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Current Advances and Future Perspectives of Cerebrospinal Fluid Biopsy in Midline Brain Malignancies

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Opinion statement

Malignancies arising in midline brain structures, including lymphomas, teratomas, germinomas, diffuse midline gliomas, and medulloblastomas typically respond to systemic therapies, and excessive surgical excision can result in serious complications, so that total surgical removal is not routinely performed. Identifying tumor specific biomarkers that can facilitate diagnosis at early stage and allow for dynamic surveillance of the tumor is of great clinical importance. However, existing standard methods for biopsy of these brain neoplasms are high risk, time consuming, and costly. Thus, less invasive and more rapid diagnosis tests are urgently needed to detect midline brain malignancies. Currently, tools for cerebrospinal biopsy of midline brain malignancies mainly include circulating tumor DNA, circulating tumor cells, and extracellular vesicles. Circulating tumor DNA achieved minimally invasive biopsy in several brain malignancies and has advantages in detecting tumor-specific mutations. In the field of tumor heterogeneity, circulating tumor cells better reflect the genome of tumors than surgical biopsy specimens. They can be applied for the diagnosis of leptomeningeal metastasis. Extracellular vesicles contain lots of genetic information about cancer cells, so they have potential in finding therapeutic targets and studying tumor invasion and metastasis.

Introduction

The term midline brain structures refer to the thalamus, pineal body, brainstem, spinal cord, and cerebellum, all of which surround the ventricular system (Fig. 1). Numerous malignancies are known to originate from this critical region including lymphomas, teratomas, germinomas, diffuse midline gliomas, and medulloblastomas all of which are often lethal brain tumors with the patients facing a grim prognosis.

Primary or secondary intracranial lymphomas often occur in the periventricular region (Fig. 2) and show infiltrative growth and as such patients have a poor outcomes [1]. Most intracranial lymphomas are diffuse large-B cell lymphoma, most of which have mutations in the MYD88 and CD79b genes [1]. Currently, a pathological diagnosis of lymphoma is required prior to the initiation of any systemic therapy. While MRIs are helpful in diagnosing lymphoma, they are not sufficient. In some cases, the main purpose of surgical resection or biopsy is merely limited to pathological diagnosis; however, this brings a high risk for patients due to the fact that tumors are often located in crucial regions of the brain and also delays the initiation of conventional therapies.

Intracranial germ cell tumors (GCTs) are a group of brain tumors with different origin. Pathologically, they can be divided into two types: germinomas and non-germinomatous GCTs. The latter is a large group that includes several tumors of different origins [2]. Currently, treatment options vary for the different types of tumors. Because of the sensitivity of germinomas to radiotherapy, focal or whole brain radiotherapy is the main treatment for pure germinomas. In addition, chemotherapy can reduce the radiological dose. For non-malignant mature and immature teratomas, total resection is the best option for a chance of a cure. For other malignant germ cell tumors, surgical resection, if feasible, combined with radiotherapy and chemotherapy is the best option to avoid recurrence [3]. Overall, early diagnosis leads to a better management of GCTs.

Diffuse midline gliomas (DMGs) tend to affect the pons (Fig. 2) and are known as diffuse intrinsic pontine gliomas (DIPGs), whose 2-year survival rate is extremely low [4]. DMGs often harbor mutations in the H3 gene encoding H3.3(H3F3A), H3.2(HIST2H3C), and H3.1(HIST1H3B/C) [5•]. The presence of these mutations is associated with a much poorer outcome compared with tumors without these mutations. Recent studies have also identified other genetic alterations including PPM1D, IDH1, TP53, and ATRX [6••]. These results suggest that DMGs are comprised of multiple genetic subgroups. To clarify the intra-tumoral heterogeneity of DMGs, dynamic monitoring tumor is needed. In this regard, novel therapeutic strategies targeting the enzymes responsible for chromatin modifications such as histone methylase, demethylase, and deacetylase have shown the most promise [4, 7–9]. Immunotherapy is rapidly becoming the treatment of choice for selected malignancies. At present, several clinical trials are currently being conducted to assess its safety and efficacy for the treatment of DIPGs [4].

