Cerebrospinal fluid tumor DNA for liquid biopsy in glioma patients' management: close to the clinic?

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Cerebrospinal fluid tumor DNA for liquid biopsy in glioma patients' management: close to the clinic?

Running head: Liquid Biopsy in diffuse malignant gliomas

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Highlights

- ✓ Liquid biopsy of circulating tumor DNA (ct-DNA) represents a promising tool for genetic diagnosis of gliomas.
- ✓ Blood levels of ct-DNA are usually low and detectable only in few cases of glioma patients, likely because of the presence of blood-brain barrier.
- ✓ The best source of ct-DNA in glioma patients is the cerebrospinal fluid (CSF) collected either during surgical procedures or through lumbar puncture.
- ✓ Proximity of glioma to intracerebral cavities or brain surface is a key factor for ct-DNA release in CSF.
- ✓ CSF ct-DNA detection and molecular analysis could be performed with PCR-based methods or NGS-based techniques.
- ✓ A CSF ct-DNA liquid biopsy would greatly assist every step of clinical management of gliomas: molecular diagnosis when surgery is not feasible, monitoring tumor response, detection of early recurrence, tracking longitudinal genomic evolution at relapse and patient selection for targeted therapies.
- ✓

Abstract

Cell-free circulating tumor DNA (ct-DNA) reflecting the whole tumor spatial and temporal heterogeneity currently represents the most promising candidate for liquid biopsy strategy in glioma. Unlike other solid tumors, it is now widely accepted that the best source of ct-DNA for glioma patients is the cerebrospinal fluid, since blood levels are usually low and detectable only in few cases. A cerebrospinal fluid ct-DNA liquid biopsy approach may virtually support all the stages of glioma management, from facilitating molecular diagnosis when surgery is not feasible, to

monitoring tumor response, identifying early recurrence, tracking longitudinal genomic evolution, providing a new molecular characterization at recurrence and allowing patient selection for targeted therapies. This review traces the history of ct-DNA liquid biopsy in the field of diffuse malignant gliomas, describes its current status and analyzes what are the future perspectives and pitfalls of this potentially revolutionary molecular tool.

Keywords: liquid biopsy; cell-free tumor DNA; malignant gliomas; glioblastoma; cerebrospinal fluid

1. Introduction

Nowadays, the genomic characterization of cancer is essential for diagnostic purposes, prognostic evaluation and treatment selection. Anticancer therapies have shifted toward the new paradigm of "personalized oncology" aiming to identify the most effective strategies for each patient according to specific molecular biomarkers. Tumor DNA for genomic profiling of solid tumors has been traditionally obtained from surgical or biopsy specimens. However, information acquired from such conventional methods provides only a spatially and temporally limited snapshot of a tumor, which may fail to reflect its heterogeneity and dynamic evolution over time. The term *"*liquid biopsy*"* refers to non-invasive tools developed to detect and analyze tumor genetic material, including circulating tumor cells (CTCs), cell-free circulating tumor DNA (ct-DNA), extracellular vesicles, RNA and non-coding miRNA, obtained primarily from peripheral blood and from a variety of biofluids [1,2]. Compared with a classic tissue-based approach, liquid biopsies present several significant advantages: provide the whole genetic landscape of cancer lesions and offer the opportunity to track longitudinally genomic evolution, minimizing procedural risks and the expenses for their collection [1,2]. Liquid biopsy has a potential application at each stage of patient management, including early cancer detection, biomarker-driven therapies, minimal residual disease assessment, early relapses identification and characterization of molecular mechanisms of resistance [1,2].

Despite a widespread experimental use for tracking mutations in several cancer types, the clinical utility of blood liquid biopsy is currently well established for detecting the epidermal growth factor receptor (*EGFR*) resistance T790M mutation in advanced non-small-cell lung cancer (NSCLC) patients who progressed to first-generation EGFR tyrosine kinase inhibitor treatment with erlotinib or gefinitib [3]. In this clinical scenario, it showed moderate sensitivity and a variable degree of specificity (40−78%) in mutation identification [3]. Positive blood test results can be used to guide therapy, whereas negative ones should be confirmed by tissue sample analysis, if possible [3]. Beyond this defined indication, liquid biopsy applications are now being increasingly tested in clinical research.

Here, we review the perspectives and pitfalls of ct-DNA liquid biopsy in the field of diffuse malignant gliomas [1,2].

2. The potential application of liquid biopsy in the era of glioma molecular profiling

Historically, the diagnosis of central nervous system (CNS) malignancies has been based primarily on histopathologic features. However, over the past decade, large-scale DNA sequencing efforts have allowed the identification of several key genomic alterations.

