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Keywords: glioblastoma, genomics, precision medicine, non-invasive biomarkers, ctDNA, CTC, microvesicles, liquid biopsies.

Abstract

Glioblastoma (GBM) is the most common and devastating primary malignant brain tumor in adults. The past few years have seen major progress in our understanding of the molecular basis of GBM. These advances, which have contributed to the development of novel targeted therapies, will change the paradigms in GBM therapy from disease-based to individually tailored molecular target-based treatment. No validated circulating biomarkers have yet been integrated into clinical practice for GBM. There is thus a critical need to implement minimally invasive clinical tests enabling molecular stratification, prognosis assessment, as well as the prediction and monitoring of treatment response. After examination of data from recent studies exploring several categories of tumor-associated biomarkers (circulating tumor cells, extracellular vesicles, nucleic acids, and oncometabolites) identified in the blood, cerebrospinal fluid and urine, this article will discuss the challenges and prospects for the development of circulating biomarkers in GBM.

Introduction

Glioblastoma (GBM, World Health Organization grade IV astrocytoma) is the most common and aggressive primary malignant brain tumor in adults. GBM accounts for ~50% of primary brain malignancies with an annual incidence of 3.19 per 100,000 in the United States [1]. Despite intensive multimodal therapy comprising surgery, concomitant radio-chemotherapy and adjuvant chemotherapy [2], patients with GBM continue to have a dismal prognosis with a median survival between 10 and 15 months [3-5], resulting in approximately 175,000 GBM-related deaths each year worldwide [6]. Therefore, efforts are urgently needed to accelerate progress in neuro-oncology and improve the outcome of patients with GBM.

The past decade has seen remarkable strides forward in the molecular characterization of GBM. These advances – achieved through collaborative works of international research groups [7-13] – have provided a tremendous opportunity to improve the management of GBM patients [14-17]. Several molecular alterations such as the *IDH1* mutation and *MGMT* promoter methylation have been associated with diagnostic, prognostic and predictive values [18-21], suggesting that such biomarkers could be used to improve the diagnosis and enhance the effectiveness of cancer care, by allowing physicians to tailor therapy to individual molecular profiles. With the rapid advances in this field, and the recent progress that has occurred during the past few years in non-invasive tests that detect circulating tumor cells (CTCs) and tumor DNA fragments [22-26], a growing body of research indicates that circulating biomarkers would facilitate the clinical management of GBM patients [27-30].

Main molecular features of glioblastoma

GBM is a highly heterogeneous and invasive malignant tumor. The definitive diagnosis requires the histological analysis of tumor tissue – obtained through surgical resection or biopsy –, and typically shows poorly differentiated glial tumor cells with increased density and mitotic activity, hyperplastic vasculature, and areas of necrosis [31]. Several studies have described the genomic landscape of GBM, using integrated analysis from multi-omics datasets including epigenome, genome, transcriptome, metabolome and proteome of large cohorts of GBM [7-13]. In most cases, genomic alterations act in concert to disrupt several fundamental cellular processes simultaneously: retinoblastoma and p53 tumor-suppressor pathways, receptor tyrosine kinase – mitogen-activated protein kinase – phosphoinositide 3-kinase signaling pathways, chromatin remodeling, and telomere maintenance mechanisms. Several types of molecular aberrations are found, including copy number variations, mutations, deletions, translocations, dysregulated gene expression, and epigenetic reprogramming. Gene expression analysis has allowed GBM to be subclassified into at least four distinct molecular subtypes (proneural, classical, neural and mesenchymal), with better survival for the proneural subtype [7,10-12]. Moreover, several molecular alterations have been associated with a significant prognostic and/or predictive value. The best described and known is the methylation of the *MGMT* promoter, which encodes a DNA repair protein that counteracts the cytotoxic effect of temozolomide, the main chemotherapeutic agent used to treat newly diagnosed GBM [2,3]. Methylation of the *MGMT* promoter results in silencing of MGMT protein expression and is associated with sensitivity to temozolomide [18,19]. Other recurrent cancer genome aberrations – including *IDH1*, *PIK3CA*, *EGFRvIII*, *BRAF* and *FGFR* oncogenic alterations – are currently investigated as theranostic

biomarkers for molecular targeted therapies in clinical trials [17,32-39].

It is important to underline here that GBM typically displays a great degree of spatial and temporal intratumor heterogeneity (i.e. extensive genetic diversity both between different regions of the tumor and tumor subpopulations over time) [12,17,40-46]. This tumor heterogeneity has important implications for the mechanisms of cancer progression and resistance to therapy and represents a major challenge to personalized cancer medicine [47-49]. Indeed, this intrinsic feature of GBM may affect the ability of molecular signatures to predict therapeutic response and clinical outcome, and may explain – at least partially – the inefficacy of targeted therapies despite the use of robust biomarkers as inclusion criteria in clinical trials [17,34,50,51]. Non-invasive longitudinal tumor sampling approaches using circulating biomarkers (*e.g.* CTCs or cell-free circulating tumor nucleic acids) have the potential to provide real-time information on tumor heterogeneity and changes in the tumor subclonal architecture along the disease course, allowing clinicians to predict and circumvent tumor adaptation and drug resistance [47,48].