Medulloblastoma is a highly aggressive neoplasm that originates mainly from the cerebellar vermis (Fig. 2) [10]. According to genetic alterations and abnormal signaling pathways, it can be divided into four subgroups [10]. Since medulloblastomas are found in the posterior fossa and frequently involves vital midline brain structures, total resection is unlikely and may result in serious complications. Despite this, it is well documented that tumor recurrence or metastasis along the ventricular system are the leading causes of death [11]. Thus, identifying tumor recurrence and metastasis during the initial stages can provide therapeutic benefits for some patients. However, the most common neuroimaging tests, such as CT and MRI scans, have relatively low sensitivity (i.e., they can be slow to indicate tumor progression or relapse) and low specificity (i.e., it is difficult to distinguish between pseudo-progression and true progression following radiotherapy).

Collectively, since these midline brain tumors typically respond to systemic therapies, early and accurate diagnoses are key to achieving a satisfactory outcome. However, the current means of detecting these tumors are limited. While histopathology remains the gold standard for diagnosis, surgical resection or traditional biopsies may pose huge risks to patients due to the sensitive anatomic location of these tumors. Worse still, these approaches cannot achieve dynamic monitoring of intracranial tumors. The commonly used neuroimaging tests, cerebrospinal fluid (CSF) cytology and flow cytometry are of relatively low sensitivity and specificity. It is widely believed that CSF circulates in a certain direction. The driving force behind this circulation is the pressure gradient between the production and absorption of CSF [12–14]. However, a novel concept has challenged this hypothesis: CSF circulation is dominated by reciprocating pulsations from the cerebrovascular

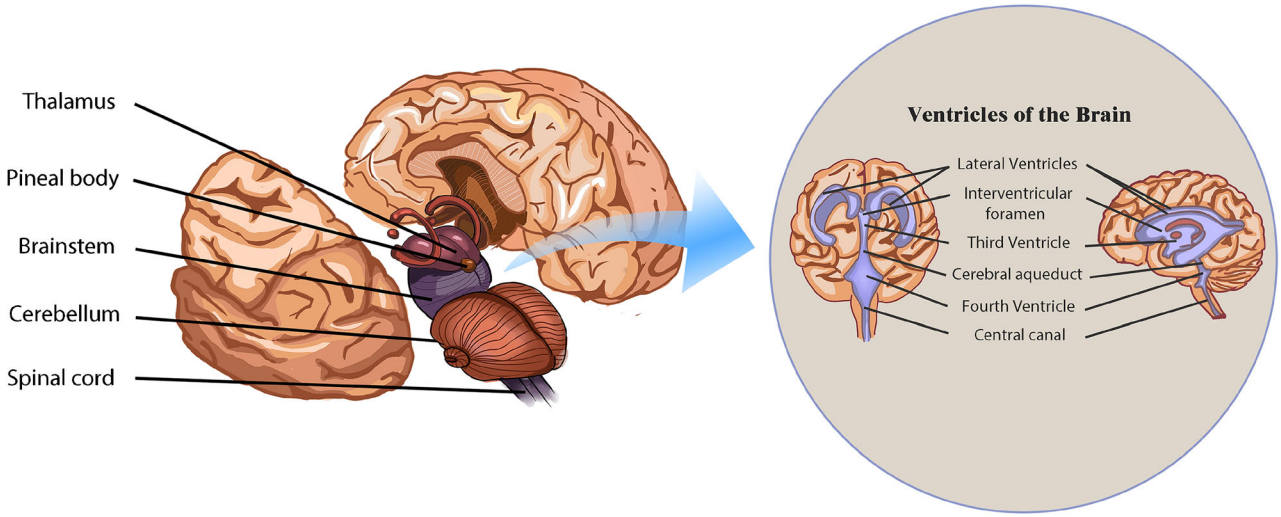

Fig. 1. Schematic overview of midline brain structures, including thalamus, pineal body, brainstem, spinal cord, and cerebellum, and ventricular system.

