

Liquid Biopsy Strategies to Distinguish Progression from Pseudoprogression and Radiation Necrosis in Glioblastomas

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Liquid biopsy for the detection and monitoring of central nervous system tumors is of significant clinical interest. At initial diagnosis, the majority of patients with central nervous system tumors undergo magnetic resonance imaging (MRI), followed by invasive brain biopsy to determine the molecular diagnosis of the WHO 2016 classification paradigm. Despite the importance of MRI for long-term treatment monitoring, in the majority of patients who receive chemoradiation therapy for glioblastoma, it can be challenging to distinguish between radiation treatment effects including pseudoprogression, radiation necrosis, and recurrent/progressive disease based on imaging alone. Tissue biopsy-based monitoring is high risk and not always feasible. However, distinguishing these entities is of critical importance for the management of patients and can significantly affect survival. Liquid biopsy strategies including circulating tumor cells, circulating free DNA, and extracellular vesicles have the potential to afford significant useful molecular information at both the stage of diagnosis and monitoring for these tumors. Here, current liquid biopsy-based approaches in the context of tumor monitoring to differentiate progressive disease from pseudoprogression and radiation necrosis are reviewed.

1. Introduction

Glioblastoma is the most common malignant primary central nervous system tumor. Glioblastoma (GBM) is highly aggressive and the median overall survival is only 15–23 months

despite aggressive treatment.^[1] Currently, maximal resection followed by radiation therapy with concurrent temozolomide (TMZ) and adjuvant TMZ treatment is the standard of care. Post-treatment surveillance involves serial MRI. A challenge faced by clinicians is the diagnosis and management of a gadolinium enhancing lesion on a follow-up MRI post-treatment. This suspicious lesion could be progressive disease (PD) or a mere post-treatment radiation effects such as pseudoprogression or radiation necrosis (RN). Pseudoprogression and RN are distinct clinical entities, which when identified and managed appropriately result in better outcomes, while PD of the tumor is often dismal. Patients with PD have a median survival of 3–6 months,^[2] and there is no standard of care. Systemic options include TMZ rechallenge, lomustine, and antiangiogenic therapy such as bevacizumab, but their effectiveness is limited. Reradiation and resection can be considered

depending on the location of the tumor and the condition of the patient.^[3] Conversely, antiangiogenic drugs like bevacizumab or cediranib decrease contrast enhancement by altering permeability of tumor vasculature without actual reduction in tumor burden, referred to as pseudoresponse. Distinguishing these clinical entities from PD is crucial to avoid unnecessary reoperations, premature discontinuation of adjuvant TMZ or its substitution with second line agents.

MR imaging based monitoring is the current standard of care for post-surgical monitoring. Contrast enhancement on imaging is indicative of disrupted blood brain barrier (BBB), but not tumor presence.^[4] Currently, MRI-based Response Assessment in Neuro-Oncology (RANO) criteria is used to monitor treatment response in GBM patients. The criteria included T1 gadolinium enhancing disease, T2/FLAIR changes, new lesions, corticosteroid use, and clinical status.^[5] Adoption of RANO criteria for monitoring response is not without limitations. There is ambiguity in identifying radiation effects, enrolling patients into clinical trials and monitoring immunotherapy response.^[6] Advanced imaging modalities including diffusion-tensor imaging, perfusion imaging, MR spectroscopy (MRS), and positron emission tomography (PET) imaging

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have been used to identify true PD.^[7,8] Although, MRS^[9] and dynamic susceptibility contrast methods^[10] show promise, imaging modalities cannot establish a definitive diagnosis nor capture the heterogeneous molecular landscape of the evolving tumor which is crucial in the setting of PD. Moreover, repeated biopsies cannot be performed to monitor tumor progression due to high risk, surgical inaccessibility and life-threatening complications.^[11] Furthermore, focal sampling cannot capture the true tumor heterogeneity.

As such, there is a great need for tools that can allow early diagnosis, molecular characterization, and assess response to therapy as well as distinguish PD from pseudoprogression and RN with higher sensitivity and specificity compared to current imaging-based technologies. Liquid biopsy refers to analysis of biofluids of patients to detect disease specific genomic or proteomic cargo for diagnostic and prognostic purposes. Liquid biopsy encompasses circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and extracellular vesicles (EVs). A longitudinal liquid biopsy based patient monitoring could provide better perspectives into the tumor presence, molecular status, tumor evolution, response to therapy and also distinguish PD from post-treatment radiation effects and ultimately strategize appropriate therapies to improve patient outcomes (Figure 1a).^[12,13] In this review, we briefly discuss the clinical entities of pseudoprogression and RN in the context of various liquid biopsy platforms to distinguish PD from pseudoprogression and RN.

