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REVIEW ARTICLE

Translational Therapeutics

Liquid biopsy: creating opportunities in brain space

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In recent years, liquid biopsy has emerged as an alternative method to diagnose and monitor tumors. Compared to classical tissue biopsy procedures, liquid biopsy facilitates the repetitive collection of diverse cellular and acellular analytes from various biofluids in a non/minimally invasive manner. This strategy is of greater significance for high-grade brain malignancies such as glioblastoma as the quantity and accessibility of tumors are limited, and there are collateral risks of compromised life quality coupled with surgical interventions. Currently, blood and cerebrospinal fluid (CSF) are the most common biofluids used to collect circulating cells and biomolecules of tumor origin. These liquid biopsy analytes have created opportunities for real-time investigations of distinct genetic, epigenetic, transcriptomics, proteomics, and metabolomics alterations associated with brain tumors. This review describes different classes of liquid biopsy biomarkers present in the biofluids of brain tumor patients. Moreover, an overview of the liquid biopsy applications, challenges, recent technological advances, and clinical trials in the brain have also been provided.

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INTRODUCTION

Biopsy as a diagnostic approach has been routinely used for molecular testing and deciding the precise therapeutic strategies in various human diseases including cancer [1]. The gold standard, conventional tissue biopsy provides high yields of analytes to study cancer-specific alterations and the opportunities to perform the histological examination and staging. However, tissue biopsy implementations have limitations such as surgical interventions, accessibility of tumor tissue, sampling bias, localized analysis, lack of serial monitoring, and inability to study tumor heterogeneity and evolution. In contrast, liquid biopsy provides a minimally invasive alternative for systemic and real-time tumor progression monitoring and therapeutic interventions [2].

Liquid biopsy in cancer patients primarily aims at detecting, analyzing, and monitoring diverse tumor-derived analytes circulating in different biological fluids, including blood, urine, saliva, cerebrospinal fluid (CSF), pleural fluid, stool, semen, bone marrow, and ascites [3–5]. Tumor-derived materials in these biofluids that have shown promising future in studying diverse aspects of tumor biology involve circulating tumor cells (CTCs), cell-free DNA (cfDNA), cell-free RNA (cfRNA), tumor-specific proteins, tumor-educated platelets (TEPs), extracellular membrane-bound vesicles, and other metabolites of tumor origin [6–11]. These analytes possess immense biological information, thus empowering the researchers to perform cancer-type-specific investigations from genomics, epigenomics, transcriptomics, proteomics, and metabolomics perspectives as shown in Fig. 1, and is also frequently reviewed in the literature [12]. Consequently, in recent years, liquid biopsy has emerged as one of the diagnostic methods for monitoring and subtyping solid tumors [13]. This strategy is of additional significance to central nervous system (CNS) tumor patients because of the risks posed by the available diagnostic procedures [14].

Brain malignancies include various types of neoplasms originating from either primary brain tumors or metastatic cancers. Glioblastoma is the most frequent and aggressive one in adults, with a 5-year survival rate of less than 5% [15, 16]. The similar appearances of different CNS and non-CNS tumors on neuroimaging scans, and the compromised life quality associated with the neuropathological examination techniques limit the CNS malignancies-specific diagnostic strategies successful otherwise [17]. In addition, the blood-brain barrier (BBB), the microvasculature system of CNS, significantly lowers the amounts of circulating analytes shed by the brain tumors in different biological fluids, thus posing limitations to the liquid biopsy enactments [18, 19]. Therefore, the development of specific and sensitive liquid biopsy-based protocols to diagnose brain tumors is currently of utmost need.

In light of the most recent World Health Organization (WHO) classification of tumors of the CNS edition 5 (WHO CNS5) proposed in 2021, the clinicopathologic significance of molecular profile alterations both in providing ancillary and defining diagnostic knowledge has increased [20]. WHO CNS5 advocates the adoption of integrated diagnostic approaches containing both classical histology and tissue-based tests (e.g., immunohistochemistry, ultrastructural) as well as novel molecular characteristics [21]. The liquid biopsy strategies in brain malignancies can be instrumental in capturing and defining the underlying discrete

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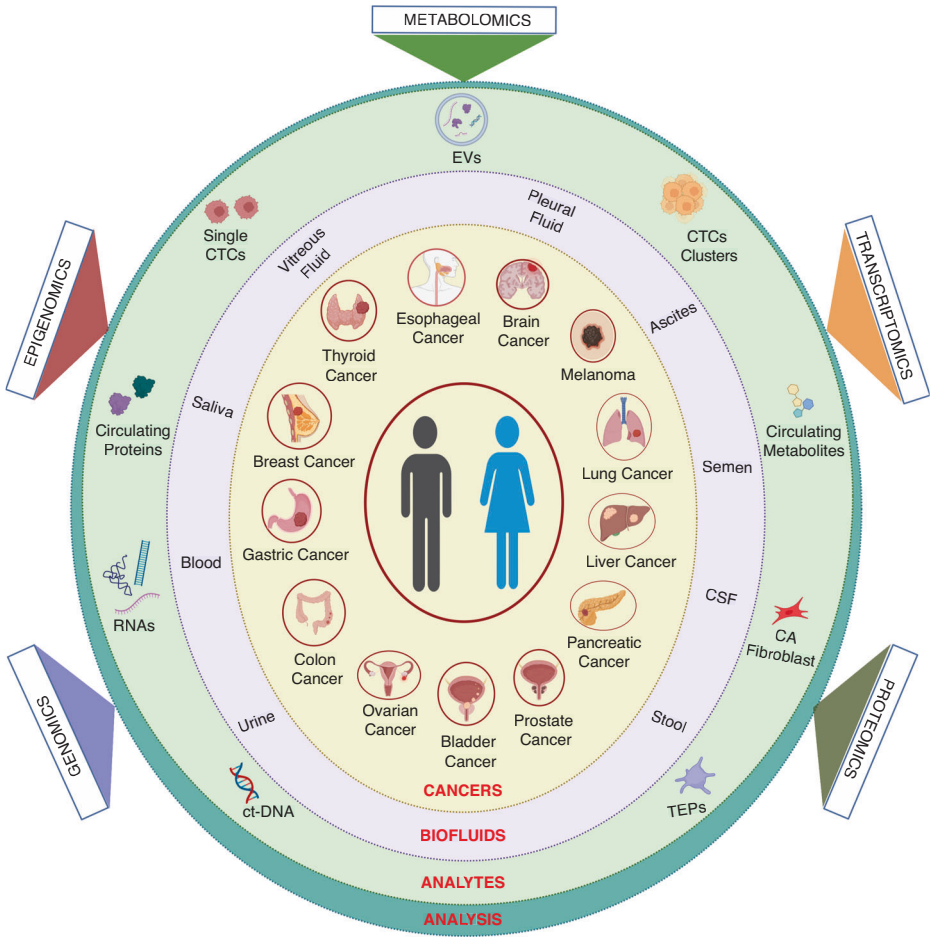

Fig. 1 Schematic representation of liquid biopsy approaches in cancer patients. In different types of cancers, biofluids such as blood, urine, CSF, ascites, pleural fluid, saliva, stool, semen, vitreous fluid, etc. are routinely collected to study circulating analytes which include cell-free DNA (cfDNA), cell-free coding, and non-coding RNA species (cfRNA), circulating proteins (CPs), CTCs (single or clusters), extracellular vesicles (EVs), circulating metabolites (CMs), cancer-associated (CA) fibroblast, tumor-educated platelets (TEPs), etc. Each of these circulating analytes can be investigated for cancer-specific alterations from genomics, epigenomics, transcriptomics, proteomics, and metabolomics perspectives at resolutions ranging from the single cell to the whole organism. This figure has been created using BioRender software and a publication license is obtained.

molecular changes, thus complementing the precision of diagnostic measures. This review describes brain tumor-specific biomarkers with a focus on glioblastoma that are present in various biological fluids, especially blood, and CSF. In addition, clinical applications, and technological advances toward the detection of analytes using the liquid biopsy approach in CNS tumors are explored.

MOLECULAR BIOMARKERS IN BRAIN TUMORS

Over the past few decades, international standards for brain tumor diagnosis have been improvised in a timely manner based on the new developments in the field. The latest molecular feature-centric proposal of WHO has led to a major paradigm shift in brain tumor classification, nomenclature, and grading [21]. For instance, adult-type diffuse gliomas with isocitrate dehydrogenase (IDH) mutation originally identified as glioblastoma in the previous 2016 WHO classification have now been categorized as astrocytoma [22]. Henceforth, for the remainder of this review, the term glioblastoma will refer to adult-type diffuse gliomas characterized either by IDH status or histologically defined by neuropathologists. An updated list of various brain tumor-specific molecular biomarkers has been summarized in Table 1. These genetic markers have now become an integral part of pathological reports, and their applications have transformed the brain tumor routine clinical diagnostic prototype [23, 24]. In this section, a few of the well-characterized molecular signatures of brain tumors are described.

