RESEARCH ARTICLE

MMP-2 AND NGAL AS BIOMARKERS IN GLIOBLASTOMA: A PILOT STUDY

Authors

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Abstract

Pseudoprogression and radiation necrosis prevent confident interpretation of magnetic resonance imaging (MRI) results and lead to challenges in the management of glioblastoma (GBM). We examined the utility of matrix metalloproteinase-2 (MMP-2) and neutrophil gelatinase-associated lipocalin (NGAL) in the serum and urine as biomarkers of tumor burden in patients with GBM, with the goal of improving the interpretation of MRI and predicting overall survival (OS) and progression free survival (PFS). Expression of MMP-2 and NGAL was analyzed by immunohistochemistry in GBM and non-tumor epileptic control tissues. Serum and urine samples were collected pre and postoperatively and at each MRI. MMP-2 and NGAL levels in the serum and urine were measured by enzyme-linked immunosorbent assay. MRI results, tumor volumes, survival, symptoms, and quality of life were assessed using repeated measures and autoregressive models correlation structure. Although the staining intensity of NGAL was indistinguishable, GBM tissues exhibited significantly higher number of NGAL positive cells as compared to control epileptic brain tissue. Serum MMP-2 was significantly higher in GBM patients than control subjects ($p=0.0112$) and was highest in patients who underwent biopsy compared to maximal resection ($p=0.0038$). Elevated levels of both NGAL and MMP-2 in the preoperative samples indicated a trend towards shorter PFS and OS, while preoperative urine NGAL levels were marginally predictive of PFS. Trajectory of symptoms and quality of life results were too variable to adequately correlate with the biomarker levels. Although biomarkers did not aid in differentiating between pseudoprogression, radiation necrosis, and tumor growth, preoperative levels correlated with survival.

Keywords: Glioblastoma, biomarkers, NGAL, MMP, pseudoprogression, radiation necrosis

Abbreviations: GBM, MMP, NGAL, OS, PFS

1. Introduction

Gliomas are the most frequently occurring primary brain tumor in adults, and are graded by the World Health Organization (WHO). As a WHO grade IV glioma, glioblastoma (GBM) represents the majority of malignant brain tumors in adults at 46.1%. The estimated number of new GBM in the United States for 2019 is 13,000 [1]. Therapy is not curative, and tumors recur despite resection and treatment. In a recent study of 750 patients who underwent standard therapy, the median overall survival was 17.5 months [2].

Magnetic resonance imaging (MRI) is most frequently utilized to follow GBM progression. As the standard of care for newly diagnosed GBM is radiation given concurrently with chemotherapy (CRT) in the form of temozolomide (TMZ) followed by TMZ maintenance therapy, therapy-related effects often complicate the interpretation of MRI. In such patients, pseudoprogression (PsP) is frequently seen in the first few months post CRT and treatment-related necrosis peaks six to nine months after completing CRT. PsP describes the presence of increased contrast enhancement in the absence of true tumor growth. Brandes and colleagues determined that the majority of PsP occurs within 90 days of CRT completion, and occurs in approximately 30% of patients, however, up to 50% of patients have increased contrast enhancement at their first post-CRT MRI. Additionally, they found a 91.3% probability of PsP in patients whose tumors had methylated O6-methylguanine–DNA methyltransferase (MGMT) promoter regions and a 59% probability of early progressive disease in unmethylated tumors [3]. Despite this correlation of PsP with methylation status, a more universally sensitive and specific marker for PsP is needed.

Radiation necrosis is desribed as acute cellular injury in both disease-free and tumor tissue exposed to high dose radiation leading to cerebral vascular injury from occlusion of microvessels. Endothelial cell injury also occurs, resulting in further vessel damage, leakage, and brain edema [4]. As the resulting edema and inflammation occurs at the targeted site of the tumor and leads to the efflux of contrast, radiation necrosis can also mimic tumor recurrence. Positron emission tomography (PET), MRI with perfusion and MR spectroscopy have all been studied to differentiate treatment effect from tumor progression, but these imaging modalities are costly and results often equivocal [5]. The limitations of the current approaches to accurately discriminate between recurrent disease, PsP and radiation necrosis mandate the need for a biomarker that parallels true disease burden.

Not only is there a need to more accurately assess tumor burden, there is an equally important need to objectively predict functional decline and worsened quality of life. The Glioma Outcome study included data from 565 persons with malignant gliomas, and found the most common symptoms at diagnosis were headaches, memory loss, neurocognitive changes, motor issues, language disturbances, seizures, and personality changes. In addition, symptoms at diagnosis can improve, worsen, or progress over time [6, 7]. Quality of life (QOL) is another important endpoint during treatment for gliomas. Bergo et al reviewed literature related to health-related quality of life in persons with gliomas, and described a number of factors that are important aspects of quality of life [8] . These

factors include the tumor, treatment (surgery, radiation, chemotherapy, and other drugs such as corticosteroids and antiepileptics), and mood disorders (such as anxiety, depression, and uncertainty about the future). In addition, QOL is affected by gender (women report lower QOL), tumor location (left-sided tumors are associated with more depression), memory problems, and verbal fluency and communication problems. Functional inventories and quality of life assessments are time consuming for patients and are not routinely done in most clinics. Correlation of function and quality of life with objective biomarkers would save time, and better stimulate the medical team to refer to therapy, social work, and palliative care.

