



Liquid biopsy: early and accurate diagnosis of brain tumor

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Abstract

Noninvasive examination is an emerging area in the field of neuro-oncology. Liquid biopsy captures the landscape of genomic alterations of brain tumors and revolutionizes the traditional diagnosis approaches. Rapidly changing sequencing technologies and more affordable prices put the screws on more application of liquid biopsy in clinical settings. In the past few years, extensive application of liquid biopsy has been seen throughout the whole diagnosis and treatment process of brain tumors, including early and accurate detection, characterization and dynamic monitoring. Here, we summarized and compared the most advanced techniques and target molecules or macrostructures related to brain tumor liquid biopsy. We further reviewed and emphasized recent progression in different clinical settings for brain tumors in blood and CSF. The preferred protocol, potential novel biomarkers and future development are discussed in the last part.

Keywords Liquid biopsy · Gliomas · Brain metastases · Cancer biomarkers

Abbreviations

AUC	Area under curve	EV	Extracellular vesicle
BBB	Blood–brain barrier	FTIR spectroscopy	Fourier transform infrared spectroscopy
BEAMing	Beads, emulsion, amplification, and magnetics	GBM	Glioblastomas
BM	Brain metastases	GSC	Glioblastoma stem cell
cfDNA	Circulating free DNA	HGG	High-grade glioma
CNS	Central nervous system	IDH	Isocitrate dehydrogenase
CNV	Copy number variations	LC–MS	Liquid chromatography-mass spectrometry
CSF	Cerebrospinal fluid	LGG	Low-grade glioma
CTC	Circulating tumor cells	LM	Leptomeningeal metastases
ctDNA	Circulating tumor DNA	MB	Medulloblastoma
ddPCR	Droplet digital polymerase chain reaction	MGMT	O6-methylguanine-DNA methyltransferase
DMG	Diffuse midline glioma	MV	Microvesicle
DSC	Differential scanning calorimetry	nanoDSF	Nano differential scanning fluorimetry
EGFR	Epidermal growth factor receptor	NGS	Next-generation sequencing
		NSCLC	Non-small cell lung cancer
		PAP	Pyrophosphorolysis-activated polymerization
		SCNA	Somatic copy number alteration
		SVM	Supporting vector machine
		TAM-seq	Tagged-amplicon deep sequencing
		TCA	Tricarboxylic acid cycle
		TEP	Tumor-educated platelet
		TERT	Telomerase reverse transcriptase

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WES	Whole-exosome sequencing
WGS	Whole-genome sequencing

Introduction

Brain and other CNS tumors encompass a broad range of different histology, molecular types and subtypes. Gliomas account for the most common primary cancer originated from neuroepithelium, among which glioblastoma (GBM) and medulloblastoma, respectively, make up the first leading type of adult and pediatric malignant brain tumors, with high intratumoural heterogeneity and low 5-year survival of 36% for GBM patients overall (Miller et al. 2021). Moreover, brain metastases (BMs) represent one of the most frequent and adverse events during or after the treatment of their extracranial primary tumors, and the top three types harboring intracranial metastatic tendency are lung cancer, breast cancer and melanoma. As one of the top ten cancer death causes, especially for men less than 40 years and women less than 20 years in the USA in 2018, brain and other CNS tumors are estimated to cause 18,600 cases of death in 2021 (Siegel 2021).

Most brain tumors are diagnosed at an advanced stage, thus making it crucial for secondary prevention when the tumor remains benign and significant mutations can be identified. The clinical diagnosis of brain tumor is highly dependent on radiological outcomes or histology of tumor tissue after resection, which either lacks sensitivity and specificity or is often delayed. Emerging nuclear medicine and magnetic resonance can resolve the early evaluation of the lesion localization, morphology and intensity; however, information at the molecular level remains limited. Other problems such as tumor evolution, heterogeneity and pseudoprogression (PsP), which presents as expanded and/or new regions of edema and enhancement on images, largely resembling tumor recurrence and radiation necrosis, also hamper the accurate evaluation and characterization by biopsy and radiology. As significant mutations like isocitrate dehydrogenase (IDH), BRAF or histone K27M have been discovered since 2008 and fundamentally changed the diagnostic approach in neuropathology to an integrated diagnosis that combines histological diagnosis and molecular profile (Capper et al. 2010, 2011; Khuong-Quang et al. 2012), early and accurate diagnosis of brain tumors on a molecular level becomes essential to reduce patients' mortality and extend survivorship.

While genotyping of informative alterations is becoming more and more frequently involved in routine clinical oncology practice, the availability of cancer tissue is largely limited. The emergence of liquid biopsy opened up the potential of noninvasive sampling and dynamic genotyping. Liquid biopsy was first introduced in a meeting as an approach for

the analysis of therapeutic or prognostic tumor biomarkers like circulating tumor cells (CTCs) in blood sample (Lianidou et al. 2010; Scher et al. 2009; Pantel and Alix-Panabières 2010). According to the NCI Dictionary of Cancer Terms, liquid biopsy refers to a test done on a sample of blood to search for cancer cells from a tumor that are circulating in the blood or for DNA fragments from tumor cells released into blood. Actually, liquid biopsy can be performed using other molecules and macrostructures (e.g., RNAs and extracellular vesicles (EVs)), from other tissue (e.g., premalignant lesions that are often precursors for malignancy) and in other body fluid, including cerebrospinal fluid (CSF), urine, saliva and even breast milk (Serrano et al. 2020).

Here, we briefly exemplify the procedure and techniques of liquid biopsy, and then focus on targets and clinical studies of liquid biopsy.

General procedure and analysis techniques

The general procedure of liquid biopsy typically involves the acquisition of body fluid, sample centrifugation, target isolation, extraction, quantification and a series of subsequent analyses. Subsequent procedure of different analysis platforms is quite parallel, usually involving three stages: library construction, sequencing, and data analysis.

Analysis techniques are mainly divided into PCR-based methods and sequencing-based methods. The next-generation sequencing (NGS)-based broad panel combining hundreds of genes and whole-genome approaches are optimal sequencing methods for liquid biopsy, since they are high throughput and time efficient, and can detect unknown mutations with high single base resolution and coverage (Kilgour et al. 2020).

PCR-based methods have advantages in sensitivity, cost, operation and analysis difficulty; however, incapability in detecting unknown mutations and PCR artifacts are shared drawbacks. Common artifacts can be divided into two main categories: amplicon biased variants (PCR bias) and mis-paired primers (PCR errors) (Acinas et al. 2005). PCR also participates in enriching targeted regions before library construction and is a known source of false positives in some sequencing-based methods.

Since the workflow of EVs liquid biopsy is the most complex and challenging, we use it as an example as shown in Fig. 1. In Table 1 we compared more detailed characteristics of various assays.

Liquids

Due to the special anatomical structure of the brain and other CNS tumors, most studies utilize blood (serum and plasma) and/or CSF. Serum and plasma are the most common source

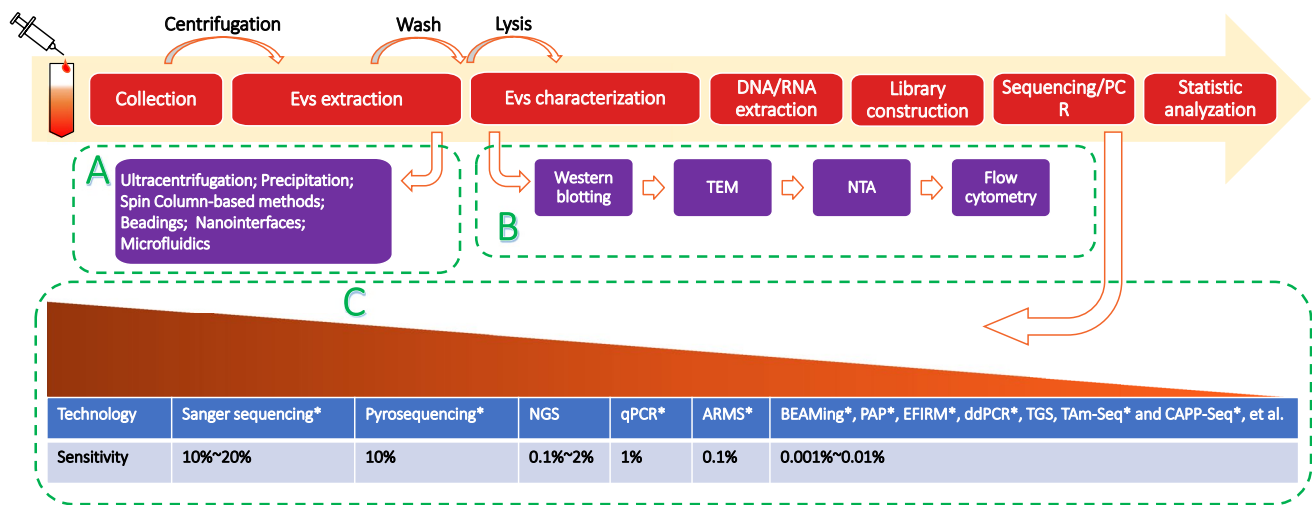


Fig. 1 The general workflow of liquid biopsy to examine extracellular vesicles (Evs). **A** The techniques of extracting Evs: ultracentrifugation is recommended, but it is time-consuming and requires higher standard for techniques. Spin column-based methods are to filter by size or membrane affinity of EVs. **B** Four steps to evaluate the quality of extracted Evs. (i) Western blotting is to detect the specific protein markers such as CD81/CD63. (ii) TEM (transmission electron microscopy) allows direct EV characterization of structure and size of Evs. (iii) NTA (nanoparticle tracking analysis) can visualize parti-

cles ranging from 10 to 1000 nm to show the concentration and size of Evs. (iv) Flow cytometry supplements the information towards Evs' size and markers. **C** The sequencing or PCR techniques ranked by sensitivity: sequencing of point mutation, epigenetic changes, copy number variations, structural changes or rearrangements. *ARMS* amplification refractory mutation system, *BEAMing* beads, emulsification, amplification and magnetics, *PAP* Pyrophosphorolysis-activated polymerization, *EFIRM* electric field induced release and measurement. * Targeted mutation, - unknown mutation

of liquid biopsy, and investigation on novel techniques usually start from blood. Martignano et al. recently conducted the first successful third-generation sequencing (Nanopore-seq) for copy number profiling from six lung cancer patients' plasma DNA (Pittella-Silva et al. 2020). CSF may be more meaningful for primary brain tumor. Blood-to-CSF comparison by liquid biopsy is also critical for identifying tumor heterogeneity (Table 2).

Serum and plasma

Serum and plasma are completely different blood components that have distinct isolation stages. Several studies have agreed that plasma samples are more desirable than serum samples regarding cfDNA in liquid biopsy (Wong et al. 2016; Messaoudi et al. 2013; Thierry et al. 2010). The main point is that although a larger quantity of cfDNA can be extracted from serum samples (Jung et al. 2003), there is a higher percentage of DNA derived from white blood cell lysates, which lessens the purity of ctDNA (Umetani et al. 2006; Lui et al. 2002; Chan et al. 2005; Gautschi et al. 2004; Pittella-Silva et al. 2020). Before centrifugation, serum samples generally need clotting at room temperature, which adds to lysis of cells and even the degradation of ctDNA.

