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Characterization of MGMT and EGFR protein expression in glioblastoma and association with survival

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Abstract

Purpose—Understanding the molecular landscape of glioblastoma (GBM) is increasingly important in the age of targeted therapy. *O-6-methylguanine-DNA methyltransferase* (*MGMT)* promoter methylation and *EGFR* amplification are markers that may play a role in prognostication, treatment, and/or clinical trial eligibility. Quantification of MGMT and EGFR protein expression may offer an alternative strategy towards understanding GBM. Here, we quantify baseline expression of MGMT and EGFR protein in newly diagnosed GBM samples using mass spectrometry. We correlate findings with *MGMT* methylation and *EGFR* amplification statuses and survival.

Methods—We retrospectively identified adult patients with newly diagnosed resected GBM. MGMT and EGFR protein expression were quantified using a selected reaction monitoring mass spectrometry assay. Protein levels were correlated with *MGMT* methylation and *EGFR* amplification and survival data.

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Compliance with Ethical Standards:

Conflict of Interest: Authors Schaff, Rosenblum, Reiner, Panageas, and Lin have no conflict of interest. Authors Thyparambil and Hembrough have a patent for the SRM assay discussed in this manuscript and are employees of NantOmics. Authors Yan, Tian, and Cecchi are employees of NantOmics.

This article does not contain any studies with human participants or animals performed by any of the authors. This study was approved by the Internal Review Board at MSKCC.

Results—We found a statistically significant association between MGMT protein expression and promoter methylation status (p=0.02) as well as between EGFR protein expression and *EGFR* amplification (p<0.0001). EGFR protein expression and amplification were more tightly associated than MGMT protein expression and methylation. Only *MGMT* promoter methylation was statistically significantly associated with progression-free and overall survival.

Conclusions—Unlike EGFR protein expression and *EGFR* amplification which are strongly associated, only a weak association was seen between MGMT protein expression and promoter methylation. Quantification of MGMT protein expression was inferior to *MGMT* methylation for prognostication in GBM. Discordance was observed between *EGFR* amplification and *EGFR* protein expression; additional study is warranted to determine whether EGFR protein expression is a better biomarker than *EGFR* amplification for clinical decisions and trial enrollment.

Keywords

glioblastoma; MGMT; EGFR; mass spectrometry; proteomics

Introduction

Standard of care treatment for glioblastoma (GBM) includes radiation, chemotherapy with temozolomide, a DNA alkylating agent, and, often, alternating electric fields. Response to treatment is inadequate, yielding an overall survival (OS) of up to 21 months [1, 2]. Understanding the molecular landscape of GBM tumors is increasingly important for prognostication and clinical trial enrollment.

O^6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein that removes DNA alkyl groups. Its expression is thought to be predominantly regulated by epigenetic silencing through methylation of the gene promoter region. Methylation of the *MGMT* promoter is predictive of response to alkylating chemotherapies, including temozolomide [3, 4]. Because of the differences in response, temozolomide is often held in patients who are *MGMT* unmethylated. For this reason, accurate and reliable assessment of *MGMT* status is important in therapeutic decision-making and often determines trial eligibility. Increasingly, there is a lack of standardization in methylation testing and setting a threshold for stratifying patients, which contributes to considerable intra- and inter-assay variability. Moreover, there is often a “gray zone” of patients whose tumors are neither strongly methylated nor completely unmethylated[5, 6]. Such issues have resulted in poor reproducibility with inter-laboratory discordance–in one study as high as 39% [7] despite evidence suggesting *MGMT* promoter methylation is relatively homogeneous throughout a tumor [8]. Theoretically, methylation serves as a surrogate marker for gene expression but attempts to quantify MGMT protein through immunohistochemistry (IHC) have been largely unsuccessful. Studies of concordance between methylation status and response to temozolomide, including survival, have yielded mixed results [8–10]. Interpretation of IHC is qualitative and staining is dependent on the operator as well as antibody used, suggesting an imperfect solution.