IDH mutations have been observed both in CNS and non-CNS tumors, predominantly in secondary adult-type diffuse gliomas (astrocytoma and oligodendroglioma) [20, 25]. The IDH protein forms a dimer in the activated state and prevents intracellular oxidative injury by catalyzing the reversible oxidative decarboxylation of the substrate isocitrate to α-ketoglutarate (α-KG) in the tricarboxylic acid (TCA) cycle [26]. In brain malignancies, the most common IDH mutations identified include arginine residues (R132 for IDH1, R140, or R172 for IDH2) that are crucial for the identification of the substrate isocitrate [27]. A homodimer resulting from an IDH-mutant gene shows neomorphic activity and catalyzes the conversion of isocitrate to an oncometabolite i.e., D-2-hydroxyglutarate (D-2-HG), which has long been associated with altered cellular metabolism and oncogenesis, mostly through DNA and histone methylation [28, 29]. The inhibitors-mediated direct targeting of the IDH mutant-enzyme prevents the manifestation of this malignant transformation, but it also results in the increased resistance of the tumor cells to genotoxic therapies such as radiation and chemo agents [30, 31]. Therefore, the IDH mutations-associated prolonged median survival is

Table 1. A comprehensive list of brain tumor-specific molecular markers.

	Molecular marker gene/pathways	Description	Reference
1	Alpha Thalassemia/Mental Retardation Syndrome X-linked (ATRX)	ATRX is a histone chaperone and mutations lead to loss of protein function Correlated with telomere dysfunction, IDH1/2, and TP53 mutations Mutually exclusive with 1p/19q- codeletion Prevalent in both low-grade and high-grade gliomas	[309, 310]
2	B-Raf protein kinase (BRAF)	BRAF point mutations V600E and fusion with the KIAA1549 gene leads to constitutive activation of the MAPK pathway which regulates cell proliferation, differentiation, apoptosis, and migration Occur frequently in ~85% of pediatric low-grade gliomas	[23, 311]
3	Cyclin-Dependent Kinase 4/6 (CDK4/6)	Copy number amplifications of CDK4 lead to altered cell cycle progression and Temozolomide (TMZ) drug resistance Common in proneural brain tumors	[312, 313]
4	Cyclin-Dependent Kinase Inhibitor 2A (CDKN2A)	Homozygous deletion of tumor suppressor CDKN2A protein leads to uncontrolled cell proliferation Astrocytoma's grades are determined based on the presence and absence of homozygous CDKN2A deletion Occur across different grades of gliomas	[314, 315]
5	Glioma-CpG Island Methylator Phenotype (G-CIMP)	Methylation of tumor suppressor and mismatch repair genes leading to tumor phenotype Occur frequently in IDH mutant gliomas but rare in glioblastomas	[316, 317]
6	Epidermal Growth Factor Receptor (EGFR)	Amplification and deletion of EGFR leads to the constitutively activated state of the EGFR pathway, which in turn facilitates tumor development and progression Key biomarker gene for glioblastomas, diffuse midline gliomas and diffuse pediatric high-grade gliomas	[318, 319]
7	Epidermal Growth Factor, Latrophilin, and 7 Transmembrane Domain-Containing Protein 1 (ELDT1)	Expressed at significantly higher levels in brain tumors Prevalent in mesenchymal and high-grade gliomas	[320, 321]
8	Fibroblast Growth Factor Receptor 3-Transforming Acidic Coiled-Coil containing protein (FGFR3-TACC3)	Fusion protein activates non-canonical pathways to apply mitotic and chromosomal instability and endorses oxidative phosphorylation and reliance upon mitochondrial metabolism Occur in 3% of gliomas	[322, 323]
9	Glial Fibrillary Acidic Protein (GFAP)	A higher ratio of GFAP δ/α isoforms expression is associated with high-grade gliomas GFAP serum levels can act as WHO grade 4 brain tumors diagnostic marker	[324, 325]
10	Glioma-associated oncogene transcription factor (GLI2)	GLI2 confers tumorigenicity through aberrant activation of the Hedgehog pathway, which in turn is responsible for the stemness and proliferation of Glioblastoma Stem Cells (GSCs)	[326, 327]
11	Histone (H3) Protein	H3.1 (K27M) and H3.3 (G34R/V) mutations alters post-translational modification of H3 protein causing impaired DNA methylation, which in turn is considered to be the driver of gliomagenesis Both pediatric and adult high-grade gliomas	[328, 329]
12	Hypoxia Inducing Factor 1 subunit α (HIF1-α)	HIF1-α promotes aggressiveness of GBM by regulating expression of several proangiogenic genes, such as VEGF, PlGF, PDGF, Ang -1 and -2, EPO, and IGF2 Higher HIF-α levels in astrocytoma than in glioblastoma	[311, 330]
13	Isocitrate Dehydrogenase (IDH)	R132 and R172 mutations of IDH1 and IDH2 respectively leads to the accumulation of oncometabolite D-2-Hydroxygluterate (2HG), which can promote tumorigenesis 2HG inhibits 2-oxogluterate dependent enzymes, leading to CIMP phenotype In WHO CNS5, IDH mutation is basis of classifying adult-type diffuse gliomas into subtypes: glioblastoma, astrocytoma and oligodendroglioma.	[331, 332]
14	Loss of Heterozygosity (LOH)	Tumor suppressor genes (CIC,PTEN etc.) loss results in malignant phenotype Most common LOH in glioma is codeletion of short arm of Chr 1 and long arm of Chr 19 (1q/19q) Loss of long arm of Chr 10 [10q] most common in high-grade gliomas	[63, 333]

Table 1. continued

	Molecular marker gene/pathways	Description	Reference
15	MET	Amplification of MET proto-oncogene (a type of receptor tyrosine kinase gene) leading to alteration of growth factor receptor/PI3K/MAPK signaling Common in mesenchymal brain tumors	[311]
16	MYC/MYCN	In brain tumors, overexpression, and amplification of MYC and MYCN proteins results in altered cell cycle regulation and metabolism Extrachromosomal amplifications of MYC and MYCN are frequently found in glioblastoma patients	[334–337]
17	Neurofibromatosis 1 (NF1)	Mutation or deletion of inhibitory signaling protein NF1 result in activation of RTK signaling pathway leading to increased cell survival Occur in both low and high-grade glioma	[47, 311, 338]
18	O^6-Methylguanine-DNA-Methyltransferase (MGMT)	Promoter methylation leads to silencing of MGMT expression, which provides endurance to alkylating agents such as TMZ Act as both predictive and prognostic marker in glioblastoma patients	[21, 23, 339]
19	Platelet-Derived Growth Factor Receptor (PDGFR)	Amplification of PDGFR results in altered growth factor receptor/PI3K/MAPK signaling through regulation of transcription factors involved in cell survival, proliferation, differentiation, and apoptosis Signature of proneural type of glioblastoma	[340, 341]
20	Phosphoinositide 3-Kinase (PI3K)	Mutation in PI3K leads to over-activation of PKB/AKT pathway involved in the tumor cell survival, proliferation, and invasion PI3K is a prognostic marker of glioblastoma	[342, 343]
21	Phosphate and Tensin Homolog (PTEN)	Mutation or deletion leading to defective localization or loss of function of PTEN (tumor suppressor gene) results in increased proliferation and migration of tumor cells due to overactivation of AKT signaling High frequency of the PTEN gene loss in glioblastoma patients	[343, 344]
22	p53	Mutations resulting in altered p53 pathway promote tumorigenesis by promoting cellular responses such as angiogenesis, maintenance of genomic stability, cell apoptosis etc. p53 alternations are associated with poor OS in glioblastoma patients	[343, 345]
23	Retinoblastoma (Rb)	Rb is a negative regulator of cell cycle, therefore altered Rb function promotes tumor growth Mutations as well as promoter methylation of Rb gene occur in high-grade glioma patients	[346, 347]
24	Sonic Hedgehog (SHH) Pathway	Higher levels of SHH activate hedgehog pathway which in turn confers stemness to GSCs and help in tumor progression	[348, 349]
25	Telomerase Reverse Transcriptase (TERT)	Mutations in TERT promoter leads to the aberrant activation of telomerase and elongation of telomeres ATRX mutations mutually exclusive More frequent in glioblastoma patients than astrocytoma	[350–352]
26	Vascular Endothelial Growth Factor (VEGF)	Increased levels of VEGF promote angiogenesis and tumorigenesis Increased level corresponds to increased glioma grade Overexpressed in mesenchymal glioblastoma	[353, 354]
27	WNT Pathway	Dysregulation of WNT signaling pathway confers high self-renewal capacity and aggressiveness to GCSs	[355, 356]
28	YKL-40	Through activation of MAPK and AKT pathways, YKL-40 mediates glioma cells proliferation Overexpressed in mesenchymal subtype of glioblastomas and responsible for TMZ resistance	[357, 358]

considered a positive prognostic biomarker in grade 2–4 gliomas [32]. Furthermore, distinct mutations and amplifications in the epidermal growth factor receptor (EGFR) gene have also been classified as glioblastoma prognostic biomarkers [15, 21]. These genomic changes have been reported to confer a ligand-independent constitutive activated state to EGFR in cancer cells, which in turn stimulate responses and pathways required for tumorigenesis [33]. In brain tumors, the most studied EGFR variation is transcript variant III (EGFRvIII) (exons 2-7 deletion), and its expression has been reported to promote cell proliferation, invasion, and angiogenesis [34]. In recent years, the EGFRvIII variant has emerged as one of the preferred candidates for glioblastoma-targeted therapy as it is exclusively present in tumor cells [35, 36]. Moreover, the elevated levels of glial fibrillary acidic protein (GFAP) in blood have been correlated with the size of the tumor, the extent of necrosis, and the intratumor expression [37]. GFAP is the principal component of the cytoskeletal intermediate filaments produced by different cells of the central nervous

system including astrocytes and is currently used as a prevalent marker for the identification of CTCs across gliomas [37–40].

