The "Liquid Biopsy": the Role of Circulating DNA and RNA in Central Nervous System Tumors

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Abstract

The detection of tumor-derived circulating nucleic acids in patients with cancer, known as the "liquid biopsy," has expanded from use in plasma to other bodily fluids in an increasing number of malignancies. Circulating nucleic acids could be of particular use in central nervous system tumors as biopsy carries a 5–7 % risk of major morbidity. This application presents unique challenges that have limited the use of cell-free DNA and RNA in the diagnosis and monitoring of CNS tumors. Recent work suggests that cerebrospinal fluid may be a useful source of CNS tumor-derived circulating nucleic acids. In this review, we discuss the available data and future outlook on the use of the liquid biopsy for CNS tumors.

Keywords

cfDNA; miRNA; Brain tumor; Liquid biopsy; Circulating tumor cells

Introduction

A "liquid biopsy," sampling of a brain tumor's genetic profile through access to blood, urine, or cerebral spinal fluid (CSF), would obviate the need for a percutaneous or open biopsy of certain central nervous system (CNS) tumors. A reliable test identifying a specific tumor's genetic signature could spare many patients an invasive and potentially morbid

CNS procedure. Over the past decade, a wide variety of "liquid biopsies" have made their way from the laboratory into the clinical setting: the detection of viral DNA in CSF for the diagnosis of herpes encephalitis in 1990, circulating tumor cells (CTCs) for cancer patients in 2004, and fetal DNA aneuploidy screening for pregnant women in 2011 [1–3]. These techniques may have additional advantages over the traditional biopsy, including the ability to obtain multiple, sequential samples for earlier detection of tumor alterations conferring treatment resistance. Further, liquid biopsies may even be a more sensitive and quantitative assessment of disease progression than clinical imaging. Increasing evidence suggests that single biopsies may not be an adequate representation of the heterogeneous genetic landscape of tumors [4], whereas, a liquid biopsy may offer a more "averaged" view of the tumor environment.

Extending the use of the liquid biopsy to CNS tumors has proven to be more challenging than cancers of other organ systems. One major limitation has been the extremely low concentrations of CTCs and cell-free nucleic acids in the serum of patients with primary CNS tumors. In the last 5 years, however, a number of publications have demonstrated the ability to detect and sequence the low concentrations of nucleic acids found in the serum and CSF of these patients [5•, 6•, 7, 8•, 9–11]. These techniques hold a great deal of promise, but their ultimate role in the diagnosis and treatment of patients with CNS tumors has yet to be defined. In this article, we review how the liquid biopsy has evolved over time (Fig. 1), its current applications, and future directions in the care of patients with CNS tumors.

CTCs: the First Liquid Biopsy

Perhaps owing to their larger size, CTCs can be considered one of the first major frontiers related to the liquid biopsy. The first description of these cells is widely attributed to Ashworth in 1869, who documented cells that appeared like tumor in the blood of a metastatic breast cancer patient at the time of autopsy [12, 13]. These cells, which are thought to migrate or shed into the circulation from a primary or metastatic tumor, are found in extremely low concentrations, making them difficult to detect in the background of native blood cells (Fig. 2) [14]. Almost a century later, the development of immunomagnetic assays, which attach specific antibodies to a magnetic bead, allowed for single cells to be detected in 1 ml of blood [15]. It was not until 2004 that Cristofanilli et al. clearly demonstrated the prognostic value of CTCs in metastatic breast cancer patients in a prospective trial [2]. The presence of CTCs in peripheral blood has now been described in many common carcinomas and in CNS metastases. Its prognostic value has been demonstrated in breast, prostate, and colorectal cancer [2, 16, 17]. In 2004, the FDA approved the Cell-Search system (Viridex) for CTC enrichment and detection in the blood of breast cancer patients. This technology can now be used for patients with metastatic breast, colorectal, and prostate cancers [18].

