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Blood-based biomarkers for malignant gliomas

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Abstract

Malignant gliomas remain incurable and present unique challenges to clinicians, radiologists and clinical and translational investigators. One of the major problems in treatment of these tumors is our limited ability to reliably assess tumor response or progression. The most frequently used neuro-imaging studies (contrast-enhanced MRI and CT) rely on changes of blood-brain barrier (BBB) integrity, providing only an indirect assessment of tumor burden. In addition, the BBB can be altered by commonly used interventions including radiation, glucocorticoids and VEGF inhibitors, further complicating the interpretation of scans. Newer radiologic techniques including PET and magnetic resonance spectroscopy (MRS) are theoretically promising but thus far have not meaningfully changed the assessment of patients with malignant gliomas. A tumor-specific, blood-based biomarker would be of immediate use to clinicians and investigators if sufficiently sensitive and specific. This review discusses the potential utility of such a biomarker, the general classes of tumor-derived blood-based biomarkers and it summarizes the currently available data on circulating tumor cells, circulating nucleic acids and circulating proteins in patients with malignant gliomas. It is unclear which marker or marker class appears to be the most promising for these tumors. This article provides thoughts on how novel candidate blood-based markers could be discovered and tested in a more comprehensive way and why these efforts should be among the top priorities in neuro-oncologic research in the coming years.

Keywords

Malignant glioma; glioblastoma; circulating tumor DNA; circulating tumor cells; circulating biomarker; blood-based biomarker; pseudo-progression

Introduction

Accurate assessment of disease burden is crucial for appropriate clinical decision making for cancer treatment. This is particularly challenging in patients with malignant gliomas, since standard imaging techniques, including CT, contrast MRI and MR spectroscopy rely on changes in blood-brain barrier (BBB) integrity and only indirectly reflect the extent of these tumors. Treatment-related changes, including surgery, radiation, steroids and antiangiogenic therapies can therefore complicate the interpretation of these imaging studies. Classic response criteria for solid tumors that rely on two- or three-dimensional measurements (such

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as the RECIST criteria) are not adequate for the measurement of malignant gliomas. Specific radiographic response criteria for assessing brain tumors have therefore been developed. The McDonald criteria integrated clinical status and steroid dose into the assessment parameters and were used in clinical trials for many years [1]. The Revised Assessment in Neuro-Oncology (RANO) criteria, a further development of the McDonald criteria, also include measurement of non-enhancing areas [2]. These criteria have not yet been formally validated. Novel imaging techniques including PET scans and MR-spectroscopy are being developed. These imaging techniques are limited by relatively poor spatial resolution and a lack of specificity; to date, PET scans and MR spectroscopy have added little to the radiographic assessment of patients with malignant gliomas.

Consequently, alternative ways to assess tumor burden and response to treatment are needed for patients with malignant gliomas. A circulating blood-based tumor marker could circumvent limitations of imaging and become an adjunct in clinical decision-making in the situations summarized below.

1) Assessment of tumor burden

Autopsy reports have shown that malignant gliomas are deeply infiltrative tumors without a clear border [3]. Even maximum surgical resection is unable to eliminate all tumor tissue and, unlike other solid tumors, ‘clear margin’ status cannot be achieved. The extent of resection appears to be a prognostic factor for survival, and radiation therapy fields are designed to encompass radiographically visible tumor after surgery. However, it is impossible to ascertain the actual extent of residual tumor due to our inability to differentiate contrast-enhancing postsurgical changes from tumor. Furthermore, microscopic tumor deposits may be present in areas of brain parenchyma that do not enhance and are undetectable with current imaging techniques. Assessment of tumor burden becomes even more difficult when patients undergo treatment with medications that affect BBB permeability, such as steroids and antiangiogenic agents [2].

2) Determination of treatment response or disease progression

In malignant gliomas, imaging cannot reliably distinguish treatment-related changes such as radiation necrosis from actual tumor growth. As a result, it is often impossible to make *ad hoc* decisions about whether a certain therapy is effective or whether the tumor has progressed through therapy. A well-described diagnostic dilemma is the phenomenon of so-called “pseudo-progression” after radiation of high-grade gliomas that can lead to premature discontinuation of adjuvant chemotherapy and in some cases to unnecessary repeat surgery [4]. The mirror image of this problem is the so-called “pseudo-response”, seen frequently when edema and BBB permeability decrease without actual reduction in tumor burden. This is commonly observed with steroid treatment and treatment with antiangiogenic therapies such as bevacizumab.

