients by lumbar puncture. cfDNA concentrations isolated from the CSF of the DMG patients ranged from 4.8 to 37.5 ng/ml. For non-DMG diffuse gliomas, the authors performed amplicon sequencing of *IDH1*, *IDH2*, *ATRX*, and *TP53*. However, for DMG patients, ddPCR assays for *H3F3A* K27M and *HIST1H3B* K27M were designed and applied to tumor DNA, germline DNA from peripheral blood lymphocytes, and CSF-tDNA. *H3F3A* K27M was detected in the CSF of all 3 DMG patients, with variant allele fractions (VAFs) ranging from 7% to 17.3%.[29] Stallard et al. also used a ddPCR approach to detect mutant *H3F3A* K27M in the CSF of 4 pediatric brain tumor patients (DIPG, n = 2; glioblastoma [GBM], n = 2).[49] In the 2 DIPG patients, VAFs were markedly higher (43% and 65%) than those in the ddPCR-based study by Martínez-Ricarte et al. Notably, while Martínez-Ricarte et al. collected CSF via lumbar puncture, Stallard et al. collected CSF directly from the ventricles (Rickham reservoir, n = 1; endoscopic third ventricle, n = 1). This point is further reinforced by the finding of Stallard et al. via multifocal sampling of CSF from the autopsy of a DIPG patient—the quantity of K27M copies was twice as high in lateral ventricle CSF than in lumbar puncture CSF.[49]

Pan et al. took a different approach, using a 68-gene NGS panel covering specific brain tumor–related genes.[39] They applied this panel to profile tumor DNA, germline DNA, and CSF-tDNA from 57 patients with brainstem tumors. Their cohort included 12 pediatric patients with grade IV tumors (DMG, n = 3; pontine GBM, n = 9). CSF was obtained by ventriculoperitoneal shunt, during resection, or by lumbar puncture. These authors reported detection of *H3F3A* mutations in the CSF-tDNA of 3/3 DMG and 8/9 pontine GBM cases. In the pontine GBM case without *H3F3A* mutations detected, a *HIST1H3B* mutation was detected in the CSF-tDNA. The median *H3F3A* CSF-tDNA VAF was 35.6% (IQR 19.2%–45.4%). The authors also attempted to detect mutations in the peripheral blood of a subset of their entire cohort (n = 8; DMG, n = 1). No ctDNA was detected in the DMG patient, and overall the sensitivity of their assay was markedly lower in peripheral blood than in CSF.[39]

ctDNA for DMG

We identified one recent publication reporting the results of plasma ctDNA assays in DMG.[40] Panditharatna et al. applied an H3K27M ddPCR assay to CSF and plasma samples from 48 DMG patients (79% with H3.3K27M, 15% with H3.1K27M) through an ongoing clinical trial (NCT02274987). The authors detected CSF-tDNA in 3/4 patients at the initial collection time and in 19/21 patients postmortem. Histone mutations were detected in the upfront/diagnostic plasma sample of 16/20 patients with known H3K27M mutations. H3K27M ctDNA was detected in 2 additional patients at a post-RT point. As anticipated, VAF in the CSF was significantly higher than in matched plasma samples. The comparatively lower ctDNA levels in plasma, compared to CSF, underscore a key limitation to the use of peripheral blood instead of CSF to monitor the disease status of patients with CNS tumors.

Beyond demonstrating the feasibility of detecting H3K27M mutations in plasma, Panditharatna et al. provided initial data describing how serial ctDNA sampling can be used to monitor treatment response.[40] They showed that ctDNA levels in DMG appear to decrease following RT and may correlate with decreased post-RT tumor volume on MRI. These authors present an additional compelling vignette of a different DMG patient with progression on panobinostat and mebendazole, suggested by an increasing H3K27M VAF. They then observed a decreasing H3K27M VAF after enrollment in a different clinical trial, potentially suggesting response to therapy.

