Published in final edited form as:

Cancer Invest. 2014 October ; 32(8): 423–429. doi:10.3109/07357907.2014.933237.

Evaluation of Eight Plasma Proteins as Candidate Blood-Based Biomarkers for Malignant Gliomas

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Abstract

Eight brain-derived proteins were evaluated regarding their potential for further development as a blood-based biomarker for malignant gliomas. Plasma levels for glial fibrillary acidic protein, neurogranin, brain-derived neurotrophic factor, intracellular adhesion molecule 5, metallothionein-3, beta-synuclein, S100 and neuron specific enolase were tested in plasma of 23 patients with high-grade gliomas (WHO grade IV), 11 low-grade gliomas (WHO grade II), and 15 healthy subjects. Compared to the healthy controls, none of the proteins appeared to be specific for glioblastomas. However, the data are suggestive of higher protein levels in gliosarcomas ($n = 2$), which may deserve further exploration.

Keywords

Brain Tumors; Cancer Biomarkers; Circulating Biomarkers; Proteins; Glioma

INTRODUCTION

The assessment of disease progression in malignant gliomas is challenging as it relies on contrast enhanced MRI and CT, which detect changes in the blood-brain barrier (BBB) rather than directly measuring tumor size or burden. Such indirect measurements of the BBB are problematic since cancer treatments (e.g., surgery and radiation) can disrupt the BBB (1). Imaging cannot reliably distinguish between changes in BBB permeability caused by the

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DECLARATION OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content of writing of this paper.

tumor and cancer treatment, so using this modality to determine tumor progression can lead to potentially poor or inconclusive clinical decision-making. Several response criteria have been developed to better interpret these radiographs, such as the McDonald criteria and the RANO criteria, but they continue to suffer from the aforementioned radiographic limitations (2,3).

An alternative means of assessing tumor progression may take the form of blood-based tumor-derived biomarkers. Such markers might circumvent the inherent limitations to imaging and could possibly serve as an adjunct metric alongside imaging. Blood-based markers are an established component of detection and managing for other cancer types (i.e., PSA for prostate, CEA for colorectal, and AFP for hepatocellular carcinoma) (4–6). Several pilot studies have reported on the relationship of various circulating proteins to tumor progression with variable success, but these studies have been narrow and small in scale (7,8) and to date, no promising blood-based biomarker has been identified and developed for patients with malignant gliomas. We have chosen to study a panel of eight brain-related candidate proteins for their potential as blood-based biomarkers for glioblastomas (Table 1).

Candidate proteins were selected based on prior studies on brain injury, or BBB disruption that suggested that these markers are brain specific, and based on availability of the respective assays for detection in our laboratory (10, 24, 30, 46, 47, 55). The goal of this study was to determine whether there are differences in plasma concentrations of these markers between patients with malignant gliomas and those without and to assess whether our findings would warrant further investigation of these markers as potential biomarkers for gliomas.

MATERIALS AND METHODS

Study subjects

Preoperative plasma samples of 34 patients with gliomas were collected between 2008 and 2013 using an IRB approved protocol, with 23 high-grade tumor patients (WHO grade IV) and 11 low grade tumor patients (WHO grade II). Grade III tumor patients were not included in this study. All patients were treatment naïve at time of plasma collection with regards to chemotherapy, radiation, and surgery.

Plasma samples of 15 healthy subjects were used as controls. These were healthy adults without a history of brain tumor and without neurological symptoms who were presumed not to have a glioma.

Blood collection and processing

All samples were collected pre-operatively using EDTA-coated tubes. Samples collected prior to the day of surgery, i.e., definitively prior to induction of anesthesia, are reported as “preanesthesia” samples, and samples collected on the day of surgery are reported as “postanesthesia” samples, as they were collected from the arterial line after the start of anesthesia but before the first incision. All blood samples were obtained, processed and frozen in less than 60 min. Samples were brought to the laboratory within 30 min of the

blood draw, centrifuged at 814×g for 10 min, and then the supernatant aspirated and centrifuged at 18,000 g for 10 min. Plasma was stored as 1 mL aliquots at –80°C.