The existence of the blood–brain barrier (BBB) restricts the releasing of biomarkers from a primary brain tumor into circulation and subsequently reduces the accuracy of liquid biopsy for genomic alteration detection. Transcranial

MR-guided focused ultrasound (MRgFUS) is capable of temporarily discharging the BBB with safety, resulting in enrichment of brain-derived cfDNA in circulation. Meng et al. applied MRgFUS to compare biomarkers in whole blood samples from nine GBM patients and healthy controls, and found acutely enhanced plasma cfDNA, neuron-derived EVs, and brain-specific protein S100b (Meng 2021).

When detecting ctDNAs in low abundance, the blood volume is another important consideration. To target de novo a single mutation with cumulative variant allele frequency (VAF) of 0.01% with 95% confidence would require 150–300 ml blood (Haque and Elemento 2017). However, the blood sample volume only required 15–30 ml for detecting any of ten independent VAF = 0.01% mutations or a single VAF = 0.1% mutation. Therefore, the sensitivity of a specific ctDNA test to local cancer patients depends not only on the amount of blood analyzed, but also on the number of mutations screened (McDonald et al. 2019).

Cerebrospinal fluid (CSF)

CSF appears to have an advantage over blood, perhaps partly due to the reduced background cells, for example, less EVs from leukocytes when analyzing tumor-derived EVs. Compared to plasma and serum, CSF is in direct contact with primary brain tumors or brain metastases (BMs), thus harboring a larger proportion of positive patients and more abundant source for liquid biopsy (Pan et al. 2019;

Table 1 Comparison of different assays in liquid biopsy

Assay	Applications	Advantages	Disadvantages
BEAMing-PCR	Known point mutations and CNVs	1 Low input with low frequencies. 2 Specific mutations can be separated and screened by flow cytometry for further analysis and research 1 Low cost. 2 High sensitivity. 3 Quantitative. 4 No need for complex bioinformatics analysis	1 Possible PCR artifacts. 2 High cost. 3 Low throughput
ARMS-PCR	Known point mutations and CNVs	1 Low cost. 2 High sensitivity. 3 Quantitative. 4 No need for complex bioinformatics analysis	1 Possible PCR artifacts. 2 Low throughput
ddPCR	Known point mutations and CNVs	1 Low cost. 2 High sensitivity with pre-amplification. 3 Quantitative. 4 No need for complex bioinformatics analysis	1 Possible PCR artifacts. 2 Specific primer for each target mutation. 3 Limited genome loci
Sanger sequencing	Customized SNV, SCNA and rearrangements	1 Golden standard. 2 Widely available. 3 Low cost	1 High input with abundant mutation fragments. 2 Low throughput and time consuming
Panel sequencing	Customized SNV, SCNA and rearrangements	1 Balanced cost. 2 High sensitivity. 3 High sequencing depth	1 Less comprehensive. 2 Low DNA input limits library complexity
WGS	Genome-wide SNV, SCNA and rearrangements	1 Comprehensive genomic loci including non-coding mutations	1 Expensive. 2 Time consuming. 3 Complex analysis
lcWGS	Genome-wide SNV, SCNA and rearrangements	1 Ideal for tumors with frequent chromosomal/focal copy-number changes while lacking hotspot. 2 Balancing cost and sensitivity	1 Less comprehensive. 2 Requiring tumor purity and ploidy of the sample. 3 Low depth
WES	Genome-wide SNV, SCNA and rearrangements in coding regions	1 Moderate coverage, depth and cost	1 Expensive. 2 Low DNA input limits library complexity
TGS	Genome-wide SNV, SCNA and rearrangements	1 Capable of high GC content and low SNP genotype. 2 No amplification. 3 More integrated and continuous genome assembly	1 High cost 2 High error rate or bias to shorter sequences. 3 Lack effectiveness and standardized workflows in liquid biopsy
Mass spectrometry	Metabolite biomarkers discovery and quantification	Detecting an almost unlimited number of different proteins from the same clinical sample	Requiring more technical knowledge, expensive devices and complex workflow
ELISA	Metabolite biomarkers discovery and quantification	Relatively cheap and easy to perform in clinical laboratories	Limited targeted proteins from the same clinical sample due to antigen-antibody reaction

SNV single-nucleotide variant, SCNA somatic copy-number alterations, BEAMing beads, emulsion, amplification, and magnetics, ARMS-PCR amplification refractory mutation system polymerase chain reaction, LcWGS low-coverage whole-genome sequencing, TGS third-generation sequencing, ELISA enzyme-linked immunosorbent assay

Table 2 Comparison of different source in liquid biopsy

Source	Collection/isolation techniques	Analytical techniques	Advantages	Disadvantages
Blood (serum/ plasma)	Venipuncture (EDTA tubes)	–	1 Extensive source of biomarkers. 2 Minimal invasiveness. 3 Mature techniques for extraction	1 Low sensitivity of CNS tumor
CSF	Craniotomy and lumbar puncture	–	1 High sensitivity and specificity of CNS tumor due to direct contact. 2 No interference by blood cells	1 More invasiveness due to lumbar puncture 2 Many contraindications
Urine	Urine (EDTA tubes)	–	1 Easy and convenient sampling. 2 Indeed non-invasiveness	1 Few investigations with neurological tumors. 2 Lack satisfactory methodologies
ctDNAs	Column-based and bead-based methods	Targeted and untargeted methods	1 Extensive source of biomarkers with detectability in vast kinds of liquid. 2 Relative stability ex vivo. 3 Genetic profile of all tumor subclones. 4 Less demanding in isolation	1 Low ctDNA and tumor-specific mutation ratio at early-stage cancer. 2 Limited researches on its origin and function. 3 Short half-life. 4 Not all detectable mutations are relevant to cancer biology and/or therapy
cfRNAs	Column-based and bead-based methods	Targeted and untargeted methods	1 Identification of fusion gene. 2 Epigenetic alterations. 3 Transcriptional profile of all tumor subclones	1 Instability due to RNase. 2 Difficulty in removing ribosomal and mitochondrial RNA
*miRNA			1 Correlation between cancer types and miRNA expression levels. 2 Stable in blood and CSF	1 Needs normal reference
CTCs	Immunological or physical methods (ultracentrifugation and density gradient centrifugation, polymer precipitation, size-based isolation techniques, and immunoaffinity capture techniques)	Targeted and untargeted methods	1 High specificity. 2 Indication of metastases in different body liquid	1 Low sensitivity due to low concentration. 2 Difficulty between processing and preserving
Evs	Immunological or physical methods	Targeted and untargeted methods	1 High quality and abundance of tumor RNA. 2 Available in various body liquid. 3 Correlation with metastatic disease	1 Difficulty in differentiating EVs from cancer and non-cancerous cells. 2 Limited isolation techniques. 3 Background from normal cells in blood (e.g., leukocytes)
TEPs	Immunological or physical methods	Targeted and untargeted methods	1 High quality and abundance of tumor RNA 2 Correlation with metastatic disease	1 Difficulty in differentiating TEPs and non-cancerous platelets. 2 Early in development
Metabolites and proteins	–	Chromatography, mass spectrometry and immunological methods	1 More clinical accessibility	1 Low specificity due to inflammation and other non-cancerous diseases

Panditharatna et al. 2018; Jiang et al. 2017a; Romero et al. 2021; Aldea et al. 2020). For advanced tumors with leptomeningeal metastases (LM), CSF is the most representative liquid and CSF cytology has been the diagnostic gold standard. Wang et al. examined 35 primary CNS malignancies and found detectable levels of ctDNA in 74% cases of patients' CSF, as all medulloblastomas, ependymomas, and high-grade gliomas that existed directly adjacent to a CSF reservoir, indicating that CSF could be useful in analyzing primary CNS tumor (Wang et al. 2015a). In another study exploring the role of CSF as a source of liquid biopsy in patients with LM, Li et al. enrolled 28 patients with *EGFR*-mutated non-small cell lung cancer (NSCLC) and compared the performance of detecting driver gene by liquid biopsy in 26 patients, yielding 100% (26/26) of CSF cfDNA, 84.6% (22/26) of CSF lysis, and 73.1% (19/26) of plasma cfDNA (Li et al. 2018). The highest number of copy number variations (CNVs), *EGFR T790M* and *MET* were detected in CSF cfDNA, with the major CNVs being exclusive to CSF cfDNA rather than primary tissue or plasma (Table 3).

Limitations of CSF analysis mainly lie in the acquisition and purification. The acquisition of CSF sample is usually performed during surgery to obtain maximal levels of biomarkers or in routine lumbar puncture with radiological surveillance. Though CSF liquid biopsy has shown minimal invasiveness, symptoms of tumor-induced CNS space occupation, or abnormal coagulation are contraindicated for lumbar puncture due to risk of herniation. Contamination by blood in the CSF sample is another technical issue to be solved (Seoane et al. 2019).

Target molecules or macrostructures

Circulating cell-free DNA (cfDNA)/ circulating tumor DNA (ctDNA)

Due to cellular turnover, active secretion, apoptotic and necrotic cells surrounding or in the tumor tissue, a mixture of nucleic acids, which is termed cfDNAs, released in a tumor patient's blood is elevated. A small tumor-derived fraction is held in cfDNA that carries cancer-specific genetic and epigenetic aberrations, which is termed ctDNA and can provide actionable information toward subsequent diagnosis and treatment (Diaz and Bardelli 2014). Identification methods of cfDNA usually involve one or more aspects of common DNA-based changes, including cfDNA levels, cfDNA integrity, copy number variations (CNV), tumor-specific DNA mutations, gene methylation, and gene fusions, reflecting the status of DNA in the tumor cells (Meo et al. 2017; Wu et al. 2020). The increase of cfDNA level and copy number in tumor patients are explained by the raised number of apoptotic and necrotic carcinoma cells. High

cfDNA integrity (> 400 bp) usually results from normal tissue necrosis in addition to the typical oligonucleosomal DNA fragments, whereas shorter DNA fragments (< 400 bp) indicate ctDNA due to apoptosis, which allows selection for the shorter cfDNA to increase ctDNA sensitivity (Mouliere 2011, 2021; Underhill 2016; Jiang et al. 2015). Human GBM ctDNA identified in xenograft rat plasma was typically shorter than the background rat cfDNA (134–144 bp vs. 167 bp, respectively) (Underhill 2016). Size difference was also identified in CSF, plasma, and urine of glioma patients (median size of 148 bp for mutant cfDNA vs 169 bp for non-mutant cfDNA; 160 vs 169 bp; and 101 vs 133 bp, respectively) (Mouliere 2021).

Standard methods optimized for cfDNA isolation include column-based and magnetic bead-based methods, and many DNA isolation kits are practical for both serum/plasma and CSF cfDNA with different pre-processes. The column-based approach refers to using spin columns with usually a silica matrix to bind DNA fragments during the pre-process, and subsequently applying a vacuum pump or a minicentrifuge to remove contaminants. The magnetic bead-based approach is based on nano-beads with magnetic coating able to bind nucleic acids that can be regulated under a magnetic field. Automatable manipulation is highlighted, which allows large-scale processing and omits manual centrifugation that may damage DNAs due to shear forces (Martignano and Cell-Free 2019; Ali et al. 2017).