Unfortunately, many of the techniques used to isolate CTCs from blood rely on epithelial markers, such as EpCAM, which are not present on cells from primary brain tumors. To overcome these limitations, researchers have used a combination of physical separation techniques and immunofluorescence for more CNS specific markers, such as GFAP and Nestin, to identify CTCs in glioma patients [9, 19, 20]. Although it has been speculated

that the blood-brain barrier (BBB) plays a role in the dissemination of CTCs, it appears that CTCs still retain some ability to spread even in the presence of an undisrupted BBB [9]. Primary CNS tumors rarely metastasize outside of the CNS, so the application of plasma CTCs is unclear. The use of CTCs for patients with primary brain tumors may ultimately lag behind circulating nucleic acid techniques due to the technical challenges of cell capture.

Cell-Free DNA (cfDNA) and Cancer

Originally described in healthy individuals, cell-free DNA (cfDNA) has evolved into a promising diagnostic modality. Its clinical application areas have expanded over time, as it has continued to be described in various bodily fluids and in various clinical applications. cfDNA consists of short 70–200 base pair fragments of genomic DNA (both coding and non-coding) and mitochondrial DNA found extracellularly in bodily fluids [21]. cfDNAwas first described in 1948 by Mandel et al. in blood samples collected from healthy individuals [22]. It was not until 30 years later that Leon et al. discovered increased levels of cfDNA in serum of cancer patients using an antibody-based detection system [23]. This technique did not clearly identify the cfDNA as tumor-derived, but did suggest a correlation between cfDNA quantity and disease metastases. The authors then aptly predicted that testing serum for cfDNA would play a role in tracking treatment response [23].

Several key advances have facilitated the investigation of cfDNA for the liquid biopsy. The development of polymerase chain reaction in 1983 allowed scientists to precisely amplify and map the genetic mutations in DNA. This boon to cancer research also led to the rapid development of "liquid biopsies" for infectious diseases. In the 1990s, several groups reported the presence of herpes simplex virus and varicella zoster virus DNA in the CSF of symptomatic patients [1, 24, 25] and PCR for the viral DNA quickly replaced viral culture and brain biopsy. Early PCR techniques could easily detect the foreign viral genome, but could not reliably distinguish mutant cancer DNA signatures from the background noise of normal DNA in plasma. Despite these challenges, groups began to recognize genetic features of cancer in cfDNA, such as mutations in oncogenes, tumor suppressors, and microsatellite repeats [26, 27].

Next generation sequencing technology refers to a multitude of techniques that increase the sensitivity and speed of nucleic acid sequencing. Additional methods that have developed around sequencing workflows have made it easier to detect tiny fractions of abnormal cfDNA and cfRNA in bodily fluids. For example, in droplet digital PCR (ddPCR), DNA templates are diluted into thousands of droplets (one DNA template or less per *picoliter* droplet) in which independent PCR reactions occur [28]. Compartmentalization of the reaction reduces background DNA and contamination, creating an increased signal-to-noise ratio. BEAMing (Beads, Emulsions, Amplification, Magnetics), a similar digital PCR-based method, utilizes magnetic beads and flow cytometry to quantify small amounts of nucleic acids. Introduced in 2005, this method involves mixing products from an initial PCR reaction with primer-coated magnetic beads [28]. After running a second PCR reaction on the beads, products are hybridized to fluorescent probes and measured. Pan et al. described two approaches to interrogate brain tumor cfDNA mutations in CSF using these techniques [6•]. They first utilized ddPCR and targeted amplicon sequencing to search for known tumor

driver mutations, then globally characterized the mutations using cancer panel sequencing. Another promising technique known as "cancer personalized profiling by deep sequencing," or CAPP-Seq, used optimized library preparation methods to capture genomic regions that frequently contain recurrent mutations [29•]. Once captured, these regions underwent deep-sequencing, which allows for the ultra sensitive detection of low mutant fractions of plasma cfDNA. An advantage of this approach is its scalability since it does not require patient specific customization [29•]. Although only implemented for non-small-cell lung carcinoma in the study, this approach should be applicable to other cancers.