system in the subarachnoid space [15, 16]. Therefore, it is reasonable to believe that the closer the CSF is to the ventricular system, the more genetic information associated with brain tumors is enriched, suggesting that CSF

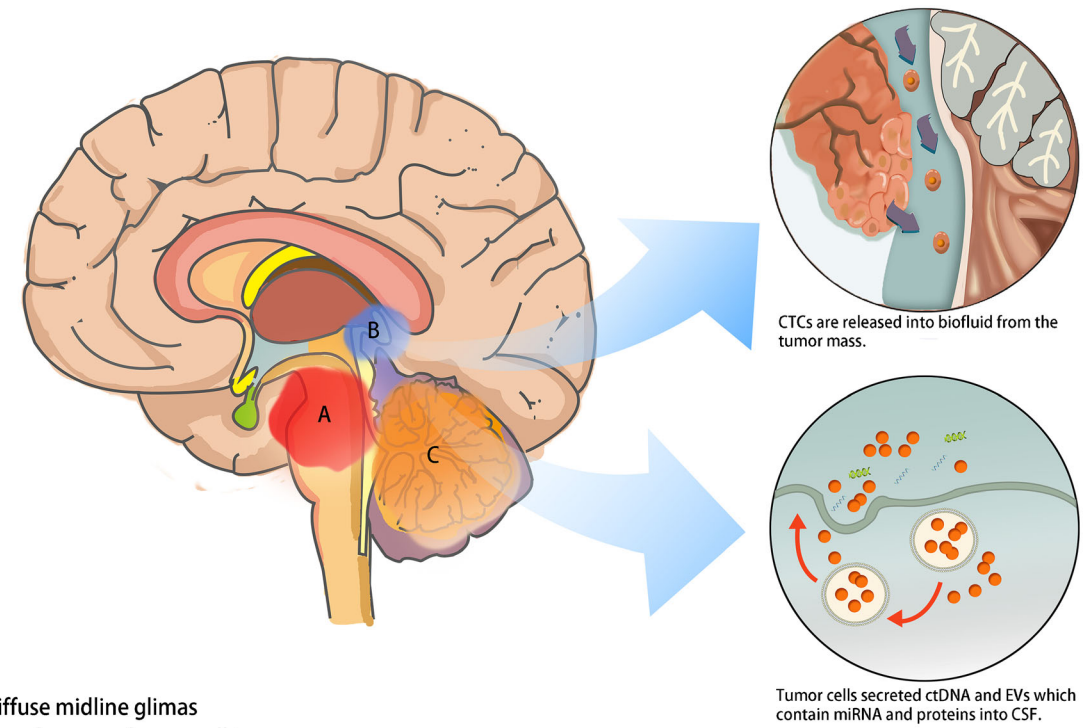

Fig. 2. The most common malignancies arising in midline brain structures. Diffuse midline gliomas often located in thalamus and brainstem; lymphoma and germ cell tumors usually occur in periventricular and pineal body; the majority of medulloblastoma are derived from cerebellum. And proposed mechanisms for CTCs, ctDNA, and EVs secretion.

biopsies for midline brain malignancies may be an attractive approach. This review describes recent advances in liquid biopsy in midline brain malignancies and provides a fundamental for the selection of sampling sites for liquid biopsy.

Circulating tumor DNA (ctDNA)

Circulating DNAs are DNA fragments that are released into body fluids by somatic cells. The mechanism behind the release of circulating DNAs has not been fully elucidated. Previous studies have shown that DNA fragments enter the circulation during the process of cell death or are derived from living cells (Fig. 2) [17]. When an inflammation, infection, or neoplasm occur, the levels of circulating DNA are increased significantly [18–20]. The levels of circulating DNA in tumor patients which have the same genetic alterations as the primary tumor lesion are considered to be derived from cancer cells [21, 22] and are referred to as circulating tumor DNAs (ctDNAs). As the presence of the blood-brain barrier, the detection rate of ctDNA in blood sample can be extremely low [23], although the use of peripheral blood as a source of ctDNA can be used for detecting several tumors [23–26]. Increasing evidence has shown that compared to plasma, direct CSF sampling is of greater superiority for the sensitive detection of tumor-specific mutations in CNS tumors [27, 28]. Moreover, several alterations that could not be found in primary cancer samples could however be identified in the CSF, highlighting an additional potential benefit of a CSF biopsy over a primary tumor [6••]. Thus, CSF might be the best source of ctDNA for detecting tumor-specific mutations in brain tumors [29].