CtDNAs contain small fragments between 70 and 200 base pairs, and large fragments up to 21 kilobases in size (Jahr et al. 2001). The concentration of cfDNA and ctDNA is influenced by anatomical location, volume, and vascularity of the tumor body, which is, in general, lower in patients with early-stage disease than in patients with metastatic disease. For example, the detection rate of ctDNA is over half of most cancer types, even almost 100% of certain primary cancers (bladder, colorectal, and ovarian) and as low as 10% of glioma cases (Butler et al. 2017). For patients with extracranial localized tumors, only 49–78% of them present detectable ctDNA levels, while the proportion is 86–100% in patients with metastatic colorectal, gastroesophageal, pancreatic, and breast cancers (Bettegowda et al. 2014). The amount of cfDNA and ctDNA is also influenced by clearance, degradation and other physiological filtering events of the blood and lymphatic circulation. Their clearance from the circulation goes through the liver and kidney and their half-life in the blood varies from 15 min to several hours (Fleischhacker and Schmidt 2007).

Circulating free RNAs (cfRNA)

While mRNAs code for proteins, microRNAs (miRNA) are usually 21–24 nucleotides in length, representing small single-stranded non-coding RNA molecules that participate

Table 3 Recent progression in different clinical settings for brain tumors

Authors	Objective	Patients' population	Source and input volume	Target molecule	Biomarker detected	Analysis method	Performance	Main findings
Atocha Romero et al. (2021)	Identifying diagnostic performance of CSF	72 EGFR-mutant advanced NSCLC patients	2 mL plasma	cfDNA	EGFR mutation (deletion exon 19, exon 21, T790M)	BEAMing, ddPCR, Therascreen, and cobas EGFR Mutation Test	42 (sensitivity) and 91 (specificity) (using GeneReader cobas, T790M)	EGFR-sensitizing mutations were detected by ddPCR, BEAMing, or NGS-based assay at significantly lower MAFs in 72 patients with EGFR-mutant advanced NSCLC, exclusively at the CSF compared to other anatomical locations
Mihaela Aldea et al. (2020)	Identifying diagnostic performance of CSF	247 NSCLC patients (54 isolated CNS progression, 99 extra-CNS only, and 94 both)	10–20 mL blood and 2 mL CSF	cfDNA	EGFR, ALK, BRAF, HER2, KRAS, MET, PIK3CA, STK11, and TP53	InVisionFirst-Lung (NGS)	50% (plasma) and 83% (CSF) (sensitivity)	Although NGS has high detection rates of genomic alteration in plasma cfDNA in patients with NSCLC with extra-CNS disease, detection rate of genomic alterations (52%) is lower in the subset of patients with isolated CNS disease
Li et al. (2018)	Identifying diagnostic performance of CSF	45 patients with suspected LMs from NSCLC	10 mL CSF and 8 mL plasma	cfDNA	ERBB2, KRAS, ALK, MYC, MET, TP53 and EGFR T790M, etc	NGS	n.a	CSF cfDNA could reveal the unique genetic profiles of LMs and should be considered as the most representative liquid biopsy medium for LM in EGFR-mutant NSCLC
Yotaro Kitano et al. (2021)	Identifying diagnostic performance of urine	100 noncancer individuals and 102 patients with CNS tumors (i.e., diffuse gliomas, meningiomas, schwannomas, and BMs)	1 ml urine	miRNA	2565 miRNAs	3D-Gene Human miRNA Oligo chip	1.0 (AUC)	"A sterilizable and mass-producible ZnO nanowire-based device was developed that could extract a significantly greater variety and quantity of miRNAs from urine. The diagnostic model based on the urinary miRNA expression could distinguish patients with CNS tumors from noncancer individuals accurately."
Florent Moulriere et al. (2021)	Identifying diagnostic performance of urine	30 HGGs, 5 LGGs, 26 healthy volunteers and 27 patients with other CNS diseases	10 ml plasma, 10 ml urine and 8 ml CSF	cfDNA	/	WES and sWGS	0.80–0.91 (AUC)	The non-invasive nature of plasma and urine may permit more regular and less restrictive monitoring for GBM patients than CSF sampling

Table 3 (continued)

Authors	Objective	Patients' population	Source and input volume	Target molecule	Biomarker detected	Analysis method	Performance	Main findings
Peiyao Li et al. (2021)	Identifying diagnostic performance of circRNAs	3 primary tumor cells	serum	EV-derived circRNA	8537 circRNAs	NGS	100% (sensitivity) and 100% (specificity)	The characteristics of HGA cell circRNAs and exosome circRNAs were demonstrated. Exosome circRNAs can serve as liquid biopsy targets in HGA screening, and a serum exosome panel of three circRNAs was identified
Wang et al. (2020)	Identifying fusion transcripts in mRNAs	9 GBM, 7 healthy	2 mL plasma	EV-derived RNA	FGFR3-TACC3 and VTI1A-TCF7L2	targeted NGS and ddPCR	n.a	Two novel fusion transcripts in GBM tissue and five novel fusions in the matched plasma of GBM patients were identified. The fusion transcripts FGFR3-TACC3 and VTI1A-TCF7L2 were detected in both tissue and matched plasma
Yang Zhao et al. (2020)	Evaluating a CTC positive sorting marker	32 brain tumor patients	0.1 ml blood and 7.5 ml CSF	CTC-derived DNA	GFAP and EGFR	qPCR, Sanger sequencing and WES	92.4% (separation efficiency of GFAP-IMLs)	The constructed GFAP-IMLs separation system with DAPI/EGFR-FITC immunofluorescence assay was effective in the detection of CTCs from CSF and peripheral blood samples
Rocco Lucero et al. (2020)	Identifying the association between EV-derived miRNAs and treatment resistance	GBM8 cells	100 mL from approximately 20 million GBM8 cells	Extracellular RNA and DNA	8 miRNA including hsa-miR-9-5p, hsa-miR-22-3p, and hsa-miR-182-5p, and 28 genes, etc	NGS	n.a	The angiogenic gene regulatory responses of brain endothelial cells to growth factors and to miRNAs in EVs secreted by GBM stem-like cells. MiR-9-5p in GBM stem cells supports metabolic activity and may influence resistance to therapeutic intervention on LGGs
Syben L. N. Maas et al. (2020)	Discriminating tumor-derived EV subgroups	30 GBM	1 µl plasma	EV-derived miRNA	PpIX, miR-21, miR-10b and Let-7a	ddPCR	n.a	Analysis indicated the presence of GB-derived PpIX-positive EVs and GB-associated miRNAs after oral administration of 5-ALA
Mahsa Jalali et al. (2021)	Discriminating tumor-derived EV subgroups	glioblastoma cell lines (U373, U87) and non-cancerous glial EVs (NHA)	500 µL of purified EVs	EVs	/	Surface enhanced Raman spectroscopy (SERS)	n.a	The plasmonic nanobowtiefluidic device discerns the sub-population GBM EVs and non-cancerous glial EVs

Table 3 (continued)

Authors	Objective	Patients' population	Source and input volume	Target molecule	Biomarker detected	Analysis method	Performance	Main findings
Abhimanyu Thakur et al. (2021)	Discriminating tumor-derived EV subgroups	Human neuroblastoma SH-SY5Y cells	n.a	Evs	EV marker (CD9) and MV marker (integrin)	SAM-AuNIs LSPR biosensor	n.a	Directly detecting biophysical difference and differential membrane properties of tumor-derived exosomes and MVs reflects disease progression
Leomar Y. Bal-lesler et al. (2018)	Identifying IDH-mutant gliomas and metabolite alterations in CSF	GBM IDH-WT (n=7), IDH-mutant (n=4), metastatic lung cancer (n=7) or metastatic breast cancer (n=5) to the CNS, n=8 controls	n.a	metabolites	/	LC-MS	0.724–0.888 (AUC)	Differences in 43 metabolites between CSF from control patients and primary or metastatic CNS tumors were identified. Patients with IDH-mutant gliomas demonstrated higher levels of TCA metabolites alterations in the CSF than other tumor types, or controls
Florent Moulriere et al. (2018)	Reducing the cost	13 gliomas	10 ml CSF	cfDNA	EGFR, PTEN and 26 genes	sWGS	n.a	The number of shorter DNA fragments (145 bp) correlated with the presence of tumor-derived somatic copy number alterations (SCNAs) in the CSF. The combination of SCNAs screening and fragmentation analysis of cfDNA by sWGS provides important genomic information at low cost and more rapidly when compared with WES or targeted sequencing
Farshad Nassiri et al. (2020)	Differential diagnosis	70 IDH mutant gliomas, 52 IDH WT gliomas, 60 meningiomas, 9 hemangiopericytomas, 14 LGG and 15 BM of unknown primary cancer	20 ml peripheral blood	cfDNA	Differentially methylated regions (DMRs)	cfMeDIP-seq and WGBS	0.99 (AUC, in distinguishing gliomas from extracranial cancer types and healthy patients)	The cfDNA from plasma helps distinguish gliomas from extracranial cancer types that may metastasize to the brain and healthy controls, and can accurately discriminate different primary brain tumors

Table 3 (continued)

Authors	Objective	Patients' population	Source and input volume	Target molecule	Biomarker detected	Analysis method	Performance	Main findings
Nik Sol et al. (2020)	Differential diagnosis	Asymptomatic controls, n = 353; GBM, Resection n = 89, Follow-up n = 151; Multiple sclerosis, n = 86; BM, n = 126	4–10 mL whole blood	platelet RNA	/	thromboSeq	0.81–0.97 (AUC)	TEPs have potential as a minimally invasive biosource for blood-based diagnosis for GBM and monitoring of GBM progression
Feng-Xiang Wang et al. (2020)	Differential diagnosis	Nontumorous brain diseases (nt, n = 36), PCNSL, (n = 59), secondary CNS involvement of systemic lymphoma (SCNSL, n = 11), lung adenocarcinoma patients with BMs (MBT, n = 34), and lung adenocarcinoma patients without BMs (NIMBT, n = 23)	n.a	metabolites	/	LC-MS	0.77–0.91 (AUC)	CSF-based metabolomics showed 27 metabolites were selected as diagnostic markers to discriminate different brain tumor types
Huayi Wang et al. (2019)	The WHO Classification of Tumors of the CNS	23 glioma patients and 12 healthy individuals	250 μ L serum	EV-derived protein and mRNA	EGFR, NLGN3 and PTTG1	latex-bead assisted flow cytometry	0.9 (AUC, EGFR)	The expression of EGFR in serum EVs can accurately differentiate HGG and LGG patients. And EGFR in EVs positively correlates with ki-67 labeling index in the tumor tissue. There is potential of NLGN3 and PTTG1 mRNA in EVs for detecting glioma patients
Tina Y Huang et al. (2017)	The WHO Classification of Tumors of the CNS	11 children with brain tumors and 1 control	0.4–2 mL CSF	cfDNA	H3F3A and HIST1H3B	Sanger sequencing and NGS	87.5% (sensitivity) and 100% (specificity)	Histone H3 gene mutation is detectable in CSF-derived tumor DNA from children with brain tumor including diffuse midline glioma

Table 3 (continued)