2. Pseudoprogression

Pseudoprogression is a reversible subacute post-treatment radiation effect identified as an increase in the size of the contrast enhancing lesion, with or without neurological deterioration following completion of radiation therapy (RT) alone or concomitant RT-TMZ, mimicking PD.^[14,15] pseudoprogression most often occurs within the first 3 months following RT/RT-TMZ, but can present up to 6 months afterward. Nearly half the patients with an enhancing lesion within 1 month post-RT have pseudoprogression.^[16] Unlike patients with PD, patients with pseudoprogression remain asymptomatic. Some present with complications due to transient demyelination including worsening of pre-existing symptoms, transient cognitive decline, subacute rhombencephalitis, or somnolence syndrome.

Pathologically, pseudoprogression corresponds to gliosis and reactive radiation-induced changes without evidence of viable tumor tissue.^[17] This may represent an exaggerated response to therapy involving changes to the vascular endothelium and the BBB as well as oligodendroglial injury leading to inflammation and increased vascular permeability.^[11] Treatment-related cellular hypoxia could also contribute to this abnormal enhancement.^[11,18] Some groups suggested pseudoprogression to be an active “inflammatory” response against the tumor considering the association between pseudoprogression and increased survival.^[19] Interestingly, patients with MGMT methylation show pseudoprogression twice as often.^[20] Considering the fact that MGMT methylation status is associated with response to TMZ and thus favorable prognosis,^[21,22] identification of MGMT status is useful in predicting pseudoprogression



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sion and differentiating it from PD.^[15,16,23] Conversely, patients without MGMT methylation have higher rates of PD, with rates of 60% occurrence.^[22] Recent studies have demonstrated a correlation between P53 overexpression and pseudoprogression.^[24] As such, P53 status could also be a potential biomarker for pseudoprogression. Emerging reports have suggested the association of higher expressions of interferon regulatory factor 9 (IRF9) and X-ray repair cross-complementing 1 (XRCC1) in pseudoprogression.^[25]

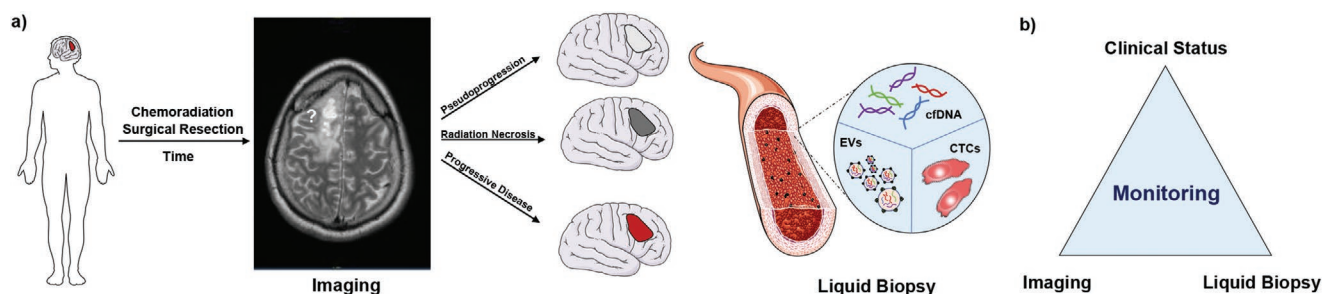


Figure 1. a) Patients with glioblastoma, post-surgical resection, and chemoradiation are monitored using serial MR imaging. A gadolinium enhancing lesion on contrast enhanced magnetic resonance imaging (MRI) could either be true progressive disease (PD), or radiation effects such as pseudoprogression or radiation necrosis (RN). Liquid biopsy strategies including circulating tumor cells (CTCs), circulating free DNA (cfDNA), extracellular vesicles (EVs) can provide minimally invasive modalities of monitoring brain tumors. b) Three-pronged monitoring approach using integrating clinical status, imaging modalities and liquid biopsy strategies could be a potential solution for tracking the tumor evolution over time and therapy. Blood vessel reproduced under the terms and conditions of the Creative Commons Attribution 3.0 Unported License. Copyright Servier Medical Art.

Conventional MR imaging is unable to distinguish between pseudoprogression and early progression, and alternative techniques have not been validated in prospective trials.^[7,11,26] The current method to distinguish the two is to perform follow-up examinations of patients comparing MR images at different points in time. Asymptomatic cases of suspected pseudoprogression are followed up by serial imaging, but when there is worsening of the symptoms due to transient cerebral edema, short course of corticosteroid treatment is initiated with close clinical surveillance and serial imaging.^[7]

3. Radiation Necrosis

Radiation necrosis is a permanent post-treatment radiation effect characterized by an increase in the size of the contrast enhancing lesion occurring 3 months to years after RT.^[11] Pseudoprogression and RN are often considered a spectrum of post-treatment radiation changes. Unlike pseudoprogression, RN progresses without treatment, and has not been associated with better prognosis. With improvements in overall survival of patients with GBM, there is growing usage of reradiation, radiation surgery, and hypofractionated radiotherapy adding to the cumulative dose of radiation received by a single patient contributing to the increasing incidence of RN of about 5–40%.^[27] Patients with RN can be asymptomatic or present with symptoms and signs of necrosis including stroke-like migraine attacks after radiation therapy (SMART syndrome), radiation induced cavernous malformations or aneurysms, Moya–Moya syndrome, mineralizing microangiopathy, tissue calcification, atrophy, leukoencephalomyelopathy, or rarely endocrine dysfunction.^[28–32]