General principles of tumor-derived, blood-based biomarkers in cancer

The ‘perfect’ blood-based tumor marker

The ‘ideal’ blood-based biomarker for any given tumor is a simple blood test that gives precise information about disease status and tumor burden. An ideal marker would be 100% sensitive (i.e., even minimal disease can be detected), 100% specific (i.e., it only correlates with the respective tumor type and no other cancers or tissues), dynamic (i.e., able to perfectly and proportionally reflect tumor burden), and cost-effective. The most specific markers are identical to a unique structure of the tumor itself (e.g., circulating tumor cells or circulating tumor-specific DNA). Improved methods of detection can increase marker sensitivity. However, changes in cell turnover rates, the variable half-life of markers in

plasma, and changes in BBB integrity may significantly affect marker levels in peripheral blood.

Does the marker need to be perfect?

A clinical biomarker and its corresponding assay do not need to be 'perfect' in order to be clinically useful. This becomes evident when looking at blood-based biomarkers that are routinely used in other cancers. For example, PSA can be dramatically elevated in patients with bacterial prostatitis but still provides considerable information about tumor response and progression [5]. Similarly, CEA is not elevated in all patients with colorectal cancer and yet can be a helpful adjunct for clinical decision-making in patients with an abnormally elevated CEA (nccn.org). Similarly in malignant gliomas, a marker may still be useful if it is detectable in a subset of patients and if it reflects relative changes in tumor burden.

Summary of previous studies of blood-based tumor markers in malignant glioma

The three main classes of brain tumor-derived candidate markers that are presently being evaluated in malignant gliomas are circulating tumor cells, circulating nucleic acids (including analysis of both genetic and epigenetic alterations in DNA and RNA), and circulating proteins. Every biomarker class has its own advantages and challenges that are intrinsically related to underlying biology (Table 1). It is of note that also non-tumor-derived circulating markers are being explored to assess response to treatment in gliomas, including circulating endothelial cells and circulating hematopoietic progenitor cells [6, 7]. Given that tumor-derived biomarkers are the most likely to be beneficial for following disease burden, the following three sections will be focused on the currently available published data on glioma-derived circulating biomarkers.

Circulating tumor cells in malignant gliomas

Tumor cells that are shed from the primary tumor and circulate in the blood stream are intrinsically tumor-specific, as these cells are part of the tumor itself. The classic example of a circulating tumor cell is a leukemic blast cell. In solid tumors, has been successfully investigated in a variety of malignancies, including breast, lung and prostate cancer [5]. Flow-cytometry based systems are usually used to detect and quantify CTCs; however, assessments of circulating tumor cells are not yet part of the standard of care for any solid tumor.

Indirect evidence suggests that CTCs are present in patients with malignant gliomas. Although metastatic glioblastoma is rarely observed in clinical practice (0.4-0.5% of glioblastoma cases [8-10]) it can occur, with numerous cases of metastatic glioblastoma reported in the literature. This indicates that glioblastoma cells can disseminate via the blood stream. Between 1928 and 2009, at least 88 cases of extracranial metastasis have been described for glioblastoma and gliosarcoma [11, 12].

In addition, at least 17 instances of GBM transmission have been reported in patients who received organ transplants from donors with GBM, and it has been estimated that between 12.5 and 25% of donors with GBM might transmit the tumor [13, 14]. These cases provide direct evidence that GBM tumor cells were present in donated organs at the time of transplant surgery; these cells must have migrated out of the brain via the bloodstream. Unlike in other solid tumors, however, CTCs have not yet been successfully detected in patients with gliomas. However, as there is evidence that these cancers spread hematogenously, further research needs to be done to evaluate whether CTCs may be a clinically useful biomarker in malignant gliomas or not.

Circulating tumor-derived nucleic acids in malignant gliomas

Recently, there has been a significant increase in knowledge about the genetic and epigenetic changes associated with cancer. Somatic alterations in DNA, including mutations, deletions, and translocations, may be unique to a particular tumor and can function as a specific tumor marker as these alterations are not found in non-cancerous tissue. It is known that cancers shed DNA into the bloodstream, and circulating tumor DNA (ctDNA) has been demonstrated in a number of solid tumors, including colorectal cancer and breast cancer. ctDNA can be a highly sensitive and specific biomarker [15, 16]. Several highly sensitive detection methods can specifically detect even very small amounts of tumor DNA within a significantly higher amount of wild-type DNA in plasma. These include the digital detection methods of BEAMing and PARE [17, 18].