Authors	Objective	Patients' population	Source and input volume	Target molecule	Biomarker detected	Analysis method	Performance	Main findings
P. Billard et al. (2021)	The WHO Classification of Tumors of the CNS	284 gliomas	blood	cfDNA	the telomeric sequence (referencing (HeLa) and short-telomere (HeLa) and long-telomere (U2OS)), ATRX loss and TRET mutation	dTeloPCR	100% (sensitivity) and 97.3% (specificity)	The TeloDIAG can detect telomere maintenance mechanisms and help glioma diagnosis
Vineesh Indira Chandran et al. (2019)	The WHO Classification of Tumors of the CNS	65 patients with WHO IV GBM and 17 with WHO II astrocytoma	500 µl plasma	EV-derived protein	SDC1	LC-MS/MS	0.81 (AUC)	Syndecan-1 (SDC1) as an EV constituent originating directly from GBM tumors can discriminate between glioblastoma multiforme (GBM; WHO grade IV) and low-grade glioma (WHO grade II)
Changcun Pan et al. (2019)	The WHO Classification of Tumors of the CNS	57 with primary tumor in the brainstem or fourth ventricle	5–10 ml CSF and 5 ml blood	cfDNA	H3F3A, TP53, ATRX and PDGFRA, etc	NGS	91.9% (sensitivity, half of the alterations)	Deep sequencing of CSF cfDNA is a reliable technique for detecting tumor-specific alterations and profiling molecules in brainstem tumors
Koushik Muralidharan et al. (2021)	Profiling genetic mutation for targeted therapy	88 TERT Mutant, 26 TERT WT, 34 matched healthy controls and 9 non-tumor MRI positive	2 mL plasma	cfDNA	TERT promoter mutation (C228T, C250T)	ddPCR	62.5 (sensitivity) and 90% (specificity)	The detection of circulating cfDNA TERT promoter mutations in glioma patients shows clinically relevant sensitivity and specificity
Noemi Garcia-Romero et al. (2019)	Profiling genetic mutation for targeted therapy	29 pediatric patients	500 µL of plasma, serum and CSF	cfDNA and EV-derived DNA	BRAF V600E	digital PCR	25% (sensitivity) and 77.8% (specificity) in plasma, 50% (sensitivity) and 100% (specificity) in serum	The cfDNA found in serum and plasma could provide the genetic characteristics of the original tumor. Serum is the most promising alternative rather than plasma for BRAF V600E by dPCR detection
Keiko M Kang et al. (2021)	Profiling genetic mutation for targeted therapy	5 BRAF V600E, 4 BRAF WT, and 4 healthy controls	2 mL plasma	cfDNA and EV-derived mRNA	BRAF V600E	ddPCR	80% (sensitivity) and 100% (specificity)	It is feasible to detect circulating BRAF V600E in patients with known BRAF mutant tumors and detect the circulating BRAF V600E hotspot mutation for longitudinal global disease monitoring in patients with metastatic disease

Table 3 (continued)

Authors	Objective	Patients' population	Source and input volume	Target molecule	Biomarker detected	Analysis method	Performance	Main findings
Bing Wei et al. (2019)	Profiling genetic mutation for targeted therapy	375 with advanced or recurrent TKI sensitive metastatic NSCLC	5 mL plasma	cfDNA	T790M	ddPCR	63.6% (sensitivity) and 83.3% (specificity)	Primary EGFR 19del, brain metastasis, and longer progression-free survival of initial EGFR-TKI treatment are associated with acquired T790M resistance
Cecile L Maire et al. (2021)	Evaluating early treatment response	GBM stem-like cells and non-tumorous temporal lobe tissue	conditioned medium	EV-derived protein and DNA	MGMT promoter, 47 genes, TRET, and copy number variations (CNV)	Methylation: 850 k arrays; Mutation: ddPCR, NGS; Protein: spectrometric proteomics	n.a	Tumor-specific mutations and copy number variations (CNV) were detected in EV-DNA with high accuracy
Thais Sabedot et al. (2021)	Evaluating early treatment response	Glioma patients	n.a	cfDNA	n.a	WGS	100% (sensitivity) and 97.78% (specificity)	A cfDNA-derived methylation signature and a score metric called GeLB that is associated with the presence of gliomas and related immune features was verified
Eshini Panditharatna et al. (2018)	Evaluating early treatment response	48 DMG patients, and 36 non-CNS diseased controls	1 ml plasma, 500 µl CSF, and 200 µl cyst fluid	cfDNA	H3F3A (p.K27M), HIST1H3B (p.K27M) and partner mutants	ddPCR	n.a	H3K27M, additional driver mutations and responses to radiotherapy were successfully predicted using patient's tumor tissue and matched CSF
Laura Escudero et al. (2020)	Accessing minimal residual disease	13 paediatric MB	plasma and CSF	cfDNA	PTCH1, TP53, MYCNamp and GLI2amp, SUFU, 17p loss and SRCAP etc	ddPCR and WES	n.a	cfDNA is more abundant in CSF than in plasma and reflects the tumour genomic alterations, which allows monitoring minimal residual disease, genomic evolution and the characterisation of tumours at recurrence
Anthony P Y Liu et al. (2021)	Accessing minimal residual disease	71 non-metastatic MB, 34 metastatic MB and 17 non-MB CNS embryonal tumor	1–2 ml CSF	cfDNA	CNVs	lcWGS	85% in patients with metastatic MB and 54% for those with non-metastatic disease (measurable residual disease (MRD) detectability)	lcWGS of serial CSF-derived cfDNA allows measurable residual disease (MRD) detection and predicts treatment response in children with MB. Future MB management may personalize therapy according to MRD response

Table 3 (continued)

Authors	Objective	Patients' population	Source and input volume	Target molecule	Biomarker detected	Analysis method	Performance	Main findings
Sabela Bobillo et al. (2021)	Assessing minimal residual disease	6 restricted CNS lymphomas, 1 systemic lymphoma involving CNS, and 12 systemic lymphomas	1–2 mL CSF and plasma	cfDNA and CTCs	300 genes and MYD88 L265P	NGS, WES and ddPCR	n.a	CSF ctDNA can better detect CNS lesions than plasma ctDNA and CSF cytology. In addition, CSF ctDNA predicted CNS relapse in CNS and systemic lymphomas
Christian Grommes et al. (2019)	Assessing minimal residual disease	15 PCNSL or SCNSL	4 mL CSF	cfDNA	585 cancer genes including MYD88_L265P, CD79B_Y196, CARD11, MALT1, PLCG2 and TNFAIP3 etc	NGS	n.a	A considerable fraction of patients with recurrent or refractory CNSL harbor tumor DNA in CSF, even if CSF involvement is undetectable by conventional techniques (MRI, CSF cytology, CSF flow cytometry). CSF liquid biopsy may be useful to monitor disease burden and evaluate treatment response in CNSL
Ben-Yuan Jiang et al. (2017a)	Tracking evolution of resistance in real time	21 NSCLC patients with suspected LMs	7.5 mL blood and 5–7.5 mL CSF	CTC-derived DNA	EGFR T790M, MET amplification and ERBB2 mutation	NGS	95.2% (sensitivity) and 100% (specificity)	The results showed significantly higher sensitivity and specificity of CSF CTCs as compared with traditional methods. CTCs in peripheral blood were found at much lower concentrations than in CSF detected by CellSearch
Alexandra M. Miller et al. (2019)	Tracking evolution of resistance in real time	85 gliomas	3.5 mL CSF and 3.5 mL plasma	cfDNA	IDH1 p19q, TRET promoter, CIC, ATRX and TP53	MSK-IMPACT assay (NGS)	n.a	The ctDNA was detected in CSF from 49.4% of patients and was associated with disease burden and adverse outcome. Liquid biopsy showed the ability to monitor the evolution of the glioma genome
Ailiang Zeng et al. (2018)	Tracking evolution of resistance in real time	75 GBMs and 81 Grade III gliomas	serum and CSF	EV-derived protein and RNA	miR-151a	qPCR and RNA-ChIP	n.a	Low miR-151a levels of GBM patients correlate with poor response to TMZ therapy. Overexpression of miR-151a restores TMZ response of resistant GBM cells via inhibiting XRCC4-mediated DNA repair in vivo

Table 3 (continued)

Authors	Objective	Patients' population	Source and input volume	Target molecule	Biomarker detected	Analysis method	Performance	Main findings
Jia Li et al. (2020)	Predicting survival time	4 pediatric MB, 4 hydrocephalus, 2 acute lymphoblastic leukemia and 2 normal cerebellum tissues	200 µl CSF	cfDNA	DNA methylation and hydroxy-methylation	WGBS and CMS-IP-seq	n.a	MB patients with a high DNA methylation level at this CpG site showed significantly lower survival rates compared with patients with a low DNA methylation level at this site

n.a. not available, *WT* wild type, *BM* brain metastases, *GBM* glioblastoma, *DMG* diffuse midline glioma, *HGG* high-grade glioma, *LGG* low-grade glioma, *LM* leptomeningeal metastases, *MB* medulloblastoma, *NSCLC* non-small-cell lung cancer, *CSF* cerebrospinal fluid, *WES* whole-exome sequencing, *sWGS* shallow whole-genome sequencing, *WGBS* whole-genome bisulfite sequencing

in post-transcriptional regulation. MiRNAs are more predominant species of cfRNA and a number of studies on CNS tumor liquid biopsy have revealed certain informative types of miRNAs. For instance, overexpression of miR-15b and miR-21 was found correlated with the presence of gliomas by Baraniskin et al. (Baraniskin et al. 2012), and enrichment of miR-10b and miR-21 in patients' CSF might indicate GBM (Tepluyk et al. 2012), while assessing miR-21, miR-19, and miR-92a CSF levels led to a combined diagnostic accuracy with 95.7% sensitivity and 96.7% specificity for primary central nervous system lymphoma (PCNSL) diagnosis (Baraniskin et al. 2011).

Circular RNAs (circRNAs) mainly function as miRNA sponges or peptide-coding RNAs and regulate alternative splicing of parental genes, which are characterized by their covalently closed structure and high tolerance to exonucleases (Meng et al. 2017; Li et al. 2015; Hansen et al. 2013). An increasing number of studies on circRNA deep sequencing have shown a correlation between a global reduction of circRNAs and different types of cancers compared to their normal tissues, such as GBM and colon rectal carcinoma (Zhang et al. 2018; Xu et al. 2018; Yin and Liu 2020). Differential expression levels of certain circRNAs have been found to be in gliomas, indicating their potential function in tumorigenesis (Zhang et al. 2018; Xu et al. 2018). From newly pathologically diagnosed high-grade astrocytoma (HGA) samples, Li et al. identified abundant circRNAs in both HGA cells and corresponding cell-derived exosomes, with a higher level in HGA cells (Li 2021). Additionally, three serum exosome circRNAs were chosen as circulating biomarkers screening of HGA and were promising in distinguishing HGA patients from healthy individuals (Fig. 2).

Although cancer is believed to be mainly driven by changes in DNA, changes in some driving factors may be manifested in RNA rather than mutations in DNA sequences (Calabrese et al. 2020). Therefore, RNA analysis may exhibit changes in cancer-related pathways that have not been detected by pure DNA methods. Another benefit of RNA-based sequencing is the ability to detect fusion genes, which refer to hybrid of two or more coding gene sequences in a common regulatory region that harbors a distinct function from the two pre-fusion genes. They are pathognomonic rearrangements which are commonly reported in about 30–50% of GBM patients (Shah et al. 2013). Wang et al. applied targeted RNA sequencing and unveiled two novel oncogenic fusion transcripts (FGFR3-TACC3 and VTI1A-TCF7L2) in EVs extracted from GBM tissues, and five novel fusions including two tissue-derived fusion transcripts in the paired GBM patients' plasma ($n=9$) (Wang et al. 2020).