However, not all intracranial tumors can be detected by a CSF biopsy. A study conducted by Wang et al. demonstrated that tumors which are located far from the ventricular system did not show the presence of ctDNA in the CSF [30]. CSF ctDNA tends to be enriched in malignant brain tumors [6••]. From this perspective, midline brain malignancies are continuously being bathed by the CSF and could be ideal targets for CSF ctDNA sequencing. Notably, even for malignancies which surround the ventricular system, the best CSF sampling site remains to be determined. For instance, in patients with diffuse midline gliomas, CSF sampled from the 4th ventricle harbored a significantly higher mutant allelic frequency (MAF) than CSF sampled from the subdural space near the brainstem, lateral ventricles, cervical spine, and lumbar spine [5•], a finding which is not consistent with the results from Pan et al. [5•] who found that tumor-specific mutations could be detected by sequencing the ctDNA from three different CSF samples, including an open biopsy, a V-P shunt, and traditional lumbar puncture. If these data are correct, a lumbar puncture should be considered as the first-line detection method for midline brain malignancies, since it is less invasive and more rapid than CSF from biopsy or V-P shunt. In addition to the sampling site, timing is another critical issue. It has been shown that the level of ctDNA in the CSF is positively correlated with tumor burden and adverse outcomes [31••, 5•]. Taken together, CSF ctDNA is of potentially great value for the dynamic monitoring of tumor progression and relapse and for assessing the efficacy of treatment.

Currently, the techniques used to analyze ctDNA include targeting known tumor-specific mutations and whole-genome sequencing. Traditional Sanger sequencing and droplet digital polymerase chain reaction (ddPCR) are the main techniques used for the analysis of known tumor-specific mutations [1, 5•, 32]. Replacement of lysine 27 with methionine in the gene encoding histone H3 is a characteristic mutation found in diffuse midline gliomas [33–36]. In light of this, one study used nested PCR and targeted Sanger sequencing to analyze ctDNA from CSF samples. Histone H3 mutations were successfully detected in 4 out of 6 CSF samples from patients with DMG with a test sensitivity of 87.5%. These were further validated by immunohistochemical staining and Sanger sequencing of a matched tumor sample [32]. Another study achieved higher detection rates by using ddPCR and found a significant decrease in ctDNA H3K27M MAF in CSF samples from patients with DMG after treatment [5•]. Also, testing CSF ctDNA for the myeloid differentiation factor 88 (MYD88) p.L265P mutation promises to become a novel approach to diagnosis patients with periventricular lymphoma [1]. Wang et al. proved that assessment of O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation in CSF using methylation-specific PCR may provide a promising clinical methodology for the early diagnosis and individual treatment of glioma patients [37]. Yang et al. used amplification refractory mutation system-PCR to analyze EGFR gene amplification in 30 brain metastases in lung cancer with a sensitivity of 67% and specificity of 82% [38]. Importantly, most patients with the EGFR mutation identified in cerebrospinal fluid responded well to EGFR-tyrosine kinase inhibitor therapy, showing that EGFR analysis of CSF can be used as an indicator for treatment with an EGFR-tyrosine kinase inhibitor. Although ddPCR is sensitive enough to detect and quantify rare and low MAF ctDNAs when compared with Sanger sequencing, this method is based on the identification of prior mutations in the tumor. With the development of next-generation sequencing (NGS), large-scale sequencing and even genome-wide sequencing have become feasible as tools for clinical diagnosis. Early events in tumorigenesis and mutations available for defining the different molecular subtypes of glioma, for example, co-deletion of chromosome arms 1p and 19q and mutations in the metabolic genes isocitrate dehydrogenase 1/2, were shared between CSF and matched tumor samples [6••, 31••]. Although both ddPCR and NGS can report MAF to quantitatively assess tumor burden and heterogeneity [1, 5•, 6••, 31••], NGS has incomparable advantages over ddPCR since it can cover mutations that traditional methods and ddPCR cannot detect. Thus, NGS provides an opportunity for finding new therapeutic targets and revealing novel drug resistance mechanisms. Furthermore, in terms of the sensitivity of liquid biopsy, NGS has gone far beyond traditional methods [6••]. However, NGS is a costly and time-consuming test and requires a lot of samples to generate satisfactory results.