EGFR is another protein whose detection has become increasingly important in GBM. Approximately 58% of patients harbor *EGFR* alterations in their tumor genome, the most common being amplification of the *EGFR* gene. EGFR is a receptor tyrosine kinase whose

activation stimulates cellular proliferation and survival. There are several methods in use for identifying tumors with upregulated EGFR, including fluorescence *in situ* hybridization (FISH), assessment of mRNA transcription via real-time reverse transcription-polymerase chain reaction (rt-PCR), whole transcriptome sequencing (RNAseq), and next-generation sequencing approaches. The only proteomic approach in wide use is EGFR IHC; this assay is thought to be inferior at identifying tumors that are being driven by EGFR, though the protein product of the *EGFR* gene is the target of investigation of agents such as receptor tyrosine kinase inhibitors and EGFR-directed antibodies such as depatuxizumab mafodotin ().

Our study quantifies baseline expression of both MGMT and EGFR proteins using mass spectrometry and correlates findings with *MGMT* methylation status as well as *EGFR* amplification. We then correlate protein expression data with overall and progression-free survival (PFS) in GBM patients treated upfront with radiation and temozolomide.

Methods

Sample Identification and Tissue Collection

We retrospectively identified adult patients with surgical resection of newly diagnosed GBM at our institution between the years 2000–2016. We selected only patients for whom methylation status, treatment course, and survival outcomes were known. We further selected only patients who received upfront radiation therapy and temozolomide at standard dosing as defined by the Stupp protocol[1]. Patients who received additional upfront therapy were not excluded from analysis. We found 54 patients who had tumor tissue available for testing. Samples had all been fixed in formalin and embedded in paraffin. Ten-micron tissue sections from each sample were placed on DIRECTOR microdissection slides.

MGMT Methylation

We selected only patients who had documented *MGMT* methylation testing of their initially resected tumor specimen. *MGMT* methylation was assessed by pyrosequencing or methylation-specific real-time PCR, according to our institutional practice at the time of surgery.

EGFR Amplification

We retrospectively reviewed charts to determine *EGFR* amplification status. Testing was performed via either FISH or next-generation sequencing.

Protein Quantification

MGMT and EGFR proteins were quantified using a selected reaction monitoring (SRM) mass spectrometry assay as previously described [11, 12]. Briefly, the tumor areas were marked up by a board-certified pathologist and were microdissected using a non-contact laser microdissection technique (Molecular Machines, Germany). The microdissected tumor tissue was solubilized to release peptides using the Liquid Tissue protocol (Expression Pathology, Rockville, MD) and the total protein levels were quantitated using a micro-BCA assay. Peptides TTLDSPLGK and IPLENLQIIR, were used to quantify MGMT and EGFR,

respectively. Synthetically labeled heavy peptides were mixed with the tumor-derived peptides and quantitated by SRM mass spectrometry on a TSQ Quantiva triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA) equipped with a nanoAcquityLC system (Waters, Milford, MA). Protein quantitation was normalized across all samples based on the total amount of protein analyzed in a given sample. Data analysis was conducted using the Pinnacle software (Optys Tech, PA).

To assess the analytical performance of the MGMT assay, a calibration curve of 11-nonzero points (100, 150, 200, 300, 400, 500, 600, 800, 1000, 5000 and 25000 amol) was generated in a *Pyrococcus furiosus* complex matrix (*Pfu*, Agilent Technologies) to determine the assay's quantitation linearity. CVs for the various concentration points in the calibration curve ranged from 0.7% to 9.0% for samples analyzed in quintuplicate. The calibration curve showed linearity ($R^2 = 1.0$) and low variations over the protein concentration range. Precision was assessed in 10 FFPE tumor samples across a range of spiked MGMT peptide concentrations from 125–10,000 amol/ug (n=3). The average intra-assay precision was 3.13% CV for instrument #1 and 3.0% CV for instrument #2. The average inter-assay precision (different instruments, different days, and different operators) was 3.64% CV.Similarly to MGMT, EGFR assay's analytical performance was previously determined and reported [11].

