TITLE: The Metabolomic Signature of Malignant Glioma Reflects Accelerated Anabolic Metabolism

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RUNNING TITLE: The Metabolomic Signature of Malignant Glioma

CONFLICT OF INTEREST: E.K. is a paid employee of Metabolon, Inc.

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ABSTRACT

Although considerable progress has been made towards understanding glioblastoma biology through large-scale genetic and protein expression analyses, little is known about the underlying metabolic alterations promoting their aggressive phenotype. We performed global metabolomic profiling on patient-derived glioma specimens and identified specific metabolic programs differentiating low and high-grade tumors, with the metabolic signature of glioblastoma reflecting accelerated anabolic metabolism. When coupled with transcriptional profiles, we identified the metabolic phenotype of the mesenchymal subtype to consist of accumulation of the glycolytic intermediate phosphoenolpyruvate and decreased pyruvate kinase activity. Unbiased hierarchical clustering of metabolomic profiles identified three subclasses, which we term "energetic", "anabolic", and "phospholipid catabolism" with prognostic relevance. These studies represent the first global metabolomic profiling of glioma, offering a previously undescribed window into their metabolic heterogeneity, and provide the requisite framework for strategies designed to target metabolism in this rapidly fatal malignancy.

Precis: Global metabolomic analysis identifies key features underlying the aggressive phenotype of malignant glioma, providing novel strategies for therapeutic intervention.
INTRODUCTION

The World Health Organization (WHO) classifies glioma into Grades I to IV, based on abundance of atypical cells, mitoses, endothelial proliferation, and necrosis. Tumor grade plays a central role in prognosis and guides clinical management. For example, patients with Grade I tumors are typically cured following surgical resection, while patients diagnosed with Grade IV tumors, termed glioblastoma, have a median survival of approximately 1 year despite aggressive multi-modality treatment consisting of surgery, radiation therapy, and chemotherapy. Although Grade II gliomas typically have a better prognosis than higher grade tumors and are often categorized as “benign”, this is somewhat of a misnomer since these tumors are rarely cured and typically transform to higher-grade tumors (1).

Considerable progress has been made in understanding the underlying biology of gliomas. For example, common molecular alterations identified in low-grade oligodendrogliomas and astrocytomas are allelic loss of 1p and 19q and mutations in p53, respectively, while Grade III and IV tumors typically are driven by alterations in PI3K, EGFR, VEGF and PTEN signaling (1). Further, through whole genome sequencing, recent data presented by The Cancer Genome Atlas Research Network both reinforced these previously identified mutations and highlighted tumor heterogeneity, identifying aberrant signaling through the RTK/RAS/PI3K, p53, and RB pathways being central for glioblastoma development (2).
Despite these advancements in our understanding of the upstream events signaling tumorigenesis, relationships between underlying metabolic alterations and mechanisms promoting the observed aggressive phenotype in these tumors remain unclear. The seminal observation made by Otto Warburg nearly a century ago (3, 4), described “aerobic glycolysis”, i.e. a high fermentative metabolism of glucose resulting in production and release of lactic acid, even in the presence of adequate oxygen. We have proposed that acid production provides a competitive advantage for invasive cancers (5), yet a definitive explanation for why tumor cells metabolize glucose through the seemingly inefficient process of aerobic glycolysis continues to be elusive. Nonetheless, its clear relevance to cancer biology is evident with the widespread application of 18-FDG-PET imaging, which can predict histologic grade in glioma with relatively high accuracy. Grade II tumors typically demonstrate low specific uptake values (SUV), while high-grade tumors (Grade III and IV) demonstrate high SUVs (6). Hence, there are grade-associated changes in glioma metabolism, yet these have not been extensively characterized.

Metabolomics is the global quantitative assessment of endogenous metabolites within a biological system, taking into account genetic regulation, altered kinetic activity of enzymes, and changes in metabolic reactions. Thus, compared to genomics and proteomics, metabolomics reflects changes in phenotype and therefore function (7, 8). Based on the clear metabolic shift between low-grade and high-grade tumors, we evaluated global metabolomic profiles in Grade II, III, and IV gliomas to comprehensively evaluate metabolic underpinnings of grade specific changes in glioma
beyond that of glucose consumption. Further, metabolomic signatures were coupled with gene expression profiles to evaluate for subtype specific changes in tumor metabolism and provide insight into upstream signaling networks that may be driving the observed metabolic alterations.