Plasma protein analysis

Plasma was distributed into 50 uL aliquots so that each assay used plasma that had been minimally rethawed. Plasma protein biomarker concentrations were determined using electrochemoluminescent immunoassays on a Sector 2400 plate imager (Mesocore Discovery, Gaithersburg, MD). Assay reagents were commercially available for BDNF (R&D Systems, Minneapolis, MN), GFAP (Covance, Princeton, ND and Dako, Carpinteria, CA), ICAM-5 (R&D), NSE (R&D), beta-synuclein (R&D), and S100B (Sigma, St. Louis, MO and Genway, San Diego, CA). Assay reagents for MT3 and neurogranin were developed at Johns Hopkins using bacterial expressed full length human recombinant proteins. Assays were conducted according to standard sandwich ELISA protocol according to the company's instructions. Plasma dilutions for assays were 1:1 for neurogranin, 1:9 for ICAM-5, beta-synuclein, and BDNF, and 1:19 for NSE. Plasma for GFAP, MT3, and S100B assays were undiluted. Experiments were conducted to determine optimal antibody and standard concentration. The assays produced standard curves with quantifiable linear ranges from 0.01 to 40 ng/mL for GFAP and BDNF, 0.055 ng/mL to 40 ng/mL for neurogranin, and 0.625 ng/mL to 40 ng/mL for ICAM-5, MT3, beta-synuclein, S100B, and NSE. The lower quantifiable limits for each protein were defined by the standard nearest to the blank (containing 1% BSA in PBS) with a concentration that also exceeded it. The lower quantifiable limit after applying their respective dilutions was 0.156 ng/mL for GFAP, 0.055 ng/mL for neurogranin, 0.34 ng/mL for BDNF, 1.25 ng/mL for ICAM-5, 2.36 ng/mL for MT3, 24.7 ng/mL for beta-synuclein, 0.635 ng/mL for S100B, and 49.2 ng/mL for NSE.

Definition of detectable and quantifiable levels

“Detectable” levels were defined as those which an ELISA can detect but which are within a range that could be attributed to non-specific binding. They are data points that fall below the lower limit of quantification established by the blank concentration (dotted line in Figure 1). “Quantifiable” levels were defined as those which an ELISA can quantify and measure with certainty above the levels of non-specific binding.

Imaging

The preoperative MRI scans were performed at Johns Hopkins Hospital. We collected information on presence or absence and degree of contrast enhancement of tumor (graded as “no contrast enhancement”/“contrast enhancement in less than 5% of tumor area”/“contrast enhancement in most areas of the tumor”) (56) and an area measurement of the contrast enhancing tumor (largest cross-sectional diameter in millimeters × largest perpendicular diameter in millimeters) (2, 56).

Statistics

The distribution of protein plasma levels were summarized using box plots. Comparisons of plasma protein levels between groups were made using Wilcoxon rank-sum test for two groups or Kruskal–Wallis test for three groups. Postanesthesia samples and gliosarcoma

patient samples were excluded from statistical tests when comparing among controls, and low-grade and high-grade glioma patients, and high-grade tumor patients. No samples were excluded for statistical tests comparing preanesthesia and postanesthesia samples, nor for statistical tests comparing glioblastoma and gliosarcoma samples. Since the majority of our samples were detectable, we tested for differences in absolute protein plasma values rather than dichotomously comparing protein levels as detectable or nondetectable. All p values are two-sided, and unless stated otherwise, significance is measured at $p < .05$. Statistical analysis was made using Stata (version 12.1, StataCorp).

RESULTS

We measured preoperative plasma concentrations of eight circulating proteins in 23 patients with WHO grade IV (21 glioblastomas, 2 gliosarcomas) tumors, 11 patients with WHO grade II (low grade) tumors, and 15 healthy subjects. Demographics of the glioma patients are shown in Table 2.

Plasma protein levels in glioma patients and in healthy controls

Detectable and quantifiable levels of each protein were found in patients with WHO grade IV and in WHO grade II gliomas, and also in healthy controls (Figure 1).

The interquartile ranges of protein levels show extensive overlap among WHO grade IV tumor patients, WHO grade II tumor patients, and healthy controls in all proteins studied. For most comparisons between healthy subjects and brain tumor patients, no statistical difference was observed. A statistical significance was detected only for plasma BDNF ($p = .0428$) and plasma ICAM-5 ($p = .0195$) when comparing glioblastoma patients with healthy controls; plasma BDNF levels were comparatively lower in glioblastoma patients and plasma ICAM-5 levels were elevated in patients with glioblastoma compared to controls.

Contrast enhancement on MRI was seen in all grade IV tumors, whereas grade II tumors were either nonenhancing or displayed only minimal and focal areas of contrast enhancement, as expected. The degree of BBB disruption as reflected by contrast enhancement on MRI therefore did not appear to influence the observed differences in plasma protein levels in this study.