Specific biomarkers have been studied with the hope of better estimating tumor burden and evaluating/predicting response to treatment, including MGMT and isocitrate dehydrogenase (IDH) status [9, 10]. Matrix metalloproteinases-2 and -9 (MMP-2 and MMP-9), and neutrophil gelatinase-associated lipocalin (NGAL) are overexpressed in orthotopic models and also in human primary brain tumor specimens, demonstrating their ability to be used as possible biomarkers for GBM [11-20]. NGAL has also been shown to have metastatic correlation in other types of cancers, including breast and colon cancer[21]. MMPs, specifically, have been implicated in tumor progression and metastasis in human brain tumors, especially the primary angiogenesis process. Smith and colleagues found that MMP-2, MMP-9 and NGAL complex were all elevated in samples from patients with different types of brain tumors vs healthy controls. Furthermore, they also demonstrated that the presence of these biomarkers correlated with tumor presence, decreased with therapy, and normalized in those rendered disease-free [16]. We hypothesized that MMP-2 and NGAL would correlate with tumor burden specifically in patients with GBM, allowing improved MRI interpretation and prediction of survival. We further hypothesized that changes in these biomarkers would therefore differentiate true tumor progression from both PsP and radiation necrosis. Finally, we hypothesized a negative correlation between biomarker levels with symptoms, QOL, and functional status.

2. Patients and methods

The UNMC Institutional Review Board approved the study. Patients aged 19 years or greater with suspected GBM on imaging were enrolled prior to tumor resection and continued on study if histologically confirmed. Exclusion criteria included pregnancy, HIV, active hepatitis and any contraindication for contrasted MRI. Epilepsy patients undergoing focal resection were also consented and samples from them were utilized as non-tumor tissue controls. All patients provided serum and urine samples preoperatively and 24 and 48 hours postoperatively, and those with GBM provided longitudinal samples at each MRI associated visit throughout therapy. MRIs were obtained every eight weeks or sooner if clinically indicated, and postoperatively for any patient undergoing repeat surgery. The European Organization for Research and Treatment of Cancer (EORTC) QLQ-C30v3 and the M.D. Anderson Symptom Inventory-Brain Tumors (MDASI-BT) were administered preoperatively and within 48 hours of each MRI visit to GBM patients [22,23]. All MRIs were performed with and without contrast, with T2 fluid attenuated inversion recovery sequences, diffusion weighted imaging, and perfusion as needed.

Thirteen GBM and 10 epilepsy patients were enrolled in this pilot study. ELISA was used to determine MMP-2 and NGAL levels using kits from R&D Systems according to the manufacturer's instructions. NGAL expression in brain tissue was determined by immunohistochemistry (IHC). Dr. Gould provided rabbit polyclonal anti-NGAL antibody [24]. All tissue samples were reviewed and histologically confirmed by author R.M. The intensity of the immunoreactivity of NGAL and MMP-2 was scored on a scale of 0 to 3+ with the latter representing strong immunoreactivity. Disease status was determined by MRI, confirmed by M.W., assessed by RANO criteria, and classified as stable disease, partial response, complete response, and progression. Precise tumor volume was measured by M.W. and Y.Z. via *Olea Sphere 2.3*, who were blinded to biomarker levels.

2.1 Statistical analysis

Patient characteristics were descriptively summarized using medians and ranges for continuous variables and frequencies and percentages for categorical variables. Overall survival time was defined as time from first surgery to death or last contact. PFS was defined as time from first surgery to first progression, death or last contact. Survival distributions were estimated using the Kaplan-Meier method and distributions compared between preoperative biomarker levels using log-rank tests. Preoperative biomarker levels were compared between GBM patients and controls with Wilcoxon rank sum test. Mixed effects repeated measures models were used to look at changes in biomarker levels from days from surgery as well as changes in the QOL and symptom measures over time. An autoregressive or compound symmetry correlation structure was chosen based on Akaike information criterion (AIC) values and log transformations of biomarkers and outcomes were utilized as necessary. Model fit was assessed using residual plots and non-constant variance was often seen. Similar models were used to look at tumor volume and the association with biomarkers. Mixed effects models were used to look at the association between change in biomarker from the preceding time point and the MRI result. With these models, we compared the mean change between equivocal results later found to favor PsP or radiation necrosis (EQ1) and those later found to have true progression (EQ2). P-values less than 0.05 were considered statistically significant; all tests were two-sided. Statistical analyses were carried out using SAS 9.3 (SAS Institute Inc., Cary, NC).

