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Liquid biopsy for brain tumors

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Abstract

Introduction—Minimally invasive methods will augment the clinical approach for establishing the diagnosis or monitoring treatment response of central nervous system tumors. Liquid biopsy by blood or cerebrospinal fluid sampling holds promise in this regard.

Areas covered—In this literature review, the authors highlight recent studies describing the analysis of circulating tumor cells, cell free nucleic acids, and extracellular vesicles as strategies to accomplish liquid biopsy in glioblastoma and metastatic tumors. The authors then discuss the continued efforts to improve signal detection, standardize the liquid biopsy handling and preparation, develop platforms for clinical application, and establish a role for liquid biopsies in personalized medicine.

Expert commentary—As the technologies used to analyze these biomarkers continue to evolve, we propose that there is a future potential to precisely diagnose and monitor treatment response with liquid biopsies.

Keywords

Liquid biopsy; circulating tumor cells; exosome; cell free tumor DNA; brain tumors

1. Introduction

1.1. Why try to detect brain tumors in the blood?

The current approach to assess tumor response to treatment rests heavily in neuroimaging. However, correlation of therapeutic response with imaging findings can be difficult as the intervention may interfere with the imaging properties of the tumor. For instance, chemoradiation may result in increased contrast enhancement, often termed pseudoprogression, which indicates alterations in the local blood–brain barrier or radiation necrosis [1]. Conversely, antiangiogenic agents may decrease contrast enhancement by altering permeability of tumor vasculature without a true reduction in disease burden [2]. Consequently, clinical trial enrollment or tailoring therapies for patients based on treatment

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response can be challenging in the absence of an objective method for measuring tumor burden and determining its genomic composition.

There are some intriguing reasons to give consideration to liquid biopsy for glioblastoma (GBM) tumors. Unlike many tumors, the biopsy process itself confers a non-negligible degree of risk related to the procedure. For example, there may be considerable brain swelling within and around the tumor itself. Even a moderate amount of hemorrhage that occurs in the biopsy tract could be potentially threatening to the patient's neurological function or even life. In addition, biopsies in the brain are very challenging from the perspective of obtaining longitudinal samples or repeated prospective collection of tissue material, again due to the risk of the biopsy itself. This difficulty in obtaining longitudinal samples decreases our ability to monitor treatment response, appreciate clonal evolution to determine new susceptibilities or acquired mechanisms of resistance to therapy [3], and distinguish tumor recurrence from pseudoprogession. A liquid biopsy would facilitate our ability to follow patients in a longitudinal fashion following initial diagnosis and treatment. Furthermore, the focal sampling of a lesion by biopsy may not fully capture the intratumoral heterogeneity [4], whereas integrating the analysis of circulating tumor cells (CTCs), circulating extracellular vesicles (EVs) and circulating tumor DNA (ctDNA) can potentially characterize the global tumor genome and transcriptome [5].

1.2. Current strategies for liquid biopsy for GBM and other brain tumors

In the past decade, several strategies for developing liquid biopsy for GBM have begun to gain traction in early phase studies. As shown in Figure 1, sources of macromolecules which may convey information regarding the tumor include CTCs, cell-free fractions, and EVs.

1.2.1. Circulating tumor cells—CTCs are found in the blood stream and individually they represent a cellular derivative of the original tumor mass. It is not known whether CTCs are only subpopulations of the central tumor, or rather pulled randomly representing the entire original tumor. CTCs are exceedingly rare (1 cell in 10^9 blood cells), and present huge technological challenges for their isolation. Traditionally, CTCs had been isolated using antibody-mediated capture ('positive selection') which would capture CTCs by targeting extracellular membrane proteins, such as EpCAM (reviewed by Ferreira et al. [6]). While successful for most solid tumors, for non-epithelial cancers, such as brain gliomas, identifying a tumor specific membrane protein is extremely difficult. Furthermore, only those CTCs with the target protein will be isolated using this approach. Other methods seek to enrich for CTCs by removing the unwanted components of whole blood ('negative selection'). Several microfluidic platforms have been developed to capture CTCs (reviewed by Pratt et al. [7]), but only recently has this been successfully done in GBM [8]. GBM CTCs have also been identified through other negative selection approaches, such as erythrocyte lysis and density gradient separation [9], or removal of leukocytes using magnetic beads [10].

