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Liquid biopsy in gliomas: A RANO review and proposals for clinical applications

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Abstract

Background. There is an extensive literature highlighting the utility of blood-based liquid biopsies in several extracranial tumors for diagnosis and monitoring.

Methods. The RANO (Response Assessment in Neuro-Oncology) group developed a multidisciplinary international Task Force to review the English literature on liquid biopsy in gliomas focusing on the most frequently used techniques, that is circulating tumor DNA, circulating tumor cells, and extracellular vesicles in blood and CSF.

Results. ctDNA has a higher sensitivity and capacity to represent the spatial and temporal heterogeneity in comparison to circulating tumor cells. Exosomes have the advantages to cross an intact blood-brain barrier and carry also RNA, miRNA, and proteins. Several clinical applications of liquid biopsies are suggested: to establish a diagnosis when tissue is not available, monitor the residual disease after surgery, distinguish progression from pseudoprogression, and predict the outcome.

Conclusions. There is a need for standardization of biofluid collection, choice of an analyte, and detection strategies along with rigorous testing in future clinical trials to validate findings and enable entry into clinical practice.

Keywords
ctDNA | circulating tumor cells | CSF | extracellular vesicles | gliomas

Introduction

Liquid biopsies sample tumor-derived material released into biofluids such as blood, CSF, urine, or saliva. The tumor-derived material may be in either free-form (circulating tumor nucleic acids and circulating tumor cells) or within membrane-bound vesicles (microvesicles and exosomes).[1] There is extensive literature highlighting the utility of blood-based liquid biopsies in several extracranial solid tumors, such as melanoma, breast, lung, and colorectal cancer. The utilities include early diagnosis, detection of minimal residual disease after surgery, early response or progression after treatments, identification of resistance mechanisms with subsequent therapy selection, and outcome prediction.[2–5] Liquid biopsies can better recapitulate tumor heterogeneity in small tumor specimens compared to traditional solid tumor tissue biopsies. In addition, the less-invasive nature of liquid biopsies allows for real-time assessment of the molecular changes in tumor cells over time, either occurring naturally or induced by the selective pressure of treatments. There are many liquid biopsy techniques that are rapidly evolving.[6]

In recent years there has been an increasing interest for the application of liquid biopsies in both primary and secondary brain tumors, leading to a number of retrospective studies investigating circulating tumor DNA (ctDNA), circulating tumor cells (CTC), and extracellular vesicles (EV) in blood and CSF. The RANO group undertook the first review of clinical applications of liquid biopsy in the brain and leptomeningeal metastases,[7] and now developed a multidisciplinary international Task Force to review the issue of liquid biopsies in gliomas. The aim of this review is to better define factors influencing feasibility and success in the different phases of the disease and to suggest how to better integrate liquid biopsies into clinical trials.

Gliomas in the Adult

Circulating Tumor DNA in the Blood

(Table 1)

Introduction.—Different ctDNA detection techniques have been employed in heterogeneous series including newly diagnosed and recurrent tumors, different grades of malignancy, and enhancing and non-enhancing lesions. Studies investigating ctDNA detection in peripheral blood of glioma patients have focused largely on three technologies: methylation-based polymerase chain reaction (PCR), droplet digital PCR (ddPCR), and next-generation sequencing (NGS). Below, we discuss the results of these studies, but first seek to discuss the tradeoffs that exist between these modalities.

Methylation-based PCR involves isolation of cell-free DNA, bisulfite conversion, and then PCR with primer pairs designed to amplify the methylated allele of the promoter of interest. While this technique has clear utility for gliomas with known promoter methylation status changes, the ability to quantify levels of ctDNA is limited. That is, the results from a methylation-based PCR may be able to determine the absence/presence of disease but are classically unable to determine the amount of disease at a given time point. Little is also known about the ability of methylation-based PCR to measure disease status over time. In breast cancer, methylation-based PCR has a limited detection limit of 0.1%.[8] Traditional ddPCR has a similar detection limit (0.1%)[9] although methodologic improvements drove this detection limit to 0.01%.[10] This improved sensitivity is an advantage of modern ddPCR ctDNA detection. Key limitations include the ability to only query single or few variants at a time and the inability to detect mutations not known *a priori*. NGS-based approaches abrogate the limitation of ddPCR in interrogating single or limited number of mutations. The ability to detect multiple mutations in ctDNA has been leveraged to make logarithmic improvements in ctDNA detection sensitivity, with NGS-based approaches approaching analytic detection limits of 0.001%.9 Ongoing efforts to lower the background error rate of sequencing and insights regarding DNA sequencing are likely to drive the detection limit of NGS-based approaches lower.[11,12]

Methylation-based PCR.—The methylation status of O6-Methylguanine-DNA-methyltransferase (MGMT), p16, DAPK, and RASSF1A was investigated in a cohort of 28 glioblastoma patients treated with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) or with temozolomide plus cisplatin.[13] When compared to tissue, sensitivity of detecting MGMT methylation in serum was 62.5% and specificity was 92.3%. For the other genes assayed, sensitivity and specificity were 85.7% and 85.7% for p16, 72.7% and 100.0% for DAPK, and 83.3% and 88.9% for RASSF1A. The methylation status of promoter of MGMT, p16INK4a, p73, and RARβ in serum was analyzed in a cohort of 10 patients with glioma.[14] The authors found a methylation of at least one of these promoters in 9/10 patients and at least one of the same methylated promoters of 6/9. This resulted in a sensitivity of 66.7% and specificity of 100.0%. Lavon et al. (2010)[15] analyzed methylation of MGMT and PTEN using MSP and loss of heterozygosity (LOH) on chromosomes 1p, 19q, and 10q via PCR for microsatellite sites in serum in a cohort of 70 glioma patients. Among astrocytic tumors, sensitivity/specificity was 35%/80% for 10q LOH, and 59%/100% for MGMT methylation. Among oligodendroglial tumors, sensitivity/specificity for 10q LOH was 58%/94%, for 1p LOH