3. Results

3.1 Patient characteristics

A total of 13 GBM patients were enrolled from March 2012 to July 2014 (**Table 1**)**.** The median age at diagnosis was 60 (range 44-85 years). A total of 10 non-tumor control patients were enrolled in this study, with a median age of 40 (range 22-58 years). All GBM patients initially received standard radiation therapy and TMZ. Of 13 patients, eight have died and five were alive at last contact. The median follow-up for those still alive at last contact was 1.5 years and ranged from 0.9 to 3.1 years. There were 11 progressions, seven of whom subsequently died. One patient died prior to progression. Median progression-free survival was 0.47 years and median overall survival was 1.89 years (**Table 1**).

Table 1

Characteristics	GBM Patients (*n*=13)	Controls (*n*=10)
Male	53.8%	50%
Median Age at surgery (years)	59	46
Extent of Resection		
Biopsy	23.1%	N/A
Subtotal Resection	15.4%	N/A
Near Total Resection	23.1%	N/A
Gross Total Resection	38.5%	N/A
Progression Free Survival (years) (Median survival)	0.47	N/A
Overall Survival (years) (Median survival)	1.89	N/A

Table 1: Patient characteristics

3.2 Tissue testing

To determine the expression and to investigate the clinical significance of NGAL and MMP-2 in human GBM, we first performed IHC on formalin fixed paraffin embedded tissue samples from 13 GBM and 10 epilepsy control cases. In human pancreatic cancer specimen positive staining for NGAL was seen in the neoplastic ducts, and thus was used as a positive control. (**Figure 1**). Based on a comparison of the density of positively staining cells versus non-staining cells, expression of NGAL was higher in GBM tissue compared to non-tumor brain tissue from epileptic patients, and an increase recruitment of inflammatory cells was observed in the GBM cases; however no difference in staining intensity was observed between GBM tissue and epileptic patients. The average number of NGAL positive cells per field in GBM and epilepsy control tissues were 14.32 and 4.1, respectively.

Figure 1

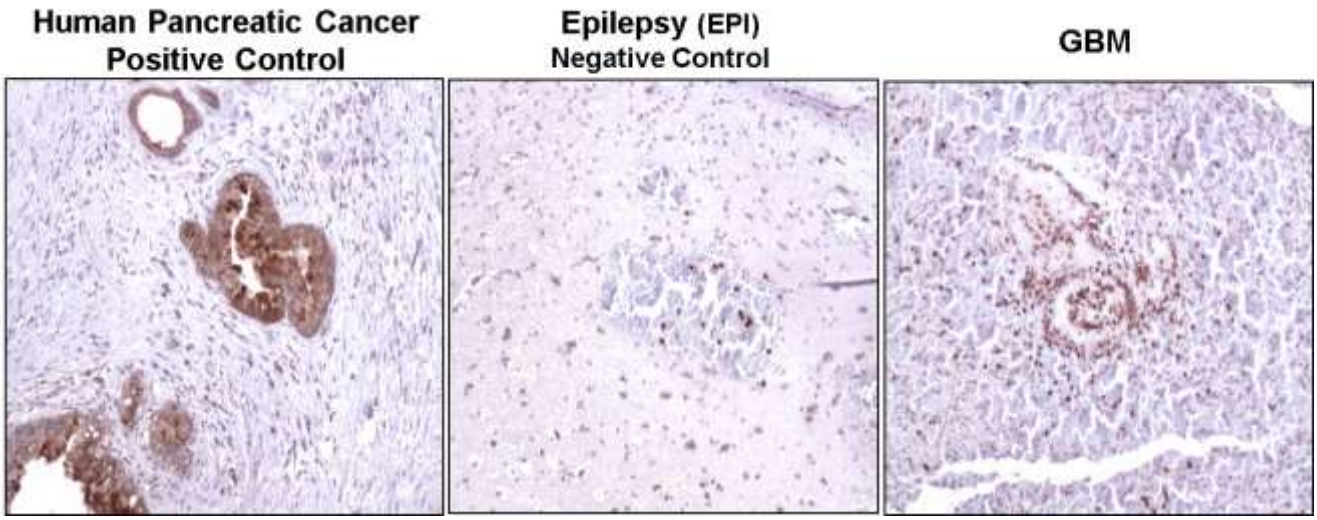

Figure 1: A representative IHC analysis of NGAL in formalin fixed, paraffin-embedded tissues. The sections were stained with polyclonal anti-NGAL antibody using the horseradish perioxidase detection method with DAB substrate. Brown-coloured product indicates positive immunoreactivity. Human pancreatic cancer tissue was used as a positive control and strong ductal expression is seen. For GBM section strong staining intensity was seen as compared to epilepsy control tissue. All sections were counterstained with haematoxylin. Magnification 20x.