General concepts related to circulating biomarkers

Circulating biomarkers refer to "measurable biological molecules found in the blood, or other body fluids that provide information on a normal or abnormal process, or of a condition or disease, such as cancer" [52]. In oncology, circulating biomarkers display several potential clinical applications [53-55]. The diagnostic value lies in the abilities of the test to: (i) estimate a risk, (ii) facilitate early and differential diagnosis, (iii) assess the prognosis, (iv) follow the tumor evolution longitudinally and (v) monitor specific treatment [48,56,57]. In addition, several circulating biomarkers –

often referred as liquid biopsies (e.g. CTCs or cell-free circulating tumor nucleic acids) – can provide specific cytologic and/or molecular information on the tumor itself, and allow non-invasive tracking of the cancer genome. Finally, biomarkers can have predictive value, when the result of the test gives an indication of the probable effect of a treatment on the patient [58]. Such biomarkers are referred as companion diagnostics.

For brain tumors, circulating biomarkers have the undeniable advantage of providing useful information via a minimally invasive procedure. Such tests would be clinically useful, especially in cases in which surgery is contraindicated or when biopsy results are inconclusive [59]. Furthermore, at recurrence – which is virtually inevitable in GBM – less than 30% of patients are candidates for second surgery [2]. For the remaining inoperable patients, circulating biomarkers could be the source of a molecular profile of the relapsed tumor, allowing clinicians to identify potentially druggable molecular alterations driving recurrence.

The development and validation of circulating biomarkers is a long process that involves multiple steps from their discovery to approval for use. These steps (Figure) aim to ensure the robustness and usefulness of the test for making decisions about effective treatments or preventive strategies, and to demonstrate its added value compared to available tools [60]. No validated circulating biomarkers have yet been integrated into clinical practice for GBM. Current standard monitoring and follow-up procedures include clinical evaluation and brain imaging (magnetic resonance imaging [MRI], when feasible) [2,61]. They are valuable for evaluating disease evolution (i.e. stable, progression or response), but have several limitations: (i) they provide no or very limited molecular information [62-64], (ii) they offer scant prognostic and/or predictive information, (iii) they are frequently challenged when

differentiating between true tumor progression and treatment-related necrosis (pseudo-progression) in case of apparent tumor regrowth [61,65] as well as between true tumor response and a pseudo-response when antiangiogenic agents are used [66]. The validation of clinically useful circulating biomarkers from blood, urine, or cerebrospinal fluid (CSF) would be a major advance in neuro-oncology, since they may improve the management of patients through multimodal diagnosis and follow-up procedures.

Sources of circulating biomarkers in patients with glioblastoma

Circulating biomarkers can be collected from several sources in patients with GBM. Blood-based biomarker testing is the fastest and easier approach. The most extensively studied circulating biomarkers are proteins, which are actively or passively secreted by tumor cells and/or their microenvironment, and can be detected in blood, urine and CSF [27-30,67]. In addition, tumor cells can release small pieces of their DNA and/or RNA into the bloodstream and/or CSF [68-72], due to cell death or active secretion [73]. These pieces of tumor nucleic acids – called cell-free circulating tumor DNA (ctDNA) and circulating microRNA (miRNA) – are relatively stable in plasma and serum [54]. Conversely, cell-free messenger RNA (mRNA) is rapidly degraded in blood (see below for analysis of mRNA). In the case of biomarkers analyzed from CSF, it is important noteworthy that although CSF collection is safe in most cases, it is a more invasive procedure than blood sampling, and may be contraindicated due to the risk of brain herniation in patients with tumor-induced increased intracranial pressure. However, CSF analysis may be more sensitive than a blood analysis to detect ctDNA, at least for a subset of patients, as

demonstrated with the *IDH1* mutation [74,75].

Extracellular vesicles (EVs) are nanometer size membrane-enclosed particles that are released from GBM living tumor cells either from the fusion of an endosome with the plasma membrane ("exosomes"), or directly from the cell membrane ("microvesicles") [76,77]. EVs are vehicles of communication between different tumor compartments and its microenvironment, as other tumor cells and normal cells take them up [78-81]. Importantly, EVs – which can be isolated from both blood and CSF [75,78,79,82-84] – are a rich source of tumor-derived molecules such as DNA, microRNA, mRNA, proteins, lipids and metabolites, because the structure of EVs protect them from nucleases and proteases [76]. Accordingly, isolation of RNA (including mRNA) from EVs can yield a greater concentration of RNA compared to circulating mRNA from whole blood, plasma or serum [74]. Platelets can sequester the content of EVs, and it has been demonstrated that tumor-specific nucleic acids can be isolated in platelets extracted from patients with GBM [85]. These additional sources of tumor RNA could be useful for mRNA-based analysis (e.g. gene expression profiling and gene rearrangements detection).