At present, malignant diffuse gliomas, which represent the most common primary brain tumors (PBTs) of adults, can be divided in two major subgroups by the presence of isocitrate dehydrogenase (*IDH*) mutations. Primary glioblastomas (GBMs) usually lack *IDH* mutations, but frequently harbor EGFR amplification and/or mutation (EGFRvIII) and telomerase reverse transcriptase promoter (*TERTp*) mutations, as well as rearrangements of chromosomes 7 and 10 [4,5]. Lower-grade gliomas, comprising WHO grades II and III, are typically *IDH*-mutated tumors: astrocytomas carry *TP53* mutations and α-thalassemia/mental-retardation-syndrome-X-linked gene (*ATRX*) loss, whereas oligodendrogliomas present 1p/19q co-deletion and frequent mutations

in homolog of *Drosophila capicua* (*CIC*), far-upstream binding protein 1 (*FUBP1*) and *TERTp* genes [4,5]. Pediatric diffuse midline gliomas are characterized by mutations in the histone deacetylase genes *H3F3A* and *HIST1H3B*, while pilocytic astrocytoma, pilomyxoid astrocytoma, ganglioglioma and pleomorphic xanthoastrocytoma often harbor alterations in the *BRAF* gene [4-7]. These advances led to a major update of the WHO classification in 2016, with the introduction of the *IDH* mutational status and the presence of 1p/19q co-deletion, in addition to histology, to define glioma subtypes [4]. Beyond their diagnostic role the clinical relevance of some of these genetic markers is currently prognostic: *IDH* mutations represents the most powerful positive prognostic factor in diffuse glioma patients, whereas the homozygous deletion of cycline-dependent kinase inhibitor A and B (*CDKN2A/B)* status has been recently proposed as a strong negative prognostic indicator among *IDH*-mutated gliomas [8]. At present, the only well-established predictive factor is O (6)-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation, which is associated to temozolomide (TMZ) response [9]. Progress is also ongoing in the field of new drug development for PBTs, and these biomarkers, along with newly discovered ones, will likely be used in the near future as selection factors to identify candidates to personalized therapies. For instance, specific IDH inhibitors are currently under development [10], while depatuxizumab, an antibody–drug conjugate against EGFR-amplified GBMs, is being evaluated in late-phase clinical trials [11]. Mutations in the mismatch repair (MMR) system induced by alkylating agents and radiotherapy (RT) may change the mutational burden and confer sensitivity to immune checkpoints inhibitors [12]. In the case of CNS malignancies, however, tumor sampling presents several limitations due to the invasive access and issues to recapitulate the entire tumor heterogeneity through the analysis of a small tissue fragment. Moreover, high-risk surgical procedures are seldom indicated in the relapsed setting, precluding a new molecular characterization of recurrent tumors. For all these reasons, a minimally invasive tool able to provide a genetic signature, such as ct-DNA liquid biopsy, would greatly help clinicians in the

management of glioma patients. ct-DNA consists of short size (150–200 base pairs) fragments shed by apoptotic tumor cells and harboring genetic and epigenetic aberrations of the corresponding tumors [1,2]. In blood, ct-DNA represents only a minimal fraction of the total cell-free DNA (cf-DNA), mostly released by normal dying cells. Thanks to highly sensitive PCR-based techniques such as droplet digital PCR (ddPCR) and beads, emulsion, amplification, magnetics (*BEAMing*) digital PCR, plasma ct-DNA based liquid biopsy has been already shown to be highly informative in many other solid tumors, tracking changes in tumor burden and mutational patterns [1,2]. Beside such targeted approaches, whole-genome or whole-exome sequencing (WGS, WES), allowing comprehensive identification of genetic alterations without prior knowledge,have also been successfully implemented, despite lower resolution and higher costs.

3. Early blood-based approaches: from optimism to disappointment

The ideal source of circulating biomarkers in PBTs has been a subject of much debate over the last years. As for other solid tumors, peripheral blood was the first source of ct-DNA to be investigated, due to intuitive advantages such as quick and non-invasive collection, and of the ability to recapitulate the genetic profile of the corresponding tumors *(Table 1)*. Pioneering experiences evaluating blood cf-DNA in CNS malignancies date back to the beginning of this century and are focused on detection through real-time PCR-based assays of a series of glioma own genomic features, such as epigenetic changes or loss of heterozygosity (LOH). In 2003, Balaña et al. assessed the methylation status of specific genes, including *MGMT*, *p16*, *DAPK* and *RASSF1A* in both tumor and serum DNA from 28 GBM patients treated with Carmustine or TMZ plus cisplatin, finding high concordance between methylation of cf-DNA and the matched tumor tissue samples [13]. Comparable results, characterized by a very high specificity, close to 100%, but by unsatisfactory sensitivity, have been reported by other groups evaluating small series of PBTs and metastatic lesions [14,15]. Following these earlier works, Lavon et al. studied 1p, 19q and 10q deletions and

methylation status of *MGMT* and *PTEN* genes in paired tumor-serum samples from a cohort of 70 patients affected by astrocytic and oligodendroglial gliomas of various grades [16]. All oligodendrogliomas and 80.5% of astrocytomas showed at least one tumor-specific aberration (LOH and/or gene methylation) [16]. However, the rate of detection of such alterations in the in blood resulted again modest, 51% and 55% for the LOH and methylation status, respectively, making the test relatively insensitive [16].