Gliosarcoma versus glioblastoma

After considering diagnostic differences of protein levels within each tumor grade, we found elevated levels (outliers) of some of the proteins in the two gliosarcoma patients that were part of this analysis (Figure 1). The first gliosarcoma patient had elevated levels of BDNF and beta-synuclein (both by more than two standard deviations above the mean) as well as ICAM-5. The second gliosarcoma patient had elevated levels of neurogranin, ICAM-5, MT3, beta-synuclein, and S100B (all five by more than two standard deviations above the mean). Both gliosarcoma patients share elevations in plasma levels of ICAM-5 and beta-synuclein, both of which are significantly elevated compared to glioblastomas ($p = .0219$ for ICAM-5, $p = .0207$ for beta-synuclein).

Influence of anesthesia on plasma levels

In order to assess whether anesthesia may have influenced plasma protein levels in these patients, we compared samples collected in the days prior to surgery relative to those collected on the day of surgery. Samples from 6 of 11 WHO grade II tumors and 6 of 23 WHO grade IV tumors were collected preanesthesia. All samples, regardless of relation to initiation of anesthesia, were collected preincision. When comparing protein levels in collected “preanesthesia” and “postanesthesia” samples, we found several noticeable differences that suggest that anesthesia may have an influence on plasma levels of these proteins (see also Figure 1). For example, significant differences were detected between “preanesthesia” and “postanesthesia” samples for ICAM-5. The median “preanesthesia” sample ICAM-5 level was 29.13 ng/mL (standard deviation, 9.17 ng/mL), and the median “postanesthesia” sample ICAM-5 level was 20.67 ng/mL (standard deviation, 3.02 ng/mL) in WHO grade II patients ($p = .0446$), and for glioblastoma patients ($p = .0430$), these values were 33.33 ng/mL (standard deviation, 7.31 ng/mL) and 21.32 ng/mL (standard deviation, 9.26 ng/mL), respectively. In both cases, ICAM-5 was found in lower plasma concentrations following anesthesia. There appears to be a similar, albeit not statistically significant, effect of anesthesia on other protein levels, with some proteins increasing and others decreasing under the influence of anesthesia (see Figure 1).

DISCUSSION

In this exploratory analysis, we measured plasma concentrations of eight brain-related proteins to assess whether they might be suitable candidates as blood-based biomarkers for malignant gliomas. In summary, we made three observations:

First, we found that all eight proteins that were studied could be detected both in plasma of patients with glioblastoma, low-grade gliomas and in healthy controls, indicating that none of these circulating proteins is a specific plasma marker for these cancers. Significant disruption of the BBB as detected by contrast enhancement on MRI did not appear to correlate with concentrations of these proteins detected in plasma (Table 2), which would have been expected as the release of tumor-derived proteins into the plasma is assumed to increase with increased permeability of the BBB. The only protein that did show statistically significantly higher marker concentrations in plasma of glioma patients was ICAM-5, a protein that had not previously been studied in this context. Still, ICAM-5 was also detectable in all healthy controls, although at lower levels, indicating that it is not specific for tumor. Perfect specificity is not a necessary prerequisite for a clinically useful tumor biomarker (i.e., PSA is not specific for prostate cancer, etc.), but the degree of specificity for ICAM-5 and other proteins does not appear sufficient to recommend further pursuit of their biomarker potential for glioblastomas. Interestingly, levels of BDNF appeared to be even decreased in gliomas compared to normal controls. The reason for this is unclear, but this further underlines that also BDNF is not a promising plasma marker for glioma. In addition, plasma GFAP, which had previously been described as a potentially more promising blood-based biomarker for high-grade gliomas (9, 10, 55), was not found to be glioma-specific based on its detectability in healthy controls.

Our second observation was that there was a different pattern of plasma protein levels in gliosarcomas compared to glioblastomas. The number of gliosarcomas was small ($n = 2$), although some of the plasma proteins (ICAM-5 and beta-synuclein) were significantly elevated in gliosarcomas compared to glioblastomas (Figure 1). One of the gliosarcoma patients had substantially elevated plasma levels of BDNF, beta-synuclein and ICAM-5, while the other showed elevated levels of neurogranin, ICAM-5, MT3, beta-synuclein, and S100B. The small sample size may have prevented finding statistically significant differences between glioblastomas and gliosarcomas for more of the studied proteins that presented as outliers. Although the patterns of plasma protein elevation were different between the two gliosarcomas, this observation raises the question of whether the protein expression pattern and or protein shedding is indeed different between gliosarcomas and glioblastomas. A more dedicated analysis of circulating proteins in a larger cohort of gliosarcomas could help characterize this further.