Pathologically, RN corresponds to white matter necrosis associated with calcification, fibrinoid deposition, vascular hyalinization, and endothelial thickening which leads to chronic inflammatory state, oxidative stress, and inhibition of neurogenesis.^[29,33,36] Radiation induced vascular injury initiates the process of necrosis; subsequently, increased tumor necrosis factor- α (TNF- α) drives endothelial cell apoptosis and increased vascular permeability, and increased vascular endothelial growth factor (VEGF) induces small vessel permeability and cerebral edema.^[7,37–40] Conventional imaging tools

cannot identify RN and alternative techniques have not yet been validated in prospective trials.^[7,11,26] Suspected RN can be managed with corticosteroid treatment, hyperbaric oxygen therapy, anticoagulation, anti-angiogenic agents like bevacizumab, laser interstitial thermal therapy, or even surgery.^[7] Corticosteroids reduce radiation induced radiation induced inflammatory response, decrease BBB leakiness and reduce cerebral edema.^[41] Hyperbaric oxygen therapy stimulates angiogenesis and restores blood supply after radiation induced vascular injury. It is even suggested as a prophylactic option in patients with high likelihood of developing RN.^[42,43] Anticoagulants like heparin and warfarin inhibit cytokine release, prevent platelet aggregation and coagulation.^[44,45] Anti-VEGF agents reduce small vessel permeability and BBB leakiness.^[46–49] Laser interstitial thermal therapy focuses on thermal coagulation of perinecrotic region of abnormal angiogenesis.^[50] Surgery reduces mass effect, edema and decreases intracranial pressure in addition to providing true tissue diagnosis.^[51,52] No controlled randomized clinical trials have been performed to establish the most beneficial regimen to manage RN.

4. Circulating Tumor Cells

Circulating tumor cells are cancer cells that leave the primary tumor and enter circulation. A fraction of these CTCs have the potential to invade distant sites and progress to metastasis.^[53] Epithelial to mesenchymal transition (EMT) within the tumor enables some cells to gain a phenotype associated with increased motility and invasion.^[54,55] CTCs are found either as single cells or in clusters, the latter of which have higher metastatic potential.^[56–58] CTCs are hypothesized to be either randomly detached cells or metastatic tumor subclones. In either case, they contain the genomic, transcriptomic, and proteomic characteristics of the primary tumor^[12] and can be valuable tools to provide insight into the primary tumor.^[59,60] Studies in multiple cancers have shown the possibility of CTC based diagnosis,^[61–63] monitoring,^[64–73] and prognosis^[74–77]. These studies have also demonstrated that the presence, phenotype, and the methylation status of markers within CTCs in peripheral circulation have prognostic significance.^[57,78,79]

Table 1. Summary of studies using circulating tumor cell-based analysis for GBM.

Author, year	Biofluid	Methodology of CTC enrichment ^{a)}	Genetic cargo evaluated	Diagnostic sensitivity	Potential role
Sullivan, 2014	Blood	CTC-iChip microfluidic technology; characterization using antibody cocktail, STEAM: SOX2, tubulin-3, EGFR, A2B5, and c-MET	SERPINE1, TGFβ1, TGFβR2, VIM; EGFR amplification	39%	Diagnosis/prognosis
Muller, 2014	Blood	Density gradient centrifugation followed by fluorescence immunocytochemistry using anti-GFAP antibody	EGFR amplification	20%	Diagnosis/prognosis
Macarthur, 2014	Blood	Density gradient centrifugation followed TERT promotor-based CTC detection assay	TERT	72%: pre-radiotherapy 8% post-radiotherapy	Prognosis/monitoring
Malara, 2016	Blood	Vimentin positive cell sorting and short time expansion	–	2/2	Prognosis/monitoring
Gao, 2018 ^{b)}	Blood	CTCs detection based on the aneuploidy of chromosome 8 examination by CEP8-FISH	Chromosome 8 aneuploidy	24 of 31 (77%) GBM (82%)	Diagnosis/prognosis/ monitoring
Krol, 2018 ^{c)}	Blood	Parsortix microfluidic system	SOX2	7/13 (53.8%)	Diagnosis/prognosis/ monitoring

^{a)}CEP8, Centromere Probe (CEP) 8; CTC, circulating tumor cells; EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridization; GBM, glioblastoma; GFAP, glial fibrillary acidic protein; SERPINE1, serpin family E member 1; SOX2, SRY (sex determining region Y)-box 2; TERT, telomerase reverse transcriptase; TGFβ1, transforming growth factor beta 1; TGFβR2, transforming growth factor beta receptor 2; ^{b)}These described cases in this series are not limited to GBM; ^{c)}The study evaluates for CTC clusters.