Table 1. Early studies evaluating serum or plasma ct-DNA liquid biopsy

Study	Patients (n)	Glioma subtype	ct-DNA source	Technique	Genes investigated	Sensitivity	Specificity
Balaña et al. [13]	28	28 GBMs	Serum	MS-PCR	MGMT p16 DAPK RASSF1A	23.8% 57.1% 38.1% 47.6%	n/a
Weaver et al. [14]	10	6 GBMs 3 AAs 1 ODGs	Plasma	MS-PCR	MGMT p16 p73	50% 60% 66%	100%
Majchrzack-Celinska et al. [15]	33	9 GBMs 8 AAs 10 BMs 6 M	Serum	MS-PCR	MGMT RASSF1A p14 p15	11% 44% 0% 44%	100%
Lavon et al [16]	70	29 GBMs 12 AAs 29 ODGs (14 GII, 15 GIII)	Serum	MS-PCR	LOH 1p LOH 19q LOH 10q MGMT PTEN	31% 7% 58% ODGs/35% AAs-GBMs 47% ODGs/59% AAs-GBMs 0%	100%
Boisselier et al. [17]	80	28 Glioma GII 42 Glioma GIII 10 GBMs	Plasma	PCR	$IDH1^{r132h}$	60%	100%
Bettegowda et al. [18]	640	41 PBTs (including 12 Glioma GII, 1 AAs, 10 GBMs)	Plasma or serum	PCR or SafeSeqS	IDH1 p53 EGFR PIK3CA PTEN	<10% for glioma	n/a
Schwaderle et al. [19]	670	152 PBTs	Plasma	NGS	54/68/70 genes panels	31.5%	n/a

AAs, anaplastic astrocytoma; BMs, brain metastases; DAPK, Death-associated protein kinase 1; EGFR, epidermal growth factor receptor; G, WHO grade; GBM, glioblastoma; HGG, high-grade glioma; LGG, low-grade glioma; IDH, isocitrate dehydrogenase; LOH, loss of heterozygosis; M, meningioma; MGMT, O (6)-

methylguanine-DNA methyltransferase; MS-PCR, methylation-specific PCR; N, number; NGS, next-generation sequencing; n/a, not applicable; ODG, oligodendroglioma; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PTB, primary brain tumor; PTEN, phosphatase and tensin homolog; RASSF1A, Ras association domain family member 1; TS, targeted sequencing, WES, whole-exome sequencing; WGS, whole-genome sequencing.

In 2012, Boisselier et al. made the first attempt to detect a glioma-specific mutation in plasma ct-DNA [17]. IDH1^{R132H} substitution represents an "ideal" candidate for a liquid biopsy approach because it is frequent (40% of all gliomas diagnosis), it is the strongest independent prognostic factor for diffuse gliomas, and it has now become a diagnostic hallmark of lower-grade gliomas [17]. Plasma samples of 80 glioma patients (28 WHO grade II, 42 WHO grade III, 10 WHO grade IV) and 31 healthy controls were collected and analyzed combining both a COLD PCR (co-amplification at lower denaturation temperature-PCR), which preferentially amplifies mutant DNA, and digital PCR, which is a highly sensitive approach to detect the IDH1^{R132H} mutation [17]. A total of 15 out of 25 mutated tumors exhibited the mutation in plasma (compared to none of the 14 nonmutated tumors), with sensitivity increasing for high-grade gliomas (HGGs; WHO grades III and IV) with enhancing tumor volume [17]. Consistently with these early findings, two subsequent studies, evaluating serum ct-DNA in huge cohorts of patients with a wide range of tumor types, clearly indicated that blood does not represent the optimal source for liquid biopsy in PBTs [18,19]. Bettegowda et al. exploited targeted sequencing, WES or WGS to fully characterize plasma ct-DNA from 640 patients with various cancer types and at different stages, including 41 patients with PBTs [18]. Overall, mutant DNA fragments were found at relatively high concentrations in the bloodstream of most patients with metastatic cancer (pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, melanoma, hepatocellular and head and neck) [18]. Conversely, <10% of the 27 patients with low-grade gliomas (LGGs; WHO grades I and II) or HGGs and <50% of the 14 patients with medulloblastoma showed detectable levels of ct-DNA in plasma [18]. Schwaderle et al. analyzed ct-DNA in 670 plasma samples, including 152 (22.7%) cases of PBTs, through digital NGS and using different-size gene panels including the most common cancer-related genes [19]. In this study, only 32% of patients with PBTs showed at least one somatic mutation in ct-DNA [19]. Among these mutations, 4% are associated with FDA-approved drugsand 11% with experimental drugs, while 85% had non-actionable alterations [19]. To explain the relative rarity of detectable

DNA alterations in the blood of PBT patients it has been proposed that the blood–brain barrier (BBB) may hinder the efficient leakage of tumor biomarkers into the bloodstream.