Compared to RNA in the lipid bilayer enclosed structures such as circulating tumor cells (CTCs), exocellular vesicles (EVs) or tumor-educated platelets (TEPs), cfRNA is single stranded and exposed to nucleases in bodily fluids with low

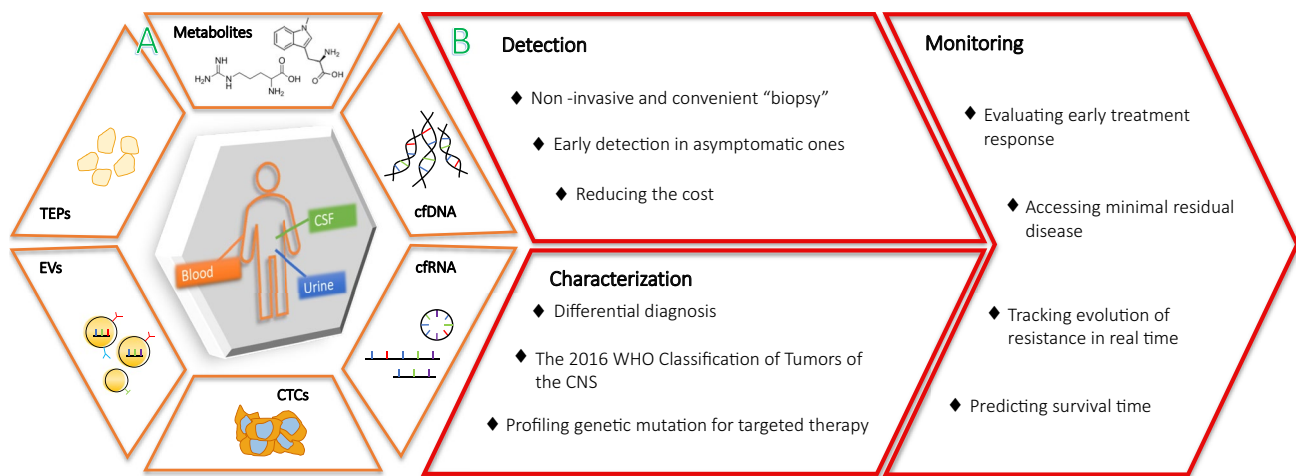


Fig. 2 **A** The circulating biomarkers in blood and CSF and **B** clinical application (detection, characterization and monitoring) in liquid biopsy

abundance and integrity; thus, it possesses limited reliability. Moreover, a large part of RNA in cells comes from ribosomes and mitochondria. This limits the number of reads of other RNAs and the accuracy of the expression levels of these RNAs (Raz 2011).

Circulating tumor cells (CTCs)

CTCs refer to a crowd of tumor cells detached from a solid tumor lesion into the peripheral circulation. As a direct source of ctDNA, they not only represent a relatively easily available sample of tumor tissue, but also a group of dynamic metastatic precursors such as epithelial and mesenchymal markers (Alix-Panabières and Pantel 2013; Aceto et al. 2015). It has been reported that approximately 39% of GBM patients harbor CTCs, and defining its ctDNA could help reveal the EGFR amplification status of the primary tumor (Sullivan et al. 2014).

CTCs may be found held together by intercellular cell–cell junctions traveling together through the bloodstream, which are called CTC clusters. Clustered formation hinders immunological surveillance and protects tumor cells from reactive oxygen species (ROS), thus possessing greater metastatic potential (Sprouse et al. 2019). For liquid biopsy, CTCs clusters exhibit dissimilar genetic profiles and metastatic characteristics from single CTCs. GBM can release CTC clusters, which can overcome the BBB and reach the peripheral circulation (Krol et al. 2018). Though the incidence of extracranial metastatic GBM is low (0.4 to 0.5% of patients), mostly to bone, lymph nodes, lungs, and liver (Pasquier, et al. 1980; Kalokhe, et al. 2012; Fonkem et al. 2011), it is reported that CTCs are positive in 20.6–39% GBM patients (Sullivan et al. 2014; Müller et al. 2014).

Enrichment or isolation is a crucial step in the liquid biopsy of CTCs. The technology approaches are divided

into two methods, respectively, based on immunological or physical characteristics. To evaluate the quality of enriched CTCs, different characteristics from the normal cell are compared by means of immunofluorescence, FISH (fluorescence in situ hybridization), PCR-based detection methods, and/or high-throughput single-cell sequencing. However, there are some special occasions when CTC enrichment may be difficult to complete. For instance, platelet-covered CTCs can shield their surface antigen and enhance the interaction between CTCs and leukocytes, which possess more metastatic potential (Weber and Springer 1997; Kitamura et al. 2015). To solve the problem, Jiang et al. performed efficient depletion of free plates in blood, then implemented herringbone CTC chip (HB-Chip), a microvortex-generating device, to enhance cell-capture ability. As a result, they observed the presence of CTCs extensively covered by platelets compared to conventional EpCAM-based capture (Jiang et al. 2017b).

Conventional CTCs positive sorting methods mainly target epithelial markers such as EpCAM, which may lead to fewer variety of molecular types of CTCs (Kulasinghe et al. 2017). GFAP is an activated protein marker located in the cytoplasm of astrocytes that is commonly expressed among brain tumors, with higher levels reported in gliomas compared to other types (Bodegraven et al. 2019). Zhao et al. utilized a novel CTCs positive sorting marker targeting the endocytic GFAP and framed an isolation system on CTCs of brain tumors derived from GFAP antibody immunomagnetic liposome beads (GFAP-IMLs), which was further combined with an EGFR immunofluorescent assay (Zhao et al. 2020). Through subsequent validation of Sanger sequencing, q-PCR and NGS, the system was shown to be feasible toward identifying CTCs from tumor tissues, CSF and peripheral blood samples. GFAP markers also outperformed those of just EpCAM or EGFR for CTC capture. One of these challenges lies in the separation of CTC, which usually requires fresh

blood samples with immediate sample processing. This may limit the initial application of CTCs in small centers that do not have the ability to process fresh samples in a short time (Shankar et al. 2017).

Exocellular vesicles (EVs)

Both cancer and non-cancer cells may release EVs into the extracellular space to transport messages between cells, by means of budding or intracellular vesicles merging with the plasma membrane (Colombo et al. 2014; Kros et al. 2015). It is intriguing that double-stranded DNA in exosomes, the smallest group of EVs, represents the entire genome and reflects the mutational status of parental tumor cells (Thakur et al. 2014). Compared to the rarity of ctDNAs and CTCs, EVs are more abundant per milliliter of blood and are present in almost all kinds of body fluids. Lipid bilayer wrapped structures also protect the contents from degradation. Cargo information is available in EV and exosome databases, such as ExoCarta, Vesiclepedia, and EVpedia (Lin 2021).

EVs play an important part in transporting messages to promote tumor progression. The tumor tissue secrete EVs and then neighboring stromal cells may take EVs and alter the cell program. For instance, glioblastoma stem cells (GSCs) derive EVs that induce non-conventional angiogenic pathways, which are free from classic anti-angiogenic drugs inhibiting the vascular endothelial growth factor (VEGF) or VEGF receptors (Geraldo et al. 2019; Kane 2019). Lucero et al. examined the RNA transfer by EVs from GSCs to human brain microvascular endothelial cells (HBMVECs) on cell lines in vitro through RNA-seq and DNA methylation profiling plus computational epigenomic deconvolution in vivo in the TCGA collection. The study provided multi-dimensional evidence that morphologic vascularization patterns of GSC EVs-mediated anagenesis were visually similar to that of VEGF, but stimulated distinct transcriptional and epigenomic alterations in HBMVECs, and that the downregulation of angiostatic genes could be mediated by miRNAs, like export of miR-9–5p inducing therapy resistance in GSCs (Lucero et al. 2020). Gliomas may produce EVs containing miRNAs, such as miR-21, miR-222, miR-124-3p, miR-221, miR-451, miR-23a, miR-29a, miR-30a, and miR-92b, which may motivate proliferation and suppress apoptosis (Colombo et al. 2014; Li et al. 2013; Godlewski et al. 2010). There is evidence that analyzing the expression of miR-21, miR-222 and miR-124-3p from serum-isolated exosomes potentially enables discrimination between HGGs and LGGs (Santangelo et al. 2018). If the tumor-secreted EVs are taken by the immune system, it may lead to immunosuppression, possibly resulting in abnormally activated myeloid-derived suppressor cells (MDSC) and regulatory T (Treg) cells (Kahlert and Kalluri 2013). Additionally, in GSC-derived EVs, PD-L1 has been identified to be involved in this process which can

be up-regulated in response to IFN- γ , as by PD1 blockade Ricklefs et al. significantly reversed the EV-mediated inhibition of T cell activation (Ricklefs et al. 2018).

Besides apoptotic bodies, the other two major classes of EVs, exosomes and microvesicles (MVs), overlap in their size and mimic surface proteins, but there is a lack a specific marker or cargo associated with MVs distinctive from exosomes (Lawson et al. 2016) and so it is challenging to isolate them. MVs are small vesicles ranging from 100 to 1000 nm that fall off the cell membrane after cell activation, injury or apoptosis (Lee et al. 2012). Exosomes are small saucer-shaped vesicles ranging from 40 to 100 nm in diameter, usually excessively produced by tumor cells, composed of proteins, lipids, DNA and RNA, which share large similarity to their original cell or tissue and harbor more diagnostic potentiality (Théry et al. 2002). In an exosome characterization study, Fernando et al. suggested that exosome-derived cfDNA makes up 90% of cfDNA and 93% of amplifiable plasma cfDNA in plasma (Fernando 2017). Thakur et al. exploited a novel nano-biosensor (SAM-AuNIs) which enables direct detection of biophysical difference, membrane property differentiation and distinguish between exosomes and MVs (Thakur et al. 2017). In conclusion, the current isolation and purification of EVs from plasma are technically more difficult, mainly concerning specificity compared to directly from cfDNA or CTCs, but harbor more sensitivity.

EVs from cancer cells and healthy cells are difficult to distinguish. The 5-ALA oral administration is performed before glioma surgery to promote tumor visualization and maximal resection, and its metabolite, PpIX, may be accumulated in gliomas-derived EVs. Maas investigated the feasibility of identifying PpIX-positive EVs from GBM patients orally administered 5-ALA (Maas et al. 2020). High-resolution flow cytometric on sorting EVs was conducted using plasma, and ddPCR analysis further presented the existence of GBM-associated miRNAs in PpIX-positive EVs, like miR-21 and miR-10b. Jalali et al. reported using a plasmonic microfluidic device with nanostructure to discriminate tumor-derived EV subgroups from different GBM cell lines, EVs from common glial cells and artificial liposomes (e.g., DOPC/Chol) that is homogenous to EVs demonstrated high sensitivity (Jalali 2021). TiN-NH-LSPR biosensor developed by Thakur et al. could detect and quantify CD44 and CD133 enhanced by GBM-secreted lactate, thus distinguishing that secreted exosomes sensitively from malignant cells (Thakur 2021).

