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). Several chemotherapy regimens have been trialed with minimal benefit achieved. 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 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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KEYWORDS ctDNA; diffuse midline glioma; diffuse intrinsic pontine glioma; liquid biopsy
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 bi-
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).35,26 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.53 In contrast, “NGS” refers to a broad range of highly scalable approaches built off the principles of Sanger sequencing.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   | 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; Martínez-Ricarte et al., 2018; Stållard et al., 2018; Panditharatna et al., 2018 |
| Emulsion PCR  |                                                                                               |                                                                                                       |                                                                                                       |                                                                                                       |
| 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                                                                 |                                                                                                       |
| Amplifying    |                                                                                               |                                                                                                       |                                                                                                       |                                                                                                       |
| NGS           | Hybrid capture                                                                                 | 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 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 ctDNA 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...
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. Panditharathna 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)43 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 prognosis 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.
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Dr. Bettegowda is a consultant for DePuy-Synthes.

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