Mutations altering the tumor-suppressing activity of the p53 protein predominantly occur in secondary brain malignancies (90%) compared to the primary lesions (30%) [41]. As an early event, mutations in the TP53 (gene encoding p53 protein) are coupled with concomitant loss of chromosome 17p in grade 2-3 astrocytoma, thus implying its significance in tumorigenesis [42]. In WHO CNS5, the clinicopathological significance of TP53 has been used to identify subtypes of molecularly defined medulloblastoma sonic hedgehog (SHH) activated brain tumors [21]. Overall TP53 gene mutations can attribute gain-of-functions, loss-of-functions, or dominant negative phenotypes, but the impact of these discrete mutational profiles in glioblastoma pathogenesis is still unclear [43–45]. The enhanced activity of the mevalonate (MVA) pathway pro-tumorigenesis enzymes MVA kinase and 3′-hydroxy-3′-methylglutaryl coenzyme A reductase is one of the suggested mechanisms of the p53-mediated glioma etiology [46]. In addition, other effector molecules of the p53 signaling pathway, such as mouse-double minute 2 homolog (MDM2), mouse-double minute 4 homolog MDM4, inhibitors of CDK4 (INK4), ADP-ribosylation factor (ARF), etc., are also known to modulate the p53 tumor suppressor activity [41, 47]. In a recent study involving glioblastoma-patients-derived brain tumor stem cells and orthotopic xenograft models, MDM2 inhibitor (BI-907828) appears as a promising new therapeutic for primary brain tumor patients having wildtype TP53 gene [48].

When compared to other adult-type diffuse gliomas carrying IDH mutations, glioblastoma patients with the wildtype IDH gene possess significantly lower levels of methylation at O^6-methylguanine-DNA-methyltransferase (MGMT) gene promoter [49, 50]. In healthy cells, the MGMT gene codes for the O^6 alkylguanine-DNA-alkyltransferase (AGT) protein involved in maintaining cell physiology and genomic stability by removing the alkylating lesions [51, 52]. The higher levels of AGT protein thus decrease both the risks of carcinogenesis and the chances of deleterious mutations on exposure to therapeutic methylating agents. In contrast, methylation of the MGMT promoter regions in brain malignancies prevents MGMT expression and increases genomic instability by enhancing the efficacy of alkylating agents. In glioblastoma patients, even the extent of MGMT promoter methylation has been shown to impact survival outcomes [53, 54]. Thus, while defining alkylating agent-based therapies in brain tumors, detecting the methylation status of the MGMT promoter region is highly recommended [55]. Furthermore, two specific point mutations (C228T and C250T) in the promoter region of the telomerase reverse transcriptase (pTERT) gene occur at higher frequency in glioblastoma patients (~80%) [56, 57]. These mutations are anticipated to enhance the telomerase enzyme activity through the formation of the TERT-activating GA binding protein (GABP) transcription factor complex, leading to the progressive increase in the length of telomeres essential for the uncontrolled proliferation of tumor cells [56, 58, 59]. In addition, single nucleotide polymorphism (C- allele of rs2853669) variants located in the proximity of TERT mutation hotspots are associated with increased risks of developing glioblastoma and reduced overall survival (OS) [60, 61]. Therefore, MGMT promoter methylation and pTERT mutation status can be used for glioblastoma patients' prognosis [62].

Loss of heterozygosity (LOH) mutations, characterized by the loss of a copy of a gene or group of genes, is also prevalent in brain tumors. Almost 60-80% of primary and secondary high-grade gliomas contain 10q LOH. The most frequently deleted genetic loci on chromosome 10 include 10q23-24, 10q25-qter, and 10q14-p15. Deletion of 10q23-24 loci results in the loss of tumor suppressor gene PTEN (phosphatase activity) along with DMBT1, FGFR2, WDRI1, LGI1, and MIX1 genes that play a critical role in inhibiting P13K/AKT/mTOR pathway involved in cell proliferation. 10q25-qter deletion is associated with the progression of low-grade brain tumors to high-grade [63]. In addition, 22q LOH is also present in 80% of secondary, and 41% of primary brain tumors. Loss of 22q12.3 loci results in the absence of tissue inhibitor of metalloproteinases-3 protein (TIMP-3) protein involved in the inhibition of tumorigenesis and induction of apoptosis [64]. Other commonly occurring LOH mutations in brain malignancies are located on chromosomes 1p, 9p, 17p, and 19q [65]. Thus, identification of the abovementioned LOH through microsatellite and PCR-based assays is very helpful in classifying and designing informed therapeutic strategies for brain tumor patients.

In combination with the aforementioned molecular features and patient characteristics, Alpha Thalassemia/Mental Retardation Syndrome X-linked (ATRX), a transcriptional regulator protein, has been now routinely used to identify biological distinct glioma subclasses. Most of the studies involving glioma and ATRX are correlation-based, and the role of ATRX is not precisely defined. For example, there exists a strong correlation between IDH canonical mutations and ATRX mutation, however, the coexistence of 1p/19q codeletion and ATRX loss has been rarely observed in the clinics [66–68]. Moreover, ATRX mutations have also been correlated to genomic instability conferring telomerase-independent telomere lengthening mechanism termed 'Alternate Lengthening of Telomeres' (ALT) [69–74]. Altogether, each of these brain tumor-specific molecular biomarkers has some advantages and disadvantages, therefore, defining a combination of multiple markers may increase the diagnostic utility of these molecular changes in clinical settings.

BRAIN TUMOR-SPECIFIC BIOMARKERS IN BLOOD

Blood is the most common source of biological samples in liquid biopsy-based oncology studies. It is of greater importance when the size and accessibility of the tumor are the constraints. Identification of blood-based biomarkers using liquid biopsy has been explored extensively in different extra-cerebral cancers such as breast cancer, non-small cell lung cancer, colorectal cancer, etc [75–77]. However, the exploration of brain space for searching blood-based biomarkers is still in its initial developmental stages. Various analytes present in a brain tumor patient's blood, and their utility in defining disease phenotype are depicted in Fig. 2 and are also a very active research domain [78]. In the following sections, specific analytes in the blood used for biomarker identification in brain tumors will be described.

CIRCULATING TUMOR CELLS (CTCS)

CTCs are phenotypically distinct rare subsets of the tumor cell population released by primary or metastatic lesions into the biofluids [79, 80]. The abundance of CTCs in biofluids is very low (<10 cells/mL of biofluid) even in metastatic conditions, and varies drastically between various cancer types [81, 82]. CTCs can be retrieved from a cancer patient's body fluids either as single cells or cell aggregates. CTC clusters have also been reported to contain WBCs, and the presence of CTC-WBC clusters suggests poor prognosis in cancer patients [83, 84]. From the disease pathology perspective, CTCs represent the potential of epithelial tumor cells to metastasize. Moreover, CTCs can attain either stem cell-like or mesenchymal phenotypes upon an epithelial-to-mesenchymal transition (EMT) [85]. At present, the identification and isolation of CTCs from biofluids are mainly based on the presence/absence of specific cell-surface epithelial markers or biophysical properties (size, deformity, etc.) of the CTCs [86]. For instance, an antibody against epithelial cell adhesion molecule (EpCAM) and cytokines are currently being used to enrich and detect CTCs in the FDA-approved CellSearch System (Veridex, Warren, NJ, USA) [80, 87].

CTCs were first identified in common non-CNS cancer types such as breast cancers, prostate cancers, etc., and more recently in