Several pilot studies have shown that circulating tumor DNA can be detected in the blood of patients with malignant gliomas. A recent study showed that mutated IDH-1 DNA can be detected in the plasma of patients with IDH1-positive gliomas, and that there appeared to be a relationship between higher rates of IDH-1 DNA detectability and blood-brain barrier disruption [19].

Epigenetic changes such as methylation can also be measured in ctDNA. Several reports have described the detection of circulating methylated DNA in patients with malignant gliomas. One study analyzed methylation of O6-methyl-guanine-DNA methyltransferase (MGMT), p16, DAPK and RASSF1A in serum and tumor of 28 patients with glioblastoma and showed sensitivity and specificity of over 75% for each of these methylated genes using a methylation-specific (MSP) PCR-based assay [20]. A similar but smaller study showed concordance between methylated tumor and plasma DNA in 6 of 9 (67%) patients who were tested for methylation of MGMT, p16, and p73 [21]. Similarly, another report showed a sensitivity of 75% of detecting methylated p16 in 12 patients in whom the promoter was methylated in tissue [22]. A more recent study on paired serum and tissue samples that analyzed a total of 70 gliomas (41 high-grade astrocytomas and oligodendroglial tumors of various grades) further supported the findings of the prior three studies; concordance of MGMT methylation was 83% in astrocytic and 72% in oligodendroglial tumors. The specificity of detection in this study was 100%. Notably, methylated tumor DNA could only be detected in a fraction of patients in each study due to variability among the different genetic alterations [23].

Another potential plasma-based biomarker is tumor-derived microRNA (miRNA). In a study of blood from 20 patients with GBM and 20 age-matched controls, 1158 miRNAs were tested. Two miRNAs were found to be significantly altered in GBM patients, miR-128 (upregulated) and miR-342-3p (downregulated) [24]. This was confirmed in a second cohort by real-time PCR. A longitudinal prospective cohort study of a group of Austrian adults has identified circulating micro-RNA-21 as a possible marker for GBM. The original intent of this study was to identify markers for Alzheimer's disease; however, the single patient in the cohort of 606 patients who developed GBM had elevated micro-RNA-21 levels 36 months before the diagnosis of GBM [25]. While these data are far from definitive, they do suggest that circulating micro-RNA is a potentially fruitful avenue for further investigation of serum GBM markers.

Tumor-derived nucleic acids (and other cellular molecules) can also be found in circulating microvesicles that are directly released from glioblastoma cells. Microvesicles can carry specific genetic information from the tumor into the periphery. In the first published pilot study, specific EGFRvIII could be detected in serum microvesicles from 7 out of 25 patients that were collected on the day of surgery (sensitivity 50%, specificity 87%, compared with EGFRvIII in tissue) [26]. Furthermore, it has been shown that in contrast to healthy controls,

microvesicle RNA from patients with glioblastoma has significantly down-regulated levels of RNAs that are coding for ribosome production [27].

To date, the available data on circulating nucleic acids in malignant gliomas are based on pilot studies that function as a proof of principle. Studies have yet to address if the detected markers are useful in detecting dynamic changes in tumor burden in patients with these cancers and to identify markers that would warrant further investigation.

Circulating proteins in malignant gliomas

Circulating proteins have been used as tumor markers in a variety of cancers. Prominent examples of protein-based tumor markers include PSA in prostate, CEA in colorectal, CA19-9 in pancreatic and CA125 in ovarian cancer. The major limitation of proteins as blood-based cancer biomarkers, in contrast to ctDNA and circulating tumor cells, is their lack of specificity. Previously investigated protein markers for malignant gliomas can be classified into three major groups: neuronal- or glial-specific markers, proangiogenic proteins, and immunomodulatory cytokines.

Many proteins that are possible biomarkers for glioma were initially identified as markers of traumatic or hypoxic brain injury. A prototype glial-specific marker is glial fibrillary acidic protein (GFAP). Serum levels of GFAP increase after stroke and traumatic brain injury and appear to also be increased in the blood of patients with high-grade gliomas [28-30]. A study of patients undergoing surgery for suspected glioma, however demonstrated that serum GFAP increases after resection regardless of tumor grade, suggesting that increased serum GFAP is a marker of brain injury and not a specific marker of tumor [30].