GBMs rarely form clinically evident extracranial metastases.^[80] This is attributed to the inability of glioma cells to survive in extracranial sites, and tolerate the immune system.^[60,81] However, recent evidence of CTCs detected in blood of GBM patients (Table 1)^[59,60,81,82] poses questions about the conventional theories of GBM dissemination, opening the field of CTC based liquid biopsy in brain tumors.^[59,60,82] Although the capability of the detected GBM CTCs to metastasize has not been established, they can be used as tools to diagnose and monitor GBM.^[59,60] Previous studies have used positive selection (surface marker based selection), negative selection (depletion of blood cells), or other novel platforms for CTC detection (Table 1).

GBM-CTCs were shown to contain tumor specific molecular characteristics and invasive mesenchymal signature.^[59] MacArthur et al., showed an increase in CTC numbers post-radiotherapy in a patient suggestive of PD, indicating the potential of CTCs in distinguishing PD from radiation effects.^[82] Gao et al. identified CTCs in all grades of glioma patients, and showed that CTC detection can reliably identify PD from RN.^[83] These studies provide a proof of principle that patients with GBM have CTCs in their peripheral blood. They demonstrate the potential of molecular characterization of these cells for minimally invasive tumor profiling and identification of PD from radiation effects. Recently, CTC clusters were also identified in the blood of GBM patients.^[81] Interestingly, Lui et al., demonstrated the capacity of intravenously injected CTCs to “reseed” the primary site using a xenograft model and showed that CTCs also demonstrated stemness phenotype more resistant to treatments.^[84] This strengthens the notion that CTCs are a subset of aggressive primary GBM cells, with EMT and stemness characteristics.

Although current studies report a very high specificity, the sensitivity of CTC detection is variable, from 20.6% to 82%.^[59,60,82] Higher sensitivities are required to establish CTCs

as a potential diagnostic modality to diagnose and monitor brain tumors. Most studies inadequately characterize CTCs, are underpowered, use limited numbers of surface markers for CTC enrichment, have samples collected at variable time points along the disease course and lack long-term followup. Furthermore, CTCs were not detectable in each of multiple samples of a given patient at a given time point. This could indicate the lack of sensitivity of current techniques in detecting CTCs or the rarity CTCs in blood (1 cell per 10⁹ blood cells). CTC analysis requires large volumes of fresh blood, and immediate sample processing. Also, detection is currently limited by technological constraints.^[12,85] Several factors including localization of the primary tumor, circulation dynamics, and entrapment in capillary beds limit CTC detection. Furthermore, EMT may alter the surface marker profiles, which may negatively affect CTC-assay performance.^[29,86] Furthermore, the role of CTCs as diagnostic screening modalities is debatable as the disease would be in an advanced stage with CTC dissemination, but it can probably be a good monitoring tool for disease progression and prognosis. Nevertheless, CTCs can provide a distant insight into the primary tumor, and analysis using complementary technology could potentially indicate the presence of a tumor, monitor disease progression, therapeutic responses, and reflect the genetic characteristics of the primary tumor.

5. Circulating Tumor DNA

ctDNA is a subtype of circulating, cell-free DNA (cfDNA) that originates from tumor cells and is composed of small fragments of DNA (180–200 base pairs in length).^[85,87] ctDNA is typically released during tumor cell death and rapidly cleared by phagocytic processes. As such, the concentration of cfDNA is very low (10–100 ng mL⁻¹) in plasma in normal individuals and in early stage cancers. However, the levels could be almost

Table 2. Summary of studies using circulating free DNA-based analysis for GBM.