3.3 Serum and urine biomarkers

There were 114 samples of both serum and urine from patients with GBM and 30 samples of both serum and urine from non-tumor control patients. The samples per patient varied based on the patient's disease progression. Each non-tumor control patient had three urine and serum samples, and each GBM patient had a minimum of three urine and serum samples as well. At a minimum, samples for both groups were taken preoperatively, 24-hours postoperatively, and 48-hours postoperatively. GBM patients had samples taken at each MRI follow-up.

NGAL levels ng/mL were not associated with extent of resection (EOR), but there was a significant association between serum MMP-2 and EOR; those only biopsied expressed (p=0.0038) (**Table 2)**.

Furthermore, preoperative MMP-2 levels were significantly higher in GBM patients than in epileptic patients (p=0.0112) (**Table 3)**.

Table 2

Outcome	Effect	Estimate	SE	DF	t Value	p-value
ln(Elisa NGAL blood)	Intercept	3.8228	0.13	11.00	29.390	<.0001
	Biopsy vs. Other EOR	0.2527	0.29	11.00	0.860	0.408
ln(NGAL urine)	Intercept	2.1917	0.33	11.00	6.720	<.0001
	Biopsy vs. Other EOR	-0.5751	0.73	11.00	-0.790	0.4458
ln(MMP2)	Intercept	-2.4716	0.12	11.00	-20.240	<.0001
	Biopsy vs. Other EOR	1.0809	0.30	11.00	3.650	0.0038

Table 2: Correlation of NGAL in blood and urine and serum MMP2 with EOR. ln=natural logarithm scale. Negative values represent any NGAL value of <1.0.

Table 3

Type	N Obs	N	Minimum	25th Pctl	Median	75th Pctl	Maximum	p-value
GBM	13	13	0.0452	0.0752	0.1255	0.2018	0.2834	
C	10	9	0.0153	0.0393	0.0654	0.0762	0.0856	0.0112

Table 3: Preoperative MMP2 (ng/mL)levels were higher in GBM vs Control (C) patients.

The higher each biomarker level, the shorter the PFS and OS overall. Prior to surgery, *urine* NGAL levels greater than 0.6ng/mL were weakly associated with shortened PFS (p=0.082 and OS (p=0.11) (**Figure 2a and 2b**);

Figure 2a

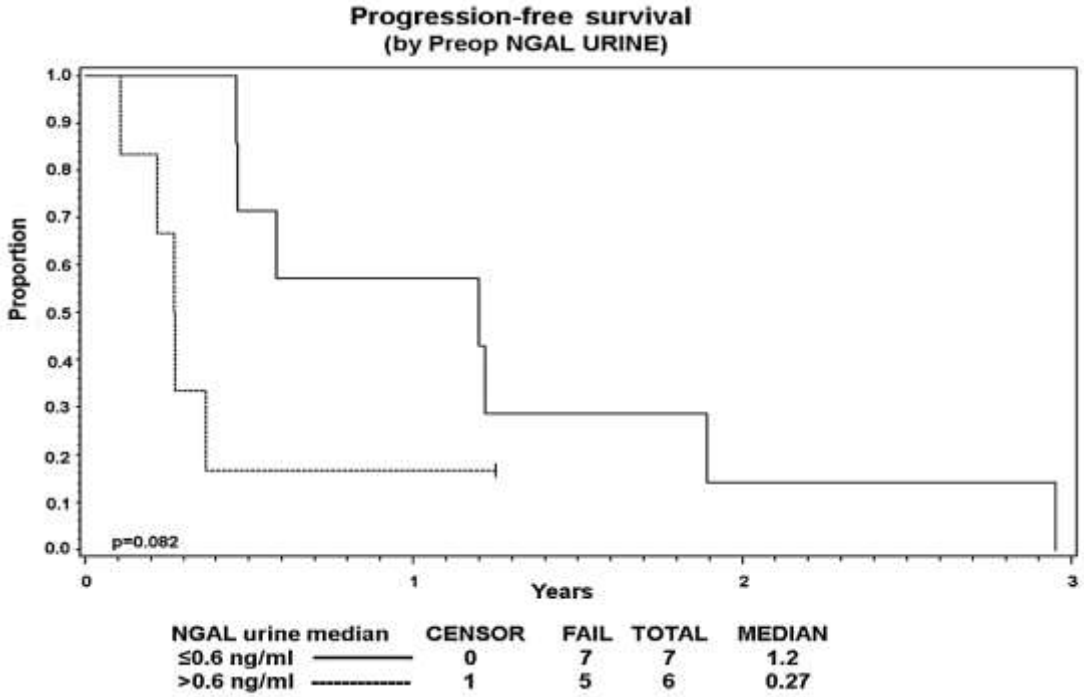

Figure 2a: Kaplan Meier curve showing PFS according to NGAL level in urine prior to surgery in GBM patients