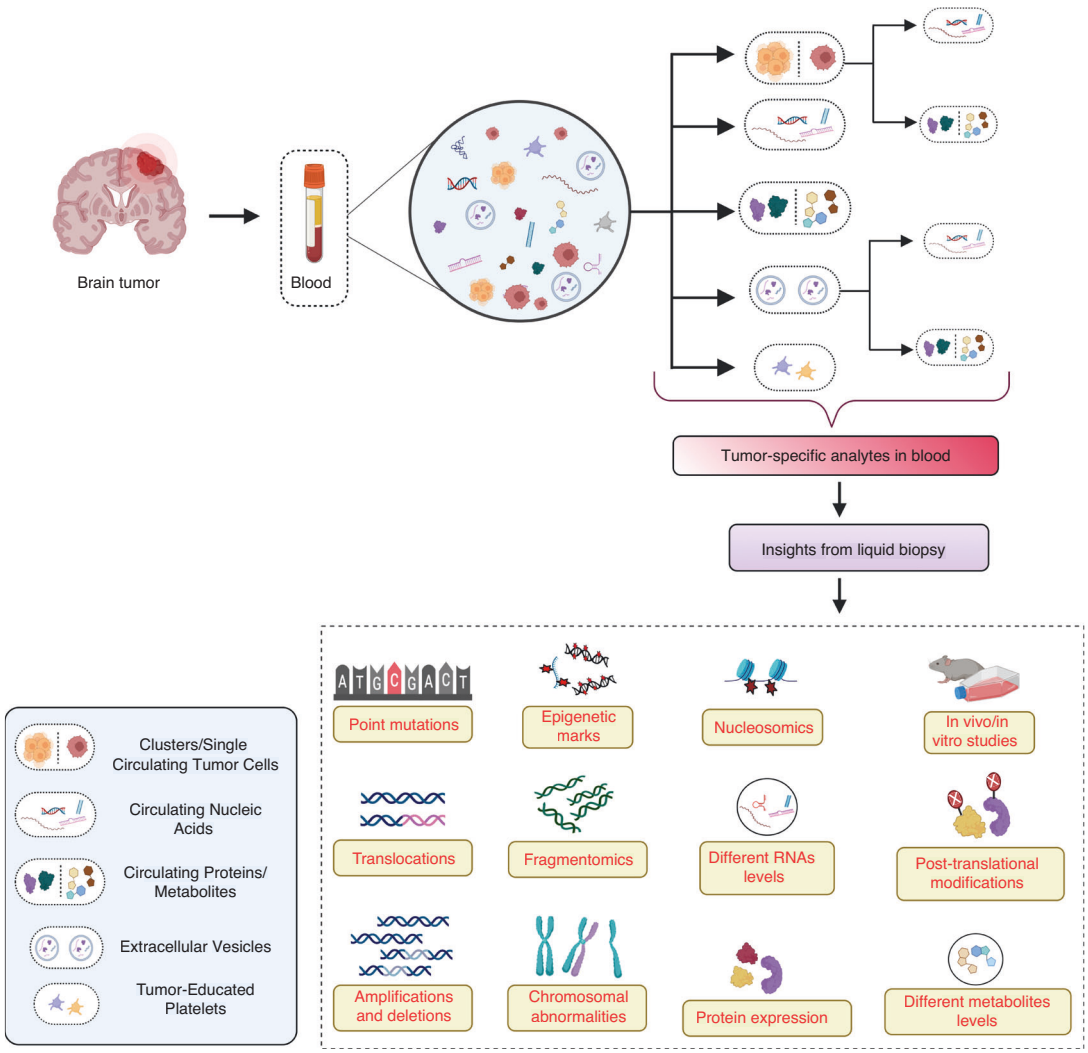

Fig. 2 Insights from the blood of brain tumor patients. Analytes such as nucleic acids (DNA/RNAs), proteins, and metabolites can be either collected as circulating cell-free entities or extracted from circulating tumor cells/extracellular vesicles/tumor-educated platelets. Each of these circulating analytes can be explored for tumor-specific alterations such as different classes of mutations, epigenetic modifications, the fragmentation pattern of DNA, nucleosome patterning, chromosomal aberrations, presence/absence/change in levels of RNAs/proteins/metabolites, post-translational modifications, etc. This figure has been created using BioRender software and a publication license is obtained.

CNS tumors [88, 89]. Since tumor cells from high-grade gliomas including glioblastoma patients preferentially adopt mesenchymal phenotype compared to epithelial, therefore, the conventional methods of CTCs identification using the CellSearch system are not very effective for the detection and enrichment of CTCs originating from brain tumors [90]. Recent studies have shown the presence of glioma CTCs in CSF and blood, suggesting that brain tumor cells are primed to breach BBB and enter systemic circulation [91, 92]. Several noteworthy attempts have been made to detect CTCs in the blood of glioblastoma patients such as the application of antibodies to target GFAP, amplification of the EGFR gene, etc, [39]. In addition, an assay detecting the increased activity of telomerase enzyme exclusively in tumor cells has been devised to identify circulating brain tumor cells. Clinical data suggest that this adenoviral detection-based strategy is capable of distinguishing pseudoprogression from true tumor progression in brain malignancies [93]. Moreover, CTCs have also been detected in the peripheral blood of glioma patients through examination of aneuploidy of chromosome 8 (CEP8-FISH) [94]. A recently developed microfluidic device, the CTC-iChip, selectively depletes leukocytes and efficiently identifies CTCs enriched in the peripheral blood of glioblastoma patients by staining with antibody cocktail referred to as STEAM (SOX2, Tubulin beta-3, EGFR, A2B5, and c-MET) [95].

Proteoglycans are complex molecules that contain a protein core with a sugar side chain. Although yet to undergo clinical testing, proteoglycans have been used to detect CTCs in many cancers. In high-grade gliomas, rVAR2 (recombinant malaria VAR2CSA protein), a ligand for the proteoglycan chondroitin can efficiently detect CTCs in the blood by binding to tumor-specific oncofetal chondroitin sulfate [91]. Glioblastoma's recurrence rate and progression of low-grade gliomas have been suggested to correlate with the presence of CTCs in gliomas, hence making it an attractive target for liquid biopsy-based diagnostic strategies [96]. In addition to the adults, CTCs can also be captured and identified from the blood of pediatric brain tumor patients [97]. The successful capture of viable CTCs from brain tumor patients has enabled us to develop CTCs-derived cell lines, xenograft, and 3D models, which in turn are useful for conducting functional analyses like therapy testing [82, 98–101]. Taken together, the CTCs obtained through liquid biopsy are suitable models to study the CNS tumors-specific molecular alterations, and thus helpful in monitoring tumor progression and defining targeted therapies [102].

CIRCULATING NUCLEIC ACID BIOMARKERS

Circulating cell-free tumor DNA

Cell-free DNA (cfDNA) is typically 180–200 bp long DNA fragments circulating in various biofluids [103–105]. In healthy individuals, cfDNA is mainly the outcome of underlying inflammatory or apoptotic processes [106]. Under physiological conditions, most of these released cfDNA pieces are cleared by phagocytosis, thus maintaining a low level of cfDNA in circulation [85]. By contrast, in cancer patients, there is an accumulation of cfDNA in circulation due to a relatively higher rate of their generation as compared to phagocytic removal. Such cfDNA fragments released by tumor cells are known as circulating cell-free tumor DNA (ctDNA). In advanced solid tumors, the proportion of ctDNA in the whole cfDNA has been correlated with tumor burden [107–110]. Therefore, ctDNA load can be used to efficiently determine the tumor burden in cancer patients as it requires less volume of biofluid compared to that of CTCs [111].

As a biomarker, ctDNA possesses several distinct characteristics. Its short half-life of approximately 1.5 h is advantageous while investigating the dynamic modifications in tumor homeostasis [105, 112]. The tumor-specific mutations harbored by ctDNA provide specificity for the detection of genetic alterations present exclusively in tumor cells [112]. Quantitatively, the ctDNA carrying tumor-specific variations such as copy number variations, chromosomal rearrangements, point mutations, etc., can contribute 0.1–90% fractions of the total circulating cfDNA [105, 113]. Additionally, the detection rate of ctDNA is much higher in metastatic and advanced disease stages compared to localized disease [114–117]. The comparative molecular profiling studies between matched ctDNA and tumor samples from colorectal, non-small cell lung carcinoma, and metastatic breast cancers have shown a high degree of concordance to the clinically significant known mutations [105, 113–115, 118–123]. Furthermore, the abundance of ctDNA has been reported to be negatively correlated with the survival outcomes in various cancer types such as melanoma, ovarian, breast, and colon cancers, etc, [105, 113, 115, 124, 125]. Therefore, ctDNA-based diagnostic assays for brain tumor patients hold a promising future in clinical settings.

In gliomas, the blood-brain barrier limits the potential of ctDNA as an efficient biomarker [112]. This structurally and functionally significant endothelial cell layer prevents the release and detection of ctDNA in circulation. Additionally, the lower frequency of ctDNA detection in CNS malignancies compared to non-CNS tumors has also been partially attributed to its extremely low concentrations in body fluids [115, 116]. For instance, in a study cohort of 419 primary brain tumor patients, ctDNA was detected only in 60% of the total 222 high-grade glioma cases [126]. Several investigations have also demonstrated the effectiveness of ctDNA in identifying brain tumor-specific molecular biomarker gene alterations, of which IDH mutations were detected at a higher frequency [127–134]. For example, in a study involving 157 adult glioma patients' plasma ctDNA, TERT promoter mutations were identified with a specificity of 90% and sensitivity of 62.5% [135]. Currently, fewer genes with mutation frequency greater than 5% in brain tumors, and the deep sequencing cost involved in their identification are the two major constraints associated with the genetic variations-based diagnosis. In contrast, genome-wide epigenetic landscapes captured on ctDNA represent the real-time dynamic changes linked to the disease phenotype. The most commonly studied epigenetics modification on DNA concerning brain tumors is methylation. The ctDNA of glioma patients has been reported to contain significantly lower levels of Alu methylation than the control group which includes benign intracranial tumor patients and healthy individuals [136]. This is significant given that Alu is the abundant interspersed element that occupies about 10% of the genomes. Several studies implementing an antibody-based capture method to isolate cfDNA have also reported a differential methylation pattern in different cancer types [137, 138]. Moreover, in an independent study, the plasma-cfDNA methylome profile has been exhibited to detect and discriminate intracranial tumors [139]. In recent years, the diagnostic significance of another epigenetic modification i.e., DNA hydroxymethylation has also been realized [140–142]. While its overall abundance is ~10% compared to methylation marks, the tissue-specificity it provides is of special interest from a liquid biopsy perspective [143, 144]. Moreover, in contrast to other tumor types, brain tumors possess a higher amount of hydroxymethylation marks and have also been correlated with the survival outcomes of glioblastoma patients [145]. Thus, blood-based liquid biopsy assays using cfDNA, especially epigenetic features, are very critical in defining the diagnosis and treatments of patients with brain malignancies, and the development of more sensitive and accurate ctDNA detection methods will help to achieve the optimum potential of these approaches.

Circulating cell-free RNA

Tumor cells also release cell-free RNA (cfRNA) in circulation [146–148]. In patients with low tumor shedding, the signals from tumor-specific overexpressed transcripts in the blood are helpful in detecting cancer. Unlike cfDNA which is contributed to the blood by necrotic cells, the cfRNA can also be added to the systemic circulation through exosome-mediated signaling by living cells. Therefore, cfDNA and cfRNA fractions in the blood due to distinct cell populations wide open the opportunities for cancer detection through their combined assessment [149]. On the technical front, several optimized protocols are currently available to capture unstable cfRNA from blood alone or in combination with cfDNA [150]. For instance, low centrifugation protocols give a higher yield of cfRNA, and the BD Vacutainer K2EDTA (EDTA) tubes are best suited for the simultaneous enrichment of cfDNA and cfRNA.