Table 1 Liquid Biopsy in Gliomas of the Adult: Studies on ctDNA in Blood

Reference	Histology	Method	Molecular alterations examined	Sensitivity	Type of comparison	Suggested clinical application
Balana et al. 2003	28 GBM	Methylation-based PCR	MGMT promoter	62.5%	with tumor tissue	Diagnosis, treatment response,
Weaver et al. 2006	6 GBM; 2AA 1 AOA; 1 O	Methylation-based PCR	MGMT, p16/INK4a, p73, and RARβ promoters	66.7%	with tumor tissue	Diagnosis
Lavon et al. 2010	29 GBM; 12 AA 15 AO; 14 O	Methylation-based PCR	MGMT, PTEN LOH 1p,19q, 10q	59%	with tumor tissue	Diagnosis
Boisselier et al. 2012	10 GBM; 42 grade III; 28 grade II;	ddPCR for single somatic mutations	IDH1 R132H mutation	60%–70%	with 31 healthy controls	Diagnosis
Salkeni et al. 2013	13 GBM	PCR, long-range, PCR amplification	EGFR VIII	23%	with tumor tissue	Monitoring
Bettegowda et al. 2014	14 GBM: 13 grade II	ddPCR for single somatic mutations	Whole-genome sequencing (point mutations or genetic rearrangement)	7.4%	with tumor tissue	Diagnosis, prognosis
Schwaederle et al. 2016	33 GBM;	Targeted sequencing of multiple somatic mutations	TP53, NOTCH1	27%	with 90 no brain tumor patients	Molecular profiling, targeted treatment selection
Piccioni et al. 2019	222 GBM 35 grade III 25 grade II 5 grade I 83 unknown grade	Targeted sequencing of multiple somatic mutations	ERBB2, MET, EGFR	55% 30% 28% 20%	with tumor tissue	Molecular profiling, targeted treatment selection, prognosis
Muralidharan et al. 2021	114 grade II, III or IV	ddPCR for single somatic mutation	TERT promoter mutation	62.5%	with tumor tissue	Diagnosis, monitoring

GBM: glioblastoma, AA: anaplastic astrocytoma; AOA: anaplastic oligoastrocytoma; O: oligodendroglioma.

31%/100%, for 19q LOH 7%/50%, and for MGMT methylation 47%/100%.

Droplet digital PCR for single somatic mutations.—An alternate approach to methylation-based PCR for detection of ctDNA is the use of droplet digital PCR (ddPCR) for known or recurrent somatic mutations. Boisselier et al. (2012)[16] designed primers to detect the IDH1R132H mutation via ddPCR and applied this approach to plasma extracted from blood samples of 80 patients with glioma and 31 healthy controls. Sensitivity resulted in 60% for all gliomas and 70% for WHO grade 3 or 4 gliomas. ddPCR technologies were applied to plasma samples from a cohort of 640 patients with advanced cancer, including 27 patients with glioma.[17] Targeted, whole-exome, or whole-genome sequencing was applied to tumor tissue and ddPCR assays were done on plasma samples based on tumor genotyping. Compared to the other solid tumors, ctDNA in glioma was more difficult to detect, (2 of 27 cases, 7.4%).

Recently, a novel ddPCR probe-based assay was developed to detect two TERT promoter mutations (C228T and C2250T) in the plasma of 114 patients with gliomas. A sensitivity of 62.5% and a specificity of 91% of detecting ctDNA TERT promoter mutations in plasma compared to a matched tumor tissue were reported.

Leveraging multiple somatic mutations.—The limit of ctDNA detection has been driven lower through refinements of laboratory protocols, deeper sequencing, and computational error-correction methods. One important advance has been the integration of multiple somatic mutations by applying targeted sequencing to cfDNA extracted from plasma. A commercially available targeted-sequencing panel for 54 cancer-related genes was applied to plasma samples from a variety of cancers, including 33 GBMs and 79 healthy controls: at least one somatic mutation was detected in 9/33 (27%) GBM patients, most commonly TP53 or NOTCH1.[18] The same commercially available targeted sequencing panel was applied to plasma samples from 419 patients with primary brain tumors.[19] Somatic alterations in plasma were detected in 20% of grade I astrocytomas and oligodendrogliomas, 28% in grade II tumors, 30% in grade III, and 55% in grade IV.

In a study of glioma evolution, a small targeted sequencing panel was used on plasma samples from 19 patients, who had mutations detected in CSF[20]: shared mutations between CSF and plasma were found in 3/19 (15.8%) patients.

Important limitations of studies are the small numbers and the absence of matched cfDNA from white blood cells. Even with accurate cfDNA assays, ctDNA detection can be confounded by "real" biological signal arising from somatic mosaicism, most notably clonal hematopoiesis.[21] A publication determined that the majority of cfDNA mutations (53.2% in cancer patients, 81.6% in healthy controls) had features suggestive of clonal hematopoiesis.[22] An alternative approach to enhancing sensitivity of ctDNA detection was recently reported.[23] The authors built upon prior observations about variation in cfDNA fragment length,[24,25] and detected significant enrichment of ctDNA in more fragmented, shorter, cfDNA molecules. Leveraging this finding, they performed in silico and in vitro size selection to enrich for the shorter cfDNA molecules, including 34 GBM patients in their "low ctDNA" group. They found that GBM patients had markedly fewer short cfDNA molecules compared to other solid cancers, and fragment length alone was of limited value in increasing the sensitivity of detecting low ctDNA levels. However, when integrating other features of cfDNA and training a supervised machine learning model to classify samples as cancer or not cancer, 65% of low ctDNA samples were classified as cancer, while maintaining 95% specificity. In particular 22 samples from GBM patients were included in the validation set and 13 of them would be classified as having cancer (59.1%).

Global methylation profiling.—Recent technological advances[26] have revealed that global methylation profiling of cfDNA may have significant utility in the development of liquid biopsies for gliomas.[27–30] Nassiri et al. (2020)[31] apply an established method, cell-free methylated DNA immunoprecipitation and high-throughput sequencing (cfMeDIP-seq),[32] to blood samples from glioma patients (N = 59) and healthy controls and other malignances (N = 388). The authors achieved high discriminative performance for glioma vs non-glioma (area under the curve (AUC) 0.99). They further used cfMeDIP-seq to investigate the ability of methylation profiles to discriminate between extra-axial (meningioma and hemangiopericytoma) and intra-axial (low-grade glioneuronal, IDH wild type glioma, and IDH mutant glioma) tumors. Their findings suggest that plasma cfDNA methylation profiles may be able to discriminate each of these tumors against the others.[31]

Sabedot et al. (2021)[33] used bisulfite-converted serum cfDNA and applied commercially-available methylation arrays to generate serum cfDNA methylation profiles. Using a discovery cohort (glioma, N = 38, non-glioma, N = 42) and a supervised epigenome-wide approach to identify CpG sites where the methylation corresponded well between serum and matched tissue, they developed a glioma-epigenetic liquid biopsy (GeLB) score.[33] They present preliminary data that suggests the GeLB score can be used for longitudinal monitoring and may be able to discriminate between true progression and pseudoprogression. Taken together, these findings from Nassiri et al. (2020)[31] and Sabedot et al. (2021)[33] implicate an exciting potential role for leveraging peripheral blood methylation profiles to non-invasively diagnose and monitor gliomas.