Author, year	Biofluid	Methodology of cfDNA analysis ^{a)}	Genetic cargo evaluated	Diagnostic sensitivity	Potential role
Balana, 2003	Plasma	Methylation specific PCR assay	MGMT methylation status	81%	Prognosis; treatment selection
Liu, 2010 ^{b)}	CSF, serum	Methylated DNA immunoprecipitation RT-PCR analysis	MGMT, p16INK4a, TIMP3, THBS1 promoter hypermethylation	CSF, 50% serum 50%	prognosis
Lavon, 2010 ^{b)}	Serum	Methylation specific PCR assay	MGMT promoter methylation status	51%	Diagnosis
Boisselier, 2012 ^{b)}	Plasma	DNA amplification by COLD PCR and further characterization by digital PCR	IDH1 mutation	60%	Diagnosis
Salkeni, 2013	Plasma	Long range PCR amplification	EGFRvIII deletion variant	23%	Monitoring
Majchrzak-Celińska, 2013 ^{b)}	Serum	Methylation specific PCR assay	MGMT, RASSF1A, p15INK4B, p14ARF promoter methylation	81%	Diagnosis
Bettgowda, 2014 ^{b)}	Plasma	Droplet digital PCR	TP53, EGFR, PTEN	<10%	Diagnosis
Wang, 2015 ^{b)}	Serum CSF	Methylation specific PCR assay	MGMT promoter methylation	Serum, 21% CSF, 43%	Prognosis
De Mattos-Arruda, 2015 ^{b)}	CSF, Plasma	Targeted capture massively parallel sequencing	IDH1, TP53, PTEN, EGFR, FGFR2, ERBB2 mutations	–	Monitoring
Schwaederle, 2016 ^{b)}	Plasma	Next generation sequencing	TP53, NOTCH1	27%	Molecular profiling, prognosis
Juratli, 2018 ^{b)}	CSF, Plasma	Nested PCR	TERT promoter mutations	CSF, 92% Plasma, 8%	Diagnosis
Piccioni, 2019 ^{b)}	Plasma	Guardant360 cfDNA digital next generation sequencing assay	TP53, NF1, MET, APC, PDGFRA mutations MET, EGFR, ERBB2 amplifications	55%	Molecular profiling, treatment selection
Miller, 2019 ^{b)}	CSF	Next generation sequencing	IDH1, IDH2, TP53 mutations; CDKN2A, CDKN2B deletions; EGFR amplification	49% (post-therapy)	Prognosis, monitoring
Mouliere, 2019 ^{b)}	CSF, Plasma, Urine	Tumor-guided capture sequencing	Matched clonal and private mutations	CSF, 50% Plasma, 50% Urine, 13%	Diagnosis
Cordova, 2019	Plasma	Droplet digital PCR	TERT promoter mutations	46%	Monitoring

^{a)}APC, adenomatous polyposis coli; CDKN2A, cyclin dependent kinase inhibitor 2A; CDKN2B, cyclin dependent kinase inhibitor 2B; CfDNA, circulating free DNA; CSF, cerebrospinal fluid; EGFR, epidermal growth factor receptor; ERBB2, Erb-B2 receptor tyrosine kinase 2; FGFR2, fibroblast growth factor receptor 2; IDH, isocitrate dehydrogenase; MGMT, O(6)-methylguanine-DNA methyltransferase; NF1, neurofibromatosis type 1; PDGFRA, platelet-derived growth factor receptor alpha; PTEN, phosphatase and tensin homolog; RASSF1A, Ras association domain family 1 isoform A; RT-PCR, real time polymerase chain reaction; TERT, telomerase reverse transcriptase; THBS1, thrombospondin 1; TIMP3, TIMP metalloproteinase inhibitor 3; ^{b)}These described cases in this series are not limited to GBM.

10-fold higher in patients with advanced cancers.^[87] The challenge in ctDNA based liquid biopsy is two-fold, in extraction and in targeted detection. At the level of extraction, optimization of methodologies would increase the chance that these markers are detected. At the level of detection sensitive technologies, including droplet digital polymerase chain reaction (PCR), BEAMing (beads, emulsion, amplification, and magnetism), and next generation sequencing, allow identification of targeted mutations in various biofluids.^[85]

Studies in multiple cancers have demonstrated the utility of cfDNA based diagnosis^[88–91] monitoring and assessing response to therapy.^[92–96] Growing evidence also suggests that cfDNA concentration correlates with tumor burden, cancer stage, cellular turnover, and response to therapy.^[87,97] However, the application of this strategy to gliomas has been hindered by the relatively low abundance of detectable molecular alterations in plasma (<10% of patients) as compared to other tumor types, likely due to the BBB.^[87]

However, emerging studies have reported the detection of tumor specific mutations in the cfDNA of patients with glioma

(Table 2).^[87,98–110] Detection of glioma specific alterations such as telomerase reverse transcriptase (TERT),^[105,111] EGFRvIII,^[102] IDH1,^[108] and histone mutations^[112] has shown promise in minimally invasive diagnosis, molecular profiling, and classification of tumors. Epidermal growth factor receptor (EGFR) gene is amplified in 30–40% of GBMs and nearly 50% of them express the in-frame deleted variant of EGFR receptor, EGFRvIII, and represents an aggressive subtype of GBM.^[113–119] IDH1 mutations occur in 10% GBMs.^[120] TERT promoter mutations occur in 60% of GBMs, associated with poorer outcomes. Simultaneous presence of IDH1 and TERT promoter mutations confer survival benefit for GBM patients. H3K27M mutation status has both diagnostic and prognostic significance in diffuse midline glioma.^[121] Furthermore, identification of prognostic markers such as MGMT can be valuable to guide therapy.^[98,101,107,109,110] Considering the association of MGMT promoter methylation with pseudoprogression, a positive MGMT methylation status can suggest the likelihood that a contrast enhancing lesion indicates pseudoprogression. Emerging studies also suggest the possibility of using ctDNA analysis to pursue

treatment alternatives^[100] as well as assess response to immunotherapy.^[100,122] Recent studies have shown the ability of ctDNA based longitudinal follow up in GBM patients. Miller et al. showed that cerebrospinal fluid (CSF) ctDNA based sequencing analysis can be used to track the evolution of tumors.^[104] Arruda and Mourliere showed that the levels of tumor specific mutation status in the ctDNA fraction of CSF parallels the disease status, correlating with progressive disease.^[103,123,124]