4. Cerebrospinal fluid is the main source of ct-DNA in malignant gliomas

Considering its anatomical proximity to the brain parenchyma, and its usefulness for the diagnosis of other pathological conditions of the CNS, the CSF has been explored as alternative source of ct-DNA. The CSF is produced by specialized ependymal cells in the choroid plexuses of the brain ventricles, and renewed three- to five-times daily, filling brain ventricles, cisterns and sulci, as well as the central canal of the spinal cord. CSF flows out to the subarachnoid space through openings in the fourth ventricle to be finally absorbed mostly through the arachnoid granulations in the superior sagittal venous sinus. In clinical practice, the CSF collection cannot be defined non-invasive, occurring or through lumbar puncture (LP), in which a needle is inserted into the subarachnoid space of the spinal canal, or directly during surgical maneuvers. However, although LP is not entirely risk-free, nowadays is considered a relatively safe procedure and is routinely performed for staging and follow-up of some PBTs, such as CNS lymphoma and medulloblastoma. Moreover, a theoretical advantage is that the fraction of ct-DNA should be higher in CSF than in plasma, owing to the relative scarcity of normal cells releasing their DNA directly or indirectly into the CSF.

Three relevant works clearly showed that CSF provides greater chance for detection of PBT genetic alterations compared with blood [20,21,24] *(Table 2)*.

Table 2. Studies comparing blood-based and CSF-based approaches for ct-DNA detection

Study	Patients (n)	Tumor type	Technique	Method of collection	Genes investigated	Sensitivity of plasma/serum	Sensitivity of CSF
Pan et al [20]	10	7 BTs, 3 LC	ddPCR/TAS	Surgery	Mutational panel including 578 cancer genes	Depending of systemic disease burden	85% (BTs)
Wang et al [21]	35	4 PAs, 3 DAs, 2 AAs, 11 GBMs, 6 medulloblastoma, 7 ependymomas	TS followed by WES	Surgery	Mutational panel including IDH1, TERT, PTEN, NF2	n/a	74% (overall)
De Mattos Arruda et al [24]	12	4 GBMs, 8 brain metastases	ddPCR/WES	LP	MSK-IMPACT	Depending of systemic disease burden	100% (overall)

AA, anaplastic astrocytoma; BT, brain tumor; CSF, cerebrospinal fluid; DA, diffuse astrocytoma; ddPCR, droplet digital polymerase chain reaction; G, WHO grade; GBM, glioblastoma; HGG, high-grade glioma; LC, leptomeningeal carcinomatosis; LGG, low-grade glioma; n/a, not applicable; ODG, oligodendroglioma; PA, pylocitic astrocytoma; TAS, targeted amplicon sequencing; TS, targeted sequencing, WES, whole-exome sequencing.

In a first pilot study, Pan et al. applied ddPCR or targeted amplicon sequencing to quantify tumor-specific hotspot mutations in both CSF and plasma cf-DNA collected from ten patients, including seven with solid brain tumors (five metastases and two PBTs: a vestibular schwannoma and an atypical meningioma), and three patients with leptomeningeal disease [20]. Tumor mutations were detected in CSF from six of seven patients with solid brain tumors, while the only patient who did not harbor mutations in the CSF had a vestibular schwannoma [20]. Concentrations of ct-DNA were higher in the CSF than in plasma when the systemic disease burden was low [20]. Moreover, using a cancer panel sequencing, seven somatic mutations were identified in the CSF of one patient with leptomeningeal disease, and the result was concordant with genetic testing on the primary tumor tissue [20].

In 2015, Wang et al. investigated the presence of tumor DNA in the CSF (indicated in this study as CSF-tDNA) of 35 patients with different PBTs (ten LGGs, 13 HGGs, six medulloblastomas, six ependymomas) and anatomical location (14 in the posterior fossa, eight in the supratentorial compartment and 13 in the spinal cord) [21]. Most of the CSF samples were collected directly from the CNS cavities at the time of the initial surgery, and a targeted sequencing followed by WES was used to the identify mutations in tumor tissues [21]. The amounts of DNA isolated varied significantly (average of 417 ng and standard deviation of 553 ng) [21]. At least one mutation for each of the 35 tumor samples was identified [21]. The detection rate of such mutations in the DNA purified from the matched CSF using PCR amplification and sequencing was 74% (95% CI: 57–88%), a sensitivity very close to that observed in body fluids adjacent to other tumor types, such as urine in urothelial cancer [22] or bronchial washing in lung cancer [23]. The average detectable mutant allele fraction in CSF was 12.2%, lower than the fraction in tumor tissues, but far exceeding the detection limit of the sequencing assay used (0.01%) [21]. Of note, 18/19 HGG cases had detectable t-DNA levels in CSF, which were significantly higher than in LGGs (mutant allele fraction of 16.3±21.2% vs 2.8±6.8%) [21]. Remarkably, CSF-t-DNA was found in all tumors (13 WHO grade III

or IV gliomas, five medulloblastomas, three ependymomas; 100% of 21 cases) that abutted a CSF reservoir or cortical surface, whereas no ct-DNA was present in the CSF of tumors encased into brain tissue ($p<0.0001$) [21]. On multivariate logistic regression analysis to identify clinical features associated with the detection of CSF-t-DNA, only tumor proximity to CSF reservoirs and tumor grade were significant [21]. These findings suggest that DNA released by HGG cells invading CNS cavities could represent the major source of CSF-t-DNA [21]. Although in this study CSF was collected during initial or repeated surgery from reservoirs adjacent to the tumor, given the modalities of CSF circulation through internal ventricles and subarachnoid spaces, it was proposed that LP would have been equally effective in retrieving CSF enriched in ct-DNA [21].