Correlations of blood ctDNA with clinical variables.—Few studies on a limited number of patients have investigated the correlations between ctDNA in blood and clinical variables. An association with tumor volume and contrast enhancement on MRI has been observed.[16,34] The level of EGFRvIII DNA in peripheral blood has been correlated with the extent of resection.[35] A longitudinal monitoring of ctDNA in blood could reflect the clinical course with levels decreasing after surgery and adjuvant therapy and increasing at tumor progression.[34,36] Interestingly, ctDNA was of help in some patients with suspected pseudoprogression.[36] An improved response and time to

progression after treatment with alkylating agents was reported earlier in patients with increased serum levels of MGMT promoter methylation.[13] Thus far, ctDNA studies have focused on achieving sufficiently low limits of detection to be able to consistently detect the ctDNA. As the sensitivity of glioma ctDNA assays improves, studies using serially collected blood samples at multiple stages of therapy will be required to determine the utility of ctDNA in a surveillance context. Important parallels can be drawn from minimal residual disease (MRD) studies in other solid tumors. CtDNA has been used to predict recurrence and detect MRD in several solid cancers.[37–42] Common features of high-quality ctDNA MRD studies are real-world patient cohorts managed with standard-of-care, serial blood samples collected at key decision-making time points, well-validated ctDNA detection methods, and cfDNA from healthy controls to investigate specificity.

Factors influencing yield and future approaches.—Several studies have found that in primary CNS cancers CSF is enriched for tumor-derived ctDNA compared to plasma.[20,43,44] This is because the blood-brain barrier, even if is disrupted, limits the transit of glioma ctDNA into the peripheral circulation. The role of lymphatic drainage in the shedding and detection of glioma ctDNA also warrants investigation.[45,46] In order to build a sufficiently sensitive peripheral blood ctDNA assay for glioma, alternative approaches are required. One route may be the integration of other analytes, such as circulating proteins, alongside ctDNA.[47] This approach has shown promise in other cancers with low ctDNA levels, such as pancreatic cancer.[47] Another avenue of active investigation is the use of genome-wide methylation profiles to detect ctDNA and classify tissue of origin.[32,48–50] The detection and the distinction of gliomas from extracranial cancer types, that may metastasize to the brain, and healthy controls, using plasma cell-free DNA methylomes, is promising.[31] Another option that is being investigated is to transiently disrupt the blood-brain barrier using focused ultrasounds. This concept has been explored in preclinical models[51] and is being actively explored in glioma patients.

Circulating Tumor DNA in the CSF

(Table 2)

Discovery of tumor-derived DNA in CSF from patients with diffuse glioma.—The detection of tumor DNA in the CSF from glioma patients has been pursued for over twenty years Rhodes et al. (1994, 1995)[52,53] provided the first evidence in CSF by documenting two of the most common genetic alterations in glioblastoma (GBM), namely increased copies of the epidermal growth factor receptor gene and a missense mutation in the p53 gene. CSF was collected during autopsy and analyzed using PCR-based methods with prior knowledge of genetic alterations in the patient's tumor.

Efforts to characterize the tumor genome in CSF from glioma patients have markedly intensified in recent years. In a study on 35 patients with primary tumors of the brain and spinal cord 18/19 high-grade gliomas had detectable ctDNA levels which were significantly higher than in low-grade gliomas (3/10).[44] Telomerase reverse transcriptase promoter (TERT) mutations were detected in 35/38 (92 %) CSF samples from patients with TERTpmutant GBM as compared to 3/38 (7.9%) in plasma.[54] Both studies collected CSF during surgery and used PCR-based methods to detect genetic variants which had first been detected in the tumor specimen. A sequencing platform (amplicon sequencing and droplet digital PCR) for missense or nonsense mutations in seven commonly altered genes (IDH1, IDH2, TP53, TERT, ATRX, H3F3A, HIST1H3B) was developed to investigate CSF ctDNA from 20 glioma patients: specific somatic mutations were detected in 17/20 (85%) CSF samples.[55]

Lower rates of CSF ctDNA have been reported for next-generation sequencing approaches. A custom FDA authorized hybridization capture-based next-generation sequencing clinical assay[56] was used to evaluate 341 cancer-associated genes: tumor-derived genetic alterations in CSF ctDNA were found in 6/12 (50%) glioma patients.[57] In a follow-up study with a larger number of patients, using the same next-generation sequencing assay tumor-derived genetic alterations were detected in 42/85 (49%) symptomatic patients who underwent lumbar puncture.[20] By using shallow whole-genome sequencing (sWGS) somatic copy number alterations were detected in 5/13 (38%) high-grade gliomas, whose CSF samples were collected through a lumbar puncture.[58] The detection of tumor-derived alterations through next-generation sequencing appears to be more successful in CSF samples collected during neurosurgical procedures, as shown by two studies with mutation detection in 36/37 (97.3%)[59] and 14/17 (82%)[60] CSF samples.

Correlations of CSF ctDNA with clinical variables.—Several studies have explored the correlations between ctDNA in CSF and clinical variables in gliomas. An association between CSF ctDNA levels and tumor location near a CSF reservoir or cortical surface has been suggested.[44,60] In glioma patients, who had received prior surgery, radiation, and at least one systemic chemotherapy before CSF collection, several radiographic findings were associated with CSF ctDNA levels, including tumor size, tumor enhancement, tumor progression, and tumor spread towards the ventricular system or subarachnoid space.[20,61] The relationship between CSF ctDNA levels and tumor size in the CNS has also been reported in a smaller study.[43]

Lower-grade gliomas release a smaller amount of ctDNA into the CSF in comparison to glioblastomas.[17,20,43,44,55] The presence of ctDNA in the CSF has also been associated with shorter progression-free survival.[20,54,62] Of note, CSF ctDNA positivity remained a statistically significant adverse prognostic factor after adjustment for the extent of resection, tumor size, and IDH status.[20]

An important question is to what extent CSF ctDNA is representative of the tumor genome and could perhaps be used for diagnostic purposes. The level of genetic concordance between CSF and tumor samples seems to be high.[20,43,55] In this regard, genetic alterations were congruent between CSF and tumor in 10/10 LGGs[56] and CSF and tumor samples