Circulating tumor cells (CTCs)

Cells in primary tumors or metastatic lesions often enter into the circulation or other bio-fluids during the invasion of surrounding tissues and blood vessels (Fig. 2). These cells are called circulating tumor cells (CTCs). It is documented that CTCs could promote tumor metastasis,

but the detailed mechanisms how CTCs drive metastasis remain largely unknown [39]. Since metastatic colonization needs to overcome a series of obstacles, the number of CTCs in circulation far exceeds the number of metastatic lesions [40]. Therefore, an analysis of CTCs may be more sensitive than a biopsy of metastatic lesions. To date, the main source of CTC samples is peripheral blood (PB), whereas the detection of CTCs in CSF for managing intracranial tumors is much less clear.

In peripheral circulation, the concentration of CTCs is low making detection by conventional approaches unlikely. Currently, the main strategy for capturing CTCs in circulation is the CellSearch® system, approved by the Food and Drug Administration in 2004. This technique uses magnetic particles bound to antibodies that target epithelial cell-adhesion molecules (EpCAM) thus allowing for the adsorption of EpCAM-positive cells. Under the action of magnetic field, these positive cells loaded with magnetic particles are adsorbed by the system. The captured cells are then labeled with fluorescently labeled epithelial cell markers and identified [41]. This system can only be utilized for the detection of tumor cells that express EpCAMs, such as breast cancer, prostate cancer, and colorectal cancer [42–44]. Epithelial cancers undergoing the epithelial-mesenchymal transition and non-epithelial tumor cells including gliomas and other primary brain tumors tend to down-regulate EpCAM, making them unrecognizable by the CellSearch® system [45]. One immunocytochemical detection system targeting glial fibrillary acidic protein (GFAP) on the surface of glioblastoma cells has been successfully used to capture CTCs present in a peripheral blood sample [46]. But this system has some limitations: not all glial cells express GFAP [47, 48] and for these GFAP-negative GBM cells, no specific markers have yet been found. In addition, another method using a "negative-depletion" CTC-iChip was able to successfully isolate GBM cells from peripheral blood by removing leukocytes from the blood samples [49, 50]. Nevertheless, the detection rates of these methods are relatively low and are mainly limited to GBM, and are still far from clinical application.

Based on early data, the CTC burden is considered to be an independent prognosis predictor in breast, prostate, and colorectal cancers [41, 51–53]. After surgery plus radiotherapy and chemotherapy, CTCs in peripheral blood decreased significantly. Those patients without any CTC in circulation survived longer than those who retained detectable CTCs, suggesting that CTCs may serve as a promising method for the early evaluation of tumor progression and relapse. Interestingly, in the field of tumor heterogeneity, CTCs may better reflect the genome of tumors than surgical biopsy specimens. Fehm et al. successfully found human epidermal growth factor receptor 2 (HER2) mutations in CTCs while the primary lesion was HER2 negative, thus broadening the treatment options [54]. To date, the diagnosis of leptomeningeal metastasis from a primary lesion is the major application of CTCs in CNS tumors. It is well documented that CTCs have a higher sensitivity and specificity for leptomeningeal metastasis (LM) compared with a neuroimaging test and CSF cytology [55–57]. Unfortunately, a study comparing the use of CSF and peripheral blood has yet to be conducted. More studies are

therefore needed to explore the feasibility of CSF-derived CTC in assisting the diagnosis and treatment of midline brain malignancies.