Sullivan and Nahed et al. [8] demonstrated that CTCs were identified in at least one blood specimen from 13 of 33 patients (39%; 26 of 87 samples) with GBM. In addition, single GBM CTCs isolated from both patients and mouse PDX models demonstrated enrichment

for mesenchymal over neural differentiation markers compared with primary GBMs. This finding sets a proof of principle that CTCs may be identified in the blood of brain tumor patients and may exhibit a specific pattern of gene expression consistent with an invasive subtype of GBM. A study by Lohr et al. [11] demonstrated that up to 90% of mutations present in prostate cancer could be detected in blood samples using exome sequencing of immunopurified CTC patients with metastatic prostate cancer. This demonstration suggests that CTCs may allow for precise oncologic monitoring of patients with secondary CNS tumors. *Ex vivo* culture of CTCs has also been demonstrated in a small number of cancers [12], establishing the potential of using CTCs to create patient-specific tumor models for individualized treatment planning. While CTCs offer considerable promise for analysis in the context of liquid biopsy, there are some challenges in using this technology. One of these challenges is that the isolation of CTCs often requires a fresh blood sample and relatively immediate sample processing. This may limit the initial application of CTCs to major centers which are equipped to perform this type of analysis in the short time frame required to process a fresh sample. The frequency of CTC release into the peripheral blood from brain tumors has yet to be definitively established, and may not be a ubiquitous biomarker throughout treatment.

1.2.2. Circulating tumor DNA—ctDNA has also been the subject of investigation in terms of liquid biopsy for brain tumors. There are some technical challenges associated with the use of ctDNA in plasma or serum as biomarkers in general. For example, typically there is a very low concentration of nucleic acid in biofluids as compared to cells or tissue. This may range to less than 100 ng/mL in plasma, thus leaving only a few molecules per sample to be detected. Furthermore, circulating DNA is highly fragmented and may exist in sizes of less than 1000 base pairs. This technical challenge requires optimized methods for DNA extraction. Higher sample volumes permit isolation of sufficient absolute amounts of ctDNA for assay, a low elution volume is required to obtain higher concentrations in the eluate after purification, and there is a need to efficiently recover smaller fragments. Nonetheless, despite the technical challenges, there have been proof-of-principle studies which have shown that it is possible to detect tumor associated nucleic acid in various biofluids in brain tumor patients. In a study of ctDNA obtained from 640 patients, Bettegowda et al. [13] found a correlation between the concentration of tumor-specific variants detected in plasma and survival for those with metastatic disease burden. However, in localized tumors, such as glioma, the ability to detect tumor alleles, such as those present in IDH1, TP53, EGFR, and PTEN, was limited to a subset of patients.

A few studies have analyzed ctDNA in brain tumor patients. Huang et al. [14] showed that in cerebrospinal fluid (CSF), cell-free DNA could be captured and specific mutations in histone H3 gene variants could be detected. Test sensitivity (87.5%) and specificity (100%) were validated via immunohistochemical staining and Sanger sequencing in available matched tumor tissue specimens ($n = 8$). Chen et al. [15] showed that the ctDNA from glioma patients showed lower levels of Alu methylation than the controls. Wang et al. [16] demonstrated that all patients with intracranial tumors adjacent to a cisternal space or those with high grade glioma were found to have detectable ctDNA in CSF. Though CSF may provide a liquid biopsy with a higher detection yield, there is often a clinical hesitation to

perform a lumbar puncture on patients who are noted to have an intracranial mass given the concern of inducing a herniation syndrome.

1.2.3. Extracellular vesicles—EVs, sometimes referred to as ‘exosomes,’ have also been put forth as a possible source of macromolecules for analysis in liquid biopsy for brain tumors. In an earlier report, Skog et al. [17] showed that EVs could be isolated from the serum brain tumor patients and the specific genetic alterations in the EGFR gene could be detected in the serum-derived EVs of these patients. Additional investigations in the serum of patients with brain tumors versus controls showed that distinct RNA expression patterns could be detected in the EVs of GBM patients as compared to controls [18].