Tumor-educated platelets (TEPs)

Platelets are blood cells forming blood coagulation in response to vascular endothelium damage. However, platelets also respond to a series of inflammatory events, cancer genesis and metastasis. When tumor-associated

biomolecules are transferred into platelets, they become “educated”. For years high platelet count and expression of coagulation factors by cancer cells have been recognized as negative prognostic markers in a variety of cancers (Nash et al. 2002; Erpenbeck and Schön 2010; Gay and Felding-Habermann 2011). Firstly, platelets can regulate the tumor vasculature through which circulating tumor cells (CTCs) disseminate by releasing angiogenic and vascular remodeling factors in granules upon activation by thrombin triggered by tumor tissue-associated factors (Gay and Felding-Habermann 2011; Sabrkhany et al. 2011). For instance, GBM stem cells can transdifferentiate into endothelial cells (GECs) which are aberrantly responsive to increased levels of VEGF released by TEPs and boost tumor progression and angiogenesis (Vito et al. 2017). Increased expression of its receptors VEGFR1 and VEGFR2, VWF, and S1P was also identified in another study as predictive and monitoring biomarkers (Campanella et al. 2020). Next, platelets can generate cloaks covering tumor cells, which together with fibrin can provide a physically protective shield (Nieswandt et al. 1999; Palumbo et al. 2005) or MHC class I that confers a pseudonormal phenotype that disrupts recognition of tumor cell, to shelter tumor cells from cytotoxic activity of natural killer cells (Placke et al. 2012). Finally, platelets also play a role in the establishment of metastases by enhancing interaction of tumor cell with vascular endothelium under hemodynamic shear environment of circulation (Konstantopoulos and Thomas 2009) and recruit granulocytes to tumor cells to form early metastatic niches (Labelle et al. 2014).

Since platelets themselves do not have a nucleus, all RNA in platelets comes from the original megakaryocytes, interacted cells and circulation. Nilsson et al. demonstrated that cancer cells can transfer mutant RNA through EVs-like membrane vesicles into blood platelets, and identified a distinct group of cancer-associated RNA alterations including *EGFR*-vIII mutations in the TEPs from GBM patients compared to normal controls by gene-expression profiling (Nilsson et al. 2011). By liquid biopsy, the detection of tumor-related proteins, cfDNA and RNA sequestered by platelets enables blood-based cancer diagnostics and treatment monitoring. RNAs in platelets advance in their abundance, easy isolation, high quality, and platelets’ capacity to take up EVs comprising RNAs originated from tumors (Best et al. 2018; Sol and Wurdinger 2017). What is more, the life span of a common platelet is 7–10 days, thus it can be a promising source of RNA analysis, revealing a real-time status of tumor dynamics.

Recently, Best et al. reported a different liquid biopsy approach called thromboSeq by detecting TEP-absorbed tumor RNA and swarm-intelligence-enhanced algorithms to optimize biomarker gene lists, with its accuracy up to 90% (Best et al. 2017). Researchers investigated blood samples from patients diagnosed with advanced non-small cell lung

cancer ($n = 518$), patients in early stage ($n = 106$), and controls without known cancers ($n = 155$). In the study, thromboSeq scanned about 5000 different RNA molecules found in platelets, continuously optimized its RNA gene panel, and found a few RNAs that can predict tumors. The results confirmed that the accuracy of thromboSeq in diagnosing early cancer was 81%, and the accuracy in diagnosing advanced cancer was 88%. In the matched group controlling age, smoking status and blood storage time, the accuracy rate was as high as 91%. The protocol and details of thromboSeq were updated in 2019 and it is promising in future pan-cancer and multiclass tumor diagnostics (Best et al. 2019).

There are some limitations that platelet mRNA profiles may be affected by non-cancerous systematic factors such as inflammatory or cardiovascular diseases; therefore, it makes it difficult to differentiate between TEPs and normal platelets and requires follow-up studies for further evaluation (Best et al. 2015). During the platelet-isolation procedure, residual cfDNA contamination can potentially introduce concomitant sequencing of reads mapping to intergenic regions, thus reducing the number of spliced intron-spanning platelet-RNA sequencing reads (Best et al. 2019).

Metabolites and proteins

In the absence of CSF cytology findings, analyzing patients’ CSF metabolites may help diagnose and distinguish different types of CNS tumors. However, identifying specific protein markers for CNS cancer is more challenging than cfDNA or cfRNA sequencing. Elevated protein levels may attribute to inflammation, unrelated diseases or responses to stress/hypoxia, making it hard to enter clinical practice.

Metabolomics and proteomics are promising in combination with liquid biopsy to identify novel biomarkers, thus providing disease screening and diagnosis. Ballester et al. examined 43 metabolites derived from CSF in primary or metastatic brain tumors patients by utilizing different spectrometries (Ballester et al. 2018). Furthermore, they found that *IDH*-mutant gliomas exhibited metabolite alterations of the tricarboxylic acid cycle, such as higher concentration of D-2-hydroxyglutarate, malic acid and succinate in CSF, when compared with healthy controls and other types of tumors. More potential protein biomarkers are summarized in a systematic review of eight proteomics articles (Shen et al. 2014). Out of these 19 differentially expressed proteins (B2M, CA2, CA12, CALD1, DDAH1, MYCN, PPIA, SPP1, VEGFB, ALB, MAPT, SERPINA3, SPARCL1), only attractin was identified twice among these 8 proteomic studies on glioma CSF, which may function as a mediator in malignant gliomas, representing a novel biomarker as well as a potential therapeutic target (Khwaja et al. 2006, 2007).

Intracranial germinomas occur in the pineal and suprasellar region, arising from abnormal migration and differential of various germ cell layers during embryogenesis. β -Human chorionic gonadotropin (bHCG) and alpha-fetoprotein (AFP) are relevant proteins representing germ cell origination, and the levels of both proteins have been shown to be significantly elevated in germinomas patients' CSF. Both bHCG and AFP have been used to diagnose malignant germ cell tumors, monitor response to treatment and predict recurrence (Nishizaki et al. 2001; Qad-doumi et al. 2012). Placental alkaline phosphatase (PLAP) holds clinical potentiality for the diagnosis and monitoring of intracranial germinomas as a biomarker. Watanabe tested PLAP in 28 patients' CSF with a chemiluminescent enzyme assay using antibodies, which presented high sensitivity and specificity of 94% and 97% (Watanabe et al. 2012).

Clinical applications

Detection

Non-invasive and convenient “biopsy”

As for tumor tissue pathological biopsy, which is still the golden standard for tumor diagnosis, there are many limitations. Patients with CNS tumor usually undergo surgery and the resected tumor tissue can be utilized for pathological examination, though brainstem glioma biopsy is not routinely performed, which ensures the quantity of tumor cells regardless of biopsy area and size. For those who do not meet the criteria of surgery, such as with disseminated systemic disease, the biopsy is costly and may induce adverse events. Surgical biopsy adds inconvenience from a scheduling perspective and there are difficulties in sample preservation. Tumor heterogeneity is another problem hampering the biopsy. For primary CNS tumor, there is intratumoral heterogeneity, and for BM, there is intermetastatic heterogeneity within the same patient.

Compared to tissue biopsy, liquid biopsy is noninvasive and convenient, allowing for early assessment and follow-up. Furthermore, since it is easy to acquire the samples, the preservation of samples can be omitted, and fresh samples enable monitoring the progression in real time through molecular and epigenetic changes profiling. Liquid biopsy samples contain synonymous or even more comprehensive genetic information than biopsy, thus providing more insights into molecular mutation status, intratumoral and intermetastatic heterogeneity, even predating changes seen on MRI.

Early detection in asymptomatic patients

For those without clinical symptoms or radiological findings, liquid biopsy helps evaluate the tumor existence or progression risk, consequently implementing early intervention to reduce the risk. The majority of cancer genes mutate at moderate rates (2–20%) or lower, despite some genetic changes happen at high incidence (Lawrence et al. 2014), and mutation rate is not perfectly correlated with driver gene potency. Though genetic mutations and chromosomal changes are seemingly random, they may actually follow specific patterns. Early monitoring of known patterns may predict the tumor onset in healthy ones or progression in diagnosed ones. Moreover, by revealing unknown patterns, it is capable to successively link mutant genotypes to cellular and clinical genotypes and obtain deep understanding of the tumor evolution and its mechanisms (Wang et al. 2015b).

A recent study by Fanfani et al. suggested that germline and somatic mutation information may help explain cancer heritability in the more comprehensive population with higher tumor risk, and could provide guidance on formulating plans for early detection and progression surveillance (Fanfani 2021).

For instance, Kammesheidt et al. successfully detected TP53 and KRAS mutations by liquid biopsy using cfDNA in healthy volunteers (Kammesheidt et al. 2016), which showed the ability of liquid biopsy in early-stage tumor detection.

Reducing the cost

There are occasions that early diagnosis definitely leads to better overall prognosis (Lennon et al. 2020), either because of currently incurable cancer types, or indolent cancers that would never progress. In both occasions, the early diagnosis has very limited impacts on improving treatment effects, but causes adverse economic, physical and psychological burdens, which is termed “overdiagnosis” (Srivastava et al. 2019).

As liquid biopsy is simple, rapid, sensitive and specific for low levels of target molecules or macrostructures to achieve early detection, low incidence of cancer occurrence in the general population is a parallel problem since current screening methodology and many analysis techniques are not cost-effective. Based on the analysis of thermodynamic and kinetic parameters reflecting complex molecular interactions in biological fluids, the DSC (differential scanning calorimetry) analysis has made promising progress in discriminating healthy from glioma patients (Tsvetkov et al. 2018; Engh 2011; Chagovetz et al. 2013). When DSC thermograms are applied in liquid biopsy, it is termed “thermal liquid biopsy”. Compared to sequencing liquid biopsy, thermal liquid biopsy has shown approximate performance but is more economical

(Velazquez-Campoy et al. 2018; Hermoso-Durán et al. 2020; Rodrigo et al. 2019).

Likewise, FTIR (Fourier transform infrared) spectroscopy, nanoDSF (differential scanning fluorimetry) or CCP-based FRET (cationic conjugated polymer-based fluorescence resonance energy transfer) may substitute sequencing to reduce the cost, yet requiring more abundant studies to validate sensitivity and specificity (Tsvetkov et al. 2021; Sala et al. 2020; Ma et al. 2021). A new liquid biopsy technique, CancerSEEK [NCT04213326], not only detects mutant genes in the blood, but also locks on abnormal proteins in the blood, with an average price lower than 500 dollars. In about 1,000 cancer patients comprising common extracranial tumors like ovarian cancer and liver cancer, its accuracy rate reached 70%, which holds promise in expanding to screen brain tumors (Cohen et al. 2018). Other few commercial assays are also under development, such as GRAIL [NCT03085888], focusing on early detection.

Mouliere et al. implemented untargeted shallow whole-genome sequencing (sWGS, $<0.4\times$) of ctDNA in CSF derived from 13 patients (Mouliere et al. 2018). Through cfDNA size profiles analysis, shorter DNA fragments (145 bp) were shown to be in positive association with the presence of tumor-derived somatic copy number alterations (SCNAs) in CSF. Combining cfDNA SCNAs screening and fragmentation analysis using sWGS is feasible to provide information on the tumor genome at lower cost and more rapidly, which can also prepare for further sequencing in larger scales, such as whole-exome sequencing or targeted sequencing.

Characterization

Differential diagnosis

N6-methyladenosine (m6A) modification was widely found in peripheral blood cfRNA and the dysregulation of m6A modification level was closely linked with tumor occurrence and progression (Patil et al. 2016; Zhang et al. 2020). Based on 14,965 serum samples (gliomas = 185) of 12 cancer types, Zhang et al. screened a total of 228 m6A target miRNAs and selected 18 candidate miRNAs for the construction of a diagnostic signature (Zhang et al. 2021). The signature was proven to have no interference by gender, age or benign disease and yielded excellent performance, with an AUC of 0.979 in distinguishing cancer samples from non-cancer controls, and an AUC of 0.703 in distinguishing GBM samples from all the mixed samples in the training cohort.