Statistics

Descriptive statistics such as frequencies, medians, and ranges were used to characterize the cohort under study. The Wilcoxon two-sample test was used to associate MGMT protein expression with promoter methylation and to associate EGFR protein expression with *EGFR* amplification status. OS was defined as time from GBM diagnosis until death or date of last follow up for those who were censored. PFS was defined as time from GBM diagnosis until death, progression as determined by RANO criteria, or date of last follow up, whichever occurred first. Cox modeling was used to associate variables of interest with OS and PFS. All p-values were two-sided with a level of statistical significance less than 0.05. All statistical analyses were performed in SAS (version 9.4, Cary, NC).

Results

Patient Characteristics

We obtained archival tissue samples from 54 patients. Three samples contained insufficient tumor tissue to yield accurate mass spectrometry results, and these patients were therefore excluded from further analysis. Of the remaining 51 patients, 17 were women (33%) and 34 were men (67%) (Table 1). The median age at diagnosis was 63 (range 33–80 years) and the median Karnofsky Performance Status (KPS) was 90 (range 60–100). All patients had either a gross total (30, 59%) or a subtotal (21, 41%) resection at diagnosis, defined by post-operative MRI.

One patient in the cohort was known to have a mutation in isocitrate dehydrogenase (IDH) 1 (2%). Twenty-four patients were wildtype for IDH 1 and 2 by sequencing (47%). The remaining 26 patients did not have sequencing data and IHC for IDH1 R132H, if performed,

resulted as negative. These patients were therefore considered IDH not otherwise specified (NOS) (51%). All patients had *MGMT* promoter methylation testing results. Testing was performed via methylation specific real-time PCR (26, 51%) or pyrosequencing (25, 49%) in Clinical Laboratory Improvement Amendments certified environments at or near the time of diagnosis. Seventeen patients were determined to be *MGMT* methylated (33%) and 34 were *MGMT* unmethylated (67%). None of the patients in this series had indeterminate results.

EGFR status was known in 44 patients (86%). Twenty-three patients were *EGFR* amplified (45%) while 21 patients were not (41%). *EGFR* status was determined by FISH (36), genetic sequencing (18) or both tests (10) at or near the time of diagnosis. For the 10 patients who had both FISH testing and sequencing, results were always concordant. Amplification status was unknown in 7 patients (14%). Full patient details are available in Supplementary Table 1.

Correlation between MGMT expression levels and *MGMT* methylation status

MGMT protein expression quantified by mass spectrometry was correlated with results of *MGMT* methylation testing. Mass spectrometry data was obtained for 51 patients. Protein concentration ranged from non-detectable (ND) in 26 patients (51%) to 608.8 amol/μg (Figure 1). The median concentration was ND. Of the 17 patients whose tumors tested positive for *MGMT* methylation, 13 had ND levels of MGMT protein by SRM mass spectrometry (76%). The remaining four patients (24%) expressed protein at concentrations of 130.5, 139.0, 285.4, and 608.8 amol/μg. Of the 34 patients whose tumors tested negative for *MGMT* promoter methylation, 21 had detectable levels of MGMT protein (62%) ranging in concentration from 108.3 – 487.4 amol/μg while 13 (38%) had undetectable levels. The median concentration of MGMT protein expression in unmethylated tumors was 160.4 amol/μg. Overall, *MGMT* methylation status was considered concordant with protein expression detectability in 34 of 51 patients (67%). Association between MGMT protein expression and promoter methylation status was statistically significant (p=0.02).

In five tumors, SRM analysis was performed on tissue from two separate tumor blocks. Four of 5 patients (80%) had consistent results between the two samples analyzed. Of these, 2 patients had ND MGMT protein concentrations in both samples. Two patients had detectable MGMT protein concentrations from both samples (134 amol/μg and 175.3 amol/μg in one patient; 142.3 amol/μg and 173 amol/μg in the other). The final patient had ND MGMT concentration in one sample and a detectable concentration of 130.5 amol/μg in the other.