Figure 2b

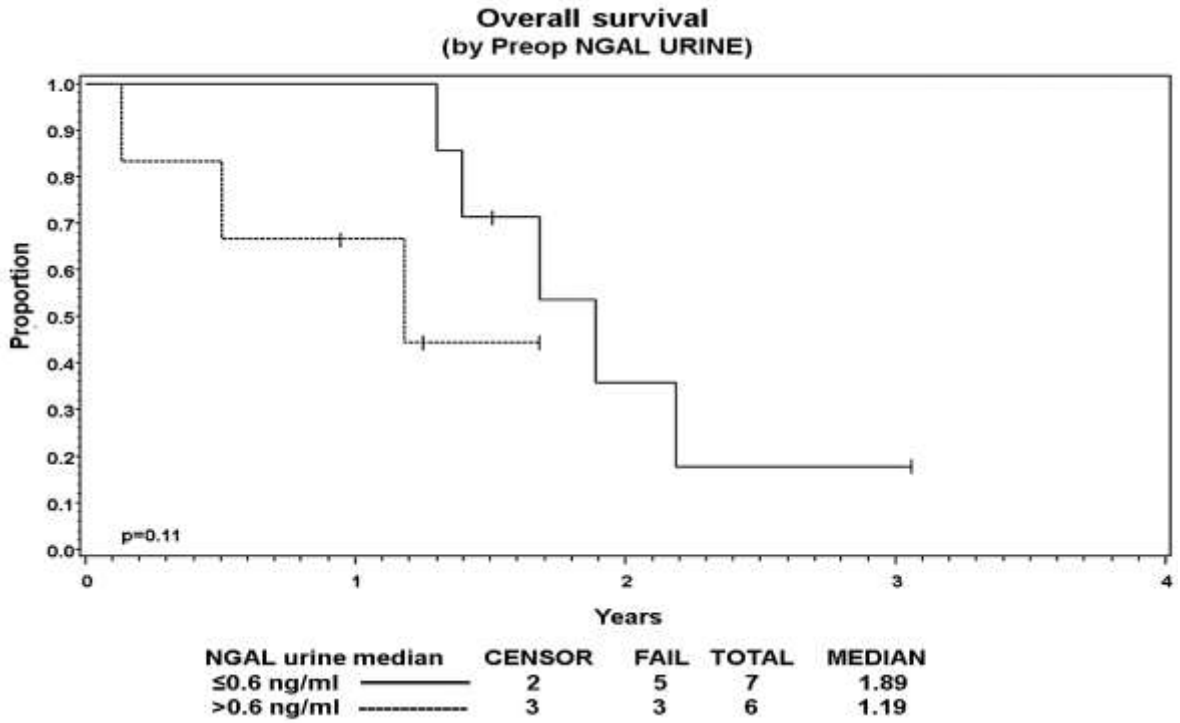

Figure 2b: Kaplan Meier curve showing OS according to NGAL level in urine prior to surgery in GBM patients

those with *serum* NGAL less than or equal to 80 ng/mL trended toward longer PFS (p=0.38) and lived longer at 2.7 years compared with 1.4 years OS (p=0.10) (**Figure 3a and 3b**).