Table 2 Liquid Biopsy in Gliomas of the Adult: Studies on ctDNA and CSF

Reference	Histology	CSF Collection	Method	Molecular alterations examined	Sensitivity	Type of comparison	Suggested clinical application
Liu et al. 2010	43GBM AA 18 3 AO 3 AOA	LP Blood in parallel	Methylation based PCR	MGMT, p16INK4a,TIMP3,THBS1 (promoter hypermethylation)	62–89%	with 20 healthy controls	Prognosis
De Mattos-Arruda et al. 2015	4 GBM	LP	ddPCR	IDH1,TP53,PTEN, EGFR, FGFR2, ERBB2	/	with tumor tissue	Diagnosis Prognosis Monitoring
Wang et al. 2015	19 high grade 10 low grade	Intraoperative	PCR	TP53, IDH1,TERT, MF2, PINKR1, PTCH1, PTEN	18/19 (95%)	with tumor tissue	Diagnosis
Pentsova et al. 2016	4 GBM 3 AO 3 AA 1 BSG 1 AE	LP	NGS	300 + genes	6/12 (50%)	with tumor tissue	Diagnosis Monitoring
Juratli et al. 2018	38 GBM	Intraoperative	ddPCR	TERTp,	35/38 (92%)	with tumor tissue	Diagnosis Prognosis
Martínez-Ricarte et al. 2018	10 GBM 2 AA 1 A 4 O 2 DMG	LP (85%)	ddPCR	IDH1, IDH2,TP53,TERT, ATRX, H3F3A, HIST1H3B	17/20 (85%)	with tumor tissue	Diagnosis
Mouliere et al. 2018	13 GBM, A, O, AO	LP	NGS	EGFR	5/13 (39%)	with tumor tissue	Diagnosis
Miller et al. 2019	46 GBM 6 grade III 13 grade II	LP	NGS	IDH 1-2,TP53, EGFR, CDKN2A, CDKN2B,TERT	42/85 (49%)	with tumor tissue	Diagnosis Prognosis Monitoring
Zhao et al. 2020	4 GBM 3 AO 4 AA 6 A	Intraoperative	NGS	PTEN,TP53, IDH, EGFR, RB1	14/17 (82%)	with tumor tissue	Diagnosis

GBM: glioblastoma; AA: anaplastic astrocytoma; AO: anaplastic oligodendroglioma; A: astrocytoma; O: oligodendroglioma; BSG: brain stem glioma; AE: anaplastic ependymoma; LP: lumbar puncture.

showed near-identical genetic profiles in CSF and tumor tissue.[20,60] However, some degree of molecular discordance has been observed when CSF collection is done long after surgery,[20,43] and this may reflect glioma genetic evolution. In such cases, molecular discordance does not involve genetic alterations occurring early during gliomagenesis (IDH1, 1p/19q codeletion, TP53, TERT, ATRX) but genes regulating growth factor signaling pathways.

Future approaches.—Limitations in determining accurate sensitivity and specificity of CSF ctDNA evaluation in adult gliomas include a small number of patients in many studies, differences between studies in the method of CSF collection (ie. intraoperative, shunts/reservoirs, lumbar puncture), differences in depth and breadth of sequence coverage (ie. PCR based, targeted exome sequencing, shallow whole-genome sequencing), differences in patient populations (ie. newly diagnosed, recurrent), and the absence of clear benchmarks for assay positivity and negativity.

Nonetheless, many groups have documented the general feasibility of obtaining informative ctDNA profiles from CSF and the current literature supports the following preliminary conclusions: (1) a considerable fraction of adults with diffuse glioma harbors tumor-derived genetic alterations in the CSF; (2) most glioma patients with ctDNA-positive CSF do not have detectable malignant cells in the CSF; (3) the ability to detect genetic alterations appears to be greatest using PCR-based single gene assays; (4) CSF ctDNA from patients with diffuse glioma may contain the full spectrum of genetic alterations found in the disease, including missense mutations, gene copy number alterations, and structural alterations; (5) CSF and tumor samples from the same patient demonstrate good concordance, suggesting that CSF ctDNA can provide an accurate "snapshot" of the tumor genome; (6) in patients with primary brain tumors, detection of tumor-derived genetic alterations in CSF is far more sensitive than detection in plasma.

PCR-based assays are generally more sensitive than NGS-based approaches for the detection of specific SNVs, but are generally unable to reflect the broader genomic changes associated with glioma progression.

Circulating Tumor Cells

(Table 3)

Circulating tumor cells (CTC) may retain specific molecular signatures from the primary tumor, but in peripheral blood CTCs are rare in comparison to normal cells. Thus far, research on CTC in gliomas has been limited and with small sample sizes and variable sensitivities. Moreover, the use of different technologies to isolate and characterize these cells in the blood makes it difficult a comparison of results.

CTC isolated by density gradient centrifugation and characterized with either GFAP staining and/or EGFR amplification and/or gain or loss in chromosomes 7 and 10 were detected in 29/141 (20.6%) patients with GBM.[63] By using CTC-iCHIP technology, CTC were detected in 13/33 (39.3%) of patients with GBM, and the majority showed the molecular signature of mesenchymal phenotype.[64] Interestingly,

Table 3 Liquid Biopsy in Gliomas of the Adults: Studies on Circulating Tumor Cells in Blood

Reference	Histology	Method	Sensitivity	Type of comparison	Suggested clinical application
Muller et al. 2014	141 GBM	Density gradient centrifugation	20.6%	with 23 healthy controls	Diagnosis Prognosis
Sullivan et al. 2014	33 GBM	CTC- i-CHIP technology	39.3%	none	Monitoring
Macarthur et al. 2014	11 GBM	Density gradient centrifugation	72% baseline 8% after radiotherapy	with 30 healthy controls	Monitoring
Gao et al. 2016	31 (grade II, III, and IV)	Matrix isolation	77%	no with 10 healthy controls	Diagnosis Monitoring
Krol et al. 2018	13 GBM	Microfluidic technology	53.8%	with 3 healthy controls	Diagnosis Monitoring

GBM: glioblastoma.

patients with progressive disease had a higher CTC count compared to those with stable disease. CTC isolated by density-gradient centrifugation and characterized with nestin and human telomerase markers, were detected in 8/11 (72%) of patients with GBM before radiotherapy, and in 1/8 (8%) only after radiotherapy.[65] By using a matrix for isolation and staining of GFAP for characterization, CTC were detected in 24/31 (77%) patients with gliomas and correlated with the enhancing tumor component but not with histologic grade of malignancy.[66] In 1 out of 3 patients with GBM with suspected progression on MRI, CTC were absent and pseudoprogression was confirmed in a subsequent MRI. A case of GBM in the elderly, in whom the CTC count, raised shortly after surgery, predicting early tumor recurrence, has been described.[67] By using microfluidic technology for isolation and EGFR positivity for characterization, CTC were detected in 7/13 (53.8%) progressive GBM.[68] By using density gradient for isolation and olig 2 and CD 139 positivity for characterization, CTC from GBM patients were analyzed and through a mouse model, it was suggested a major chemoresistance as compared to parental cells.[69]

Overall most studies have reported low sensitivity in the detection of CTC, and it is important to develop methods with improved sensitivity before clinical usage.

Extracellular Vesicles (Exosomes and Microvesicles)

(Table 4)

Extracellular vesicles (EVs) consist of membrane-bound vesicles, that are released by cells under physiological and pathological conditions. EV content is highly heterogeneous as they can carry a broad repertoire of cargos, including nucleic acids (eg. DNA, mRNA, long and short noncoding RNA including miRNA), proteins (eg. membrane receptors and receptor ligands, growth factors, cytokines), lipids and metabolites, together with some common markers reflecting their biogenesis (CD9, CD63, CD81, eT). There are two types of EVs, which differ mainly in their size: exosomes (30–150 nm diameter) and microvesicles (MVs) (150–1000/nm). However, there are no standard protocols to specifically isolate and separate exosomes from MVs. The double-layer lipid membrane of EVs protects noncoding RNAs from ribonuclease-mediated degradation, and allows them to cross the blood-brain barrier. EVs secreted by tumor cells may be taken up by neighboring and distant cells in the microenvironment, resulting in intercellular communication.