Shen et al. developed an approach called cell-free methylated DNA immunoprecipitation and high-throughput sequencing (cfMeDIP-seq), which is bisulfite free and only requires 1–100 ng of cfDNA input (Nassiri et al. 2020). By profiling cfDNA of early-stage cancers from various body

liquids, researchers were able to identify methylation patterns unique to each cancer type. Then with the help of big data and machine learning, they could build a classifier to determine the cancer type. Subsequent discovery cohort consisted of 189 samples from seven different tumor types to classify them, respectively, from healthy controls with an AUC >0.9 for each cancer type. For further validation of plasma-based cfMeDIP-seq in CNS primary tumor, Nassiri et al. tested 220 patients with brain tumor and built a machine learning classifier (Nassiri et al. 2020). The classifier showed good performance (AUC = 0.99) in recognizing differentially methylated regions (DMRs) and distinguishing gliomas from BMs with extracranial primary tumor types along with healthy controls, and revealed highly specific signatures discriminating different primary brain tumors.

Sol et al. investigated the extended diagnostic power of thromboSeq (Best et al. 2017) to differentiate several neurological diseases (GBM, BMs and active multiple sclerosis lesions) (Sol 2020). By comparing the spliced RNA profile of TEPs, they successfully identified GBM patients from healthy controls (AUC = 0.97), and other neurological diseases (AUC = 0.81). They further developed a machine learning algorithm called digitalSWARM that scores on TEP to improve monitoring of individual GBM patients, which correlates with tumor behavior and allows distinguishing false positive progression from true progression (AUC = 0.86).

To discriminate tumor and non-tumor diseases and cross-compare different types of brain tumor, Wang et al. enrolled 163 patients with either nontumorous brain diseases, systemic lymphoma involving CNS, and metastatic or non-metastatic lung adenocarcinomas and analyzed their CSF using liquid chromatography–quadrupole time-of-flight spectrometric (LC-Q/TOF-MS) (Wang et al. 2020). As a result, 27 representative metabolites were proven diagnostic in discriminating different brain tumor types and yielded good performance with the highest AUC of 0.91 for the metastatic status of lung adenocarcinomas.

The WHO classification of tumors of the CNS

For those with unidentified brain mass, liquid biopsy helps ascertain the malignancy and correctly classify the tumor in time. In 2016, WHO published a new guideline on classifying CNS tumors; the molecular markers were especially spotlighted to describe brain tumor entities histology features, which were further refined especially in pediatric subtypes in 2021 (Louis et al. 2016). Furthermore, target therapies and treatment strategies for malignant brain tumor patients are predominantly dependent on specific molecular markers, emphasizing the importance of precision oncology. Recent clinical studies using liquid biopsy below approved the feasibility.

Wang et al. found using serum EVs for assessing the expression level of EGFR provided accurate prediction on HGG and LGG differentiation, and found positive correlation with the EGFR level and ki-67 labeling index, by implementing a flow cytometry method with microbead-based EV enrichment for detection, isolation and analysis of proteins and mRNAs in EVs. The analysis of NLGN3 and PTTG1 mRNA in EVs also revealed their potentiality as diagnostic markers (Wang et al. 2019).

Syndecan-1 (SDC1) mRNA can be detected in several GBM cell lines, but remains undetectable in normal brain tissues (Watanabe et al. 2006), and its expression level is related to a stroma hyperplastic, hypoxic and pro-angiogenic phenotype. Chandran firstly identified SDC1 as a plasma EV component which can help classify gliomas into GBM or LGG in a cohort ($n = 82$) (AUC: 0.81) and further in an independent cohort from TCGA (AUC: 0.91). They also provided robust evidence that the EVs containing SDC1 mRNA originated from GBM cells (Indira Chandran et al. 2019).

Huang et al. studied 11 patients with pediatric gliomas, including diffuse midline gliomas (DMGs) and successfully assessed H3 gene mutations in their CSF ctDNA for the first time, through targeted *H3F3A* and *HIST1H3B* via Sanger sequencing, and targeted H3.3K27M mutant *H3F3A* allele detection via nested PCR (Huang et al. 2017).

Billard et al. proposed a novel approach to classify diffuse gliomas by detecting telomeric DNA in blood samples and revealing telomere maintenance mechanisms (TMM) (sensitivity: 100%; specificity: 97.3%) (Billard et al. 2021). They further combined TMM with IDH mutation status and histological grading to develop a simple and rapid classification tool called the TeloDIAG, which efficiently defines five glioma subtypes corresponding to five different classes by the 2016 WHO classification (sensitivity of 56% and specificity of 97% for IDHwt astrocytoma).

Medulloblastoma (MB) is the most common form of malignant pediatric brain tumor, with a much lower frequency of oncogenic genomic mutations than other pediatric cancers or adult cancers. During MB progression and clinical treatment, distinct epigenetic signatures and altered DNA methylation were found by Northcott et al. who epigenetically analyzed 1256 cases (Northcott et al. 2017). Different subtypes display distinct transcriptional and epigenic profiles (WNT and SHH, driven by Wingless and Sonic hedgehog signaling pathways, and Group 3 and Group 4, with less clear underlying genetic and biological mechanism). The prognosis of MB is correlated with the subtype, for example, approximately 30% of patients with Group 3 or 4 MBs presented signs of metastasis at initial diagnosis (Srinivasan et al. 2016), and relapsed MB still maintains the original molecular subtype. Furthermore, they integrated this data with large public datasets (training cohort: 438;

validation cohort: 189) and set up a reliable survival score for each patient.

Profiling genetic mutation for targeted therapy

Gliomas of the brainstem are molecularly heterogeneous and difficult for resection. Pan et al. assessed alterations in 47 brainstem glioma patients by NGS with ctDNA in CSF for potential targeted therapies, which include mutations in *H3F3A/HIST1H3B*, *IDH*, *BRAF*, *PDGFRA*, *PPM1D* and *MET*. Among all CSF ctDNA samples, 82.5% (47/57) yielded at least one tumor-specific mutation. Subsequently, the patient cohort went through surgical resection or open biopsy, and the matched DNAs were sequenced to verify the sensitivity and specificity of liquid biopsy. The results showed all primary tumor alterations by biopsy were detected in over 83% (31/37) of matched CSF ctDNA samples. For those with primary tumor DNA biopsy negative for mutations, they also found 30% (3/10) exhibited detectable mutations exclusively by CSF ctDNA sequencing. Finally, they carried out liquid biopsy using cfDNA from plasma, which showed lower sensitivity than matched CSF ctDNA (plasma: 38%, CSF: 100%) (Pan et al. 2019).

While previous study has exhibited that *TERT* promoter mutations are among initial genetic alterations in *IDH-WT* GBM (Barthel et al. 2018), they commonly happen and act as a precondition for rapid growth. *C228T* and *C250T* account for two of the most frequent alteration subtypes in *TERT* promoter mutations, but technically difficult in amplification due to the GC-rich regions in the promoter loci. Recently, Muralidharan and his colleagues developed a ddPCR assay to assess gliomas patients with *TERT* promoter mutations in cfDNA from plasma, independently in discovery and blinded validation ($n = 157$) cohorts from multiple centers (sensitivity: 62.5%; specificity 90%) (Muralidharan et al. 2021).

BRAF V600E mutation activates the MAPK/ERK pathway (Carlino et al. 2015) and contributes to a 10-year progression-free survival rate decrease by 33% compared to wild type in patients with pediatric LGGs (Lassaletta et al. 2017). As one of the inhibitors of the kinase BRAF, which mainly functions in MAPK/ERK signal paths, dabrafenib holds excellent effectivity against BRAF in vitro and the best diffusivity in brain tissues, thus harboring the most popularity in clinical practice. However, the use of dabrafenib is contraindicated in patients with wild-type *BRAF* due to the risk of tumor progression in these patients (Sanchez et al. 2018). To detect and quantify *BRAF V600E* mutation with a high efficiency, García-Romero et al. sequestered cfDNA and extracted EV-derived DNA from 29 pediatric CNS tumor patients from serum, plasma and CSF (García-Romero et al. 2019). The results showed that ctDNA derived from serum rather than plasma yielded the best performance

for genetic characteristics such as *BRAF V600E* mutation of the original tumor by dPCR detection. Kang et al. tested the feasibility of detecting circulating *BRAF V600E* in a cohort of nine patients with known *BRAF* mutant gliomas or metastatic disease, showing a sensitivity up to 80% and a specificity up to 100% (Kang et al. 2021).

The US NCCN guidelines recommend epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) like osimertinib for the first-line treatment of NSCLC patients with *T790M*-positive mutations in the EGFR gene, but acquired resistance of TKIs is prevalent in clinical management. BMs are frequently found in NSCLCs that possess at least one oncogenic driver mutation, which can be a potential therapy target. Wei et al. assessed 375 patients harboring either an *EGFR 19del* or *L858R* mutation that sensitizes TKIs ascertained by initial biopsy and implemented ddPCR and ARMS-PCR assay on evaluating *T790M* mutation, which approximately explains for 50% of the acquired TKI resistance in Caucasian and Asian populations, through plasma extracted ctDNA validated by NGS (Wei et al. 2019). Their results indicated that acquired *T790M* mutation is positively correlated with primary *EGFR 19 del* and brain metastasis as revealed by univariate analyses. Kaplan–Meier survival curves suggested that progression-free survival of initial EGFR-TKI treatment was significantly longer in *T790M*-positive patients than negative ones.

Another study involving 71 GBMs was reported by Figueroa et al. The CSF of patients was collected, and the EVs were isolated and assessed for *EGFR-vIII* mutation and *EGFR* amplification status, with comparison of cells from primary tumor biopsies (Figueroa et al. 2017). The results elucidated that CSF-derived EVs contain RNA signatures reflecting wild-type *EGFR* expression and *EGFR-vIII* status in GBMs, with a sensitivity up to 61% and a specificity up to 98%.

Monitoring

Evaluating early treatment response

For DMGs which are impossible for surgical resection, radiotherapy remains the standard of care at diagnosis. In a recent study aiming at differentiating pseudoprogression (PsP) and DMG progression carried out by Panditharatna et al., 88% of patients with pediatric DMGs were *H3K27M* positive using CSF and matched plasma, with the highest enrichment of ctDNA in CSF (Panditharatna et al. 2018). Following radiotherapy, a significant decrease in *H3K27M* plasma ctDNA (at least 50% decrease) consistent with MRI assessment (at least 10% decrease) of tumor volume alterations was found in 83% of patients, which indicates that liquid biopsy could function as a strong supplement.

Compared with genomic alterations, the specificity of detecting epigenetic changes is often lower, but ctDNA methylation often yields good sensitivity, since methylated DNA often occurs in early carcinogenesis. The detection of O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation status through cfDNA helps predict the treatment response of temozolomide in adults (Maire 2021). Sabedot et al. identified a cfDNA-derived methylation signature through genome-wide DNA methylation profiling of gliomas (Sabedot 2021). Based on the signature, they further verified a score metric (the "glioma epigenetic liquid biopsy score" or GeLB) that optimally distinguished patients with or without glioma (sensitivity: 100%, specificity: 97.78%). Following up patient's GeLB score can also reflect their tumor progression and treatment response.