Finally, recent works demonstrated the presence of CTCs in a subset of patients with GBM [86-88].

Circulating Tumor Cells

CTCs are cells that have shed from a tumor into the vessels and circulate in the bloodstream. CTCs are tumor-specific, and can potentially constitute seeds for subsequent growth of additional tumors (metastasis) in distant organs. CTCs are a widely studied biomarker with potential to inform on the prognosis and therapeutic

response in a variety of solid malignancies including lung, breast, and prostate cancers [89]. Furthermore, CTC analysis provides crucial diagnostic information on tumor cells, including morphology, immunocytochemical phenotype and molecular profile [90-92]. Finally, CTC isolation offers the ability to perform *in vivo* functional testing thereby providing opportunities to study the biology of metastasis, and to test drug sensitivity in *ex vivo* preclinical models [93,94].

Various technologies have been developed to detect and characterize CTCs including flow-cytometry based systems, immunomagnetic cell enrichment, and automated microscopy systems [89,95,96], but only one, CellSearch® (Janssen Diagnostics), based on clinical trials in patients with metastatic breast, prostate and colorectal cancers, has been approved by the US Food and Drug Administration (FDA) for detecting the presence of CTCs and monitoring disease evolution through CTC levels. As CellSearch® is mainly based on the detection of epithelial cell adhesion molecule (EpCAM), it is probably ineffective for the detection of primary brain tumor cells, which do not express such surface markers [86].

The presence of CTCs in GBM has fuelled debate for many years [97]. Although GBM is a highly aggressive and invasive tumor, it is mostly restricted to the brain. Unlike the case in other solid malignancies, the incidence of extracranial metastases is extremely low in patients with GBM with only 0.5%-2% of patients developing secondary lesions in the bone, lymph nodes, or liver [98-100]. Conversely, cases of GBM transmission have been reported in patients who received organ transplants from donors with GBM [101]. These rare observations have provided indirect evidence that GBM tumor cells may acquire the ability to disseminate via the bloodstream, in particular circumstances such as systemic immunosuppression. Recently, three research groups reported on the isolation of CTCs in the blood of 20-

70 percent of GBM patients (Table 1). MacArthur *et al* devised a strategy based on the evaluation of telomerase activity in a cohort of glioma patients undergoing radiotherapy [86]. Telomerase is a DNA polymerase required to replicate the ends of the chromosomes, and is aberrantly expressed through *TERT* promoter mutation in 75.7 percent of primary GBM [102]. The detection assay used was an adenoviral probe allowing GFP expression in cells with elevated telomerase activity. Initial experiments demonstrated the specificity of the probe for accurately isolating glioma cells while leaving normal cells – including normal brain and white blood cells – unaffected. In this pilot study, CTCs were detectable in 72 percent (8/11) of pre-radiotherapy patients, compared with 12 percent (1/8) of post-radiotherapy patients [86]. Interestingly, in a small subset of patients with available serial measurements, the variation of CTC levels during the disease course was correlated with tumor progression, suggesting that CTC levels may reflect the status of the tumor after resection and/or radiation therapy. In a recent study by Sullivan *et al* [88], CTCs were identified in 39 percent (13/33) of patients with GBM, using both immunomagnetic and immunofluorescence-based cell selection. First, blood specimens were enriched for CTCs using a microfluidic device developed by the authors, the CTC-iChip platform, which combines size-based removal of red blood cells and platelets, and immunomagnetic depletion of leukocytes using antibodies against leukocyte markers (CD45 and CD16). Subsequently, GBM CTCs were isolated from purified samples using immunofluorescence staining based on a panel of markers commonly expressed in GBM tumor cells (SOX2, Tubulin β-3, EGFR, A2B5, and c-MET). The analysis of CTCs isolated from both patient-derived xenografts (PDX) models and patients revealed enrichment for mesenchymal over neural subtype, suggesting that the mesenchymal subtype of GBM may harbor a greater potential to proliferate outside

the brain. Finally, Müller *et al* [87] identified CTCs in blood collected from 20.6 percent of patients (29/141), using tumor cell enrichment and screening methods based on glial fibrillary acidic protein (GFAP). The tumor-specificity of GFAP-positive and CD45-negative cells was demonstrated by the absence of such cells in healthy volunteers. GFAP-positive CTCs were further characterized with comparative genomic hybridization (CGH array) and/or next generation deep sequencing, which allowed the detection of tumor-specific aberrations such as gains of chromosome 7 regions, losses of chromosome 10 regions and *EGFR* amplification. Interestingly, there was no correlation between the presence of CTCs and survival or GBM dissemination.