Figure 3a

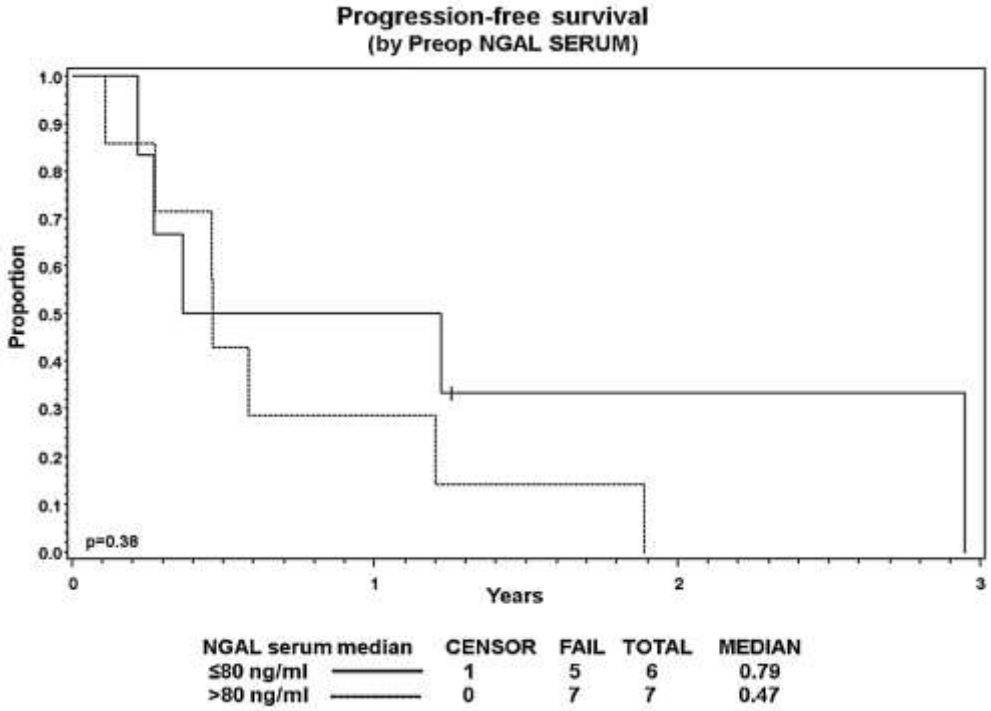

Figure 3a: Kaplan Meier curve showing PFS according to NGAL level in serum prior to surgery in GBM patients

Figure 3b

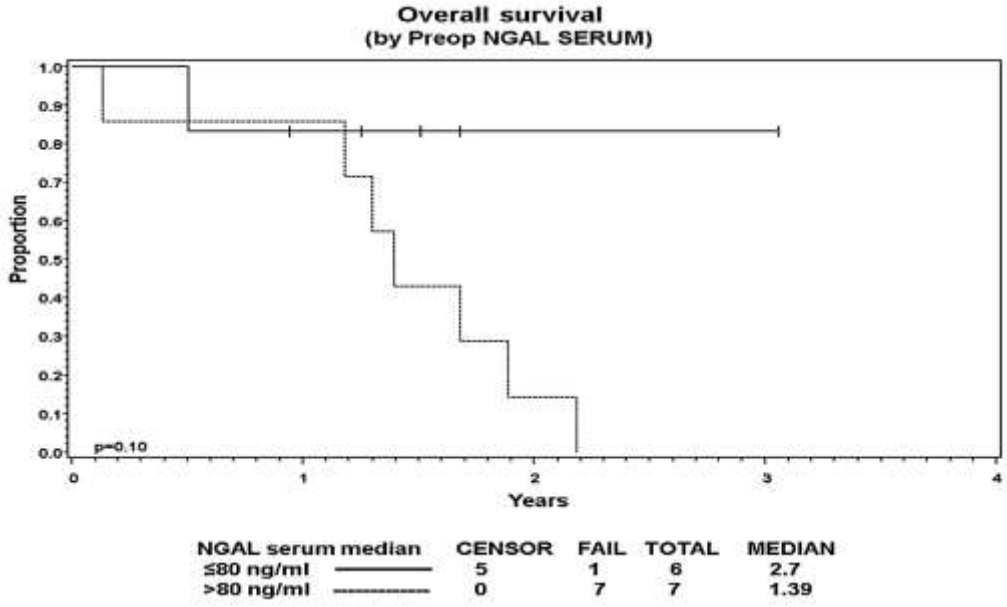

Figure 3b: Kaplan Meier curve showing OS according to NGAL level in serum prior to surgery in GBM patients

While initial levels of MMP-2 above 0.13 ng/mL led to shorter PFS and OS, this was not significant (p=0.81 and 0.45 respectively) (**Figures 4a and 4b**).

Figure 4a

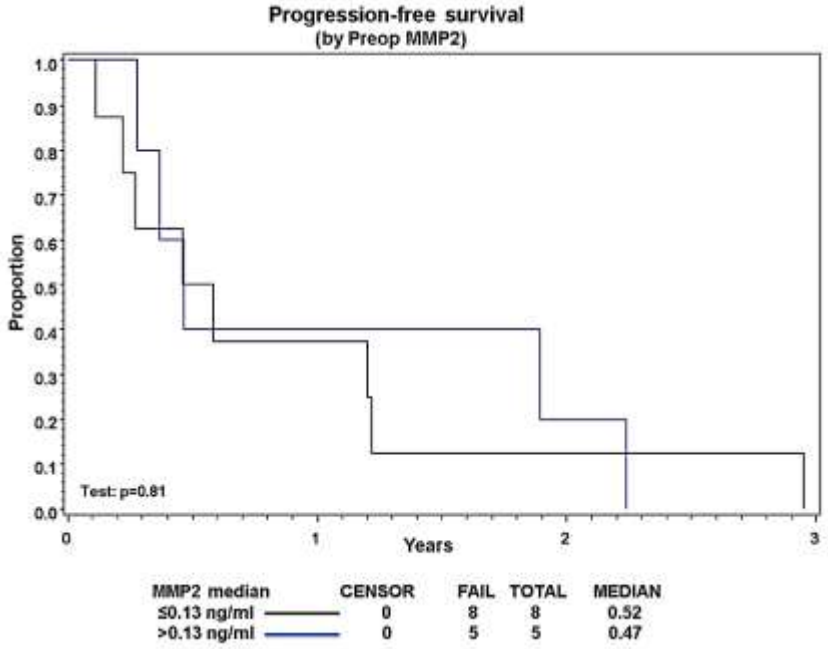

Figure 4a: Kaplan Meier curve showing PFS according to MMP2 expression (ng/mL) in serum prior to surgery in GBM patients. Those patients with MMP2 > 0.13 ng/mL trended toward decreased survival.