Most of the previous cfRNA studies are focused either on highly stable and abundant circulating micro RNAs (miRNAs) in plasma or a few of the already reported cancer-associated messenger RNA (mRNAs) [149, 151, 152]. The hypothesis-driven cell-free mRNA studies can only identify genetic alterations or expression changes of previously characterized tumor-associated genes but could miss a substantial number of potential biomarkers. Also, detecting small expression changes linked to early disease states in the presence of abundant circulating transcripts of red blood cells, immune cells, and platelets is difficult [146, 148, 149, 153]. Therefore, the knowledge of circulating cfRNA baseline concentrations in disease-free conditions, and its cancer-subtype specificity is very crucial [154]. Interestingly, transcriptome-wide cfRNA characterization studies in brain tumors are yet to be reported. Furthermore, RNA methylation as a regulatory biological mechanism has also gained extensive momentum in recent years, but the utility of cfRNA methylation has not been fully explored. It is possible that methylation of cfRNA obtained through liquid biopsy could uncover novel cancer-specific alterations. Altogether, cell-free mRNA profiling represents transcriptionally active living tumor cells and proposes a promising approach for detecting, localizing, and identifying biomarkers in different cancers [149].

miRNAs are usually 18-25 nucleotides long non-coding RNAs of endogenous origin, involved in the post-transcriptional regulation of gene expression through translational repression or mRNA destabilization, and can function either as an oncogene or tumor suppressor [155]. In healthy tissues, miRNAs regulate diverse biological functions such as cell proliferation, development, metabolism, differentiation, and intercellular communication. Therefore, perturbation in miRNA expression can significantly impact cell growth, differentiation, and tumor cell apoptosis [156]. Several studies have shown the role of circulating miRNAs in the initiation and progression of different cancer types, including brain tumors [157, 158]. For instance, the levels of miR21,

miR115b, miR-23a, and miR-146b species in pediatric juvenile pilocytic astrocytoma patients can predict the tumor nodule sizes and the response to therapies with 100% specificity and sensitivity of 86% [159]. A comparative study between matched tumor and blood samples of pediatric astrocytoma patients has revealed disease-specific miR-130 upregulation and downregulation of miR-145 and miR-335 [160]. In other independent studies, significantly higher levels of miR-21 and miR-15b, and lower quantities of miR-128 and miR-342-3p have been observed in grade 4 glioma patients [161, 162]. Moreover, high-grade gliomas can be distinguished from low-grade based on differential enrichment of miR-29, miR-125b, miR-497, and miR-16 in the blood [158, 163]. Also, the elevated levels of the miR-221/222 family of miRNAs in the plasma are negatively correlated with the overall survival of glioma patients [164]. Qu et al. performed a meta-analysis to gauge the diagnostic potential of miRNAs as a glioma biomarker and identified miR-21 as the most significant and reproducible miRNA [165]. In conclusion, the inconsistencies and reproducibility of miRNA repertoire in cancer patients limit its potential as a biomarker.

Through tumor biopsy studies, the role of non-coding RNAs and tRNA-derived fragments (tRFs) in various cancer types has been known for a long time [166–168]. For instance, higher expression of RNAs such as nlr-20-2IHZI73Z, tRF-18-HRE9XFD2, and tRF-22-WB86N7O52 are positively correlated with IDH mutant pathology (astrocytoma and oligodendroglioma) and confers improved survival of glioma patients [169]. However, the exploration of these rare RNA species as biomarkers using liquid biopsy approaches is still limited due to the lack of sensitive detection methods. Taken together, addressing the technical and analytical inaccuracies associated with the sample type, the platform used for measurements and data normalization will allow the usage of cfRNA species as a prognostic biomarker with higher confidence [170].

CIRCULATING PROTEIN BIOMARKERS

The correlation of tumor pathology with the expression of proteins and peptides has provided a new search space for novel biomarkers and therapeutic target identification [171]. Increased secretion of proteins in cancer patients may result in elevated levels of circulating proteins (CPs) in various biological fluids, including the blood [15]. The high degree of quantitative disparity in the concentrations of abundant proteins and CPs secreted in the blood limits the detection and clinical usefulness of CPs [15, 171]. Currently, most clinical centers routinely measure tumor protein markers such as PSA, CEA, CA15-3, CA125, CA19-9, CYFRA21-1, S100, NSE, ProGRP, sHER2, SCCA, HE-4, CA72-4, etc., for various cancer types. The proteomics profiling of cancer patients has suggested a few potential circulating tumor protein biomarkers including circulating nucleosomes, thymidine kinases, immunogenic cell death markers, soluble receptors of advanced glycation end products (sRAGE), DNAse activity and high-mobility group box 1 (HMGB1) [172, 173].

Previously, several attempts had been made to identify brain malignancies-specific circulating proteins. Kikuchi et al. were the first to report blood-based protein biomarkers in brain tumors. They showed higher levels of immunosuppressive acidic proteins (IAP) like alpha-1 antitrypsin and alpha-1 acidic glycoprotein, and endothelial cells-derived thrombomodulin and glycoprotein fibronectin in the glioma patients compared to non-glioma and healthy individuals [174]. Besides, the levels of circulating angiogenesis-related proteins such as vascular endothelial growth factor (VEGF), soluble EEGP receptor- (sVEGFR-1), primary fibroblast growth factor (FGF-2), etc., are significantly elevated in different glioma grades [175–177]. Other potential circulating protein biomarkers of brain tumors and metastases include tumor cells' extracellular matrix remodeling proteins such as tissue inhibitors of metalloproteinases (TIMPs) and matrix metalloproteinases (MMPs). These circulating proteins help to classify tumors based on their staging [178]. Also, the plasma levels of interleukins 2(IL-2) and its receptor, neural cell adhesion molecule (NCAM), chitinase-3-like protein 1 (CHI3L1/YKL-40), tumor necrosis factor-alpha (TNFα), neuropeptide Y (NPY) and tumor necrosis factor beta (TNFβ) have diagnostic significance in brain malignancies [179–181]. Amongst these, YKL-40 appears as a promising grade 4 glioma prognostic biomarker, inversely associated with overall survival [180, 182]. Plasminogen activator inhibitor-1 (PAI-1) is another marker of interest, as its serum levels are negatively correlated to the progression-free survival (PFS) of brain tumor patients [183]. In addition to early prognosis, CPs can also be implemented to oversee the effectiveness of cancer therapies. For instance, brain tumor patients treated with irinotecan and bevacizumab have decreased plasma levels of VEGF protein (measured after 8 weeks) with an improvement in OS and PFS [184]. Overall, the proteomics studies inferring potential circulating tumor-specific protein biomarkers in blood have contributed substantially to our understanding, but their translational relevance in clinics needs further exploration.

CIRCULATING METABOLIC BIOMARKERS

Similar to proteome studies, liquid biopsy-based metabolome profiling is also used to identify and quantify different classes of compounds in the biofluids of cancer patients [185]. The circulating metabolites include amino acids, carbohydrates, nucleosides, nucleotides, lipids, vitamins, fatty acids, etc., and are mostly involved in the maintenance of cellular architecture and signal transduction through secondary messenger molecules [186, 187]. The metabolic reprogramming in brain tumors is known to compromise both catabolic and anabolic processes and ultimately signaling pathways involved in various cellular effector functions, leading to the development of tumors and resistance to treatment in the long run [188, 189]. For instance, in plasma metabolic profiling of 159 high-grade brain tumor samples, methionine, arginine, and kynurenine were found to be significantly associated with survival outcomes. While methionine and arginine are positive prognostic markers, kynurenine (intermediate of tryptophan metabolism) is associated with poor survival outcomes [190]. In another study, the levels of metabolites uridine and ornithine were found to be significantly different between low and higher-grade glioma patients [191]. Moreover, as compared to healthy controls, the serum of primary grade 4 patients was observed to contain a higher concentration of antioxidant properties compounds i.e., α- and γ-tocopherol, proposing them as the latent markers of high-grade brain tumor progression [192].

Because of the higher amounts of lipids in the brain, and their involvement in critical biological functions like lipid membrane formation, energy metabolism, and signal transduction, lipidomics has emerged as one of the specialized arms of brain tumor metabolomics studies [193]. However, unlike other neurological disorders, studies involving a liquid biopsy approach toward identifying and characterizing brain tumor-specific lipid biomarkers are very few. For example, Zhou et al. have recently identified 11 plasma lipids as candidate diagnostic biomarkers of malignant brain tumors [194]. Moreover, a landmark lipid profiling study involving 99 glioblastoma tumor tissues has identified >500 unique significantly different lipid species, thus proposing new avenues of lipid biomarker identifications [195, 196]. In conclusion, recent advancements in metabolite detection strategies and the integration of prediction models into lipid biomarker screening workflows offer unprecedented biomarker screening opportunities [197].