Using assays based on different technologies, these independent studies established the proof of concept of the feasibility of isolating and characterizing CTCs in GBM (Table 1). The question is what is the more accurate and useful technology for identifying CTCs originating from brain tissue, especially given the heterogeneity of GBM? Larger-scale prospective studies are needed to address this question, and to demonstrate the clinical utility of characterizing CTCs in GBM. Further research is needed in this field to determine whether CTCs are: (i) relevant for understanding the clinical behavior of GBM and its mechanisms of extracranial dissemination, (ii) representative of the majority of tumor cells, and (iii) informative for the characterization of somatic genomic aberrations and the dynamics of tumor genomic diversity during the disease course.

Cell-free circulating tumor DNA

ctDNA can be easily extracted from blood plasma, using commercially available kits,

and is found at varying levels in the blood of patients with diverse solid tumor types [103]. Extensive research is ongoing to develop and implement ctDNA-based biomarkers [54,55,104]. Multiple approaches are now available, from PCR-based methods that allow the detection of known point mutations to next-generation exome or whole genome sequencing that allow characterization of mutations, copy-number changes and chromosomal rearrangements [22-25,104]. Thus, ctDNA analysis has the potential to provide similar molecular information as that obtained from invasive tumor biopsies [54-56]. However, isolating and characterizing ctDNA pose a number of challenges, especially for diseases confined to the brain, since ctDNA levels correlate with both cancer type and stage. To illustrate this point, Bettegowda *et al* [103] reported a very low rate of glioma patients with detectable ctDNA as compared with patients with other solid tumors. This is probably mainly due to the biophysical obstacle represented by the blood-brain barrier (BBB). Indeed, although being partially disrupted in virtually all GBM, the BBB may prevent the efficient passage of DNA molecules into the peripheral circulation [105]. However, with the development of ultrasensitive ctDNA detection methods [106], as well as that of novel methods to overcome the BBB [107], more sensitive ctDNA detection assays are expected in the coming years.

Several small retrospective studies have addressed the feasibility and utility of ctDNA detection in patients with primary brain tumor (Table 2). Using Polymerase Chain Reaction (PCR)-based assays, ctDNAs were successfully detected in GBM patients, and multiple molecular alterations were characterized including loss of heterozygosity (LOH) in chromosome arms 1p, 19q and 10q, *IDH1* and *EGFRvIII* mutations, as well as methylation status of promoters of *MGMT*, *PTEN*, and *CDKN2A* [105,108-113]. In addition, a recent study reported on the value of a ctDNA-based NGS panel of 54

genes using the Guardant360® assay in a prospective cohort of patients with primary brain tumors, including 34 GBM. Overall, 43 percent of GBM patients had at least one somatic alteration detected among 21 genes [114], with most common mutations affecting *TP53* (n=12). These studies indicated the potential diagnostic value of ctDNA and provided several other insights. *MGMT* promoter methylation status assessed from ctDNA exhibited both prognostic [115,116] and predictive values [111]. However concordance between tissue and serum methylation status was limited [115], with several false-negative results when status was assessed from blood. Nevertheless, compared with the analysis performed on respective tumors samples, ctDNA-based mutation testing showed an excellent specificity (100 percent in most studies), and sensitivity was between 50 and 90 percent in most studies. As expected, the sensitivity was correlated with the tumor volume and contrast enhancement [105], indicating that an intact BBB prevents ctDNA from entering the circulation. Until now, the clinical utility of candidate ctDNAs as biomarkers for patients with GBM has not been demonstrated, and larger-scale prospective studies are needed before their implementation in routine clinical practice.

Cell-free circulating miRNA and extracellular vesicles carrying tumor-derived RNA

Tumor cells can release small pieces of their RNA into the bloodstream and/or CSF [68-72], as the result of cell death or active secretion [73]. Circulating miRNA can be collected as cell-free circulating entities, as these nucleic acids are relatively stable in plasma and serum [54]. Conversely cell-free mRNAs are prone to rapid digestion due to the presence of RNA degrading enzymes which are elevated in the serum. Tumor-

derived mRNA can be efficiently isolated and analyzed after extraction from EVs. EVs are highly stable nanometer size membrane-enclosed particles that are released from both normal and tumor cells in blood, urine and CSF [75,78,79,82-84], carrying a rich source of nucleic acids (including DNA, miRNA and mRNA), proteins, lipids and metabolites from the host cells. Importantly, isolation of RNA (including mRNA) from EVs can yield a greater concentration of RNA compared to circulating RNA from whole blood, plasma or serum [74], as the structure of EVs protect RNA from circulating nucleases [76].

miRNAs are small (~ 22 nucleotides) non-coding RNAs that can be detected as cell-free entities or as the content of circulating EVs in blood and/or CSF. miRNAs are regulatory nucleic acids that modulate the epigenetic state and gene expression of cells from both the tumor and its microenvironment, thereby exerting an impact on tumor behavior [117]. Indeed, several functional studies have demonstrated that miRNAs may act as oncogenes or tumor suppressor genes and influence tumor cell proliferation, differentiation, survival, and invasion [118,119]. miRNAs are the most extensively studied circulating nucleic acids in GBM. Several groups have studied the expression patterns of circulating miRNAs, and have reported that miRNA differential expression can distinguish patients with GBM from healthy patients [69,77,84,120-125]. Altered levels of several miRNAs have been associated with GBM, and miRNA-21, miRNA-128 and miRNA-342-3p plasma levels have been associated with both prognostic and monitoring values [120,121,123,124].