There is clear evidence, in vitro and in vivo, that EVs are released by glioma cells and modulate other neoplastic (including GSC) or normal (astrocytes, microglia, T lymphocytes, etc.) cells.[70] Thus, glioma EVs can enhance tumor proliferation, migration and invasion, induce angiogenesis, reprogram metabolic activity, cause immunosuppression, and influence drug resistance.[71]

Several studies have highlighted the clinical value of EV quantification in GBM. Blood-derived MVs were investigated in 11 patients with GBM and 7 healthy controls, and the quantity of MVs from patients with pseudoprogression or stable disease was significantly lower than in patients with tumor progression.[72] In the study of Evans et al. (2016)[73] an increase in MVs number correlated with early recurrence and poor overall survival. An increased concentration of EVs in blood of patients with GBM, in comparison to healthy controls and patients with other brain tumors (brain metastases, meningiomas, neurinomas, adenomas), was reported.[74] The EVs increment disappeared after surgical resection, while increasing again at recurrence. Moreover, GBM with samples showing a high level of necrosis released fewer EVs in comparison to GBM with a low level of necrosis.

Conversely, correlations between EVs and outcomes were not found. Recent studies have investigated the potential usefulness of fluorescent-labeled EV quantification using imaging flow cytometry.[75,76]

Other clinical applications of EVs for liquid biopsy in gliomas include the investigation of specific molecular alterations, such as EGFRvIII and IDH1 mutation proteins or miRNAs.

mRNA of EGFRvIII was identified in serum EVs of 7/25 (28%) patients with GBM, while no EGFRvIII was detected in healthy controls (0/30).[77] Interestingly, the EGFRvIII was found in blood EVs even in some patients with tissue sample negative for the molecular alterations raising concerns on specificity or validity of such assessments. The expression of EGFRvIII mRNA in serum exosomes and tumor tissue was compared in 96 patients with high-grade glioma: there was a concordance in 44.7% of cases, and the presence of EGFRvIII in exosomes correlated with shorter OS (21 months vs. 28.6 months).[78] In a multicenter study on 71 GBM patients, a high specificity (98%) but lower sensitivity (<61%) for the detection of EGFRvIII in CSF exosomes was reported.[79]

Mutant transcripts of IDH1 have been found in exosomes from CSF of GBM patients (sensitivity of 62.5% and specificity of 100%), but not in exosomes derived from the corresponding blood serum.[80] A usefulness of detecting in plasma exosomes syndecan-1, a surface protein associated with the mesenchymal GBM subtype to distinguish GBM from low-grade gliomas, was reported.[81]

EVs have been correlated with treatment resistance as well. GBM patients with higher levels of tumor-related proteins in serum EVs were more likely to fail standard TMZ.[82] EVs released by a GBM patient-derived GSCs upon treatment with TMZ displayed a specific enrichment in proteins involved in cell adhesion, and ultimately in treatment resistance.[83]

MicroRNAs (mi-RNA or miR) are small non-coding RNA species, that regulate gene expression at the posttranscriptional level, and are involved in glioma initiation and progression.[84] Various studies have identified potential miRNA, in the blood and CSF of patients with gliomas, either upregulated or downregulated, that could be potentially used as biomarkers.

Exosomes secreted by glioma cells are important transporters of oncogenic miRNA.[85]

EV-miR-21 has been suggested to be a candidate diagnostic biomarker in GBM.[86] MicroRNA-21 levels in EVs isolated from CSF of GBM patients were 10-fold higher than those from healthy controls, while no differences were detected for miR-21 levels in EVs from serum. Moreover, CSF miR-21 content decreased after surgery.

Table 4 Liquid Biopsy in Gliomas of the Adults: Studies on Extracellular Vesicles

Reference	Histology	Bio-fluid	Methods	Molecular alterations examined	Sensitivity	Type of comparison	Suggested clinical application
Skog et al. 2008	25 GBM	Blood	RT-PCR	EGFRvIII	28%	with 30 healthy controls	Diagnosis
Shao et al. 2012	24 GBM	Blood	Chip-based EV protein analysis	EGFRvIII, IDH1, PDPN proteins	68% (EGFRvIII, PDPN) 16% IDH1	with tumor tissue	Treatment resistance
Chen et al. 2013	10 GBM 6 AO 2 AA 6 grade II	CSF Blood	RT-PCR and ddPCR	IDH1 mutation	62.5% 0%	with 2 healthy controls	Diagnosis
Manterola et al. 2014	25 GBM 50 GBM	Blood	RT-PCR	miR-320, miR-574-3p, RNU6-1	65% 59% 73%	with 25 healthy controls	Diagnosis
Akers et al. 2015	24 GBM	CSF	RT- PCR	miR-21	85% initial cohort 87% validation cohort	with 20 healthy controls	Diagnosis Monitoring
Shi et al. 2015	45 III/IV 25 I-II	CSF	qRT-PCR	miR-21	–	with 25 non-tumoral neurological patients	Monitoring
Figueroa et al. 2017	23 GBM	CSF	qPCR	EGFRvIII	61%	with tumor tissue	Diagnosis
Ebrahimkhani et al. 2018	12 GBM	Blood	Deep sequencing	miR-182-5p miR-328-3p miR-339-5p miR-340-5p miR-485-3p miR-486-5p miR-543	92% λ	with 9 healthy controls and 10 non-glioma patients	Diagnosis
Manda et al. 2018	96 HGG	Blood	RT-PCR	EGFRvIII mRNA	82%	with 50 healthy controls and 15 other neurological diseases	Diagnosis Prognosis
Ricklefs et al. 2018	21 GBM	Blood	Droplet PCR	PD-L1 DNA	67%	none	Monitoring
Santangelo et al. 2018	85 III/IV 15 I/II	Blood	RT-PCR	miR-21 miR-222 miR-124-3p	84% 80% 78%	with 30 healthy controls and 11 brain metastases	Diagnosis Monitoring
Osti et al. 2019	43 GBM	Blood	Mass spectrometry	Proteins vWF, APCS, C4B, AMBP, APOD, AZGP1, C4BPB, Serpin3, FTL, C3, and APOE	–	with 33 healthy controls and 25 non-glial tumors	Monitoring Diagnosis
Lan et al. 2020	59 III - IV 32 I – II	Blood	qPCR	miR-210	83.2%	with 50 healthy controls	Diagnosis Prognosis

GBM: glioblastoma; AA: anaplastic astrocytoma; AO: anaplastic oligodendroglioma; A: astrocytoma; O: oligodendroglioma.