Correlation between EGFR expression levels and *EGFR* amplification

EGFR protein concentration was determined in 51 tumor samples. Concentrations ranged from ND – 173,676.3 amol/μg (Figure 2). The median concentration was 1,128.0 amol/μg and three patients had ND levels (6%). Two of the three patients with ND EGFR protein were not amplified (both by FISH testing). The *EGFR* status of the third patient was unknown. Median EGFR protein expression in amplified tumors was 9,263.2 amol/μg (254.6–173,676.3 amol/μg) vs 257.0 amol/μg (0–21,121.5 amol/μg) in non-amplified tumors. The association between amplification status and protein expression held whether amplification was assessed by FISH (p=0.0005), sequencing (p=0.0083), or either method

(p<0.0001). Despite this correlation, there was significant overlap in protein expression levels among samples that were determined *EGFR* amplified and non-amplified by FISH/sequencing.

Correlations with survival

The median PFS in our cohort was 10.0 months (95% CI: 7.6–13.7) and median OS was 21.7 months (95% CI: 18.1–26.1). Clinical variables such as sex, age, KPS at diagnosis, and extent of resection were not statistically significantly associated with PFS or OS in our small cohort (Supplementary Table 2). *MGMT* promoter methylation status was the only variable statistically significantly associated with PFS and OS; patients who were =methylated had a reduced hazard of progression and death (PFS hazard ratio, 0.41; 95% CI: 0.22–0.74; p=0.003; OS hazard ratio, 0.40; 95% CI: 0.21–0.76; p=0.005).

Patients with methylated *MGMT* promoter had a median PFS and OS of 19.2 mo (95% CI: 11.6–28.3) and 36.1 mo (95% CI: 21.7–50.0), respectively vs 7.6 mo (95% CI: 5.7–9.9) and 18.2 mo (95% CI: 12.4–22.2) in patients with unmethylated tumors. There was no significant difference between PFS and OS in patients with non-detectable levels of MGMT protein by mass spectrometry as compared to patients with detectable levels of MGMT (median PFS 11.5 mo (95% CI: 7.2–17.6) vs 9.9 mo (95% CI: 5.7–14.0); median OS 22.7 (95% CI: 16.4–31.1) vs 21.1 mo (95% CI: 13.8–26.1)). MGMT protein expression was not statistically associated with PFS or OS by Cox regression analysis. Moreover, in patients whose tumors tested positive for *MGMT* methylation, presence or absence of protein expression did not significantly influence PFS or OS (Table 2). Similarly, detection of MGMT expression did not impact PFS or OS in patients whose tumors were *MGMT* unmethylated.

EGFR amplification status was not associated with survival. Patients whose tumors were not *EGFR* amplified demonstrated median PFS of 11.5 mo (95% CI: 5.8–17.3) and median OS of 21.3 mo (95% CI: 12.4–57.2) vs 9.6 mo (95% CI: 7.6–13.7) and 21.7 mo (95% CI: 14.5–27.2) in patients whose tumors were *EGFR* amplified. Cox regression analysis did not demonstrate EGFR protein expression to be significantly associated with PFS or OS.

Discussion

The relationship between MGMT expression and promoter methylation status has been explored using IHC techniques with no clear consensus emerging. Multiple studies failed to demonstrate a correlation between IHC quantification of MGMT protein with methylation testing results [9, 10, 13–15]. While some studies have reported associations between protein expression and patient outcomes with alkylating chemotherapy [13, 16–19], these results are not consistent [9, 15, 20, 21].