In the same years, a NGS analysis through a targeted panel (MSK-IMPACT) and/or WES, coupled with ddPCR, was applied by De Mattos-Arruda et al. on simultaneously collected CSF and plasma ct-DNA, and tumor tissues, from 12 patients with PBTs or metastatic lesions (4 GBMs, 6 brain metastases from breast cancer, 2 brain metastases from lung cancer) [24]. Detectable levels of CSF ct-DNA were identified in all cases, while plasma ct-DNA was present only in patients with bulky visceral disease, but not in patients with disease restricted to the CNS [24]. GBM cases showed the lowest rate of genomic alterations in CSF ct-DNA when compared to the corresponding tumor tissue [24].

Overall, these early studies clearly demonstrated that CSF is a potentially valuable source of ct-DNA, where a wide-range of genomic alterations, including putative actionable mutations and copy number alterations (CNAs) can be detected with high levels of sensitivity [20,21,24]. These data paved the way for the use of CSF ct-DNA as a biomarker to provide molecular diagnosis, prognostic and predictive information, irrespective of the availability of the primary tumor tissue. Moreover, a minimally invasive and repeatable procedure, such as CSF collection by LP, would allow unprecedented monitoring opportunities, such as the longitudinal track of tumor burden, and the assessment of genomic evolution in tumors relapsed after CT and RT.

5. Comprehensive and longitudinal genomic analysis of CSF in PBT

In recent years, state-of-the-art genomic profiling technologies have been assessed for the comprehensive characterization of CSF ct-DNA *(Table 3)*.

Table 3. Comprehensive genetic landscape of CSF ct-DNA

Study	Patients (n)	Glioma subtype	Technique	Method of collection	Genes investigated	Sensitivity
Pentsova et al [25]	53	1 brainstem glioma, 1 ODGs, 3 ODGs GIII, 1 anaplastic ependymomas, 1 AAs, 1 HGGs nos, 4 GBMs, 4 LM, 37 BMs	NGS	LP	MSK-IMPACT	50% (overall)
Miller et al [26]	85	46 GBMs, 26 gliomas GIII, 13 gliomas GII	NGS	LP	MSK-IMPACT	49.4% (overall)
Moulliere et al [27]	13	10 GBMs, 1 ODGs GIII, 1 ODGs, 1 DA	sWGS	LP	SCNAs and DNA fragmentation	39%

AA, anaplastic astrocytoma; BMs, brain metastases; CSF, cerebrospinal fluid; DA, diffuse astrocytoma; G, WHO grade; GBM, glioblastoma; HGG, high-grade glioma; LM, leptomeningeal metastases; LGG, low-grade glioma; NGS, next-generation sequencing; nos, no otherwise specified; ODG, oligodendroglioma; PTB, primary brain tumor; SCNAs, somatic copy number alterations; sWGS, shallow whole genome sequencing; WHO, World Health Organization.

The Memorial Sloan-Kettering group systematically explored whether a high-throughput sequencing assay (MSK-IMPACT), which included 341 cancer-associated genes, was able to identify ct-DNA in CSF samples, collected through LP from 53 patients with suspected or known CNS involvement [25]. Of these patients, 12 had PBTs, and the remaining brain and leptomeningeal metastases [25]. High-confidence somatic mutations were detected in 63% of patients with CNS metastases, but in none of the patients without CNS involvement (n=9) [25]. Among the 12 patients with diffuse gliomas, mutations were observed in 50% of the CSF samples collected [25]. However, as the matched tumor specimens were not analyzed with the same methodology, it is difficult to conclude whether CSF ct-DNA faithfully mirrors the genetic features of the original

tumor [25].