Tumor-specific mRNAs – including EGFRvIII and MGMT – can be isolated from EVs and/or platelets from GBM patients [77,82,85], allowing analysis using technologies such as NGS and RT-qPCR. Several pilot studies [77,82,85] have demonstrated that tumor-derived EVs isolation is feasible in GBM, providing the

opportunity for mRNA-based molecular profiling (e.g. gene expression profiling and characterization of tumor-specific molecular alterations such as mutations and translocations).

Overall, these studies established the feasibility of noninvasive tumor-derived RNAs analysis in patients with GBM. Such tests have several potential applications from differential to molecular diagnosis. Like ctDNAs, circulating RNAs, have not been evaluated in large-scale prospective studies. Further validation of these biomarkers is needed before their integration into clinical practice.

Circulating Tumor-associated Oncometabolite and Protein Biomarkers

Circulating Oncometabolites

Mutations of the isocitrate dehydrogenase genes (*IDH1* and *IDH2*) are found in 6 percent of primary GBM and more than 70 percent of secondary GBM [12,127]. *IDH1/2* mutations are independent prognostic factors [10-12,128,129], and assessment of the *IDH1/2* status has been implemented in routine clinical practice for patients with gliomas. *IDH1* and *IDH2* encode enzymes that catalyze the oxidation of isocitrate to alpha-ketoglutarate in the tricarboxylic acid cycle [130]. Mutant IDH enzymes acquire neomorphic enzymatic activity, thereby catalyzing the production of D-2-hydroxyglutarate (D2HG), an oncometabolite that accumulates at high levels and inhibits several enzymes notably involved in histones and DNA demethylation [131]. Elevated circulating D2HG levels have been reported in patients with D-2-hydroxyglutaric aciduria (an extremely rare neurometabolic inborn disease), and with *IDH1/2*-mutant cancers, including acute myeloid leukemias (AML), and cholangiocarcinomas [132,133]. Importantly, D2HG levels have been demonstrated to

be of interest for both the diagnosis and monitoring of patients with *IDH1/2*-mutant malignancies [132-135]. However, in most patients with *IDH1/2*-mutant gliomas, plasmatic D2HG values are in the normal range [136], suggesting that the BBB prevents D2HG from entering the circulation. A recent study showed that the plasma/urine ratio of 2HG may increase the sensitivity and specificity for *IDH1/2* mutation detection in glioma [137]. However, in the latter study, the authors explored the value of the total 2HG level – which include both enantiomers L-2-hydroxyglutarate and D2HG – although previous studies have demonstrated that only D2HG level is predictive for *IDH1/2* status. Whereas the clinical value of noninvasive detection and monitoring of D2HG levels has been well established in AML [132], the feasibility in glioma remains unclear. Brain imaging-based methods [62] are currently under investigation. To our knowledge, the diagnostic value of the D2HG level in the CSF has not been evaluated.

Non-tumor specific proteins

Whereas CTCs, tumor EVs, circulating tumor nucleic acids, and oncometabolites are tumor-specific, most of the circulating proteins that have been studied as putative GBM biomarkers are not tumor-specific because they are also closely associated with diverse types of brain parenchymal injury [138-142], suggesting that these proteins are released into the bloodstream in case of disruption of the BBB, whatever the mechanism of disruption. Indeed, when BBB disruption occurs due to tumor development, these circulating proteins could originate either from tumor cells or other cells in the tumor microenvironment, such as endothelial, inflammatory and stromal cells. That circulating GFAP levels are reported to be significantly increased in patients with glioma compared with healthy controls is a case in point, as GFAP

values were also elevated in several other conditions such as head trauma, intracerebral hemorrhage, brain infarction and multiple sclerosis [138-142].

Several circulating proteins have been evaluated, and include proteins associated with: (i) cell lineage such as GFAP [143-147], NCAM [148] and S100B [145,147,149], (ii) matricellular proteins and matrix metalloproteinases such as YKL-40 [150-153], MMP2 [154-159], MMP9 [151,154-156,159-161], TIMP-1 [78,157,158,162-164] and osteopontin (OPN) [164,165], and (iii) cytokines/growth factors and growth factors receptors such as VEGF [159,160,166-173], FGF-2 [157,158,162,171,174], PIGF [147], IGFBP-5 [175], epidermal growth factor receptor (EGFR) [176], VEGFR1 [161,162,171], and TGFβ-1 [177].

Further validation of these biomarkers is warranted, and several ongoing prospective studies are examining the clinical utility of candidate circulating proteins in patients with GBM.