Though we now have many techniques to identify cfDNA, we do not yet understand exactly how and why concentrations of nucleic acids vary in various bodily fluids. cfDNA may arise from apoptotic or necrotic cells [30], rapidly dividing cells [31] or CTCs. A recent review by Crowley et al. suggests that low numbers of circulating tumor cells cannot account for the quantity of cfDNA typically seen in blood [32]. Some authors have suggested that macrophages may actively release cfDNA into the serum [33, 34].

cfDNA and Primary CNS Tumors

Naturally, research on cfDNA and the liquid biopsy has expanded into the realm of neuro-oncology as the CNS represents a high-risk area for biopsy. In 2003, one of the first studies focusing exclusively on patients with glioblastoma (GBM) used serum cfDNA to detect promoter hypermethylation of specific genes. In this study, Balaña et al. [35] used methylation specific PCR to interrogate the promoter methylation status of MGMT, p16, DAPK, and RASSF1A in circulating serum DNA from GBM patients. Promoter methylation of MGMT, p16, DAPK, and RASSF1A was detected in 11 of 28 (39.3 %), 15 of 27 (53.6 %), 9 of 26 (34.3 %), and 13 of 26 (50 %) serum samples, respectively. The methylation status of serum cfDNA corresponded well with the methylation status of the matched tumor tissue samples. Their findings also corroborated other studies of methylation status in GBM tissue; MGMT methylation status was predictive of response and time to progression in BCNU-treated patients.

Balaña's work was followed shortly by a study focusing on mitochondrial DNA mutations (mtDNA) in matched samples of tumor tissue, blood, and CSF in patients with medulloblastoma [36]. While mtDNA mutations in seven of eight CSF samples were detected, in only one of these cases did the CSF mtDNA mutations exactly match those in the corresponding tumor. In this patient, the CSF sample was obtained one month after the completion of radiation therapy. Though there was no sign of disease on the patient's magnetic resonance imaging at the time, this patient developed recurrent disease 5 months later. The authors suggested this may be due to the presence of a small number of therapy resistant cells whose presence was otherwise undetectable via conventional methods such as magnetic resonance imaging and cytology. Exactly why there was disparity between tumor and CSF mtDNA mutations in the majority of patients, however, remains unclear.

The ideal source of cfDNA has been a topic of much debate. In 2010, Lavon et al. published a paper supporting Balaña's earlier work, re-demonstrating the high specificity of serum cfDNA promoter methylation patterns and allelic chromosomal losses such as 1p19q

deletions in gliomas of various grades [7]. Despite nearly 100 % specificity, the authors noted that the rate of detection of deletions in the serum was between 50–60 %, making the test relatively insensitive [7]. Liu et al. also reported nearly 100 % specificity detecting aberrant promoter methylation patterns in matched tumor, serum, and CSF samples of patients with malignant gliomas [37]. In addition, their techniques demonstrated differences in tumor, serum, and CSF detection rates. While neither paper directly addresses the concern, together they suggest that broader use of serum cfDNA for primary brain tumor mutation detection may be limited, due either to thresholds in technique sensitivity, or permeability of these molecules through the BBB or blood-tumor barrier (Fig. 2). The use of CSF rather than serum might circumvent this problem. Liu et al. highlighted this possibility with an example of a patient in their cohort who eventually developed leptomeningeal involvement after negative cytologic testing but positive EGFR mutation status in cfDNA isolated from CSF. The notion that CSF is a better source of circulating nucleic acids for primary brain tumors was further supported by Bettegowda and colleagues in 2014 [8•]. Their study, which included 640 patients of various cancer types, included 41 patients with primary brain tumors. Less than 50 % of patients with medulloblastomas or metastatic cancers of the kidney, thyroid, or prostate had detectable levels of tumor-derived cfDNA in plasma. Additionally, tumor derived cfDNA was found in plasma in less than 10 % of patients with gliomas. The authors hypothesized that these results could be explained by barriers such as the BBB or organs coated with mucin impairing the release of cfDNA into the circulation [8•].