Accessing minimal residual disease

A traditional way of monitoring the dynamics of tumor is to access the sugar, cholesterol and/or protein circulating biomarkers, such as prostate-specific antigen (PSA) for prostate cancer and cancer antigen (CA) 19-9 for pancreatic cancer. However, these biomarkers are found only in a small portion of tumor and lack specificity for an individual. The analysis of cfDNA deals with the above problems, since the genetic alteration happens in every tumor. To discriminate ctDNA from normal cfDNA, genetic alterations in cfDNA must be detected and compared with DNA in normal cells in the same individual. Also, circulating biomarkers are not accurate for onset time prediction, since the protein biomarkers are persistent in circulation for several weeks. The half-life of cfDNA is comparatively short (approximately 2 h), so changes in cfDNA can be identified before being seen on regular imaging or employing protein biomarkers with weeks to months ahead (Diehl et al. 2008; Dawson et al. 2013). This characteristic allows liquid biopsy to be utilized in early detection and monitoring of tumor progression during or after treatment.

During routine cancer surveillance, MRI and CTCs found in CSF cytology and flow cytometry analysis are highly specific of CNS relapse, but harboring low sensitivity. Ignatiadis et al. introduced a term 'ctDNA relapse' for those with detectable ctDNA, but without obvious imaging evidence after completion of primary cancer surgery and neoadjuvant and/or adjuvant chemotherapy (Ignatiadis et al. 2021). EscuderoIn et al. reported that CSF ctDNA analysis not only facilitated MB subgroup and risk stratification, but also helped to identify minimal residual disease in two cases, as ctDNA obtained in the follow-up CSF samples had similar mutations and/or loss of chromosome arms (Escudero et al. 2020). Liu enrolled 123 MB patients and 476 CSF samples, and applied low-coverage whole-genome sequencing for cfDNA detection of tumor-associated CNVs, which were

used as a biomarker for measurable residual disease (MRD) and showed good detectability (85% in patients with metastatic MB, 54% for those with non-metastatic disease and 64% for those with negative CSF cytology findings) (SHH, 32%; WNT 63%; Group 3, 77%; Group 4, 75%). Persistent MRD positivity at the end of treatment in patients, regardless of initial risk grouping, is correlated with shorter PFS, which is detectable months ahead of radiographic progression (Liu et al. 2021).

Bobilo et al. evaluated CSF and plasma from 19 patients with CNS B-cell lymphomas and observed that CSF ctDNA levels increased earlier than CTCs by flow cytometry, which confirmed CNS relapse after months (Bobillo et al. 2021). Besides, ctDNA also showed better performance in monitoring CNS tumor burden and response to treatment. In another trial exploiting the effect of ibrutinib with HD-MTX-based chemotherapy in CNS lymphomas, Grommes applied MSK-HemePACT targeted panel to sequence CSF ctDNA and observed a considerable fraction of patients with subsequent recurrence or refractory harbor tumor DNA in CSF (Grommes et al. 2019).

Tracking evolution of resistance in real time

GBM is characterized by a high infiltration rate and treatment resistance, remaining the most fatal brain tumor. While GBMs may be primary, or secondary ones that evolve from LGGs, it makes tracking gliomas evolution of great importance. Zeng et al. looked into the mechanism of TMZ resistance by characterizing exosomes from two TMZ-resistant cell lines, patients' serum and matched CSF using qPCR, and demonstrated that miR-151a in exosomes not only plays as a positive predictor to TMZ response, but also potentially serves as a target for treatment of refractory GBMs. The results suggested that recurrent GBM samples and TMZ-resistant cells usually present lower levels of miR-151a, while high levels of miR-151a can strengthen the response to TMZ *in vitro* via inhibiting XRCC4-mediated DNA repair (Zeng et al. 2018).

Brain tumor may directly release ctDNA into CSF and its level is related to tumor burden and adverse outcome. In extensive research carried out by Miller et al., 85 patients with WHO II-IV gliomas were evaluated using CSF-derived ctDNA (Miller et al. 2019). The data suggested that the alterations detected in CSF ctDNA from diffuse gliomas represent the genome landscape found in tumor biopsies. They exploited this technique to track the pattern of genomic evolution within the same patient and found out that truncal genetic alterations are characterized by *IDH1* and 1p/19q codeletion in early tumorigenesis and subsequently in *TP53*, *TERT* and *ATRX*, which mostly correlate with the core pathways of gliomas, especially growth factor receptor pathways.

To investigate liquid biopsy techniques and mediums in leptomeningeal metastases (LM) frequently reported in NSCLC with EGFR mutations, Jiang et al. detected the driver and resistance mutations in 21 NSCLC patients diagnosed with LMs and investigated the performance of CellSearch Assay™, the Thinprep cytologic test (TCT), and brain MRI (Jiang et al. 2017a). The results supported CellSearch as it yielded the highest sensitivity (95.2%) and specificity in capturing CSF CTCs among other single methods. By CellSearch, they also found significantly higher concentration of CTCs in CSF than peripheral blood samples.

Predicting survival time

To noninvasively detect MBs and discriminate the subtypes, Li et al. performed whole-genome bisulfite sequencing (WGBS) and CMS-IP-seq analysis that elucidated 6598 differentially methylated CpGs in the CSF-derived ctDNA paralleling the mutation direction of MB tumors. By associating DNA methylation level at this CpG site with survival rates, they found that high DNA methylation may function as a poor prognostic marker for pediatric MB patients (Li et al. 2020).

Since IDH mutations are common in LGGs and the mutated enzymes result in increasing abundance of tissue total 2-HG, including R-2-hydroxyglutarate (R-2-HG) and S-2-hydroxyglutarate (S-2-HG), Sim et al. quantitated R-2-HG and S-2-HG and calculated the individual ratio of R-2-HG/S-2-HG (rRS) in tissues and blood samples from glioma patients intraoperatively, based on HPLC–mass spectrometry (Sim et al. 2019). The tissue rRS proved sensitive and specific for *IDH* mutations, while blood rRS did not; it is rapid and can be implemented during surgery. Higher rRS is associated with poorer prognosis, which may be an additional target of CSF liquid biopsy.

Future development

Tissue-based multi-panel gene analysis has been contributing to the clinical application of liquid biopsy. As more and more novel genes are discovered, gene panels in panel sequencing are rapidly developing, for instance, TAM-Seq (tagged-amplicon deep sequencing) and CAPP-Seq (cancer personalized profiling by deep sequencing) both harbor high sensitivity and coverage depth with balanced cost and coverage mutation number (Thierry et al. 2010). The earliest application of liquid biopsy in brain tumor may take place in most prevalent mutation points or panels of different miRNA, such as IDH mutation, TERT mutation, a marker correlated with ATRX loss on total blood cells, and miR-21.

For example, GliomaDx assay, an LNA-based allele-specific qPCR method developed by Diplas et al., has yielded

much sensitivity and rapidity in diffuse glioma classification (Diplas et al. 2019). Based on the sensitivity analysis, GliomaDx targets recurrent mutations in the TERT promoter and codon 132 of IDH1 in samples, which is over 200 times more sensitive than Sanger sequencing and can be performed within an hour. Besides exploiting CTCs or ctDNA in plasma, voided urine, or CSF, GliomaDx also showed ability in genotyping surgically resected tissue or frozen specimens of variable tumor purity, making intraoperative diagnosis more practical. Other researches like the circTeloDIAG, a liquid biopsy tool combining detection of three markers for glioma, are ongoing (NCT04931732).

Recently, urine is emerging as a CNS tumor liquid biopsy source. With the development of a nanowire device that needs only 1 mL of urine from mainly glioma patients and noncancer individuals, an artificial intelligence (AI) diagnostic model detecting miRNAs of such tumors in urine samples was established at a sensitivity and specificity of 100 and 97% (Kitano et al. 2021). Mouliere et al. detected tumor-derived DNA in vast samples (in CSF, 7/8; in plasma, 10/12; and in urine, 10/16) (Mouliere 2021). Based on cfDNA fragmentation features in urine samples, they further built a random forest (RF) to classify gliomas and noncancer patients that exhibited a median AUC = 0.91.

Combining liquid biopsy with machine learning is promising to improve the accuracy and efficiency in recognizing mutational patterns and identifying key features predictive of progression, which has been proven by several studies (Zhang et al. 2021; Nassiri et al. 2020; Sol 2020; Li et al. 2020). With the development of machine learning and the prevalence of big data from multiple centers, it is also feasible to uncover the unknown driver genes by sequencing multiple mutational patterns and further utilize these patterns for the construction of predictive models. However, aging, chemo and popular germline mutations may affect the analysis of patterns and they have been not extensively catalogued. Like other noninvasive diagnosis tools, artificial intelligence is promising to be involved in future liquid biopsy workflow and accelerate liquid biopsy into clinical practice. Multiple studies have utilized machine learning for sequencing analysis. However, deep learning requires a large quantity of data. While deep learning in radiomics can easily gain images for analysis, liquid biopsy lacks extensive clinical application which limits the source of learning material. A public database with standard criteria of reporting is crucial for future development of liquid biopsy.

Based on multiple noninvasive diagnostic tools, somatic mutation accumulation and driver genes can be evaluated, the characteristics of tumor can be assessed, and progression of tumor can be recorded and visualized. For example, Cucchiara et al. integrated liquid biopsy and radiomics to monitor clonal heterogeneity of *EGFR*-positive NSCLC (Cucchiara 2020). As a result, more individualized treatment

plans can be tailored and patients with imperceptible premalignant lesion or those who undergo surgery can also benefit. Future studies should focus on improving the sensitivity and specificity.

Conclusion

The emergence of noninvasive detection techniques such as liquid biopsy has yielded much progress in recent years. The liquid biopsy, analyzing molecules or macrostructures in low concentration from body liquid, shows minimal invasiveness toward patients who are susceptible to tumor or cannot withstand biopsy. The blossoming of sequencing technique, such as ddPCR and NGS, ensures more accurate molecular profile, higher specificity, and more clinical practicability. The plasma is the most preferable source for brain tumor liquid biopsy due to convenient sampling. For patients with primary brain tumor, the CSF may be a better source of liquid biopsy, since the BBB blocks the spreading of the target molecule or macrostructure, which may be safely and transiently opened by techniques like MRgFUS. For patients with metastases, the comparison among CSF, blood and normal tissue may provide more information regarding intratumoral and intermetastatic heterogeneity. Though cfDNA is more convenient, EV-derived DNA may hold inherent stability due to its lipid bilayer enclosed structures, yet isolation and purification of EVs are associated with more technical difficulties. Due to the limitations in the low concentration, discrimination and quantification of the target molecules, the overall profiling is still challenging, thus needing more reliable source and more sophisticated sequencing techniques. Another barrier hindering liquid biopsy's extensive clinical application is its high cost. Shallow WGS or targeted extreme including ddPCR may be relatively cost-effective (Mouliere et al. 2018). Other noninvasive detection techniques like DSC, FTIR, nanoDSF or CancerSEEK (Tsvetkov et al. 2018; Tsvetkov et al. 2021; Engh 2011; Chagovetz et al. 2013; Sala et al. 2020; Cohen et al. 2018) may initially replace sequencing to reduce the cost and may prepare for further larger-scale sequencing, but require more studies to prove feasibility.

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Data availability The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that there are no competing interests.

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Consent for publication All listed authors have actively participated in the study and have read and approved the submitted manuscript.

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