Conclusion

Circulating biomarkers represent an exciting area of research that holds promise for better management of patients with GBM. They have the potential to expand our knowledge of GBM biology, to improve the diagnosis of this entity and to identify novel therapeutic targets, which should ultimately help improve the outcome of GBM patients. Circulating biomarkers can be studied from several types of biofluids such as blood, CSF and urine. Current research is at an exploratory stage and shows that the potential applications for circulating biomarkers encompass early diagnosis, molecular stratification, prognosis assessment, prediction of treatment response, and disease course monitoring. Several promising circulating biomarkers identified in

small studies require further evaluation and validation in larger-scale prospective studies before their implementation in routine clinical practice. Compared to tissue analysis which is the 'gold-standard', CTCs, tumor-derived circulating EVs and nucleic acids, and oncometabolites have the advantages of being tumor-specific and of providing genomic information that will help physicians to tailor treatment to individual molecular profiles along the disease course. The translation of circulating biomarkers into a routine clinical test is hampered by the relatively low sensitivity of some candidate biomarkers for disease confined to the brain. The advent of such a test is in the offing, since novel ultrasensitive methods for detecting circulating biomarkers are under development.

Expert commentary

Despite numerous attempts to improve the outcome of patients with GBM, overall survival remains stagnant. Translating progress achieved in our understanding of the biology of GBM into a clinical benefit is an unmet need, since recent clinical trials evaluating targeted therapies have failed to demonstrate efficacy in overall population of enrolled GBM patients. Several particularities of neuro-oncology may explain these failures. First, GBM has an extremely complex biology that thwarts our ability to: (i) identify and prioritize actionable alterations, (ii) understand intratumor heterogeneity and the dynamics of clonal and subclonal tumor architecture during disease progression, and (iii) prevent tumor adaptation and drug resistance. Second, we lack the noninvasive tools to more accurately monitor disease progression. Third, GBM is a unique challenge with regard to difficulties in drug delivery due to the BBB. Circulating biomarkers – especially tumor-specific biomarkers – may help clinicians overcome some of the above-mentioned challenges. From the viewpoint of

a neuro-oncologist, circulating biomarkers have the major advantage of providing real-time molecular information without recourse to brain surgery, particularly when tumor recurrence occurs.

Key issues in this field of research must be addressed in order to maximize the potential of circulating biomarker-based profiling for treatment selection and monitoring. Ensuring that the process and technologies implemented will exhibit greater sensitivity in patients with GBM is one of the most pressing issues. Specifically regarding antiangiogenic agents such as bevacizumab – one of the main agents used to treat progressive GBM –, studies are needed to better determine the impact of therapies "normalizing" the BBB on our ability to detect circulating biomarkers. As normalization of tumor vasculature may be associated with decreased levels of circulating biomarkers, some of these may not be adequate for monitoring patients treated with antiangiogenics. Finally, combining several approaches of noninvasive tumor profiling (*e.g.* concomitant analysis of tumor-derived RNA and DNA) may enhance our ability to characterize tumor genome at diagnosis and during disease progression, which may help to match patients to targeted therapies. The integration of data collected from noninvasive diagnostics tests with clinical and radiological evaluation will be undoubtedly pivotal in upcoming years to characterize specific tumor processes (*e.g.* discriminate between tumor progression and pseudo-progression) within the setting of clinical trials or daily routine medical management of patients.

The sensitivity, accuracy and prognostic and predictive value of the technologies remain to be evaluated, and larger-scale prospective studies are required before these novel methods can be integrated into routine clinical practice. Collaborative research efforts worldwide are needed to this end.

Five year view

Two scenarios can be expected. In the first scenario, with continued research, the clinical utility of more informative circulating biomarkers will be demonstrated, resulting in their use in routine clinical practice as liquid biopsies to: (i) perform non-invasive molecular stratification, (ii) improve patient treatment stratification based on genomic characterization, (iii) offer assistance for real-time disease monitoring, and (iv) identify changes in the tumor genomic architecture and mechanisms of drug resistance. Liquid biopsies – integrated with sequential analysis of advanced imaging –, will provide a noninvasive and relatively easy way to sample tumors in real-time. This will lead to earlier diagnosis of cancer or recurrence, and will ultimately improve the survival of GBM patients. Due to their lack of specificity and their limited value to inform on molecular profiles and tumor heterogeneity, it is less likely that circulating proteins will demonstrate clinical utility in the coming years.

In the second scenario, circulating biomarkers will fail to demonstrate clinical utility, due to their low sensitivity for diagnosis and inefficient prediction of disease progression or response to therapies. Tissue-based analysis will remain the standard strategy at diagnosis. Clinicians will need to obtain multiple tissue samples from patients during surgery, in order to maximize the chances of identifying the most relevant and/or actionable target among clonal and subclonal molecular alterations. Whenever possible, the same strategy will be repeated at relapse, in order to address resistance mechanisms and to adapt the therapeutic strategy. As multiple tumor sampling will not be feasible for all patients during the disease course, progress in noninvasive advanced imaging procedures [62-64] will contribute to therapeutic decision-making.