In the past year, two papers have demonstrated a high throughput sequencing approach for the detection of brain tumor-derived cfDNA in CSF. A study by our group applied both ddPCR with targeted amplicon sequencing and cancer panel sequencing to search for cancer mutations in cfDNA of CSF of primary and metastatic brain tumor patients [6•]. Cancer panel sequencing specifically targets regions previously identified as containing genes of interest and several sequencing kits are available commercially. Using this technique, we were able to identify seven SNV mutations in the CSF of a patient with leptomeningeal disease from non-small-cell lung cancer that were concordant with the primary lung tumor. For the remainder of the patients, mutations of interest were first identified through exome sequencing of normal and brain tumor DNA, and then were compared to CSF and serum samples. Tumor mutations were detected in the CSF of 6 of 7 patients. The only patient who did not have detectable mutations in the CSF had a vestibular schwannoma, a benign tumor encased in arachnoid layers. Interestingly, the ratio of CSF to plasma mutant concentration of cfDNA corresponded with burden of CNS versus systemic disease. Therefore, in patients with quiescent systemic disease, but active brain disease, CSF may be a more reliable source of brain tumor-derived cfDNA than plasma.

Our paper was quickly followed by a study by Wang et al. which investigated the presence of brain and spinal cord tumor mutations in CSF [5•]. They found detectable levels of tumor-derived cfDNA in 26 of 35 cases (74 %). In cases where no tumor cfDNA was detected, the tumors were either low-grade or not directly adjacent to a large CSF reservoir. Prospective, longitudinal studies that correlate cfDNAwith disease activity and treatment response will ultimately be required before CSF or serum cfDNA can be used clinically as a liquid biopsy for primary brain tumors.

Circulating RNA and Brain Tumors

Circulating RNAs have also been a topic of much investigation. However, this paradigm has matured rather recently due to the complex and transient nature of RNA expression. Circulating RNAs consist of messenger RNA and noncoding micro RNA (miRNA). These exist in surprisingly stable form in plasma, perhaps due to association with subcellular particles or exosome packaging [38, 39]. The majority of mRNA found in plasma consists of short fragments [40]. Lo et al. were the first to describe the detection of tumor-derived cell-free mRNA in patients with nasopharyngeal carcinoma in 1999 [41]. This was followed shortly by another study describing the detection of tumor-derived cell-free mRNA in the serum of patients with malignant melanoma [42]. Since then, a variety of other tumor-derived cell-free mRNA products have been found including TERT, Her2/neu, and CCND1 [43–45].

Despite these promising studies on circulating mRNA, the cancer research community has gravitated toward miRNAs. This may be due to the high level of variability seen in circulating mRNA levels. miRNA fragments, usually 19–25 base pairs in length, are thought to be derived from 70–100 base pair hairpin precursor molecules [21]. They regulate post transcriptional gene expression and are involved in a variety of cell processes such as apoptosis, proliferation, and differentiation [21]. Surprisingly, the use of miRNA as a liquid biopsy has made rapid progress since 2008, when circulating miRNAs were detected for the first time in the blood of diffuse large B-cell lymphoma patients and prostate cancer patients [46, 47]. In 2010, Hu et al. demonstrated that, like cfDNA, a panel of specific miRNA in blood can predict survival in patients with non-small-cell lung carcinoma [48]. Circulating miRNA has subsequently been found in a number of other body fluids including urine, saliva, fecal material, and CSF [49–52].

Research on cell-free RNA has recently expanded into tumors of the CNS. One of the first papers on circulating RNA and brain tumors was published in 2011 by Baraniskin et al. [52]. In this study, the authors identified miRNAs characteristic for primary diffuse large B-cell lymphoma of the CNS in CSF. This was quickly followed by a similar study in gliomas by the same group, which showed characteristic overexpression of miR-15b and miR-21 [53]. Together, these markers were able to distinguish patients with gliomas from patients with primary central nervous system lymphomas, brain metastases, or leptomeningeal carcinomas. Tepyluk et al. corroborated this finding, demonstrating that the CSF of patients with GBM was enriched for miR-10b and miR-21 [54]. Patients with brain metastases were distinguished by high expression of members of the miR-200 family. In the short time since these studies, a number of authors have used next-generation sequencing related techniques to increase the sensitivity of miRNA detection in plasma, serum, and CSF [10, 11]. For example, Chen and colleagues used BEAMing and ddPCR to detect IDH1 glioma mutations in mRNA transcripts isolated from CSF [11]. In this study, mutated transcripts were not reliably detected in serum.