Exo-miR-21 from CSF of GBM patients was associated with poor prognosis and tumor recurrence.[87] Levels of miR-301-a in serum exosomes from GBM patients were shown to be higher as compared to those from low-grade gliomas, decrease after surgery and increase at tumor recurrence.[88] Serum exosomal miR-210 allowed a differentiation between low-grade and high-grade gliomas.[89] Moreover, the levels decreased following surgical resection, increased at the time of recurrence, and correlated with poor survival. Interestingly, overexpression of miR-210 was suggested to reflect high levels of tumor hypoxia. The role of several mi-RNA in predicting response to radiotherapy has been recently investigated in gliomas.[90] miR-574-3p, already reported as a biomarker in solid extracranial tumors, was significantly decreased after radiotherapy.

Multiple mi-R signature could increase the sensitivity and specificity. In this regard, miR-21 from serum exosomes was able to differentiate glioma patients from healthy controls, but failed to distinguish high-grade gliomas from brain metastases: conversely, this was made feasible when combining the detection of miR-21 with that of miR-222 and mi-R124-3p.[91] Several other studies have reported high sensitivity (up to 91%) of panels of multiple micro-RNA for differentiation between GBM and healthy controls.[92,93] Interestingly, Akers et al. (2017)[94] noted that the sensitivity of the signature for glioblastoma detection was higher for cisternal CSF than lumbar CSF (67% vs 28%). Comparable results were obtained from the analysis of CSF extracellular vesicles and crude CSF. Next-generation short non-coding RNA sequencing on GBM EVs has recently reported the expression of many additional non-coding RNA classes.[95]

Genome-wide methylation profiling of glioblastoma-derived EVs has been reported to correctly identify the methylation class of the parental cells and original tumors, including the MGMT promoter methylation status.[96]

This experimental finding needs validation in a clinical liquid biopsy setting.

Overall, all studies on EVs in gliomas suffer from a limited sample size and still the correlations with clinical parameters need validation.

Diffuse Intrinsic Pontine Gliomas in Children

(Table 5)

Patients with diffuse intrinsic pontine glioma (DIPG) and its molecularly defined counterpart, diffuse midline glioma, H3K27M mutant, are amongst those that may benefit most from the development and application of liquid biopsies for disease management. Because of the location of these tumors, biopsies are only selectively performed with tissue samples that are generally small: thus, obtaining tumor tissue before and after treatment to interrogate for response is not yet an accepted practice. The ability to non-invasively diagnose, identify mutations, and assess changes in response to therapy would be an important clinical advance to assist in the management of this patient population.

Historically, the diagnosis of children with DIPG has been determined radiographically in the setting of a typical clinical presentation and characteristic findings on MRI.[97] This practice has recently begun to change as biopsy of the brainstem has been shown to be relatively safe and feasible when performed by experienced neurosurgeons in the setting of a clinical trial.[98–100] The majority of DIPG harbor mutations in the histone H3 gene (H3.3 or H3.1), that are found in every tumor cell and across the disease course.[101] Thus, the H3K27M mutation is a genetic biomarker in patients with suspected DIPG, who have supporting clinical and radiographic findings[102]: these information could be useful for diagnosis as well as stratifying or selecting patients for clinical trials, particularly those involving histone deacetylase inhibitors.

The analysis and measurement of ctDNA, CTCs, and EVs may represent a potential non-invasive means of assessment of DIPG, also for the risk of leptomeningeal spread.[103,104]

Liquid biopsy utilizing CSF has been evaluated in several studies of patients with CNS tumors including brainstem tumors.[105] For children with DIPG, liquid biopsy has been investigated as a means (1) to confirm diagnosis, (2) to identify the presence of the histone H3K27M mutation, and (3) to assess response to therapy.

While the body fluids evaluated and methods of assessment are not standardized and issues with sensitivity and specificity remain, these studies have nonetheless demonstrated the feasibility and therefore potential utility as these interrogations mature. Most liquid biopsy studies in children with DIPG have focused on the identification of the H3K27M mutation. The feasibility of detecting the H3K27M mutation has been demonstrated in both the blood and CSF of children with DIPG.[44,59,106,107] The initial study by Wang et al. 2015,[44] evaluating tumor DNA in CSF from patients with various primary CNS malignancies, demonstrated that all tumors abutting CSF space, including a pontine-based malignant glioma, had detectable cell-free tumor DNA in CSF using a tiered tumor mutational profiling technique. Of note, CSF from the single patient with a pontine lesion was obtained from the basal cistern. Additional studies, specific for DIPG, have been performed. The most commonly evaluated liquid biomarker in DIPG is ctDNA. Using Sanger sequencing and nested PCR with mutation-specific primer, H3K27M mutations in the CSF were detected in 83% of children with DIPG.[108] Although promising, the number of patients (N = 5) evaluated was small; one additional patient had insufficient ctDNA detected in the CSF collection to perform analysis. This study reported that the site of CSF collection mattered, with CSF adjacent to tumor yielding higher sensitivity, and that detection depended upon sufficient quality and quantity of ctDNA to prevent false-negative results. A strategy based upon nested PCR was utilized for selective amplification of H3K27M mutant alleles. As Sanger sequencing does not allow for quantitation of ctDNA, further technical refinements and improvement in sensitivity are necessary before utilizing these approaches for most clinical applications. Digital droplet PCR is being utilized more recently given its increased sensitivity and ability to detect single nucleotide variants as well as differentially methylated cfDNA.[109] Stallard et al. (2018)[106] found that ddPCR was able to detect

the H3K27M mutation in patient CSF, and there was a close relationship between H3K27M copies and contrast-enhancing cross-sectional tumor area on MRI. Moreover, the number of H3K27M copies was twofold higher in CSF from the lateral ventricle compared to that from the lumbar puncture. Overall, the sensitivity of detection of ctDNA in CSF in patients with biopsy-proven H3K27M is 93% (43 of 46 samples) compared to a sensitivity of 77% (30 of 39) in blood plasma samples.[102]

The utility of liquid biopsy testing has moved beyond feasibility and detection of the H3K27M mutation to the identification of driver mutations as well as quantitation and assessment of response. One of the first studies to incorporate circulating tumor DNA assessment in DIPG was PNOC003, a pilot precision medicine trial.[107] In this study, plasma ctDNA was collected at baseline and at MR imaging timepoints, that is post-radiation therapy, during treatment, at the time of progression and at end of therapy. ctDNA was processed using a droplet digital PCR method and pre-amplified using forward and reverse primers for the histone mutations, H3F3A and HIST1H3B. Both H3F3A and HIST1H3B wild types and mutant alleles were assessed allowing assessment of mutation allele frequency (MAF) (with MAF >0.001% considered positive). In this study, 11 of 13 patients with biopsy-proven H3K27M mutation had plasma ctDNA detected at diagnosis; moreover, ctDNA at subsequent time-points included 6 of 6 patients at the post-radiation time-point and 5 of 7 patients at the time of progression.[107]