In a subsequent study, the same group further characterized the glioma genetic landscape applying the same NGS-based assay (MSK-IMPACT) to 85 CSF samples derived from adult patients with diffuse gliomas of various grade (46 GBMs, 26 WHO grade III and 13 WHO grade II) [26]. CSF collection occurred through LP as part of patients' clinical management for signs or symptoms suggestive of CNS infection, leptomeningeal spread or increased intracranial pressure [26]. Notably, CSF was gathered long after initial surgery and in all cases after the completion of adjuvant oncological treatments (RT and CT) [26]. Multiple radiological parameters, such as tumor progression, tumor burden, intra-ventricular spread, were strongly associated with DNA leakage in the CSF [26]. Conversely, no correlation was observed with glioma grade, disease duration, or prior therapy [26]. The presence of CSF ct-DNA established a powerful independent negative prognostic factor in the multivariate analysis, even after adjustment for extent of resection at diagnosis, tumor burden at CSF collection and *IDH* mutational status [26]. Tumor-derived genetic alterations were observed in CSFs from 42 out of 85 (49.4%) patients, and the most common were *TERTp*, *TP53* or *IDH* mutations, CDKN2A/CDKN2B deletions, EGFR amplifications, and in-frame EGFR-variant III deletion [26]. No mutations were detected in plasma cf-DNA from 16 out of 19 patients who were positive for CSF ct-DNA, confirming once again that blood is not a reliable source of glioma DNA [26]. All available tumor specimens obtained at the time of initial surgery, belonging to patients with positive CSF ct-DNA, and including 20 GBMs, 10 lower grade gliomas and 6 tumors with hypermutated profile, were sequenced with the same gene-panel [26]. This genetic comparison allowed investigating whether CSF displayed signs of a genetically evolved tumor. The median percentage of shared mutations between tumor and CSF in the 30 cases without a hypermutated profile was 81.7%, while considerably less common (3–49%; median 19.6%) in the six hypermutated tumors [26]. Lower-grade gliomas molecular diagnostic signature was concordant between CSF and tissue in all ten cases analyzed [26]. A significantly higher mutational

burden along with a genetic signature suggestive of prior exposure to alkylating agents (G:C→A: T transitions) was observed in five CSF ct-DNA samples of patients treated with TMZ before CSF collection [26]. Interestingly, as the interval between tumor and CSF collection increased, the genetic profile of CSF tended to diverge from that of the primary tumor [26]. However, in accordance with established patterns of glioma evolution, divergence between tumor tissue and CSF usually did not involve early "truncal" alterations such as 1p/19q co-deletion, or *IDH1*, *TP53*, *TERT* and *ATRX* mutation, but alterations involving growth signaling pathways, such as EGFR, PDGFRA, MET, reflecting a presumptive convergent evolution [26].

An untargeted and relatively low-cost shallow WGS at low-depth coverage (<0.4×), was used by Moulière et al. with the aim to identify ct-DNA in CSF without *a priori* knowledge of tumor tissue alterations [27]. Specifically, CNAs and DNA size profiles distribution were analyzed in 13 CSF samples of patients with primary HGGs [27]. Despite the small cohort size and limited sensitivity of sWGS, CNAs were observed in 5/13 (39%) patients [27]. In one case, for which there were available multiple biopsies representative of different tumor areas, most of the CNAs were shared by CSF and tumor tissue [27]. Interestingly, loss of chromosome 10 and gain of chromosome 7 were detected in CSF but missed in one of the four tumor specimens, indicating that CSF ct-DNA may better recapitulate the spatial heterogeneity of tumor [27]. Of note, in this work an analysis of the CSF fragmentation pattern, inferred from WGS data, was reported for the first time [27]. A specific fragmentation signature with enrichment in fragments smaller than 150 bp and low or absent peak at ~167 bp, different from that observed in plasma and urine, was observed in most of the CSF samples [27].The methodology implemented in this work may represent an alternative way to detect glioma ct-DNA in CSF at low cost, able at the same time to provide significant genomic information and to be the premise for further more expensive larger-scale sequencing in those patients with high levels of ct-DNA [27].

Important variables affecting the successful detection of tumor genetic alterations are the quality

(fragmented vs. high-molecular weight) and the quantity of DNA found in LP-retrieved CSF. Studies published so far did not present a systematic characterization of these parameters, or their relationships with data concerning tumor volume and localization. Pentsova *et al.* [24] reported in CSF supernatants (cleared by cell centrifugation) a mean of 27 ng of DNA (N = 8). However, high variability among different samplings is expected, as shown by Moulière [26]. The sensitivity of current PCR-based or NGS techniques would allow to provide information starting from less than 20 ng of DNA.

6. Focus on specific diagnostic and prognostic biomarkers

Given the well-known ability of *TERTp* and *IDH* mutations to predict survival, Juratli et al. conducted a prospective pilot study to assess the feasibility of detecting these hotspot mutations in CSF and serum ct-DNA collected during surgery of a cohort including 60 patients with different gliomas subtypes [28]. In order to reduce potential confounding factors, only patients with *TERTp*-mutant/*IDH* WT GBM (n=38) were selected for the analysis and studied through nested and ddPCR [28]. Remarkably, *TERTp* mutations were detected in CSF samples with a 92% sensitivity (35/38 cases), far higher compared to that obtained in plasma ct-DNA (3/38; sensitivity 8%) [28]. All tumors were close to a CSF reservoir, while the only case of a tumor entirely encapsulated into brain parenchyma did not show *TERTp* mutations in the CSF [28]. Patients with *TERTp* low variant alleles frequencies (VAF) in CSF-tDNA had a significantly longer overall survival, compared with patients with high VAF [28]. Applying a cut-off of 13% the *TERTp* mutation VAF represented an independent negative prognostic factor for overall survival [28]. A lower sensitivity of *TERTp* mutation detection in CSF was observed in a subset of oligodendrogliomas (*TERTp*-mutant/*IDH*-mutant), consistent with previous findings that low-grade tumors may shed only modest amount of tumor DNA in in CSF [18,28].