While it appears that cfDNA in CSF may be more ample and may have less background noise than serum cfDNA, there is less agreement on the optimal source of brain tumor-derived miRNA. Studies have reported widely varying sensitivity and specificity. A meta-

analysis conducted in 2014 included 23 studies from six articles that profiled miRNA in serum, blood, or CSF for a variety of diseases of the CNS [55]. The authors concluded that CSF-based assays produced more accurate results than those of blood; pooled sensitivity increased from 0.83 to 0.86, specificity increased from 0.81 to 0.88 in blood versus CSF. A second meta-analysis concluded that panels including miR-21, regardless of source, may be more specific for glioma, with sensitivity and specificity of 0.82 (95 % CI 0.72–0.89) and 0.94 (95 % CI 0.85–0.98), respectively [56]. These studies suggest a role for miRNA in the diagnosis and treatment of brain tumors, but like cfDNA, prospective, longitudinal studies are required to clearly define the utility of miRNA.

Future Directions

The utility of circulating nucleic acids for the diagnosis and monitoring of brain tumors is certainly promising. While sequencing techniques have been available for decades, the ability to detect, differentiate, and sequence low concentrations of tumor-derived nucleic acids efficiently is a relatively recent innovation. As a result, there is no consensus on which nucleic acid (DNA or RNA), which bodily fluid (serum or CSF), or which technique (targeted panel or whole exome sequencing) is best suited for use as a liquid biopsy in brain tumor patients. As many of the authors included in this review have concluded, larger prospective trials are needed to solidify the role of circulating nucleic acids in brain tumor patient care.

While there are a number of small exploratory studies underway, an ideal study would prospectively enroll patients, possibly those already enrolling in larger therapeutic trials, to have samples of cfDNA and RNA from CSF and serum collected prior to and at the time of surgical resection for correlation with tumor tissue. As Wang et al. have suggested, tumors close to CSF reservoirs should be prioritized. By comparing cfDNA and RNA sequencing to surgical pathology, the current gold standard, the sensitivity and specificity can be accurately determined. Though repeat CSF sampling may not be an option for most patients, repeat serum and CSF testing at specific time points during treatment should also be performed, which would ultimately allow for correlation with survival and with treatment efficacy. Including patients who are undergoing re-operation for progression versus pseudo-progression should also be prioritized. If cfDNA or RNA techniques can demonstrate clear separation in these patients, this could dramatically alter their treatment courses. We look forward to seeing the data from Gaur and Fadul's prospective study of miRNA-10b in glioma patients, estimated to close accrual to 200 patients in May 2018 [57].

We also believe that these techniques may be used to help diagnose and treat patients with metastatic disease to the CNS. A recently published paper by Brastianos et al. used whole-exome sequencing in 86 matched samples of brain metastases and primary tumors and demonstrated that although separate brain metastases in the same patient were relatively genetically homogenous, their genomic profiles were quite distinct from the primary tumor or extracranial metastases [58]. The vast majority of patients do not undergo surgery or biopsy for brain metastases. CSF sampling could detect clinically actionable mutations in the CNS that would otherwise not be known and reduce the number of patients undergoing surgery for brain metastases of unknown etiology. This is particularly relevant for EGFR-

mutated lung cancer patients, who may have the option for second or third generation drugs that target resistance mutations. For patients with suspected leptomeningeal disease, it is also possible that cfDNA or RNA may be more sensitive than the current gold standard, CSF cytology. One potential pitfall, however, is that we do not currently have a technique that can differentiate between cfDNA and RNA from a brain parenchymal versus leptomeningeal metastases. Because many physicians make the diagnosis clinically and radiographically, a study to confirm this would require multiple sites with a special interest in leptomeningeal metastases.