CSF studies have consistently shown higher sensitivities in ctDNA detection compared to blood based analysis,^[101,105–107] however, serial monitoring may not be practical considering the invasiveness of CSF collection. While recent studies have explored the potential of alternative biofluids such as urine,^[123] blood-based detection has shown promising sensitivity and is more practical for serial monitoring. cfDNA is shed by virtually all cells in the body; it is especially difficult to identify ctDNA within this background. Furthermore, ctDNA fragments have a very short half-life and require rapid processing.^[12] Most cfDNA studies in glioma have small sample sizes and have used various methods of mutant detection to allow for meaningful comparisons. Lack of standardized procedures for sample collection, isolation, and analysis has been another major hurdle for the field, making it challenging to compare sensitivities across various studies. Nevertheless, development of sensitive technologies for ctDNA capture and tumor specific mutant and methylation status can provide minimally invasive diagnosis and monitoring for GBMs, providing insights into the spatiotemporal heterogeneity over time and therapy.

6. Extracellular Vesicles

Tumor cells actively release stable membrane bound nanobodies called EVs. They carry functional genomic and proteomic cargo from their parental cells and deliver that information to surrounding and distant recipient cells to modulate their behavior. EVs are identified to modulate and reprogram the tumor microenvironment to promote tumor proliferation, reprogram metabolic activity, induce angiogenesis, escape immune surveillance, acquire drug resistance, and undergo invasion.^[125] They can also be detected in biofluids including plasma, CSF, urine, etc. Their stable configuration confers a protective niche for tumor derived mRNA, miRNA, and proteins. EVs are classified according to size and biogenesis pathway: microvesicles (100–1000 nm) are formed by budding of the plasma membrane, exosomes (30–150 nm) are formed by the fusion of intracellular multivesicular bodies with the plasma membrane, apoptotic bodies (1000–5000 nm) are produced and released by dying cells, and large oncosomes (>1 µm) are formed by nonapoptotic blebs from plasma membrane.^[85,126,127] Detection of tumor specific EVs amidst the vast background of normal EVs derived from every other cell of the body is challenging. Methodologies to allow for optimal EV isolation and sensitive technologies for EV cargo analysis are being developed.

Emerging reports have demonstrated the utility of EVs as biomarkers of cancer diagnosis^[3,128–130] and prognosis. We have summarised the studies using EV based analysis for GBM in (Table 3).^[131–133] Quantitative studies demonstrated that EV numbers in plasma were higher in patients with GBM

patients compared to controls and the numbers dropped with therapy.^[134–137] Higher numbers were noted in PD compared to patients with stable disease or pseudoprogression.^[134,135] However, nanoparticle tracking analysis or flow cytometry based EV quantification methods are nonspecific and nonrepresentative of true tumor derived EV burden. Nevertheless, these studies indicate that the pattern of EV dynamics parallel the disease course in the broad sense.

Recent EV based mRNA studies have reported sensitivities between 28% and 82% for the detection of EGFRvIII in EVs extracted from serum of GBM patients.^[138,139] In addition, analysis of CSF-derived EV mRNA has shown higher sensitivities in IDH mutant detection compared to blood based EV analysis.^[140,141] Several protein based EV analysis methods have been used for tumor specific EV characterization.^[142,143] Shao et al. used micronuclear magnetic resonance system chip based EV protein analysis and identified EGFRvIII, podoplanin (PDPN), and IDH1 proteins in the plasma EVs of glioma patients. The sensitivities were higher for EGFRvIII and PDPN (68%) than they were for IDH1 (16%).^[142] Chandran et al. showed that detection of syndecan-1 in plasma (sensitivity, 71%) can differentiate high grade gliomas from low grade gliomas. Other groups have explored EV miRNAs including miR-301a,^[144] miR-182-5p, miR-328-3p, miR-339-5p, miR-340-5p, miR-485-3p, miR-486-5p, and miR-543,^[145] miR-21, miR-222, and miR-124-3p,^[146] miR-320 and miR-574-3p, as well as a small noncoding RNA, RNU6^[147] as diagnostic tools. Specifically, Lan et al. and Santangelo et al. showed that serum miR-301a levels,^[144] miR-21, miR-222, and miR-124-3p levels^[146] in serum EVs were higher in GBM patients and paralleled the clinical disease course, with levels decreasing with surgical resection and increasing with recurrence.^[144]

Recent studies have explored the potential of fluorescent labeled EV quantification using imaging flow cytometry. Ricklefs et al. used imaging flow cytometry to show that EVs with double positive tetraspanin expression (CD63+/CD81+) are enriched in patient plasma samples.^[148] Galbo et al. showed that CD9+/GFAP+/SVN+EVs can predict response to therapy.^[149] These studies highlight the possibility of monitoring GBM EVs using surface marker analysis. Jones et al. identified protoporphyrin positive EVs in plasma of patients with malignant glioma undergoing fluorescence guided surgery with 5-amino levulinic acid (5-ALA) as a potential diagnostic strategy to identify and monitor malignant gliomas. As the drug is currently approved only for surgical resection, the potential of the drug in a longitudinal setting has not yet been evaluated.^[150]