Assessment of response to treatment requires a high degree of tumor specificity and sufficient sensitivity to detect relatively small changes. Currently, response assessment in children with DIPG is performed via MR imaging and clinical examination. However, the sensitivity and specificity of these are low because treatment-related effects can mimic tumor progression and glucocorticoids may temporarily improve symptoms. Patients in the PNOC003 study were assessed for response by evaluating changes in ctDNA and correlating results to tumor size as measured on FLAIR MRI sequences. A 50% reduction of H3K27M MAF in plasma ctDNA was correlated with a ≥10% decrease in tumor volume on MRI at the post-radiation time-point as compared to baseline.[110] A decrease in tumor size correlated with decreased H3K27M plasma ctDNA in 83% (10 of 12) patients. Among patients assessed at the time of disease progression, 60% (3 of 5) had an increase in plasma ctDNA, demonstrating the need for further refinement and increased sensitivity of the technique. Although the numbers in studies to date are small, liquid biopsy utilizing plasma or CSF ctDNA may have a supporting role in assessing response to therapy.

Several studies have assessed the importance of the site of CSF collection. In general, CSF collected adjacent to tumor was associated with a significantly higher MAF. Also, in those patients with matched CSF and plasma, ctDNA was higher in CSF compared to plasma.[59,110] From a clinical perspective, CSF is not routinely collected during the disease course in children with DIPG given the potential for herniation as increased intracranial pressure is frequently encountered at diagnosis. However, with the increased role of tumor biopsy, CSF collection adjacent to the tumor at the time of biopsy may be more feasible.

Table 5 Liquid Biopsy in Pediatric Gliomas: Studies with Different Methods

Reference	Tumor type	Liquid biopsy source	Technique	What was measured	Molecular alterations examined	Suggested clinical application
Huang et al. 2017	6 DMG	CSF	Sanger sequencing or nested PCR	ct-DNA	H3F3A and HIST1H3B K27M mutation	Diagnosis Stratification Monitoring
Panditharatna et al. 2018	48 DMG	CSF Blood	ddPCR	ct-DNA	H3K27M	Monitoring treatment
Stallard et al. 2018	4 DIPG	CSF	ddPCR	ct-DNA	H3F3A H3K27M	Monitoring
Garcia-Romero et al. 2019	7 DIPG 4 thalamic glioma 8 gliomas I/ II, III/ IV	CSF Blood	ddPCR	cf-DNA, EV-derived DNA	BRAF V600E	Targeted treatment selection
Mueller et al. 2019	13 DIPG	Blood	ddPCR	ct-DNA	H3F3A, HIST1H3B	Monitoring treatment
Pan et al. 2019	23 DIPG	CSF (matched blood and tumor DNA)	Targeted sequencing	ct-DNA	HF3A, TP53ATRX, PDGRA, FAT1, HIST1H3B, PPMID, IDH1, NF1, PIK3CA, ACVR1	Diagnosis

DIPG: diffuse intrinsic pediatric gliomas; DMG: diffuse midline glioma.

Additional sites and sources of assessment may have clinical utility. Saratsis et al. (2012)[111] performed a proteomic analysis on extracellular vesicles in blood and CSF of children with DIPG. Tumor-associated proteins, including dimethylarginase and cyclophilin A, were detectable in blood, CSF, and urine, suggesting a potential source of treatment-related biomarkers. More recently it has been reported that pediatric high-grade gliomas stem cells release exosomes with a miRNA repertoire that differs from exosomes secreted by normal cells.[112] However, this has not been confirmed thus far in liquid biopsy studies.

As biopsy of DIPG is performed in a limited patient population and typically restricted to a single time-point, molecular characterization via identification of a histone mutation in a non-invasive manner, that could be reliably quantitated and followed at multiple time-points, would be a welcome tool to aid in tumor characterization, patient randomization on clinical trials and response evaluation.[113] Current limitations include lack of standardization of approaches and limited sensitivity.

Clinical Applications of Liquid Biopsy in Gliomas: Preliminary Conclusions

This review of currently available studies suggests several potential applications of liquid biopsies in the clinical care of glioma patients: (1) Liquid biopsies may help establish a diagnosis when tissue biopsy is not feasible due to the risk of an excessive morbidity, such as in deep-seated or multicentric lesions or in presence of advanced age and/or a burden of comorbidities; (2) Liquid biopsies may also be useful for longitudinal disease monitoring, in particular for surveilling minimal residual disease after surgery, for distinguishing tumor progression from treatment-associated changes (so-called "pseudoprogression") following radiotherapy or immunotherapy, and to document the presence of genetic alterations in genotype-directed clinical trials; (3) Information obtained from liquid biopsies may have prognostic and/or predictive value; (4) CSF studies have indicated higher sensitivities in the detection of biomarkers (ctDNA, exosomes) compared to blood-based analysis.

The three main approaches discussed in this article (ctDNA, CTC, and exosomes) each have advantages and disadvantages (Table 6). As for sensitivity of the different techniques in patients with glioblastomas as compared to healthy controls, most studies report values in the range of 60%–85%, with only few of them having lower values. Thus far, no clinically validated circulating biomarkers for managing glioma patients exist, due mainly to the small sample size and heterogeneity of patients' cohorts and techniques across the different studies. For future biomarker work attention to reproducibility and reliability are key as well as sensitivity and specificity. Moreover, uniform testing and validating of biomarkers are needed, and their capacity to predict the outcome should be also investigated. Importantly, the number of ongoing clinical trials that are investigating liquid biopsy biomarkers (ctDNA, CTC) as primary or secondary outcome measures are few (Table 7). This is likely a missed opportunity—neuro-oncology clinical

Table 6 Main Advantages and Disadvantages of Using ctDNA, Circulating Tumor Cells and Exosomes as Biomarkers in Gliomas

	Advantages	Disadvantages
ctDNA	• Higher ctDNA levels compared with CTC – hard to make quantitative comparisons • Potential to fully represent spatial and temporal heterogeneity of tumor • Greater sensitivity than CTC or exosomes?	• Short half-life, <1.5 h[a] • Release mainly by cells undergoing necrosis or apoptosis • Lower sensitivity of ctDNA in blood vs CSF • Lower sensitivity of ctDNA in lumbar CSF vs cisternal CSF[b]
CTC	• Information at the protein, DNA and RNA levels • Potential value of quantification • New techniques in development to optimize isolation of CTC	• CTC are rare • May represent only parts of tumor heterogeneity • Limited number of surface markers for CTC enrichment • Process of isolation challenging
Exosomes	• Carry proteins, DNA, RNA, and miRNA • Able to cross the BBB • Stable under various conditions • Content protected from degradation • Released by living cells	• Release not exclusive from tumor cells • May represent only parts of tumor heterogeneity • Possible presence of contaminants by current isolation method • No specific isolation protocols • Dexamethasone inhibits EV release.