The reasons for the failure of IHC to reliably substitute for methylation testing are unclear. Some evidence suggests the interpretation of IHC results is not standardized and is subject to interobserver variability [9, 15, 19]. Results may also differ based on the specific antibody used in IHC testing [9, 15]. We analyze MGMT protein expression with mass spectrometry, which yields a quantitative value and eliminates the subjectivity of interpretation. While we

report an association between MGMT protein expression and methylation status, the rate of discordance was 33%. Despite reported high rates of interlaboratory discrepancy with *MGMT* methylation results, this is unlikely to be the cause of the observed discordance [7]. Importantly, *MGMT* promoter methylation was strongly associated with improved OS and PFS in our cohort while MGMT protein expression was not.

We observed MGMT protein expression in 24% of methylated tumors. Prior experience with IHC testing suggests intra-tumoral quantities of MGMT can be overestimated due to contamination by healthy tissue, such as endothelial cells and lymphocytes [14]. Despite our attempts to mitigate this problem by requiring pathologic review of slides prior to processing, it is possible that a small amount of stroma or healthy tissue was still present. Conversely, 38% of unmethylated tumors did not have detectable levels of MGMT protein expression. Protein quantification at diagnosis may underestimate a cell's ability to produce MGMT protein, as radiation and alkylating therapy may induce expression. Additionally, MGMT protein expression likely depends on factors other than promoter methylation. For instance, NF-κB and p53 have both been implicated in regulating MGMT expression [22, 23]. Data indicating a correlation between RNA expression and methylation status but no correlation between RNA expression and OS suggest post-transcriptional regulation may also be at play [21, 24]. In our cohort, MGMT protein expression and *MGMT* promoter methylation were less strongly associated than EGFR protein expression and *EGFR* amplification, which supports the contention that MGMT protein expression is not exclusively regulated by epigenetics.

MGMT protein expression was not predictive in this data set. We did not detect a survival difference in unmethylated patients who expressed MGMT protein compared with patients with undetectable MGMT (19.1 mo vs 16.4 mo, p=0.54). We did not find that the combination of *MGMT* promoter methylation status and protein expression was significantly associated with survival, in contrast to a similar study performed using IHC technique [10]. It may be that methylation of the *MGMT* promoter is a surrogate marker for broader changes to the epigenome that may affect treatment response in other ways [25, 26]. Interestingly, Patient 43 in our series was *MGMT* methylated but expressed the highest concentration of MGMT protein in this series (608.8 amol/μg). This patient had a PFS and OS of 17.3 mo and 57.2 mo, respectively, far beyond the median of our cohort, which supports this idea. We might have seen a stronger correlation between MGMT protein expression and methylation status (and possibly survival), with more sensitive methods for quantifying MGMT protein (below 108 amol/μg).

Of interest, MGMT protein expression was quantified in two different tumor blocks for five patients included in the study. Testing in four patients yielded concordant results while expression in the fifth patient was discordant. As MGMT methylation is homogeneous throughout a tumor [8], we hypothesize discordance is related to regulatory factors such as transcriptional or translational processing. Additionally, the sensitivity of our assay may have resulted in a discordant result. Although MGMT protein expression did not predict patient outcomes in newly diagnosed GBM, quantification may still be useful. MGMT expression, but not promoter methylation, correlates with patient outcomes in pituitary tumors and is currently assessed via IHC [27, 28]. Moreover, therapeutic strategies to

specifically target MGMT-deficient cells are under development [29] and quantifying MGMT protein expression may be useful in this setting.

EGFR protein expression correlated significantly with amplification status whether tested by FISH or sequencing. EGFR is an investigational target in GBM and the significance of amplification is unknown, though multiple therapeutic strategies targeting *EGFR*-amplified tumors are being investigated. We did not find a correlation between PFS or OS and *EGFR* status. While some individual studies have shown shorter OS with *EGFR* amplification, a prognostic role of *EGFR* amplification has never been confirmed [30]. Further study is needed to correlate levels of EGFR expression with response to EGFR-directed therapy and to investigate expression of EGFR protein throughout a tumor. Though *EGFR* amplification and protein expression are strongly associated, there was still considerable overlap in EGFR protein expression among tumors testing both positive and negative for *EGFR*-amplification. Further study is needed to correlate levels of EGFR protein expression with response to EGFR-directed therapy. It is possible that treatment response to agents such as depatuxizumab mafodotin, an antibody drug conjugate that targets a unique conformation of EGFR in the setting of *EGFR* overexpression, is better predicted by protein expression than amplification status.