Work from the EV field has shown that CSF is also a viable biofluid for examining the macromolecular contents of EVs. Chen et al. showed that the mutant IDH1 G395A can be detected in CSF EVs with 63% sensitivity and 100% specificity [19]. More recently, Figueroa et al. [20] have published that the detection of the oncogene, EGFRvIII, in extracellular vesicular RNA can be achieved with a 60% sensitivity and greater than 98% specificity as compared to the gold standard of qPCR of the EGFRvIII transcript from brain tumor tissue. Manda et al. [21] showed 80% sensitivity and 79% specificity in a similar study in plasma. Akers et al. have also shown that both miR-21 [22] and miRNA signature [23] from CSF EVs can distinguish tumor and non-tumor disease states.

In addition to RNA or DNA, EVs also harbor cellular proteins on their surface which can be detected [24]. Shao et al. [25] showed that a four-protein panel of EV surface proteins could be used to distinguish GBM patients from controls using a novel antibody capture based technique. Microfluidics based capture and analysis of tumor-specific exosomes has also been explored with some success [26].

2. Expert commentary

There is a clinical need for minimally invasive method for diagnosis and tracking of brain tumors for both primary and metastatic disease. Liquid biopsies will provide this opportunity in the near future. Especially as technologic advancements allow for continued refinements in standardization and improved signal detection, the measurement of circulating tumor biomarkers has the potential for widespread use for tumor burden monitoring. Each of the sources discussed in this review, including CTCs, ctDNA, and EVs, has great potential to address various aspects of assessing the intracranial disease burden. However, the most reliable and comprehensive clinical information may derive from a combined analysis of these sources as each has the potential to capture a unique aspect of the alterations in the tumor genome.

3. Five-year view

While the aforementioned studies have demonstrated several proof-of-principle concepts including the fact that tumor-derived molecules are available for detection in both CSF and peripheral blood, there are several important obstacles to be overcome in the future.

3.1. Improving signal detection

A first challenge for liquid biopsy is the complexity of the most accessible biofluid which is blood. For example, in the case of EVs, we know that the total vesicle population in a cancer patient is diverse and could potentially include several subpopulations simultaneously: (1) EVs are released directly by the dividing and growing tumor cells. These vesicles contain a molecular profile similar to that of the tumor cells and can inform about tumor growth, evolution, or pathogenesis of the cancer [18]; (2) exo-somes derived from the immune cells. Understanding and profiling molecular characteristics between the immune vesicles and the tumor-derived ones, which have been shown to be immuno-suppressive, can also inform clinicians on the tumor status and/or response to therapy [27]; and (3) vesicles derived from the normal stroma around the tumor, as well as all other organs that directly or indirectly are affected by the tumor presence. In order to accurately separate, isolate, and profile these distinct subpopulations of EVs, more research is necessary to understand surface markers or other molecular distinctions to better differentiate these vesicles.

This challenge has been approached by some investigators, who have specifically sought to isolate tumor-specific vesicles from a mixed population. In two separate investigations [25,28], the Lee and Weissleder laboratories have demonstrated a novel technology for specifically detecting tumor-associated proteins on the surface of EVs. This opens up the possibility of specifically isolating such vesicles and probing their nucleic acid contents for a more specific tumor-associated signal.

Another strategy applicable to both cell-free DNA and RNA is to improve rare signal detection in a mixed background by employing next-generation sequencing (NGS) to identify low frequency tumor variants in the background of wild-type alleles present in liquid biopsies. High depth coverage allows for detection of these circulating tumor variants through increased coverage of targeted regions, which limits the proportion of the genome that can be analyzed [29]. However, the recent rapid genomic characterization of highly prevalent malignancies provides guidance as to which alterations should be targeted and analyzed by NGS from liquid biopsies.

3.2. Standardization and reproducibility

In addition, there is a need for highly standardized processes for liquid biopsy collection [30]. For example, macromolecule yield from different liquid biopsy isolation techniques can be influenced by freeze/thaw protocols, collection method, processing time, level of hemolysis, preservative agent exposure, and storage conditions [31]. In the case of EVs, the method of vesicle counting can also yield different results [32] and this variable should be accounted for in downstream analytics. Another reason for limited reproducibility of biomarker studies is a lack of guidelines and standardized protocols and each study addresses differently issues relating to cohort sizes, treatment of confounding factors, as well as the choice of control groups. Microarrays, real-time quantitative polymerase chain reaction (qRT-PCR), RNAseq, and droplet digital PCR (ddPCR) are among the most commonly used methods for biomarker studies, each one with its own advantages, limitations, and biases and efforts should be placed in ensuring the continuity of technology used from discovery to full validation of the assay.