[a]May be different in CSF versus blood.
[b]Limited amount of data.

Table 7 Ongoing Clinical Trials on Gliomas of the Adult and DIPG Including Liquid Biopsy

Study	Patient population	Type of study	Biofluid	Biomarker	Primary outcome measure	Secondary outcome measure
NCT03980249 (Phase 1)	30 newly-diagnosed GBM	Carvedilol + TTFields + chemoradiation and adjuvant TMZ (6 cycles)	Blood	CTC	- OS - PFS	- Quantification of CTC - Correlation of CTC levels and disease burden on MRI measured by RANO criteria
NCT03861598 (Phase 1)	6 newly-diagnosed GBM	Carvedilol + chemoradiation and adjuvant TMZ (6 cycles)	Blood	CTC	- Correlation between CTC and response to treatment on MRI	- Radiological response - Incidence of AEs
NCT04776980 (observational)	30 GBM	Ferumoxtyol infusion 20–28 h before brain MRI	Blood	ctDNA	- Correlation between ferumoxytol enhancement on MRI and macrophage quantification on tumor tissue	- Match number of mutations between tumor tissue and in plasma ctDNA
NCT03973918 (Phase 2)	62 with recurrent BRAF V600E/K-mutated HGG and PXAs	Encorafenib + binimetinib	Blood	CTC	- Radiological response according to RANO criteria	- PFS - OS - Duration of response - AEs - Detection of CTC
NCT03115138 (observational)	19 GBM	Treatment with surgery and chemoradiation	Blood	ctDNA	- Correlation between the molecular abnormalities of GBM ctDNA	NA
NCT02960230 (Phase 1/2)	49 children with newly-diagnosed DIPG and other Gliomas	H3.3K27M Peptide Vaccine + Nivolumab	Blood	ctDNA	- OS - AEs	- H3.3K27M expression status and infiltration of H3.3K27M specific T cells in tumor tissue - Determination of ctDNA as biomarker of tumor burden, response to treatment, or development of drug resistance - detection of H3.3K27M epitope-specific CTL response in post vaccine PBMC in HLA-A2+ children - QoL and cognitive assessment
NCT03990285 (Phase 1)	30 GBM in patients who progressed following chemoradiation and underwent second surgery	18F-fluciclovine (Axumin) administration before brain PET/CT scan and contrast-enhanced MRI	Blood	ctDNA	- To identify pseudoprogression or tumor progression by histopathology	Correlations between histo-pathology and ctDNA
NCT03593993 (observational)	8 in BRAF-V600E mutated recurrent gliomas	A biospecimen collection study to evaluate the pharmacokinetic, pharmacodynamic, and resistance profile to trametinib and dabrafenib	Blood, CSF, tumor tissue	ctDNA	- To determine concentrations of dabrafenib and trametinib in tumor tissue - To evaluate feasibility of measuring ctDNA in CSF	NA
NCT01106794 (observational)	100 DIPG and brainstem gliomas	Molecular analysis of samples	Blood, CSF, urine, tumor tissue	RNA and protein expression	- To compare RNA expression in tumor samples, normal brainstem tissue, and CSF. - Validation of results of the genome-wide analysis. - Proteomic profiling of tumor, normal brainstem tissue, and CSF - Protein expression patterns in tumor tissue compared to normal brainstem tissue.	NA

Table 7 Continued

Study	Patient population	Type of study	Biofluid	Biomarker	Primary outcome measure	Secondary outcome measure
NCT04185038 (phase 1)	70 DIPG or diffuse midline glioma and recurrent or refractory pediatric CNS tumors	B7-H3-Specific CAR T cell loco-regional immunotherapy	Blood, CSF	CTC protein expression	-Tolerability	- To assess the distribution of B7H3-CART cells in CSF and peripheral blood - Assessment of disease response using CTC and MRI - Protein expression analysis in CSF as biomarkers of CART cell functional activity
NCT03416530 (Phase 1)	130 Newly-diagnosed DIPG and recurrent pediatric H3K27M gliomas	ONC201 trial	CSF	ctDNA	H3 K27M DNA levels and drug concentrations in the CSF	- NA
NCT04539431	220 Glioma brain tumors E12513 SensiScreen glioma	Sensitive diagnosis, prognosis, and treatment Planning on the open platform.	Blood, CSF	ctDNA	To demonstrate that the E1213 - SensiScreen glioma reveals at least the same number of mutations in comparison with standard tests (digital PCR, methylation-specific PCR)	-NA

TTFields: Tumor Treating Fields; TMZ: temozolomide; GBM: glioblastoma; qRT-PCR: real-time reverse transcriptase-polymerase chain reaction; OS: overall survival; PFS: progression-free survival; CTC: circulating tumor cells; MRI: magnetic resonance imaging; RANO: Response assessment in neuro-oncology; criteria; AEs: adverse events; ctDNA: circulating tumor DNA; NAv: not available; MG: malignant gliomas; PXAs: anaplastic pleomorphic xanthoastrocytoma; NA: not applicable; DIPG: diffuse midline pontine gliomas; CTL: cytotoxic T lymphocyte; PBMC: peripheral mononuclear cells; QoL: quality of life.

trials should incorporate molecular liquid biopsy endpoints in an effort to spur the development of better liquid biopsy assays, to compare traditional end points head-to-head with molecular biomarkers, and to identify potential surrogate end points. Various local assays should be validated through some kind of centralized testing.

Several issues need to be addressed in more detail by future studies: influence of tumor type (GBMs vs. lower-grade gliomas vs DIPG), tumor location, tumor size, extent of BBB disruption, and disease stage (initial diagnosis, stability, progression) on the sensitivity, specificity, and clinical utility of individual liquid biopsy biomarkers; value of combination of biomarkers in the different settings; best site and modality of CSF collection. In this regard, there are differences in the composition of lumbar vs cisternal CSF, and it is not known whether this difference impacts the diagnostic value. The collection of lumbar CSF seems more feasible for monitoring patients in trials with medical therapies, while cisternal CSF is appealing in patients with indwelling catheters for surgical studies, yet early studies suggest sampling closest to the tumor may increase sensitivity. Since serial monitoring of CSF is not standard of care in glioma patients, well-designed prospective studies should be implemented to demonstrate patient benefit from all these diagnostic procedures which come at a cost. However, CSF does not appear as the ideal non-invasive approach for monitoring patients off treatment.

These issues underscore the need for standardization of biofluid collection, choice of analyte, and detection strategies, along with rigorous testing in future clinical trials to validate findings and enable entry into clinical practice.

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