Extracellular vesicles (EVs)

EVs are membrane-containing bodies that are secreted by eukaryotic, prokaryotic, and plant cells and range in size from 30 to 1000 nm (Fig. 2). According to their origin and size, EVs can be divided into three types: micro-vesicles/micro-particles/ectosomes (50–2000 nm), exosomes (30–100 nm), and apoptotic bodies (50–5000 nm). Cells are able to secrete micro-vesicles through fission and budding of the cyto-membrane. Exosomes are produced by endosomal network and are then released by fusion with the plasma membrane. Apoptotic bodies are produced by apoptosis. DNA, RNA, and proteins are the major components of EVs, which mainly play a role in intercellular signal transduction [58, 59]. Despite numerous studies compared plasma with CSF for separating EVs, CSF has many potential advantages as a source of EVs. Since CSF is found in the ventricular system and subarachnoid space, it is easy to exchange substances with midline brain malignancies. Compared with plasma, CSF contains fewer EVs from non-brain tissue sources such as leukocytes and platelets [60••, 61–63]. Furthermore, the expression level of EV-derived proteins decreases after treatment, suggesting that EVs can be used as an indicator of tumor progression [60••].

Recently, an increasing number of studies have focused on diagnosis and monitoring of brain tumors by analyzing EVs. Real-time quantitative PCR is most commonly used to quantitatively analyze EV-derived RNA expression levels [61, 62, 64–67]. A few studies have also used ddPCR [68] and sequencing methods [69]. Based on EV-derived proteins, one research group has created a new strategy to diagnose glioma by combining chromatographic isolation and ultrasensitive immunoprofiling of EV-derived proteins. Using this method, this group was the first to identify syndecan-1 as a diagnostic and prognostic indicator of GBM [60••]. EVs can be separated by ultracentrifugation, after which their morphology can be analyzed by electron microscopy. Their concentration and size can be determined by nano-tracing, and their protein composition was analyzed by mass spectrometry. In this way, therapeutic reaction and target of EVs for GBM therapy may obtain [70•].

Previous studies have shown that GBM-derived EVs can pass through the blood-brain barrier and enter the blood circulation [71–73]. As a result, EV levels in the blood can also reflect the progress of brain tumors. Therefore, EVs can assist in the diagnosis of suspected high-grade gliomas in patients who are difficult to assess by surgical biopsy and provide a basis for tumor staging. Secondly, because EVs contain nucleic acid, protein, and other molecules which play a role in intercellular signal transduction and contain lots of genetic information about cancer cells, they are of great value in studying potential therapeutic targets and inhibiting tumor invasion and metastasis. The expression levels of EVs and their contents are known to be significantly different

before and after treatment. Therefore, EVs can be used as an effective minimally invasive tool for monitoring tumor response and recurrence [60••, 70•].

Others

Since Warburg discovered the abnormal metabolic patterns of cancer cells a century ago, the energy metabolism of cancer cells has been an ongoing focus of the oncology field [74]. Recently, several studies have shown that gene mutations in cancer cells often lead to changes in metabolites in cancer cells [75–78]. The IDH1 mutation is the most common one found in gliomas, and it has been proven that IDH1 mutation can lead to the production of 2-hydroxyglutarate (2HG) [75]. Notably, Kalinina et al. created a novel approach to successfully measure D-2-hydroxyglutarate (D-2HG) in CSF samples collected from glioma patients using mass spectrometry. It was shown that CSF from patients with the IDH-mutant glioma contains higher levels of D-2HG [79]. Therefore, using metabolites as a biomarker seems to be a feasible way to achieve a "liquid biopsy" for brain tumors. More recently, Ballester et al. showed that through a determination of metabolites in CSF, they were able to diagnose brain neoplasms. They utilized mass spectrometry to examine 129 different metabolites in CSF samples (8 from healthy people, 23 from primary or secondary brain tumor), and found 43 metabolites that were significantly different among the two groups. In addition, a significantly higher level of D-2HG was found in CSF from patients with IDH-mutant gliomas compared to healthy controls. As a result, although more study is needed to prove its efficiency in clinical application, the analysis of metabolites in biofluids is a promising method to diagnose and monitor patients with CNS tumors [80].