EXTRACELLULAR VESICLE (EV)

EV is a lipid-bilayer-bound organelle released into the extracellular space by both tumor and healthy cells. Under physiological conditions, the rate of EV generation of tumor cells is much higher than that of normal cells [198]. Based on the size, morphology, and method of generation, EVs are broadly classified as exosomes, microvesicles, and apoptotic bodies [199, 200]. As an analyte, EV offers several advantages over other liquid biopsy-derived substrates [14]. For instance, EV contains a variety of biomolecules such as DNA, coding and non-coding RNA, miRNA, lipids, proteins, metabolites, etc., and is present in almost every biofluid including blood, CSF, semen, urine, saliva, and ascites [201–204]. The biological stability of EV allows its storage without degradation at different temperatures [201]. The contents of EV give a more precise representation of the altered biological processes than the ctDNA arising from apoptotic bodies because their parental cells are living [205]. Moreover, EV can manifest surface markers specific to the parental cell of origin, thus helping to predict organ-specific metastases [206]. Finally, the frequency of detecting cancer mutations on DNA enclosed inside EV is much higher than on ctDNA [207, 208].

In cancer, extracellular vesicles (EVs) are suggested to facilitate tumor progression and propagation by increasing cell proliferation, extracellular matrix remodeling, promoting angiogenesis, modulating immune responses, and eventually, metastasis [209–213]. Quantitatively, EVs derived from tumor cells have been shown to correlate with prognosis in various cancer types [214, 215]. Tumor origin exosome's DNA has been shown to harbor KRAS, EGFR, BRAF, and TP53 mutations in pancreatic, non-small cell lung carcinoma, melanoma, and CRC, respectively [216–218]. Several studies have also used EVs collected using liquid biopsy approaches from brain tumor patients as a source of the analyte. For example, specific EGFR gene mutations were detected in DNA isolated from serum-derived EVs in glioblastoma patients [210]. While the mRNA, microRNAs (miR-320 and miR-574-3p), and noncoding RNA (RNU6-1) expression patterns in glioblastoma patients were found to be distinct as compared to the normal healthy individuals, some studies have also shown a panel of surface proteins of EVs as distinguishing feature among brain tumor subtypes [219]. Glioma patients with a higher quantity of tumor-associated molecules such as podoplanin and EGFRvIII in their circulating microvesicles are more prone to chemoradiation therapy failures (radiotherapy plus temozolomide) [220]. In general, the tumor recurrence post-resection is also coupled with the increase in EV concentration in the plasma of patients with brain malignancies [221]. Furthermore, the high-grade gliomas-derived exosomes have been shown to stimulate tumor growth and neoangiogenesis, implying the potential of EVs to mediate metastasis [222]. Similarly, the proteomic profiling of serum-derived EVs from histologically defined medulloblastoma patients indicated their potential role in cancer cell proliferation and migration and suggested tumor-repressor activity of transcription factor hepatocyte nuclear factor 4 alpha (HNF4A) [223]. Thus, the EVs-based liquid biopsy approach provides an opportunity to explore a variety of biomolecules of tumor origin for biomarker identification and monitor therapeutic responses in CNS malignancies [14]. However, further investigation of EVs-based biomarkers is required in large and diverse cohorts to increase the sensitivity, specificity, and reproducibility before its routine clinical application can be considered.

TUMOR-EDUCATED PLATELETS (TEPS)

Recently, TEPs have been added as a cancer biomarker resource to the blood-based liquid biopsies [2]. An increase in platelet counts (thrombocytosis) is associated with poor survival in various malignant tumors, including mesothelioma, melanoma, breast, lung, and ovarian cancers. A retrospective examination of platelet concentrations in 122 glioma patients predominantly glioblastoma cases (88/122) at longitudinal time points (pre-operative, pre-RT, pre-adjuvant temozolomide (TMZ), and after 2 cycles TMZ) showed a correlation of changes in platelet concentrations during therapy with the survival outcomes [224]. Several previous studies have also shown the prognostic significance of platelet aggregation in brain malignancies. During tumorigenesis and metastasis, the platelets from the blood function as the localized and systemic responders, and get exposed to tumor cells leading to their altered behavior [225–228]. Besides education through platelet-cancer cell direct interaction, platelets can also uptake RNA and proteins released by tumor cells [227, 229–234]. In two independent studies, glioma and other cancer subtypes patients' platelets RNA profiles have been detected to be altered [229, 235]. Education of platelets in different cancer types is reflected in terms of distinct differentially spliced RNA profiles as compared to healthy individuals [230]. Using these TEPs spliced RNA profiles, the origin of different types of primary cancers, which include pancreatic cancer, breast cancer, colorectal cancer, non-small cell lung carcinoma [NSCLC], and localized high-grade brain tumors, has been successfully identified with an accuracy of 71% [230]. In a recent study, spliced RNA profiles from TEPs were shown to distinguish glioblastoma patients from healthy individuals, brain metastases, and inflammatory processes like multiple sclerosis with an average precision of 85%. Moreover, the genetic arrangement in TEPs (e.g., EGFRvIII rearrangement) can act as an onco-signature for localized high-grade brain tumors [230, 236]. Also, TEPs-specific RNA signatures of glioblastoma patients were observed to be correlated with the tumor volume and its recurrence, which in turn may help in differentiating true tumor progression from false positive advancement [237]. Hence, TEPs offer alternative blood-based diagnostic and monitoring opportunities in brain tumor patients.

BRAIN TUMOR-SPECIFIC BIOMARKERS IN CSF

In addition to peripheral blood, CSF-based liquid biopsy is critical in the diagnosis, monitoring, and biomarker identification of CNS tumors [238]. The presence of BBB restricts the movement of CNS tumor-specific analytes in the systemic circulation, thus limiting their detection in cancer patient serum/plasma. In contrast, CSF that comes in direct contact with primary CNS tumors are enriched in tumor-originated CTCs, nucleic acids, proteins, metabolites, etc, [115, 239, 240]. A major limitation of CSF-based liquid biopsy procedures is the requirement of lumbar puncturing to collect CSF from cancer patients [14].

Recent studies have revealed the presence of CTCs in CSF of adult as well as pediatric brain tumor patients. Using antibodies against cytoplasmic GFAP protein, CTCs have been successfully isolated from CSF of various types of pediatric brain tumor patients, and the overall detection has been observed to be more efficient than in systemic circulation [91, 92, 97, 241, 242]. Single-cell RNA sequencing of CTCs sequestered from CSF of lung adenocarcinoma leptomeningeal metastases has led to the identification of metastatic-CTCs signature genes enriched for cell adhesion molecules and metabolic pathway categories [243]. Compared to the reference samples, CSF of HER2+ leptomeningeal metastasis patients possess enhanced levels of CTCs, and these changes have been observed 2-3 months before they appear on magnetic resonance imaging (MRI) or CSF cytology. Thus, the enumeration of CSF CTCs may offer a more dynamic and quantitative assessment of tumor burden, leading to successful CNS tumor diagnosis [244].

As compared to plasma, there is a 10-fold enrichment of tumor-derived DNA in CSF (CSF-tDNA), and its detection partially depends on the tumor location [239, 245]. Cancer-specific mutations including single nucleotide variants of NRAS, BRAF, EGFR, AKT1, and KRAS have been identified in CSF-derived cfDNA

of primary and metastatic brain tumor patients [246]. In a cohort of 35 patients, tumor-specific mutational profiling of cfDNA from CSF successfully identified different types of CNS malignancies in 74% of cases [239]. In contrast to low-grade lesions, more mutations were observed in high-grade brain tumor lesions probably due to a higher mutational burden. Hence, the possibility of detecting cancer-related mutations in high-grade lesions is more as compared to that of low-grade lesions. In another study, matched tissue, blood, and CSF from 57 brain tumor patients were collected and analyzed. In 82.5% of these CSF-tDNA samples, at least one tumor-specific mutation was identified, which is concordant with those observed in the primary tumors. In brainstem gliomas that reside close to the CSF reservoir, a higher percentage of alterations in CSF-tDNA has been detected [246]. The utility of CSF-tDNA has also been studied concerning response to therapies and tumor evolution [3, 247]. For instance, a decrease in the levels of CSF-tDNA after resection was observed, which corroborated with the radiographic evidence in response to chemotherapy. The CSF-tDNA level increases again with increasing radiographic progression. Moreover, sequential biopsies of CSF are very helpful in understanding tumor evolution [3]. Highly diverse mutational profiles of the initial CSF-tDNA samples were observed especially within genes coding for growth factors signaling pathways [247]. Missense and amplification mutations of EGFR genes were observed in the initial sampling, whereas the platelet-derived growth factor receptor alpha (PDFRA) gene mutations appeared in subsequent CSF sampling time points without any alteration in EGFR. These outcomes thus suggest the partial concordance of mutations detected between sequential biopsies over time [248–253]. Additionally, CSF-tDNA has also been used to identify copy number variations in glioma patients [254, 255]. Somatic copy number variations in CSF-tDNA have been observed only in a subset of glioma patients [256]. Furthermore, during tumorigenesis, CSF-tDNA from matched CSF and tumor samples have also been used to study alteration in the methylation patterns of promoter regions of MGMT, THBS1, TIMP-3, TERT, and p-16 genes [127, 129, 257–259].

Like CSF-tDNA, cfRNA in CSF can also be used as one of the detection modalities for cancer. Most commonly the cfRNA species are encapsulated within extracellular vesicles such as exosomes [260–262]. The ability to detect the fusion genes is one of the prime benefits associated with these RNA-based approaches in liquid biopsy [238]. For example, the fusion gene KIAA: BRAF was identified in pilocytic astrocytoma by profiling cfRNA in CSF [263]. In addition, miRNAs isolated from CSF also provide crucial information about various CNS malignancies. While primary diffuse B-cell lymphomas of CNS are characterized by the presence of miR-21, miR-19, and miR-92a, glioma patients showed overexpression of miR-15b and miR-21 [162, 264]. The integrated analysis of miR-15b and miR-21 expression has the potential to differentiate glioma patients from primary CNS lymphomas, leptomeningeal carcinomas, and brain metastasis [162]. In grade 4 glioma patients, miR-10b and miR-21 are present at higher levels in the CSF [265].