Figure 4b

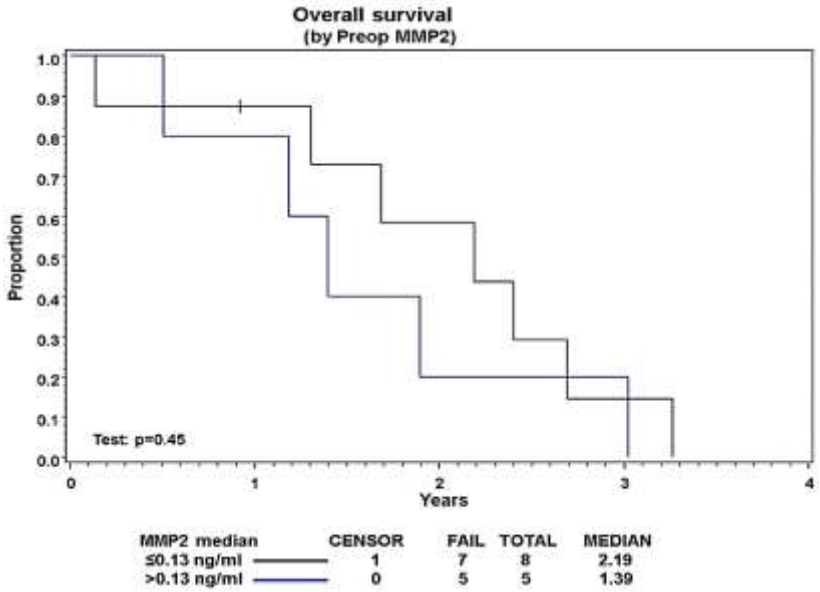

Figure 4b: Kaplan Meier curve showing OS according to MMP2 level (ng/mL) in serum prior to surgery in GBM patients. Those patients with MMP2 > 0.13 ng/mL trended toward decreased survival.

With increasing tumor volume, serum and urine NGAL, and serum MMP-2 increased, although this was not statistically significant (p=0.12, p=0.28, and p=0.82). Despite this trend for biomarkers to increase with tumor growth, all biomarker decreased over time. Serum NGAL levels were significantly decreased with disease progression (p=0.0040). There was no association between biomarker change and tumor response as defined by MRI report. Changes in biomarkers did not differentiate PsP or treatment-related necrosis from true tumor progression (data not shown).

Bevacizumab use positively correlated with greater MRI volume (p=0.012), and higher levels of urine NGAL (p=0.049) (**Table 4**).

Table 4

Outcome	Effect	Estimate	SE	DF	t Value	p-value
ln(MRI volume)	Intercept	1.4509	0.3523	12	4.12	
	Avastin vs. No Avastin	0.9809	0.3833	93	2.56	0.012
ln(Elisa NGAL blood)	Intercept	3.912	0.1268	12	30.85	
	Avastin vs. No Avastin	-0.1618	0.2152	83	-0.75	0.45
ln(NGAL urine)	Intercept	1.8793	0.2993	12	6.28	
	Avastin vs. No Avastin	0.8783	0.4404	83	1.99	0.049
ln(MMP2)	Intercept	-2.3101	0.1542	12	-14.99	
	Avastin vs. No Avastin	0.129	0.2731	87	0.47	0.64

Table 4: Correlation of bevacizumab use with MRI volume (cm^3), urine NGAL and MMP2. Bevacizumab positively correlated with increasing MRI volume and urine NGAL level. ln=natural logarithm scale. Negative values represent any value of <1.0.

Dexamethasone use also positively correlated with greater MRI volume (p=0.0005), but was not associated with urine NGAL (p=0.082) (data not shown).

3.4 MDASI and QOL Inventory

The MDASI-BT and EORTC QLQ-C30 were measured to assess functional status and QOL, respectively, at pre-op and at follow-up visits. Neither the MDASI-BT nor the QOL measures changed significantly over time and none were associated with MRI volume, NGAL or MMP-2. Although both of these tools measured dyspnea and appetite loss, only the EORTC QLQ-C30 showed changes in these over time (p=0.051 and p=0.088 respectively). Appetite loss worsened after the preoperative visit and only marginally changed over time, whereas

dyspnea increased initially then decreased. Higher urine NGAL levels were strongly associated with constipation (p=0.06) and diarrhea (p=0.016) in the QOL measures.

Bevacizumab was associated with decreased physical and social functioning, and higher levels of dyspnea, constipation, and insomnia in the EORTC QLQ-C30 (all p<0.05), but no significant associations were found for dexamethasone on either inventory.