Key issues

- The past decade has seen remarkable strides forward in the genomic characterization of GBM. Several candidate oncogenic alterations have been identified for rational drug design.
- Accurate molecular diagnosis is a major issue in precision medicine to assess patient candidacy for a smart therapy targeting specifically molecular alterations in the tumor.
- Circulating biomarkers can be collected from blood, urine and CSF. Potential applications for circulating biomarkers encompass early diagnosis, molecular stratification, prognosis assessment, prediction of treatment response, and disease course monitoring.
- Circulating tumor cells (CTCs) can be isolated and characterized from a simple blood test and their molecular characterization can inform diagnosis, prognosis and therapeutic response.
- Cell-free circulating tumor DNA (ctDNA) represents a very promising biomarker with several potential applications from molecular diagnosis to disease monitoring.
- Several circulating proteins have been associated with diagnostic, prognostic and predictive values in GBM.
- Analytical and clinical validation of technologies is required if circulating biomarkers are to become routine tests in the clinic; collaborative research efforts worldwide will be decisive in achieving this objective.

Legend to figure

Figure 1. Development and validation of circulating biomarkers – from discovery to clinical utility. The initial discovery generally involves comparing samples from patients with cancer and healthy donors (or different subgroups of patients with cancer, depending on the clinical question) matched for known confounding factors such as age and performance status. Multiple types of platforms based on ”omics” technologies (*e.g.* genomic, metabolomic and proteomic platforms) can be used to identify candidate biomarkers. Analytical and clinical validation involves analyses performed in larger patient populations in order to: (i) ensure robust biomarker detection, and (ii) demonstrate that the test accurately and reliably informs on the clinical state of interest. The final step of development is when the clinical utility of the test for making decisions about patient management is established, followed by regulatory approval for its application in the clinic. This step requires controlled studies across multiple institutions to demonstrate the usefulness and added value of the test, compared with currently available testing. The current standard diagnosis and following procedures include tumor histological analysis, and clinical and imaging follow-up. No circulating biomarker has to date demonstrated its clinical

utility in GBM.

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Aknowledgements

The authors would like to thank Lorna Saint Ange for providing editing assistance and Sebastien Di Dio for providing graphical assistance.

Table 1. Summary of clinical studies evaluating circulating tumor cells in glioblastoma.

Methodology	Type of analysis	Sensitivity (%)	Observations	Reference
GFAP-based assay	Quantitative, molecular profiling	21	CTCs shared genomic alterations with the primary tumor.	[87]
Immunomagnetic and immunofluorescence-based cell selection	Quantitative, molecular profiling	39	CTCs analysis showed enrichment for the mesenchymal subtype. CTCs shared genomic alterations with the primary tumor.	[88]
Telomerase-based assay	Quantitative, longitudinal analysis	72	Detection rate was higher before treatment with radiation therapy. Evolution of CTCs levels correlated with treatment response in a small subset of patients with available longitudinal sampling.	[86]

Table 2. Summary of clinical studies evaluating cell-free circulating tumor DNA (ctDNA) in glioblastoma.

Candidate ctDNA	Methodology	Observations	Reference
EGFRvIII	PCR, RT-PCR	Good correlation between mutational status in tumor and blood.	[109,85]
Mutant *IDH1*	COLD-PCR	Moderate sensitivity (60%), excellent specificity (100%). The sensitivity increased with both tumor volume and contrast enhancement.	[105]
MGMT promoter methylation	Methylation-specific PCR	Moderate sensitivity (50-60%) and good specificity in most studies. Prognostic and predictive values of serum *MGMT* status.	[68,108,110,111,113,115,116]
PTEN promoter methylation	Methylation-specific PCR	Sensitivity of 50% and specificity of 100%.	[116]
RASSF1A promoter methylation	Methylation-specific PCR	Hypermethylation of *RASSF1A* differentiated primary from metastatic brain tumors.	[110,111,116]
CDKN2A promoter methylation	Methylation-specific PCR	High correlation between methylation status in tumor and serum.	[68,108,110,111,112,116]

1p, 10q and 19q LOH	PCR-based LOH analysis	Sensitivity of 50% and specificity of 100%.	[113]

Abbreviations: PCR, Polymerase Chain Reaction; RT-PCT, Real-Time Polymerase Chain Reaction; COLD-PCR, co-amplification at lower denaturation temperature-PCR; LOH, Loss of Heterozygosity;

Table 3. Summary of main clinical studies evaluating circulating proteins in glioblastoma.