Our study was limited by its retrospective nature and reliance on archived tissue samples. As we only examined tumors with prior methylation testing and remaining sample, all patients in our cohort had undergone gross or subtotal resections and none had only been biopsied. This likely contributed to the relatively high KPS and the longer OS in our cohort than that of a typical GBM population. Of note, we did not detect a survival difference between patients with gross total and subtotal resections, contradictory to previously published findings [31, 32]. This is likely a result of the retrospective nature of the study, the small cohort size, and the way patients were selected. Specifically, only patients with available archival tumor tissue were analyzed, suggesting all patients had sizable tumor tissue removed. Moreover, our patients did not all receive uniform treatment. While all were treated with radiation and temozolomide, several in this cohort were enrolled in clinical trials for upfront therapy and may have received additional treatments. As our primary outcome was based on tissue analysis, these patients were included. Despite these limitations, we feel that this study is an important addition to the literature as it elucidates the pitfalls and the promise of using mass spectrometry for evaluating biomarkers in GBM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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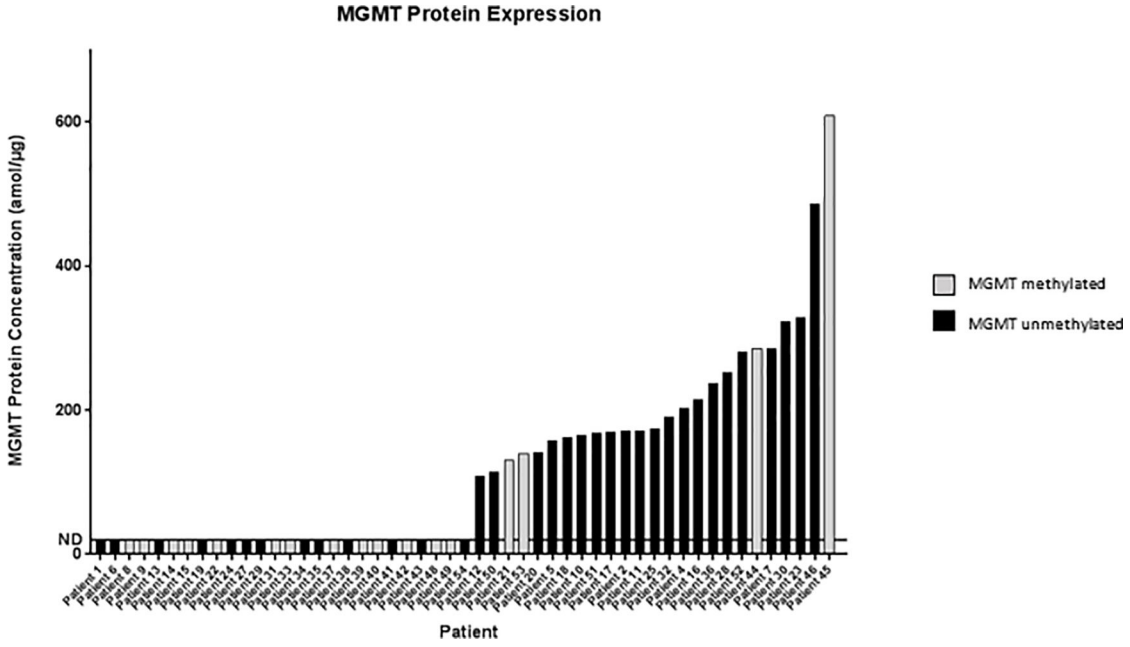

Fig. 1.