CNS tumors also release proteins into the CSF and can be detected by mass spectrometry and ELISA [238]. In contrast to cfDNA or cfRNA, identification of protein biomarkers specific to malignancies is difficult because the alterations in the baseline levels of proteins in CSF may be either because of disease or an outcome of the inflammation or unrelated sufferings [238]. Intracranial germ cell malignancies are the most significant example where specific CSF proteins have an impact on CNS tumor care [266]. For instance, elevated levels of beta human chorionic gonadotropin (bHCG) and alpha-fetoprotein (AFP) proteins have been associated with intracranial germ cell tumors [267]. Levels of both these proteins have been currently used to diagnose a tumor, monitor response to therapy, and predict recurrence. In independent studies, 19 upregulated proteins were identified reproducibly in different types of CNS malignancies [268, 269]. Further studies showed that elevated levels of four peptides (alpha- 1-antichymotrypsin, osteopontin, transthyretin, and the N-terminal residue of albumin) in the CSF of glioma patients, and the reduced levels of prostaglandin D2 synthase in the CSF of pediatric medulloblastoma patients can distinguish these disease pathologies from healthy individuals [270, 271]. Moreover, compared to control patients, CSF of glioma patients has also been reported to be differentially enriched with metabolites such as lactic acid, malic acid, succinate, phosphoenolpyruvate, etc., in an IDH-stratified manner [272]. Taken together, CSF is a highly significant and very promising biofluid for biomarkers identification, therapy monitoring, and tumor recurrence prediction, especially in the case of CNS tumors.

BRAIN TUMOR-SPECIFIC BIOMARKERS IN OTHER BIOFLUIDS

In addition to blood and CSF, vitreous fluid and urine samples have also been used as a source of analytes to determine brain tumor-specific biomarkers [85]. For example, vitreous fluid has been used for the diagnosis of lymphoid malignancies of the brain i.e., primary central nervous system lymphoma (PCNSL). The spread of PCNSL to the retina and vitreous of the eyes is known as vitreoretinal lymphoma (VRL). For PCNSL diagnostic purposes, vitreous fluid offers easy collection and decreased invasiveness compared to other liquid biopsy approaches for brain tumors. Targeted next-generation sequencing on matched vitreous and brain samples has led to the discovery of unique sets of common and distinct genetic alterations both in PCNSL and VRL patients, which in turn suggests the common origin of both the lymphomas. Therefore, vitreous liquid biopsy can be used for the treatment of PCNSL/VRL through accurate diagnosis [273]. In addition, the presence of tumor cells and interleukins ratio (IL10/IL6) ratio of more than 1 in the vitreous fluid also indicate the VRL condition [274]. The mutational profiling of ctDNA from vitreous fluid detected specific mutations in the signaling protein MYD88 in 69% of VRL patients [275]. Besides, the targeted sequencing of 16 genes of VRL patients, revealed copy number variations in CDKN2A or PTEN, and at least one-point mutations impacting the MYD88 gene [276].

Urine has been considered an ideal biomarker resource for early disease detection, as it gathers all the systemic changes within the body. As compared to healthy individuals, significantly elevated levels of MMP-2, MMP-9, neutrophil gelatinase-associated proteins (NGAP), and vascular endothelial growth factor (VEGF) have been reported in the urine of brain tumor patients [277]. These identified urinary biomarkers were used to design a panel containing MMPs and associated proteins, capable of predicting new brain tumors in the early stages of development and monitoring brain malignancies after treatment [277]. The proteomic analysis of glioma patients has revealed a considerable change in the level of twenty-seven urinary proteins after tumor resection, and several of them have been previously correlated with glioma [278]. Functions like angiogenesis and autophagy, which have been formerly associated with glioma development were significantly enriched in these identified proteins. After the targeted proteomics validation, another glioma detection biomarker panel of six proteins, including AHSG, LEG1, CALR, TSP4, MDHM, and AACT, was designed [278]. Also, a diagnostic model has recently been developed based on the expression pattern of specific urinary microRNAs. This model can distinguish CNS tumor patients from noncancerous patients with a specificity of 97% and sensitivity of 100% [279]. On the whole, additional studies need to be conducted before urine and vitreous fluid along with other alternate biofluids can be used as the preferred resource for biomarkers identification, and monitoring disease response and therapeutic efficacy.

CLINICAL APPLICATIONS, CHALLENGES AND TECHNOLOGICAL ADVANCES IN LIQUID BIOPSY

The liquid biopsy approach has opened the window of opportunities in brain tumor patient management. Similar to other cancer types, in clinical settings, this strategy can be implemented for brain tumor early detection, staging, identification of treatment targets, defining personalized therapeutic strategies, longitudinal monitoring of disease progression and therapy in advanced stages, and understanding resistance mechanisms [12]. However, most of these applications in brain malignancies are restricted due to the presence of BBB and the inherent limitations associated with each of the analytes [19]. Currently, liquid biopsy research in the context of brain tumors is mainly focused on increasing the availability of analytes in circulation and developing sensitive and specific analytical tools.

Zhu et al. used focused ultrasound (FUS) to transiently disrupt BBB, and enhance the release of brain tumor biomarkers in blood circulation. The presence of tumor-derived eGFP mRNA in blood suggested that FUS-induced BBB disruption could augment brain-blood trafficking. In another advanced study, spatial precision to create temporary brain openings was achieved using low-intensity MR in conjunction with FUS [280]. However, these transient openings allow bidirectional movement of biological entities, leading to the access of systemic circulation to the brain. Therefore, further explorations are required to achieve the regulated unidirectional release of biomarkers from the brain to the blood-only [18, 281].

In the last few years, the evolving field of liquid biopsy has witnessed unprecedented advances toward reducing technical and biological variabilities, leading to the development of more sensitive and specific analytical strategies. Collectively, these efforts aim to achieve an analyte of interest in the purest form and extract maximum insights about the tumor by minimizing the effect of confounding or background signals. Lately, integrated platforms for CTC enrichment, detection, and characterization have been developed to prevent sample loss. The in vivo detection or capture of CTCs using a capture wire, intravascular aphaeretic system, and acoustics have the potential to overcome the CTC's low count problem [282–284]. Moreover, epithelial ImmunoSPOT (EPISPOT) and its microfluidic version i.e., EPIDROP have enabled the investigations of CTCs at a single-cell resolution, thus defining tumor cell heterogeneity from biofluids of cancer patients [285, 286]. Several targeted and untargeted approaches for the precise detection of ctDNA have also been developed namely BEAMing Safe-Sequencing System (BEAMing Safe-SeqS), Cancer Personalized Profiling by deep sequencing (CAPP-Seq), Tagged Amplicon deep Sequencing (TamSeq), and digital PCR. All these ultrasensitive technologies can provide insights into tumor-related single nucleotide variations, copy number variations, changes in methylation patterns, etc [287–290]. Furthermore, recent technological advances can also filter out ctDNA mutations contributed by factors such as age, leukocytes, cfDNA-sequencing, etc. from those of tumor origin [291–293].

Unlike cfDNA reproducible isolation protocols, cfRNA sequestering and recovery from biofluids are challenging. Even from the same biofluid aliquot, different RNA isolation methods result in the enrichment of distinct RNA species. With the commercial availability of spike-ins and control libraries, the technical variations due to PCR amplifications and batch effects can be minimized now [294]. In addition, the application of cross-validation and model-simplification strategies prevents the over-fitting problem of cfRNA-based ML biomarker detection methods. Most of the efforts in cfRNA research are focused on extracting pure cfRNA fractions from EVs and lipoprotein complexes, enrichment of poly-A tail lacking distinct coding and non-coding cfRNA fragments, preventing library preparation biases due to cfRNA secondary structure and enzyme affinity, and studying differential epigenomic profiles of cfRNA [149, 294]. The lack of FDA-approved cfRNA-based tests in clinics defines both the experimental difficulties as well as immense possibilities within this research arena.

In a coherent approach, multimodal assays defining protein/metabolites and transcript composition have also been developed. For instance, advanced techniques like cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) and proximity extension assays (PEAs) can identify proteins and transcripts from CTCs simultaneously [295, 296]. Also, cleavage under targets and tagmentation (scCUT & Tag) and chromatin accessibility assay for transposase-accessible chromatin (ATAC) with select antigen profiling by sequencing (ASAP–seq) can provide insights about protein levels and chromatin accessibility in CTCs concurrently [297, 298]. Moreover, circulating proteins/metabolites in biofluids can be studied using various mass spectrometry-based techniques such as MALDI-TOF, SELDI-TOF, etc. Alternatively, the targeted approaches using antibodies and aptamers (short peptide sequence) can also be used to identify the level of known circulating proteins/metabolites biomarkers [299]. Cytometry by time-of-flight (CyTOF) that uses non-biological metal isotopes with precise mass spectrometry parameters can be used to quantify cell surface and intracellular components of tumor cells [300]. Single CTC proteome can also be studied using unique mass bar-code for multiple samples (18 samples at a time) by the technique known as isobaric labeling [301]. Plasma proteomics-specific advancements and their application to precision medicine have been covered in detail in a series of recent articles [302, 303].