Protein	Methodology	Potential values for clinical application	Reference
YKL-40	ELISA	Diagnostic, prognostic, and monitoring	[150-153]
MMP-9	ELISA	Diagnostic, prognostic, and monitoring	[151,154-156,159-161]
MMP-2	ELISA	Diagnostic, prognostic, predictive, and monitoring	[154-159]
TIMP-1	ELISA	Diagnostic	[78,157,158,162-164]
OPN	ELISA, mass spectrometry	Prognostic	[164,165]
VEGF	ELISA	Diagnostic, prognostic, and monitoring	[159,160,166-173]
PIGF	ELISA	Diagnostic, prognostic	[147]
FGF-2	ELISA	Diagnostic, prognostic	[157,158,162,171,174]
IGFBP-5	ELISA	Predictive	[175]
EGFR	ELISA	Diagnostic, prognostic	[176]
Soluble VEGFR-1	ELISA	Diagnostic	[161,162,171]
TGFβ-1	ELISA	Diagnostic	[177]

GFAP	ELISA	Diagnostic	[143-147]
S100B	ELISA	Diagnostic	[145,147,149]
2-hydroxyglutarate	Mass spectrometry	Diagnostic	[137]

Abbreviations: ELISA, enzyme-linked immunosorbent assay

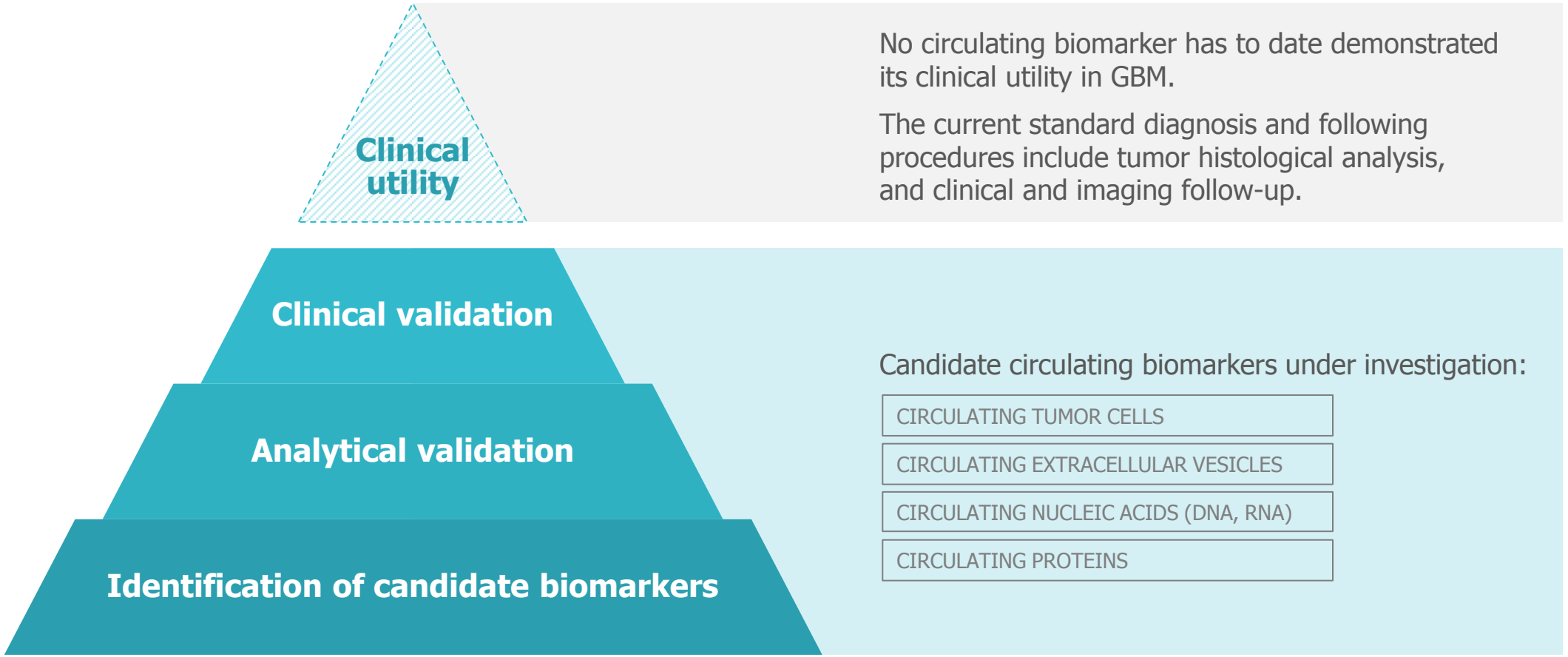
Clinical utility
No circulating biomarker has to date demonstrated its clinical utility in GBM.
The current standard diagnosis and following procedures include tumor histological analysis, and clinical and imaging follow-up.
Clinical validation
Analytical validation
Identification of candidate biomarkers
Candidate circulating biomarkers under investigation:
CIRCULATING TUMOR CELLS
CIRCULATING EXTRACELLULAR VESICLES
CIRCULATING NUCLEIC ACIDS (DNA, RNA)
CIRCULATING PROTEINS

Figure 1