Medulloblastomas often involve the posterior cranial fossa and grow around the fourth ventricle, with about one-third showing metastasis [2]. Once central nervous system metastasis occurs, the patient prognosis is poor [81]. Conventional methods for the diagnosis of CNS metastasis include magnetic resonance imaging and cerebrospinal fluid cytology, but the sensitivity of these methods is low. Studies have shown that light has different transmission properties in different tissues and cells, which are helpful in distinguishing between normal cells and cancer cells [82]. In light of this, Gershanov et al. attempted to use fluorescence lifetime imaging microscopy (FLIM) to diagnose the CNS metastasis of intracranial malignant tumors [83]. Unlike traditional fluorescence techniques, which are unable to quantify cell function at the molecular level, FLIM can identify normal cells and cancer cells based on fluorescence lifetime. Gershanov et al. collected cerebrospinal fluid samples from 17 children at diagnosis, before chemo/radiotherapy, during chemo/radiotherapy, and after chemo/radiotherapy; among these, 15 were medulloblastomas and 2 were atypical teratoid/rhabdoid tumors. Significantly higher fluorescence lifetimes were found in the CSF samples from patients with CNS metastasis compared to the control group with inflammatory lesions. Therefore, the FLIM technique may be an effective

diagnostic tool for cerebrospinal fluid dissemination of medulloblastomas and some malignant germ cell tumors.

Discussion

The outcomes for patients diagnosed with midline brain malignancies, diffuse midline gliomas, and medulloblastomas, have not changed despite the past three decades of clinical research. Recent advancements in next-generation sequencing provide new opportunities for wholly mapping the molecular profile of tumors and subsequently developing novel therapeutic interventions for patients with these tumors. However, the difficulty of performing surgical resection or repeated surgical biopsies in the sensitive location is a barrier to an accurate diagnosis.

Notably, liquid biopsies may offer an alternative minimally invasive approach for quickly and repeatedly obtaining critical genetic information for these tumors (Fig. 3). The utility of liquid biopsies in brain tumors has great potential and may have important implications for early diagnosis, guiding individual treatment, monitoring of disease progression, recurrence and metastasis, and the evaluation of the effectiveness of a selected therapy. However, some special thoughts must be considered when applying liquid biopsies. Firstly, identifying the best DNA source is the number one priority. Several studies have compared ctDNAs from plasma and CSF and found that ctDNA isolated from the plasma is not a reliable source due to low sensitivity and lack