3.3. Technology development

In addition to the challenges associated with standardizing the handling and processing of liquid biopsies is that current workflow for biomarker analysis requires resource-intensive technologies, such as NGS platforms or ddPCR. While NGS allows to explore a wide range of nucleic acids present in a sample with high sensitivity and reproducibility, ddPCR allows to determine the presence of vary rare events, from as low as picograms of RNA/DNA input, even in the presence of a large wild-type background with assay sensitivities reported as high as 0.01% [33]. Therefore, deploying the analytic workflow broadly to clinical pathology labs will require the development of cost-effective platforms. The focused and cost-effective analysis of biomarkers from liquid samples will be made possible as future studies continue to elucidate which genomic alterations provide clinical information to guide treatment strategy. Another characteristic that will evolve is the speed with which the clinical information is generated for biomarker analysis. For instance, we have recently reported a rapid Taqman-based genotyping assay to identify somatic mutations in brain tumor specimens to guide intraoperative decision-making [34]. This approach has been validated to a sensitivity of 0.1% tumor allele fraction, a dynamic range that is comparable to the lower end of ctDNA tumor purity [35].

3.4. Correlation with clinical outcomes and personalized medicine

Tumors are very dynamic and diverse entities, often with genetic signatures varying highly from one patient to the next. Tumors are also able to quickly evolve and modify their genotypic and phenotypic profiles to gain resistance to a certain treatment so having a reliable platform that allows for real-time assessment of the changes occurring in the primary tumor is highly valuable. Liquid biopsy, and the associated ease of monitoring treatment response, would allow many more discovered (targeted) drugs to be tested in clinical trials and eventually reach the clinic. Clinicians and patients would have more effective ways to evaluate treatment options in a personalized fashion. It is important to note that while we work on advancing this field, more focus has to be placed on the discrepancies among different studies and the inability to replicate biomarker signatures in independent cohorts. These discrepancies are often due to the varying source of sample (or processing method) analyzed, the differences in samples collection protocols, as well as the employment of different downstream analytical techniques.

Ongoing larger cohort studies will assess the correlation of the various circulating biomarkers with estimating disease burden and prognosis. Quantifying these temporal variations over the course of patient's oncologic history will also provide the ability to assess response to surgical and adjuvant treatment. Qualitatively, detection of branch point mutations evolving during an individual patient's treatment course will also guide clinical trial enrollment or assignment of targeted therapies.

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Key issues

- Correlation of therapeutic response with imaging findings can be difficult as the intervention may interfere with the imaging properties of the tumor.
- Unlike many tumors, the biopsy of intracranial lesions confers a non-negligible degree of risk related to the procedure and liquid biopsy would facilitate our ability to follow patients in a longitudinal fashion following initial diagnosis and treatment.
- Sources of macromolecules that may convey information regarding the intracranial tumor include circulating tumor cells (CTC), circulating tumor DNA (ctDNA), and extracellular vesicles (EV).
- CTCs are rare and difficult to detect but one study demonstrated that 39% of patients with glioblastoma had detectable CTC in blood.
- Concentration of ctDNA has been correlated with metastatic disease burden and has been detected in the cerebrospinal fluid of patients with high grade glioma, especially those adjacent to a cisternal space.
- Analysis of serum EV-derived RNA has demonstrated unique signatures, such as detection of EGFRvIII or levels of miRNA, in patients with a glioma as compared to patients without intracranial tumors.
- Future developments in the analysis of circulating tumor biomarkers will result in improved standardization of specimen preparation, greater signal detection and more cost-effective and rapid testing to guide treatment decisions.
- Larger cohort studies will provide insights as to how liquid biopsies can allow for tracking disease burden and guide treatment decisions based on tumor evolution.

Figure 1.
Examples of sources of macromolecules that are accessible in various liquid biopsy fractions.