The clinical value of CSF-based liquid biopsy strategy might be extremely high in the case of diffuse

midline gliomas. These tumors are indeed highly morbid glial neoplasms of the thalamus or brainstem that almost exclusively arise in young children, usually are not surgically accessible, and characterized by a high rate of gain-of-function histone H3 mutations. Haung et al. studied 12 CSF samples collected from children during the course of their treatment, searching *H3F3A* and *HIST1H3B* mutations via either traditional targeted Sanger sequencing or via nested PCR, depending on CSF DNA levels [29]. CSF-DNA amount varied from 0.01 to 3.76 ng/μL [29]. In this small cohort, the H3 mutations was identified with a 100% specificity and 87.5% sensitivity, demonstrating the feasibility of a CSF liquid biopsy assay for molecular diagnosis and, selection to molecularly targeted therapies [29].

Martinez-Ricarte et al. developed a sequencing platform to simultaneously detect a set of gene mutations (*IDH1*, *IDH2*, *TP53*, *ATRX*, *TERT*, *H3F3A* and *HIST1H3B*) that are crucial for the definition of histo-molecular glioma subgroups [5,30]. Tumor specimens and CSF ct-DNA from 20 glioma patients were analyzed by targeted exome sequencing and ddPCR [30]. CSF DNA concentration varied from 3.6 to 103.7 ng/mL, with a single value of 560 ng/mL collected from cisterna magna [30]. In 17 out of 20 cases, ct-DNA was found in the CSF carrying the same mutational profile as the matched tumor sample, therefore allowing the right diagnosis [30]. The three cases lacking ct-DNA in the CSF were all LGGs (one grade II diffuse astrocytoma and two grade II oligodendrogliomas), suggesting again that tumor grading may represent a key factor influencing the presence ct-DNA in the CSF [30]. As highly relevant finding, all three midline gliomas in this cohort showed the characteristic H3K27 mutation in the CSF ct-DNA [30].

Data of these studies focused on specific glioma biomarkers are summarized in *Table 4* [28-30].

Table 4. Studies focused on specific biomarkers with diagnostic and prognostic significance in CSF ct-DNA.

Study	Patients (n)	Tumor type	Technique	Method of collection	Genes investigated	Sensitivity
Juratli et al [28]	60	38 hTERT-mutant GBMs, 16 IDH-mutant/hTERT WT gliomas, 6 BMs	PCR	Surgery	hTERT	92%*
Huang et al [29]	12	12 diffuse midline gliomas	PCR	LP	H3 mutation	66.7%
Martinez Ricarte et al [30]	20	10 GBMs, 2 AAs, 4 ODGs, 1 DA, 3 midline gliomas	ddPCR/TAS	LP	IDH1, IDH2, TP53, TERT, ATRX loss, H3F3A, and HIST1H3B	85%

BMs, brain metastases; AAs, anaplastic astrocytomas; ATRX, α-thalassemia/mental-retardation-syndrome-X-linked gene; CSF, cerebrospinal fluid; DA, diffuse astrocytoma; ddPCR, droplet digital polymerase chain reaction; GBMs, glioblastomas; HGG, high-grade glioma; hTERT, human telomerase reverse transcriptase promoter; H3, histone 3; H3F3A, H3 histone family 3A; HIST1H3B, histone cluster 1 H3 family member b; IDH, isocitrate dehydrogenase; LGG, low-grade glioma; LP, lumbar puncture; ODGs, oligodendrogliomas; PCR, polymerase chain reaction; TAS. Target amplicon sequencing; WT, wild-type.
*Data referred only to 38 patients with hTERT mutant GBMs

7. Questions and Controversies

Our knowledge of the genetic and epigenetic landscape of diffuse gliomas has dramatically increased over the last few years. Key genomic alterations defining glioma subtypes are now part of the routine diagnostic workout, allowing stratification of patients' prognosis, and guiding treatment decision-making [4-10]. Currently, molecular characterization of diffuse gliomas still requires access to tumor specimens. The idea of using ct-DNA as a reliable biomarker for gliomas genomic characterization sparing risky surgical maneuvers is undoubtedly intriguing. ct-DNA-based liquid biopsy has a potential significant impact in different areas of glioma patient management. Analysis of ct-DNA could facilitate diagnosis in all those cases where anatomical location or patient's comorbidities make surgery challenging or simply not feasible, and where conventional sampling methods fail to be informative. Moreover, ct-DNA could provide a comprehensive framework of the whole tumor genome, potentially recapitulating the broad spatial and temporal intratumor heterogeneity, a well-recognized hallmark of diffuse gliomas. Beyond diagnostic purposes, ct-DNA sequencing should aim a longitudinal surveillance of genomic evolution over time allowing a new molecular characterization at recurrence or progression after anticancer treatments, usually precluded by the unavailability of serial bioptic samplings. The recent advent of a wide array of novel molecularly targeted and immunotherapeutic agents also in the field of neuroncology imposes improved tools for molecular profiling of patients and for treatment selection, especially at relapse when the genomic profile can differ from the primary. Liquid biopsy might acquire a key role also in monitoring tumor burden and response to oncological treatments. Indeed, current MRI-based neuroimaging has limited accuracy, often lacking the ability to unmask two phenomena typical of CNS tumors, such as pseudoprogression and pseudoresponse [31]. A novel tool helping physicians to evaluate glioma clinical behavior, differentiating between pseudoprogression and true progression, or identifying early relapse even before radiological evidence, would have a great clinical utility.