The ability of GBM-EVs to cross the BBB has always been a topic of debate, which could be the reason for lower sensitivities of target detection in blood based EV analysis. Garcia-Romero et al. recently demonstrated that tumor specific EVs are capable of crossing intact BBB and navigating into plasma, using an orthotopic xenotransplant mouse model of human glioma-cancer stem cells featuring an intact BBB.^[141]

Although both CSF and plasma/serum based EV analysis is promising, the superiority of a biofluid for EV based monitoring is still unclear. However, plasma/serum-based monitoring is more practical for the purposes of longitudinal monitoring as repeated CSF sampling is not feasible.^[151] Biofluids such as urine and saliva need to be explored. Small sample sizes, variable technologies, lack of a gold standard method of EV characterization

Table 3. Summary of studies using extracellular vesicle EV-based analysis for GBM.

Author, year	Biofluid	Methodology of EV analysis ^{a)}	Genetic cargo evaluated	Diagnostic sensitivity	Potential role
Skog, 2008	Serum	Nested RT-PCR EGFRvIII mRNA	EGFRvIII	28%	Diagnosis
Shao, 2012	Plasma	Micro nuclear magnetic resonance system chip based EV protein analysis	EGFRvIII, IDH1, PDPN proteins	68% (EGFRvIII, PDPN) 16% IDH1	Diagnosis
Chen and Balaj, 2013	CSF, Serum	BEAMing (beads, emulsion, amplification, magnetics) RT-PCR and ddPCR	IDH1 mutation	62.5% 0%	Diagnosis
Akers, 2013	CSF	RT-PCR	miR-21	85% initial cohort, 87% validation cohort	Diagnosis/monitoring
Manterola, 2014	Serum	RT-PCR	miR-320, miR-574-3p, RNU6-1 expression	miR-320, 65%, miR-574-3p, 59%, RNU6-1, 73%	Diagnosis
Koch, 2014	Plasma	Flow cytometry: size of 300 nm or greater and Annexin V positivity	–	–	Monitoring
Evans, 2016	Plasma	Flow cytometry: Annexin V positivity	–	–	Monitoring/prognosis
Garcia-Romero, 2017 ^{b)}	Plasma	Fast Cold-PCR	IDH1 mutation	48%	Diagnosis
Galbo, 2017 ^{b)}	Serum	Imaging flow cytometry- fluorescent labeled antibodies	CD9+/GFAP+/SVN+ EVs	–	Monitoring
Andre-Gregoire, 2018	Plasma	Tunable resistive pulse sensing analysis (TRPS)	–	–	–
Ricklefs, 2018 ^{b)}	Plasma	Droplet PCR	PD-L1 DNA	67%	Monitoring
Manda, 2018 ^{b)}	Serum	Semi-nested RT-PCR	EGFRvIII mRNA	82%	Diagnosis
Lan, 2018 ^{b)}	Serum	RT-PCR	miR-301A	–	Prognosis/monitoring
Ebrahimkhani, 2018 ^{b)}	Serum	Deep sequencing	miR-182-5p, miR-328-3p, miR- 339-5p, miR-340-5p, miR-485-3p, miR-486-5p and miR-543	92% ^Δ	Diagnosis
Santangelo, 2018 ^{b)}	Serum	RT-PCR	miR-21, miR-222, miR-124-3p	miR-21, 84%, miR-222, 80% miR-124-3p 78%	Diagnosis/monitoring
Osti, 2019 ^{b)}	Plasma	Nanoparticle tracking analysis, mass spectrometry	Proteins: vWF, APCS, C4B, AMBP, APOD, AZGP1, C4BPB, Serpin3, FTL, C3, and APOE	–	Monitoring
Jones and Yekula, 2019	Plasma	Imaging flow cytometry based monitoring of PpIX positive EVs pre and post-5-ALA based fluorescent guided surgery	PpIX positive EVs	4 out of 4	Diagnosis/monitoring
Chandran, 2019	Plasma	Mass spectrometry, nanoparticle tracking analysis, electron microscopy	Levels of Syndecan 1	71%	Diagnosis/classification
Ricklefs, 2019 ^{b)}	Plasma	Imaging flow cytometry- fluorescent labeled antibodies	CD63+/CD81+ EVs	–	–

^{a)} AMBP, alpha-1-microglobulin/bikunin precursor; APCS, serum amyloid P component; APOD, apolipoprotein D; APOE, apolipoprotein E; AZGP1, alpha-2-glycoprotein 1; C3, complement C3; C4B, complement C4B; C4BPB, complement component 4 binding protein beta; CSF, cerebrospinal fluid; ddPCR, droplet digital PCR; EGFR, epidermal growth factor receptor; FTL, ferritin light chain; IDH, isocitrate dehydrogenase; PDPN, podoplanin; PpIX, protoporphyrin; RT-PCR, reverse transcriptase polymerase chain reaction; vWF, von Willebrand factor; 5-ALA, 5 aminolaevulinic acid; ^{b)} These described cases in this series are not limited to GBM.

makes it difficult to make meaningful comparisons. However, these initial EV biomarker discovery studies show promise and their potential in longitudinal setting is yet to be explored.