Because of recent interest in anti-angiogenic agents as investigational treatments for glioblastoma, many of the most thoroughly investigated circulating proteins in glioma have been pro-angiogenic proteins, notably vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), and basic fibroblast growth factor (b-FGF). Previous studies of this biomarker class focused on establishing these proteins as predictive markers of the response to anti-angiogenic agents rather than as markers of disease burden, and the markers are not tumor-specific.

VEGF is a moderately large protein with a molecular weight of 38.2 kDa, and its primary functions in normal physiology include the stimulation of endothelial cell growth, angiogenesis, and increasing capillary permeability. Overexpression of VEGF is a common feature in glioblastoma, and its ligand is the target of bevacizumab (Avastin). However, circulating levels of VEGF were analyzed in two prospective trials of the anti-angiogenic agent thalidomide, and serum VEGF did not appear to be associated with overall survival nor with disease recurrence in patients with glioblastoma [31, 32], suggesting that circulating VEGF is unlikely to be a valuable marker of tumor burden for GBM.

EGFR has been studied as a marker for GBM in a single prospective study [33]. The main purpose of the study was to investigate whether EGFR levels had any utility in differentiating patients with GBM from healthy controls. However, this study also tracked postoperative EGFR levels in patients with proven GBM. Patients with GBM had higher serum levels of EGFR compared with healthy controls. However, EGFR levels did not drop significantly postoperatively, suggesting that it is not a reliable marker of tumor burden.

B-FGF is another circulating marker for disease status and survival in GBM patients. Four published clinical trials testing anti-angiogenic agents in patients with GBM also evaluated the prognostic impact of circulating b-FGF levels. Two trials of the antiangiogenic agent cediranib have shown an association between increased serum b-FGF levels and an

increased risk of disease recurrence [34, 35]. Another prospective study of thalidomide in the recurrent setting suggested that increased b-FGF was correlated with decreased overall survival [36].

The third major category of serum proteins that have been tested as possible tumor markers in GBM are inflammatory markers. A single study evaluated TGF-B as a candidate tumor marker, but found no correlation with overall survival or with lesion size in 28 GBM patients [37]. Three studies have investigated matrix metalloproteinase-9 (MMP-9); two of these showed that increasing MMP-9 was associated with disease recurrence [38, 39]. However, like GFAP, MMP-9 increases following brain surgery, suggesting that increases in the serum level of this protein may in fact represent brain injury and disruption of the blood-brain barrier rather than being a true measure of tumor recurrence [40].

Another possible blood-based biomarker for gliomas is YKL-40 [39]. YKL-40 is an extracellular glycoprotein whose exact physiologic function remains unknown. It was first described as a circulating marker in osteosarcoma, and elevated levels of this protein have been described in patients with metastatic breast cancer as well as non-malignant inflammatory conditions such as sarcoidosis. YKL-40 also increased after surgical resection, but low YKL-40 levels were associated with improved overall survival. Similarly to MMP-9, YKL-40 levels may be associated with brain inflammation and breakdown of the blood-brain barrier, rather than be a true measure of tumor burden.

High-throughput methods of evaluating serially, prospectively collected samples may be a better way of identifying a reliable protein marker, although this approach also has methodological and statistical pitfalls related to the large number of queries applied to small groups of samples. A recent prospective study used multiplex protein analysis to identify five novel circulating protein markers for malignant glioma [41]. However, due to a small sample size and a large number of candidate markers tested, these results must be considered to be exploratory only and require prospective validation on a large scale.

The search for a tumor-specific, protein-based, circulating tumor marker for GBM has thus far been unsuccessful and is likely to be long and difficult. Most potential protein biomarkers that have been investigated to date are not specific. This is likely due at least in part to the major BBB disruptions and ongoing changes in BBB permeability that occur in patients with GBM. Proteomic analysis may be able to identify circulating proteins that are secreted in small quantities by malignant glioma cells. Proteins identified in this fashion may turn out to be more specific tumor markers for GBM than the neuronal/glial, pro-angiogenic, and inflammatory proteins that have been studied thus far.