Conclusion

Circulating nucleic acids have been detected in a variety of bodily fluids and their diagnostic applications have continued to expand. A great amount of interest has been generated around their use for a "liquid biopsy" for cancer. This approach may be particularly beneficial for brain tumors since biopsies are often unable to be performed or repeated. The ability to successfully utilize circulating nucleic acids to better diagnose and monitor tumors of the CNS and to select targeted therapeutics would most certainly improve our ability to care for this patient population. The studies we have reviewed here confirm that circulating DNA and RNA are present and reliably detectable in the fluids of patients with primary and metastatic brain tumors. The optimal use of these tests as diagnostic and therapeutic tools, however, has yet to be defined. We are eager to see these techniques integrated into clinical trial protocols so that prognostic and predictive values can be determined. Only with these parameters defined can the liquid biopsy approach its ultimate goal, obviating the need for an invasive surgical procedure to diagnose and treat cancer patients.

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Abbreviations

BBB	Blood-brain barrier
miRNA	micro RNA
GBM	Glioblastoma
CNS	Central nervous system
CSF	Cerebral spinal fluid
cfDNA	Cell-free DNA
CTCs	Circulating tumor cells
ddPCR	Digital droplet PCR
mtDNA	Mitochondrial DNA

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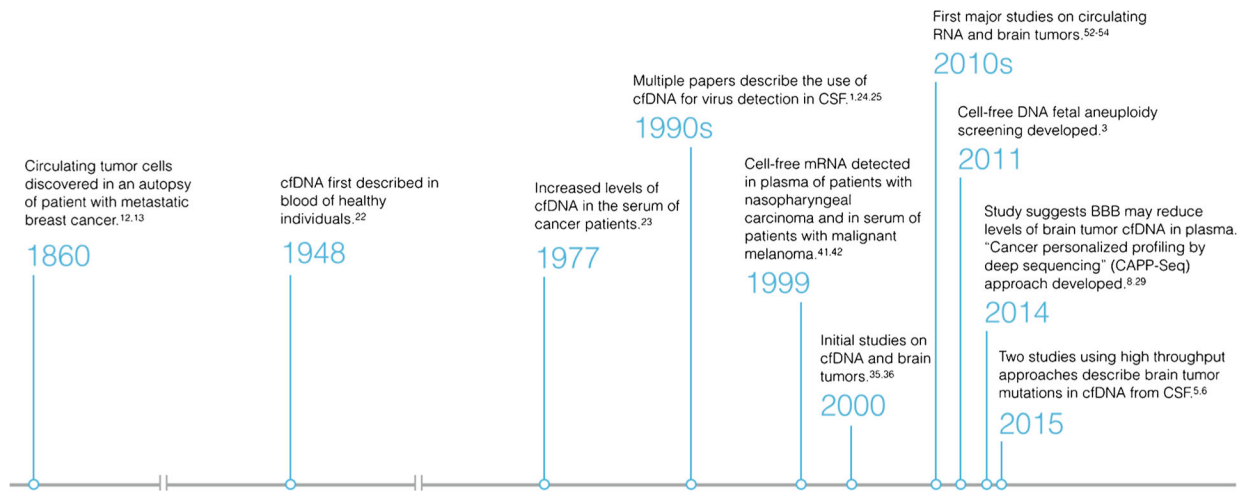
First major studies on circulating RNA and brain tumors.[52-54]

2010s

Multiple papers describe the use of cfDNA for virus detection in CSF.[1,24,25]

1990s

Cell-free DNA fetal aneuploidy screening developed.[3]

2011

Circulating tumor cells discovered in an autopsy of patient with metastatic breast cancer.[12,13]

1860

cfDNA first described in blood of healthy individuals.[22]

1948

Increased levels of cfDNA in the serum of cancer patients.[23]

1977

Cell-free mRNA detected in plasma of patients with nasopharyngeal carcinoma and in serum of patients with malignant melanoma.[41,42]

1999

Study suggests BBB may reduce levels of brain tumor cfDNA in plasma. "Cancer personalized profiling by deep sequencing" (CAPP-Seq) approach developed.[8,29]