TEP-derived RNA panels have also been frequently used for biomarker screening purposes in several cancer types. However, minimizing the contribution of potential contaminants such as erythrocytes, leukocytes and residual plasma components which include EVs, cfDNA, etc. is essential to prevent the activation of platelets during pure sample preparation, storage, and transport, and obtain TEP-specific RNA profiles [304]. The recently developed pan-cancer particle swarm optimization (PSO)-enhanced thromboSeq algorithm trained on TEP-derived RNA profiles can detect 18 different cancer types with high (99%) specificity, but for various non-cancer diseases its specificity is low (78%) [305]. Also, this algorithm and TEP RNA profiles can be used to identify the tumor site of origin. However, before bringing the thromboSeq test to clinics, independent validation studies at population strata are required.

Exosomes promote tumor development and progression by facilitating the intercellular transfer of bioactive molecules from parent cells with altered pathological states to recipient cells. Therefore, examining cancerous exosome analytes provides new opportunities for biomarker identification. While the number of exosomes in circulation is very large compared to CTCs and ctDNA, identifying and isolating cancerous exosomes and detecting different analytes they contain is challenging. In a recent review article, Yu et al. present a detailed description of the technological advances in exosome separation and analyte detection techniques in detail [306]. However, studies discussing the translational relevance of exosomes originating from brain tumors are very few and need further investigation [307, 308].

In conclusion, overcoming the limitations of the availability of brain-tumor-derived analytes in circulation and the development of ultrasensitive detection and characterization techniques will allow the successful implementation of liquid biopsy clinical applications in the context of brain malignancies.

LIQUID BIOPSY-BASED BRAIN-TUMOR-SPECIFIC CLINICAL TRIALS

In contrast to other cancer types, brain tumor clinical studies are more challenging, and there is a lack of participation. Additionally, issues like brain tumor subtype, tumor size, tumor stage, the

Table 2. A list of clinical trials involving glioblastoma patients and their present status.

Identifier	Study description	Status	Study Type	Biofluid	Biomarker
NCT04776980	Multimodality MRI and liquid biopsy in high-grade glioma patients	Withdrawn	Interventional	Blood	ctDNA
NCT05695976	GRETeL: Tumor response to standard radiotherapy and TMZ therapy in IDH wildtype malignant glioma patients	Not yet recruiting	Observational	Plasma	ctDNA
NCT05383872	Blood-Brain Barrier Disruption (BBBD) for liquid biopsy in suspected glioblastoma patients (based on pre-operative imaging scans)	Recruiting	Interventional	Blood	ctDNA
NCT04869449	Neuro-pharmacological study of ketoconazole for high-grade gliomas	Recruiting	Interventional	CSF	Metabolite
NCT04825275	Neuro-pharmacological properties of repurposed posaconazole in high-grade gliomas	Recruiting	Interventional	CSF	Metabolite
NCT03355794	A study of ribociclib and everolimus following radiation therapy in children with newly diagnosed non-biopsied diffuse pontine gliomas (DIPG) and RB+ Biopsied DIPG and high-grade gliomas	Completed	Interventional	Blood	Metabolite
NCT05281731	Sonobiopsy for noninvasive and sensitive detection of intracranial lesions with imaging characteristics of glioblastoma	Recruiting	Interventional	Blood	ctDNA
NCT05236036	Mycophenolate mofetil in combination with Standard of care for the treatment of glioblastoma and other high-grade glioma patients	Recruiting	Interventional	Plasma	Metabolite
NCT03389230	Memory-enriched T cells in treating patients with recurrent or refractory grade 3-4 glioma	Recruiting	Interventional	Tumor cyst fluid, Blood, and CSF	Metabolite
NCT01234740	Bafetinib in treating patients with recurrent high-grade glioma or brain metastases	Completed	Interventional	Plasma	Metabolite
NCT04214392	Chimeric Antigen Receptor (CAR) T Cells With a chlorotoxin tumor-targeting domain for the treatment of MMP2+ recurrent or progressive grade 4 gliomas	Recruiting	Interventional	Tumor cyst fluid, Blood, and CSF	Metabolite
NCT01177397	Study to assess safety, pharmacokinetics, and efficacy of oral CC-223 for patients with advanced solid tumors, non-hodgkin lymphoma or multiple myeloma	Completed	Interventional	Blood and Urine	Metabolite
NCT03980249	Anti-cancer effects of carvedilol with standard treatment in histologically or cytologically confirmed grade 4 glioblastoma and response of peripheral glioma circulating tumor cells	Withdrawn	Interventional	Blood	CTC
NCT03861598	Carvedilol with chemotherapy in second line grade 4 gliomas and response of circulating tumor cells	Terminated	Interventional	Blood	CTC
NCT03115138	Evaluation of circulating tumor DNA as a theranostic marker in the management of grade 4 glial tumor patients (Bi-GLAM)	Terminated	Interventional	Blood	ctDNA
NCT03990285	18F-Fluciclovine (Axumin) in post-treatment high-grade gliomas	Completed	Interventional	Blood	ctDNA

extent of BBB disruption, inherent sensitivity, specificity, and clinical utility associated with liquid biopsy markers, and biofluid used for analyte collection greatly impact the study design. Therefore, these confounding factors need to be addressed with precision. Currently, clinical trials involving liquid biopsy and brain tumors are very few, this count goes further down for glioblastoma cases. Various liquid biopsy-based clinical trials involving glioblastoma patients in the USA are listed in Table 2. Detailed description for each of these clinical trials is available at https://beta.clinicaltrials.gov (as of 22nd July 2023).

CONCLUSION AND FUTURE PERSPECTIVES

Emerging liquid biopsy strategies are currently being deployed to detect prognostic and diagnostic markers in biofluids as well as monitor disease progression, response to therapies, and treatment planning. In several disease pathologies, liquid biopsy has even replaced invasive methods like tissue biopsy and image-based methods. The significance of the liquid biopsy-based assays becomes more relevant to cancer types where the size of the tumor and its location is a major concern such as CNS tumors, especially brain tumors. Moreover, routinely used MRI scans are cost-intensive, and the resection of brain tumor tissue may lead to neurological deficits, thus compromising the quality of life of cancer patients. Therefore, liquid biopsy appears to be the promising approach toward brain tumor diagnosis and treatment management.

Liquid biopsy procedures used to explore brain malignancies are typically based on four types of biofluids (blood, CSF, vitreous, and urine), and multiple categories of biomarkers (tumor cells, nucleic acids, proteins, metabolites, extracellular vesicles, and platelets). The diagnostic and prognostic strategies, mostly aim at defining diverse genetic, epigenetic, transcriptomic, proteomic, and metabolomics alterations in brain tumor patients as compared to healthy individuals. While significant progress has been made toward clinical applications of liquid biopsies in other cancer types, these implementations have not been yet employed as a standard of care neuro-oncology exercise for brain tumor patients. Incorporation of WHO CNS5 recommended key genes/molecules/pathways involved in glioblastoma pathogenesis can also be helpful in designing high-precision diagnostic approaches. Also, addressing the technological challenges and enriching the understanding of brain tumor biology in the context of liquid biopsy will help to achieve these goals quickly.

At the technological front, increasing availability of liquid biopsy analytes in biofluids of brain tumor patients, development of standard and reproducible processes of sample collection, improved specificity and the sensitivity of tumor-associated signal detection, and the employment of analyte-specific tailored downstream analytical and bioinformatics techniques need absolute attention. Additionally, brain tumor-specific biological insights like distinct roles of CTCs as single cells and clusters (CTCs-clusters as well as CTCs-WBC clusters), estimation of genetic mutation frequency on ctDNA in context to biofluid and ctDNA size profiles, the significance of cfRNA modifications and tRNA fragments, the role of various secondary messenger molecules in altering transcriptome profiles of platelets and effector outcomes of alternate splice forms, and EVs contribution to metastasis and its utility as drug delivery vehicle in the brain will complement the efforts regarding the technology and assays development. In conclusion, all these discovery, exploration, and validation efforts will be expensive and time-consuming, but their clinical implementations are expected to be cost-effective. Besides, the liquid biopsy research fraternity intends to reach a consensus regarding analyte and biofluid with more biological information for specific cancer types.

Finally, the introduction of liquid biopsy brain tumor-specific multi-national/continental initiatives/societies/consortiums will help to orient the specialized scientific community towards common goals and increase public awareness about participation in brain tumor clinical trials. Global organizations such as IBRO (https://ibro.org) and ABTA (https://www.abta.org), etc. can act coherently to establish brain-tumor-specific liquid biopsy functional bodies similar to that of already existing initiatives such as BloodPac (https://www.bloodpac.org), Cancer-ID (https://www.imi.europa.eu/projects-results/project-factsheets/cancer-id), PANCAID (https://www.pancaid-project.eu), European Liquid Biopsy Society (ELBS) (https://www.uke.de/english/departments-institutes/institutes/tumor-biology/european-liquid-biopsy-society-elbs/index.html), Hellenic Society of Liquid Biopsy (http://en.actc-lab.chem.uoa.gr/hellenic-society-of-liquid-biopsy.html), Liquid Biopsy Consortium (https://prevention.cancer.gov/major-programs/liquid-biopsy-consortium), International Society of Liquid Biopsy (ISLB) (https://islb.info), etc. Altogether, these concerted efforts will facilitate the quick realization of the proposed milestone of 'liquid biopsy as the standard care in brain malignancies'.

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RT and KB established the review idea and finalized the framework. RT and KB were involved in the writing and editing of the review. All authors read and approved the final version of the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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