Bar graph indicating MGMT protein concentrations as determined by mass spectrometry in each patient sample. Shading indicates *MGMT* methylation status with light shading representing *MGMT* methylated tumors and dark shading representing *MGMT* unmethylated tumors.

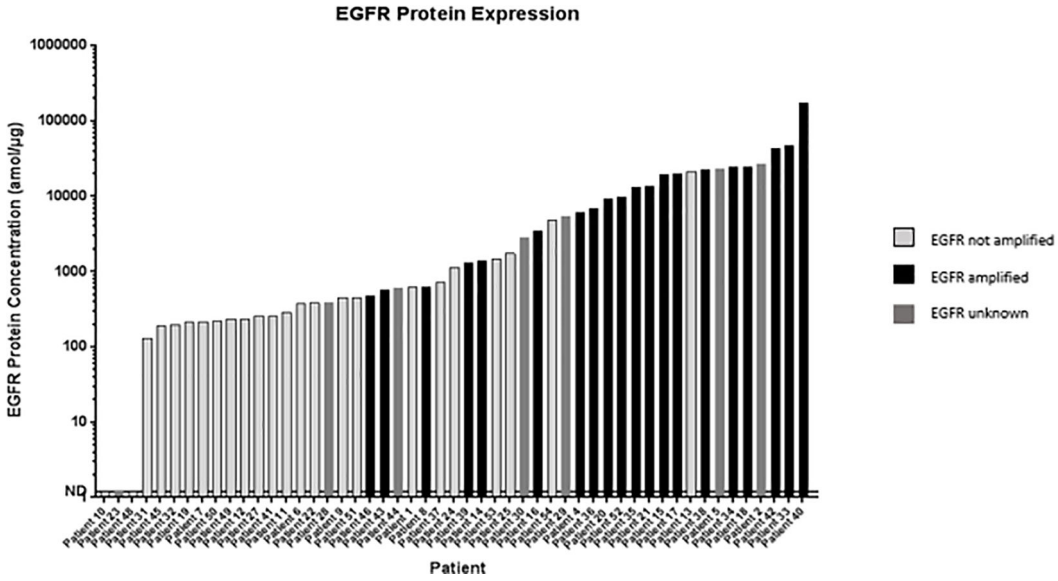

Fig. 2.

Bar graph indicating EGFR protein concentrations as determined by mass spectrometry in each patient sample. Shading indicates *EGFR* amplification status with light shading representing non-amplified tumors, dark shading representing amplified tumors, and medium shading representing unknown amplification status.

Table 1.

Patient Characteristics

Characteristic	Median	Range	Number (%)
Sex			
Female			17 (33)
Male			34 (67)
Age	63	33–80	
KPS	90	60–100	
Surgery Type			
GTR			30 (59)
STR			21 (41)
IDH Statu			
IDH mutated			1 (2)
IDH wildtype			24 (47)
IDH NOS			26 (51)
MGMT Status			
MGMT methylated			17 (33)
MGMT unmethylated			34 (67)
***EGFR* Status**			
EGFR amplified			23 (45)
EGFR not amplified			21 (41)
Amplification unknown			7 (14)

Table 2.

Association between *MGMT* methylation, MGMT protein expression, PFS and OS.

			PFS		OS	
		N	**PFS (Mo) Median, (95% CI)**	**Log Rank p**	**Median OS Median, (95% CI)**	**Log Rank p**
MGMT	Methylated promoter, ND expression	13	19.2 (11.5–29.1)	0.66	29.9 (21.7–50.0)	0.82
	Methylated promoter, detectable expression	4	18.8 (15.4–28.3)		43.8 (29.0–57.2)	
MGMT	Unmethylated promoter, ND expression	13	7.2 (5.5–9.6)	0.98	16.4 (12.3–27.2)	0.54
	Unmethylated promoter, detectable expression	21	8.6 (5.1–12.6)		19.1 (12.4–22.2)	