Current data on the use of ct-DNA in the field of brain tumors rise from small series of patients, very often including different PBTs or metastatic lesions [13-30]. Moreover, CSF sampling methods and timing differ widely between available studies, as well as detection and sequencing assays applied for analysis of CSF ct-DNA [20,21,24-30]. In a translational perspective, some important conclusions have been established, but several issues remain still open. First, in the context of gliomas, the best source of ct-DNA is represented by the CSF. Most gliomas shed ct-DNA in the CSF [20,21,24-30], whereas blood ct-DNA levels are low and detectable only in few patients, probably owing to the presence of the BBB [13-19]. Second, CSF ct-DNA liquid biopsy may not be as informative across all glioma subtypes. Given their slow growth-fraction rate and poor cellularity, only few LGGs release detectable amounts of ct-DNA into the CSF which preclude their molecular profiling [20,21,24-30]. Detection rate seems to be lower even in the case of gliomas encapsulated into brain parenchyma and who are not directly adjacent to a CSF reservoir or the cortical surface [21]. Third, CSF ct-DNA provides a comprehensive and genetically faithful representation of the corresponding tumor genome [20,21,24-30]. The level of genetic concordance between CSF and tumor DNA overall seems to be high, particularly in patients who underwent both CSF collection and tumor resection simultaneously [24,26]. Reflecting glioma genetic evolution, a certain degree of divergence is observed when CSF collection occurs long after surgery [24,26]. In these cases, molecular differences do not involve genetic alterations occurring early during the course of gliomagenesis, such as *IDH1*, 1p/19q co-deletion, *TP53*, *TERT*, *ATRX*, but rather genes encoding for growth factor signaling pathways [24,26].

Issues to be addressed in the very near future regard mainly methods and timing of CSF samples collection, as well as amplification and sequencing techniques to apply (targeted vs untargeted approach). If our intention goes beyond diagnosis, and rather we would track dynamic genomic evolution or follow tumor response, serial CSF sampling has to be repeated. In this respect LP remains the only procedure conceivable for collecting and analyzing CSF ct-DNA over the course of

treatments, despite its invasive nature, its potential risks and the entire clinical scenario in which it is contraindicated.

The choice of the best assay mainly depends on the aim to be pursued and should take into careful consideration an accurate balance between complexity, speed and costs. Looking at a future implementation in the routine clinical setting, a relatively low-cost PCR-based targeted approach evaluating a small subset of candidate genes for known hotspot mutations and amplification, with a high level of analytical sensitivity (detection level between 0.001–0.01%) and specificity seems the most reasonable strategy. Despite limitations due to low multiplexing capability, ddPCR-based methods can provide clinicians all the molecular information necessary to classify different glioma subtypes, define patient prognosis, and guide treatment. NGS technologies have introduced the extraordinary potential to reveal not only mutations, but all types of cancer-associated alterations, including gene fusions. Developing a predefined sequencing platform to simultaneously and rapidly interrogate multiple loci, including genes encoding for potential targets of the therapeutic interventions, would have a greater clinical utility. A broader untargeted ct-DNA genome-wide sequencing in absence of prior knowledge of the tumor molecular aberrations should be useful to uncover new actionable mutations or *de novo* acquired resistance mutations. These techniques are expensive, require bioinformatics expertise and longer times to process and analyze results. Nowadays, such approach should be limited for research purposes. As clear from this review, the ideal use of these innovative biomarkers has yet to be defined and only larger prospective studies focused exclusively on glioma patients and rather integrated into therapeutic clinical trials will provide definitive answers and eventually the required level of evidence for introducing CSF ct-DNA analysis into clinical practice. Key steps in this direction are represented necessary by the technical standardization of CSF collection and validation of CSF ct-DNA extraction methods.

8. Conclusion

A minimally invasive tool able to provide molecular tumor profiling would be particularly useful in the context of diffuse gliomas, since these tumors are not accessible by definition to serial tissue sampling. CSF ct-DNA liquid biopsy strategy may virtually assist all the steps of glioma management: aid molecular diagnosis, monitor tumor response, identify early recurrence, track glioma clonal evolution, molecularly characterize recurrent tumors and guide targeted therapies. Currently, there is not sufficient evidence for implementation of liquid biopsy in routine clinical practice and large prospective studies are needed to validate how reliably CSF ct-DNA can reflect the mutational profile of gliomas, especially when comprehensive genome-wide technologies are used.

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