4. Discussion

Apart from surgery, there is no sensitive and specific test to differentiate true disease progression from treatment effect-associated MRI artifact in GBM. Therefore, it is essential to identify biomarkers that correlate with tumor burden, to facilitate improved MRI interpretation and confident and timely treatment modification. Although this prospective longitudinal study did not conclusively establish the clinical relevance of MMP-2 or NGAL as biomarkers for improved MRI interpretation of GBM, it did demonstrate MMP-2 and NGAL to be significant biomarkers for tumor burden.

One objective was to find correlation between the expression of biomarkers in the serum and urine to biomarkers in the tumor tissue. Although NGAL expression was observed in both GBM and non-tumor control tissue, as seen in the study completed by Smith and colleagues, there was a higher expression of NGAL in the GBM tissue, suggesting that NGAL is upregulated in tumor tissue and is a possible marker for tumor burden. Contrary to data by Smith and colleagues, expression of MMP-2 was not detected in tumor or non-tumor control tissue, perhaps due to inadequate staining, antibody variance, and tumor type [16]. In the aforementioned study, varying grades and morphology of tumors were assessed, whereas we only examined expression in GBM.

While others have attempted to correlate biomarkers and disease with a variety of brain tumors in all ages, or several grades of glioma, we sought to do so with a more homogenous group of adult patients with GBM. Smith and colleagues reported the expression of MMP-2, MMP-9 and MMP-2/NGAL complexes in brain tumor tissue and in the urine samples in patients of all ages with any primary brain tumor. Urinary expression of NGAL/MMP-9 was 89% in primary brain tumor patients compared to 17% in controls. MMP-9 and MMP-2 were positive 57% and 82% of the time, respectively, in primary brain tumor patients. The urinary levels of all three biomarkers were elevated significantly compared with non-tumor controls (p< 0.001), and furthermore, these urinary markers resolved after gross total resection one year later in a subset of 5 patients with both benign and malignant tumors, supporting the tumor as source of the biomarker [16]. More recently, however, a large prospective longitudinal study of patients solely with glioma, including low-grade glioma, anaplastic glioma and GBM, failed to show correlation between serum MMP-9, disease status based on MRI, or survival data [17]. With these studies in mind, we prospectively evaluated adults with GBM at each MRI with concurrent serum and urine measurements, evaluated both MMP-2 and NGAL, and simultaneously obtained symptom inventories and QOL assessments. We further explored quantitative MRI measurements for greater accuracy.

Biomarkers correlated positively with tumor size over time. Furthermore, the inverse

relationship between EOR and serum MMP-2 further supports a correlation between this biomarker and tumor burden. The significantly higher level of preoperative serum MMP-2 in GBM patients compared to non-tumor control patients demonstrates its possible efficacy. In this population, the data leads to MMP-2 as a better marker for tumor presence. However, this conclusion is confounded by the overall trend of the biomarkers decreasing over the patients' course, despite tumor recurrence. This result demonstrates MMP-2 and NGAL may be more beneficial as biomarkers at the time of original diagnosis as opposed to their analysis in longitudinal samples.

NGAL may be a useful indicator for survival in GBM patients. It may also be a surrogate marker for a subset of patients with unfavorable molecular features, something that has not yet been studied. Significantly, serum NGAL decreased over time. Interestingly, patients on bevacizumab had higher urine NGAL and also higher tumor volumes. This treatment was used in this patient population typically as their last therapy, so having a correlation with increased tumor volumes is not surprising. This suggests that bevacizumab may alter the levels of NGAL, making it less advantageous a biomarker in patients receiving this therapy.

A meta-analysis of 30 randomized controlled trials from the EORTC included survival data for 10,108 patients who had a variety of cancer diagnoses, including brain cancers [24]. The importance of both symptoms and quality of life is underscored by the fact that both were found to be predictive of OS. We found, as noted in other studies, that the trajectory of symptoms and quality of life was variable. This variability and the small sample size could have contributed to the lack of correlation between these parameters, and the biomarkers. As both symptoms and quality of life are altered by medication, disease progression, and other illnesses, continued longitudinal assessment of these factors is important to ensure measures can be taken to minimize symptoms and maximize quality of life [13-17, 22-26]. Measurement and clinical use of QOL have been established to be feasible [25]. Maintaining QOL, which includes management of symptoms, is recognized as an important endpoint during treatment.

5.0 Conclusion

We present the first study to correlate MMP-2 and NGAL with not only disease burden and survival in GBM, but also QOL and patient symptoms. Although intra-patient biomarker changes did not elucidate equivocal MRI results, nor did they correlate with the MDASI-BT or EORTC QOL inventories, our data suggest that MMP-2 and NGAL correlate longitudinally with tumor size and with PFS and OS at baseline in patients with GBM. These comparisons have a low power due to the small sample size in this pilot study. Further exploration within a larger cohort is warranted.

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