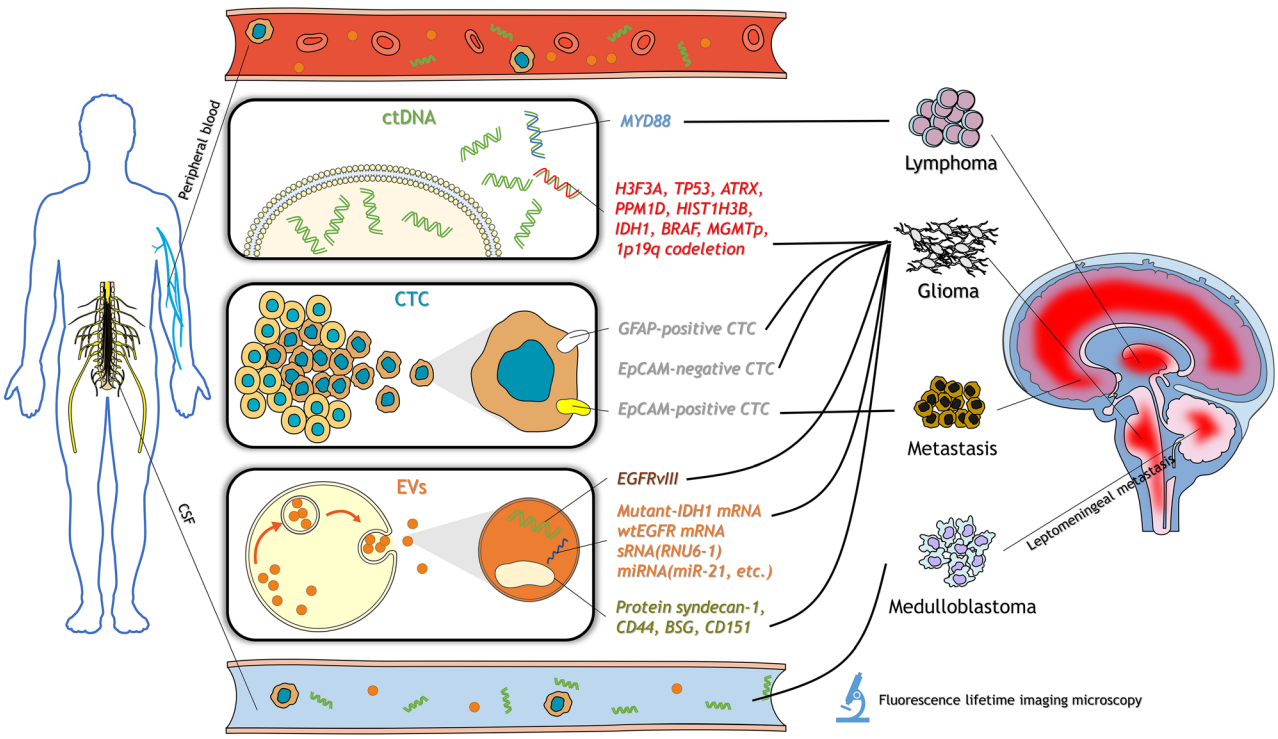

Fig. 3. Main detection methods and markers of midline brain malignancies.

of specificity. From this point of view, direct CSF sampling might be superior for the sensitive detection of tumor-specific alterations. In addition, to detect specific mutations in patients with brain neoplasms, Pan et al. designed two different approaches: one was targeted sequencing, and the other was whole-exome sequencing. Targeted sequencing needs a prior knowledge of point mutations present in the tumor. Although droplet digital PCR is an ultrasensitive method that can test low quantity ctDNAs, it has limited coverage. While whole-exome sequencing can fully characterize changes in the ctDNA profile, it is relatively costly and time consuming. Effort is therefore needed to maximize the strengths of these approaches. Recently, Mouliere et al. used shallow whole-genome sequencing of cell free DNAs from the CSF from 13 patients and successfully provided information on the tumor genome using low-cost screening. Moreover, some studies have further described the range of application using CSF biopsies. Liquid biopsy may be more appropriate for aggressive tumors which are directly adjacent to the CSF reservoir. For this reason, midline brain malignancies which are being bathed continuously by the CSF and could be ideal targets for CSF ctDNA sequencing. Interestingly, whether the CSF samples collected by open biopsy, V-P shunt, or lumbar puncture contribute to biopsy equally is contradictory. Larger studies should clarify these promising findings. A lumbar puncture is performed routinely in patients with a midline brain tumor and is relatively safe. Furthermore, the inter-tumor (primary tumors and secondary metastases) and intra-tumor heterogeneity makes it difficult for liquid biopsy to represent the entire primary lesion. Taken together, more studies are needed before establishing CSF biopsy as a feasible tool for assessment in midline brain malignancies.

Conclusion

The utility of CSF biopsy in patients with midline brain tumor is promising. CSF biopsy may offer an alternative minimally invasive approach for quickly and repeatedly obtaining critical genetic information about these tumors, which would have important implications for diagnosis, guiding individualized therapy, monitoring of disease progression, recurrence and metastasis, and evaluating the effectiveness of a selected therapy. However, further studies are needed before establishing CSF biopsy as a feasible method of assessment in patients with midline brain malignancies.

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All contributors to this study are included in the list of authors.

Author contributions

Y.P., and W.L. wrote the manuscript and drew the figures. Q.L. wrote the manuscript and supervised the entire work. All the authors provided final approval for the version to be published.

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Compliance with Ethical Standards

Conflict of Interest
Yimin Pan, Wenyong Long, and Qing Liu declare they have no conflict of interest.

Human and Animal Rights and Informed Consent
This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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