DISCUSSION

Blood-based biomarkers for malignant glioma are needed for more accurate evaluation of treatment response and failure. However, biomarker research in primary brain tumors is challenging given the rarity and genetic diversity of these tumors. Identifying a tumor marker in these patients is further complicated by variations in the permeability of the BBB that affect the amount of marker released into the bloodstream. Discovery efforts for more tumor-specific and better detectable biomarkers are therefore needed. In addition, a systematic approach to the selection and development of promising candidate markers is needed. Cells and molecules derived from primary brain tumors can be detected in the blood of patients with these tumors, and these data provide proof of principle supporting the search for a circulating tumor specific marker in brain tumors. Existing studies of brain tumor biomarkers are highly variable in quality, research design, and statistical power. Currently,

there are no promising circulating blood-based tumor markers for malignant gliomas, and much work remains to be done to identify markers suitable for clinical practice.

Ideal tumor markers are molecular structures or cells that stem directly from the tumor. It is therefore logical to interrogate tumors for the presence or absence of unique markers. Surgical specimens have been analyzed to study the genetic, epigenetic and proteomic signatures of brain tumors. The analysis of surgical specimens alone cannot determine whether a candidate marker is tumor specific and if it is shed into the circulation and detectable outside the territory of the tumor itself.

Any tumor-derived marker is expected to have its highest concentrations within the tumor itself, followed by the surrounding extracellular fluid, followed by peripheral blood. In other words, if it is not present in the extracellular fluid surrounding the tumor, it should not be present in the peripheral blood. One potentially high-yield but challenging approach would therefore be to analyze the extracellular fluid of tumors in order to screen for candidate biomarkers. A potential technique to collect small quantities of extracellular fluid in this context is microdialysis. This is an FDA-approved technique for pharmacokinetic analysis of traumatic brain injury that has been used to measure drug concentrations in and around tumors via small dialysis catheters that are temporarily placed during surgery [42, 43]. The extracellular fluid could then be further used to screen for tumor-derived molecules. Following this, blood tests could be performed to determine whether the respective biomarker can be reliably detected in blood.

CSF analysis has been suggested as a more appropriate way to screen for biomarkers; marker concentration may be higher in CSF than in circulating blood as CSF is inside the BBB. Unfortunately, obtaining serial CSF specimens from patients with high-grade gliomas is not practical as patients are not likely to participate in undergoing serial lumbar punctures for exploratory tests.

Candidate biomarkers must overcome several hurdles in order to be acceptable for routine clinical use. The three-step development model outlined below suggests a pathway for biomarker development in patients with brain tumors.

1) Feasibility

Is the marker present in the tumor?

Can the marker be detected and quantified in the blood of patients with high-grade gliomas with sufficient sensitivity and specificity?

To answer this question, surgical samples could be analyzed to identify potential tumor-specific nucleic acids and/or proteins. Blood samples from patients with significant tumor burden (e.g., patients presenting with a new enhancing lesion in the brain or with recurrent disease) could be analyzed as a first step. If it is not reliably detectable in patients who are known to have large tumor burdens, the biomarker should not be explored further.

2) Dynamic changes

Does the candidate biomarker reflect changes in tumor burden over time?

To answer this question, markers need to be tested longitudinally in patients, during treatment, ideally within a prospective clinical data set with correlative clinical and imaging data. If the marker is unable to detect dynamic changes, it should not be explored further.

3) Validation

Does the candidate marker have the potential to be implemented into clinical practice? Markers that are found to be sufficiently prevalent and measurable in blood from patients with malignant gliomas and that show dynamic changes in relation to disease burden, need to be validated in prospective clinical trials to test their clinical potential to improve assessment of disease burden, response and progression in patients.

The results of this research have the potential to significantly impact research and clinical care in patients with these cancers. This effort will require significant commitment and long-term investment; however, we believe that the development of blood-based biomarkers in CNS cancers should be of high priority in brain cancer research.

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Table 1

Classes of candidate circulating biomarkers in malignant gliomas

	Circulating tumor marker class		
	Nucleic acids	**Tumor cells**	**Proteins**
Specificity	High	High	Variable; often not tumor specific
Sensitivity	Variable	No preliminary data published	High
Examples	Circulating tumor DNA	Proof of glioma CTC existence:	GFAP
	Mutations / Deletions	Cases of gliomas with distant metastases	VEGF
	Rearrangements	Organ transplant cases	EGFR
	Methylation		b-FGF
	RNA		YKL-40
	miRNA		MMP-9
	RNA from microvesicles		