7. Blood Brain Barrier

Although CSF is considered as the ideal biofluid for liquid biopsy-based diagnosis and monitoring due to the anatomic proximity to the primary tumor, plasma and serum are easily accessible and minimally invasive. CSF collection is highly invasive, requires trained professionals and has several potential complications. The utility of blood based liquid biopsy depends on the inherent ability of the liquid biopsy substrates (CTCs,

CtDNA, EVs) to cross the BBB and reach peripheral circulation. The BBB provides both physical and biochemical barriers with a continuous network of tight and adherens junctions between brain capillary endothelial cells preventing paracellular diffusion of hydrophilic molecules.^[153] The most obvious path for these substrates is circumnavigation of the BBB at the regions of BBB disruption. Despite the fact that GBM is a highly aggressive and invasive brain tumor with a disruption of BBB, large sections of BBB remain intact.^[152] Wide scale disruptions of BBB usually occur with the progression of disease.

CTCs are large and require disrupted BBB to navigate their way into the bloodstream. This could be one of the reasons for their low abundance in blood. It is unlikely for the hydrophilic ctDNA to cross an intact BBB. CtDNA could enter the

bloodstream via the sites of BBB disruption. Studies have shown higher ctDNA levels in blood in high grade gliomas than in low-grade gliomas,^[87] which can partly be attributed to the BBB disruption in high grade gliomas. A positive correlation between the extent of BBB disruption and ctDNA levels in the blood was identified by Nabavizadeh and colleagues, indicating the ability to detect ctDNA as a function of BBB disruption strengthening this notion.^[154] Morad et al. demonstrated using in vitro and in vivo BBB models, the ability of native tumor derived EVs to breach the intact BBB and reach the circulation via transcytosis.^[155] Kur et al. showed a neuronal activity driven uptake of hematopoietic cell derived EVs by neurons across the BBB via transcytosis.^[156] These studies provide a proof of principle that tumor specific EVs navigate through the intact BBB, and reach the peripheral circulation. However, further investigation is required to unveil the ability of liquid biopsy substrates to reach peripheral circulation as well as determine the optimal biofluid for monitoring disease progression.

8. Future Directions

Promising developments in the field of liquid biopsy can aid clinicians making diagnostic and therapeutic decisions to manage GBMs. The potential of combining both liquid biopsy fractions, cfDNA from dying cells and actively secreted EVs from live cells might be a better representation of the ongoing tumor dynamics. Recent clinical application of liquid biopsy based diagnostics such as cobas EGFR Mutation Test version 2, which monitors T790M mutation status in plasma cfDNA in non-small cell lung cancer patients to aid the use of osimertinib,^[157,158] and ExoDx Prostate IntelliScore (EPI Test, Bio-Techne), a noninvasive EV based urine test measures three mRNAs considered to be important genomic RNA biomarkers that can guide urologists in determining the true need for a prostate biopsy^[159,160] have shown promise of liquid biopsy for minimally invasive diagnostics and prognostics. However, there are several challenges along the pathway of blood-based biomarker development from discovery to clinical utility, and a systematic approach to tackle these hurdles is critical to develop a blood-based biomarker with clinical utility. These aspects are extensively reviewed elsewhere.^[151,161,162] Ideally, an advanced machine learning model^[163,164] integrating clinical, imaging and liquid biopsy based molecular characterization could help decision making during follow-up (Figure 1b). With the advent of sensitive technologies, liquid biopsy could be the future of tumor diagnosis, monitoring, and therapy response.

9. Conclusion

Liquid biopsy strategies offer minimally invasive tools for diagnosis as well as monitoring brain tumors for response to therapy and for predicting treatment related changes. Despite recent advances in liquid biopsy based biomarking for brain tumors, the sensitivity of detection in brain tumors has been low. As of now, there are no clinically applicable circulating biomarkers for the diagnosis and monitoring of GBMs, but promising developments in the field with complimentary sensitive

technologies have moved the needle closer to a clinical assay. Biobanking and appropriate sample collection and handling protocols are needed to allow the field to harvest and save biofluids for development and validation of biomarkers and technologies. Ideally, a three-pronged monitoring approach correlating clinical status, imaging characteristics and liquid biopsy based molecular characterization, to provide a comprehensive clinical and molecular snapshot of the tumor in space and time, to assess the evolution of the tumor, and identify true PD from radiation effects could be a potential solution to the current challenge.

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Conflict of Interest

The authors declare no conflict of interest

Author Contributions

All authors conceptualize, write, review, and edit the manuscript.

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