Our third observation was that comparing patients whose blood was collected prior to induction of anesthesia to patients whose blood was drawn after the start of anesthesia shows an apparent influence of anesthesia on circulating protein levels. This indicates the need to pay careful attention to the timing of sample collection prior to surgery in future studies on circulating proteins as biomarkers of disease (57–59).

Our study had several limitations. These include the relatively small sample size and the retrospective nature of this study. In addition, we did not perform expression studies of the respective proteins in tumor, partly because tissue-based tests were not yet available. Moreover, the healthy control subjects did not undergo brain imaging to exclude that they could have had a brain tumor at time of sample collection. It is of note, however, that routine screening for brain cancers is not recommended based on the rarity of these tumors and that it is highly unlikely that the healthy subjects, who were not symptomatic, had undiagnosed brain cancer at time of sample collection.

CONCLUSION

In summary, these eight brain-related plasma proteins were not found to be tumor-specific markers for gliomas. Gliosarcomas appear to be associated with substantially higher levels of some of the studied proteins, which deserve further evaluation. There appears to be an effect of anesthesia on plasma protein levels, which should be an important consideration for future studies involving similar methods.

Acknowledgments

This study was supported by the Robert H. Gross Memorial Fund, and Retired Professional Fire Fighters Cancer Fund, Inc. (RPFCCF). Support also came from P30CA006973 (Sidney Kimmel Comprehensive Cancer Center core grant).

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Figure 1.

Box-and-whisker plots of the eight candidate protein biomarkers. Summary of the pre-surgical plasma concentrations of each biomarker in healthy subjects ($n = 15$), grade II ($n = 11$) and grade IV ($n = 23$). For glioma samples: Data are subcategorized into samples collected after anesthesia (blue diamonds), before anesthesia (red squares), and representing gliosarcomas (green triangles). Dotted lines represent the lower limit of quantification. The absence of a line indicates all data as quantifiable. Extreme outliers are listed above the graph beside the number representing its quantified level in the plasma (ng/mL). An asterisk (*) means the sample was too high for quantification.

Table 1
Biomarker Characteristics. Molecular weight, function, expression, and specificity of the eight protein biomarkers analyzed in this study

Protein	MW (kDa)	Function	Physiological expression	Brain specific?	References
GFAP	50	A primary intermediate filament protein, providing mechanical strength and cell shape	Astrocytes	Yes	(9–14)
NRGN	15 – 19	Post-synaptic protein regulating homeostasis of intracellular calcium	Neurons of hippocampus, cerebral cortex, amygdala, and caudate-putamen	Yes	(15–18)
BDNF	14	Supports survival, growth, and differentiation of neurons	Cerebral cortex, hippocampus, and basal forebrain; retina, kidneys, and saliva	No	(19–26)
ICAM-5	115	Transmembrane protein that attenuates inflammation and immune response; development and maturation of neuronal synapses	Telencephalon	Yes	(27–31)
MT-3	6–7	Inhibits growth and survival of neurons; scavenges free radicals; metal ion homeostasis	Cerebral cortex, hippocampus, and throughout CNS; human kidney, various forms of cancer	No	(32–38)
SNCB	14	Poorly understood; putative roles in synaptic regulation, vesicle traffic, plasticity, and neurotransmitter release	Neocortex, hippocampus, striatum, thalamus, cerebellum, brain stem; skeletal muscle (?)	TBD	(39–43)
S100B	22	Stimulates cell proliferation, inhibits apoptosis and differentiation; growth and development; cell shape maintenance, transcription, protein degradation, calcium homeostasis, energy metabolism, and enzyme functions	Astrocytes, neurons, Schwann cells, melanocytes, chondrocytes, adipocytes, skeletal myofibers, certain dendritic cells	No	(44–51)
NSE	78	Cytoplasmatic glycolytic enzyme	Neuron cytoplasm, smooth muscle, adipocytes, platelets, erythrocytes	No	(52–54)

Table 2

Patient Demographics

	WHO Grade II (<i>N</i> = 11) ¹	WHO Grade IV (<i>N</i> = 23) ²
Age: Median (range)	27 (4–65)	62 (16–83)
Sex:% Male	36%	57%
Measurable tumor contrast enhancement (%)	22% ³	100%

¹ Astrocytoma (9/11), Oligodendroglioma (1/11), Mixed (1/11).

² Glioblastoma (21/23), Gliosarcoma (2/23).

³ Pilocytic features in 1/9 (11%) of WHO Grade II gliomas.