2014

Initial studies on cfDNA and brain tumors.[35,36]

2000

Two studies using high throughput approaches describe brain tumor mutations in cfDNA from CSF.[5,6]

2015

Fig. 1.
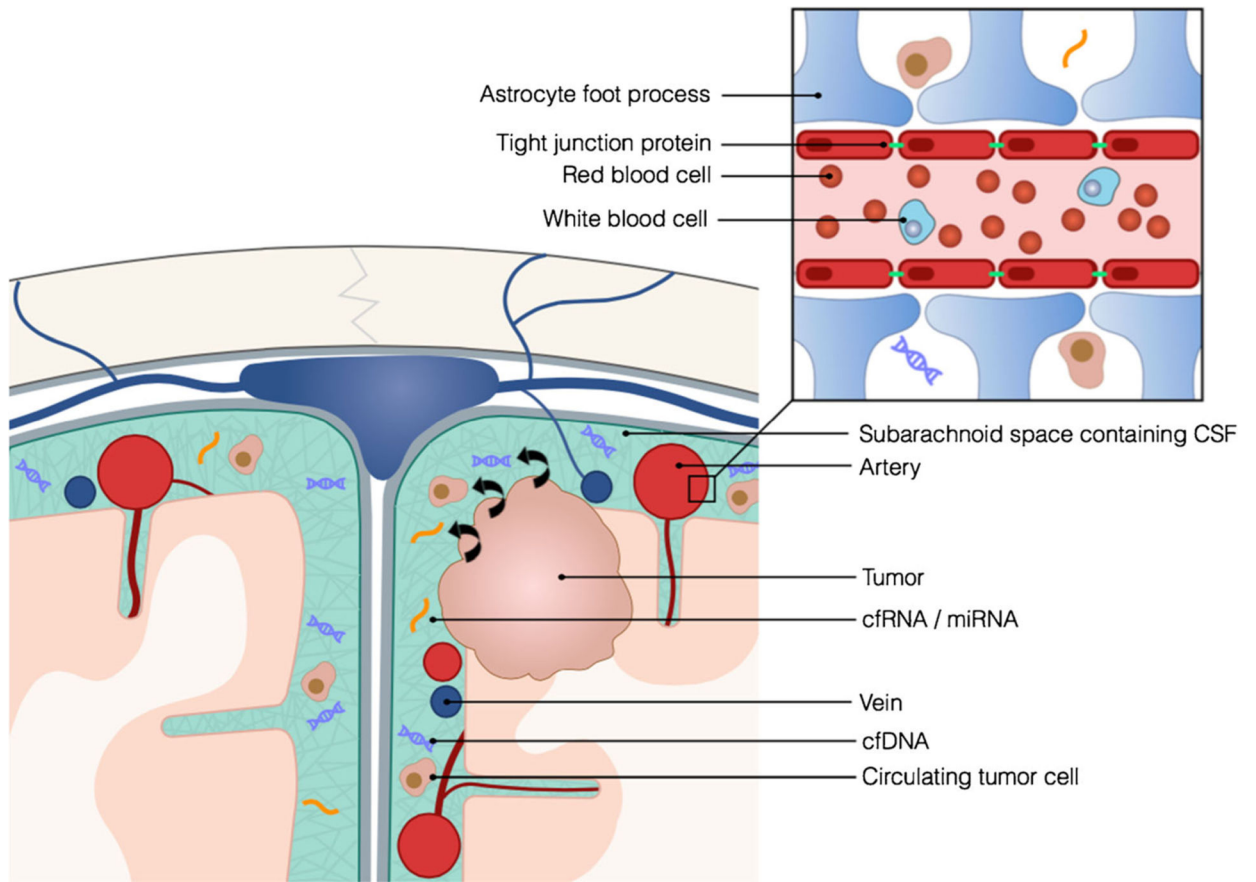Timeline depicting how the liquid biopsy has evolved

Fig. 2.
CSF may be a more optimal source of tumor-derived circulating nucleic acids than blood which may have poorer signal-to-noise ratios due to the presence of the BBB and native blood cells