Clinical Utility of ctDNA and CSF-tDNA for DMG

The utility of detecting tumor-associated cfDNA in the blood or CSF of DMG patients falls into two general categories: minimally invasive molecular profiling and longitudinal monitoring. In light of recent guidelines[30] recommending tumor NGS of cancer-related genes for pediatric high-grade glioma patients, less invasive means
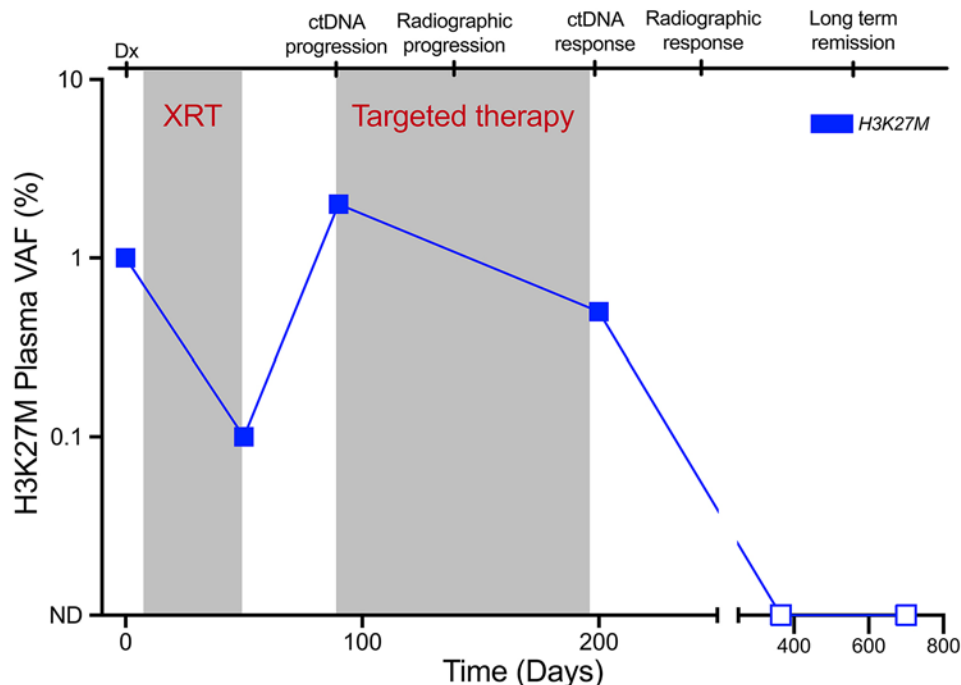
FIG. 3. Hypothetical vignette illustrating the use of ctDNA in a DMG patient to guide therapeutic decision-making. Dx = diagnosis; ND = not detected; XRT = radiation therapy.

of genotyping are warranted. While tumor tissue is likely to remain the gold standard, neurosurgical biopsy is never without risk and may not be available to all DMG patients. For DMG patients, there is clear benefit in ascertaining H3K27M status and, in the future, potentially additional genetic information that could be used to drive therapeutic choices. The ability to reliably determine these data through venipuncture or lumbar puncture would likely expedite the care of these patients. Moreover, it would facilitate enrollment in future clinical trials for DMG patients, as many recent or upcoming trials require knowledge of H3K27M status (NCT02960230, NCT02274987, NCT01182350). The feasibility of obtaining this information was demonstrated in a recent DIPG pilot trial, in which detection of *H3F3A* or *HIST1H3B* K27M mutations in peripheral blood using ddPCR was successful in 92% of H3K27M-mutant cases.[34] A key limitation of using mutation-specific ctDNA assays such as ddPCR is that molecular information about the remainder of the exome or genome, which could potentially influence treatment decisions, is unavailable. Approaches to ctDNA that survey greater swathes of the genome, such as targeted sequencing panels,[37] WES,[28] low-pass WGS,[32] or hybrid approaches,[6,9] offer avenues to overcome this limitation.

In addition to minimally invasive molecular profiling, monitoring the response to therapy is a key application of ctDNA and CSF-tDNA for patients with DMG. Panditharatna et al. began to explore this in their study of CSF-tDNA and ctDNA,[40] but further studies are required. The use of serial ctDNA measurements in diffuse large B-cell lymphoma (DLBCL)[25] has recently been shown to enable superior prediction of patient outcome and could be used to guide treatment decisions longitudinally. While

the treatment lines for DMG are markedly more limited than in DLBCL, a parallel paradigm does exist. Specifically, disease status could be monitored sequentially using CSF sampling or venipuncture. Serial CSF sampling has been a long-standing part of care for some pediatric malignancies, such as medulloblastoma, where cytology is utilized to assess disease status and response to therapy. In addition, CSF-tDNA or ctDNA levels may be able to demonstrate early disease progression that may not be radiographically detectable and would enable modifications of the treatment regimen or enrollment into a clinical trial. We illustrate this paradigm using a hypothetical DMG patient vignette in Fig. 3. The utility of ctDNA for this purpose has been demonstrated in other malignancies such as colon and lung cancer, where ctDNA changes precede clinical and radiographic progression by 5 or more months.[4,50] In lung, breast, and other advanced cancers, ctDNA-guided trials are beginning to emerge (NCT02284633, NCT02743910).[45]

Conclusions

The treatment options for patients with DMG, unfortunately, remain limited. However, the advent of targeted and cell-based therapies provides reason for cautious optimism. The ability to less invasively obtain molecular information via ctDNA or CSF-tDNA to aid in prognostication and treatment decision-making may afford a significant step forward for patients and families. Improved monitoring of therapy response and early allocation to molecularly informed clinical trials represent important potential benefits of regularly using ctDNA or CSF-tDNA in the care of patients with DMG.

Acknowledgments

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Disclosures

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