**MATERIALS AND METHODS**

**Tumor samples and patient characteristics**

A summary of all glioma cases studied is included in Table S1. All surgeries were performed at the H. Lee Moffitt Cancer Center and Research Institute and tissue was obtained from the Moffitt Cancer Center Tissue Core Facility. All of the Grade III and IV tumors used in this analysis were newly diagnosed malignancies. Tumors were fresh-frozen and their integrity and histology confirmed by a staff pathologist prior to aliquoting samples. Clinical outcomes were obtained through the Cancer Center Tumor Registry. Institutional Review Board/Human Subjects approval was obtained for this retrospective study.

**Sample Preparation and Metabolic Profiling**

Metabolomic studies were conducted at Metabolon Inc. (Durham, NC) using a non-targeted platform that enables relative quantitative analysis of a broad spectrum of molecules with a high degree of confidence (9). The metabolic profiling analysis combined three independent platforms: ultrahigh performance liquid
chromatography/tandem mass spectrometry (UHPLC/MS/MS\textsuperscript{2}) optimized for basic species, UHPLC/MS/MS\textsuperscript{2} optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS). Samples were processed essentially as described previously (9, 10). Tissue samples were homogenized in a minimum volume of water and 100 μL withdrawn for subsequent analyses. Using an automated liquid handler (Hamilton LabStar, Salt Lake City, UT), protein was precipitated from the homogenate with methanol that contained four standards to report on extraction efficiency. The resulting supernatant was split into equal aliquots for analysis on the three platforms. Experimental samples and controls were randomized across platform run days. For UHLC/MS/MS\textsuperscript{2} analysis, aliquots were separated using a Waters Acquity UPLC (Waters, Millford, MA) and analyzed using an LTQ mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA), which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The MS instrument scanned 99-1000 m/z and alternated between MS and MS\textsuperscript{2} scans using dynamic exclusion with approximately 6 scans per second. Derivatized samples for GC/MS were separated on a 5% phenyldimethyl silicone column with helium as the carrier gas and a temperature ramp from 60°C to 340°C and then analyzed on a Thermo-Finnigan Trace DSQ MS (Thermo Fisher Scientific, Inc.) operated at unit mass resolving power with electron impact ionization and a 50-750 atomic mass unit scan range. Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as associated MS spectra, and were curated by visual inspection for quality control using software developed at Metabolon.
(11). Metabolomic subtypes were generated using unsupervised hierarchical clustering on GeneCluster 3.0 (Berkeley, CA). Metabolite concentrations (excluding xenobiotics) were log transformed and clustering was performed with uncentered correlation and single linkage metrics, and visualized using Java TreeView.

For statistical analyses and data display purposes, any missing values were assumed to be below the limits of detection and these values were imputed with the compound minimum (minimum value imputation). Statistical analysis of log-transformed data was performed using “R” (http://cran.r-project.org/), which is a freely available, open-source software package. Welch’s t-tests were performed to compare data between experimental groups. Multiple comparisons were accounted for by estimating the false discovery rate (FDR) using q-values (12). Random Forest (RF) analysis, (13) was carried out on untransformed data. When data from the glioblastoma grade categories were used in comparisons for classification by RF, the number of in-bag samples was set to 50% of smallest sub-group to account for unbalanced group sizes, with 50,000 trees. Random forest analysis was performed using the R-package “randomForest” (14).

**Expression Transcriptional Subtyping**

Total RNA was extracted from snap frozen malignant glioma tissue using magnetic binding beads for cDNA and Qiagen RNeasy kits for cRNA purification. The final in vitro transcription incorporated biotin moieties that were later labeled with
phycoerythrin. Samples were fragmentated to improve hybridization sensitivity and consistency. The labeled molecules were biotinylated-cRNA. GeneChip microarrays were loaded with the fragmented target sample/hybridization buffer mix using standard techniques. Arrays were hybridized for 18 h at 45ºC with vigorous mixing. Unbound sample was removed and staining was accomplished through the binding of streptavidin conjugated phycoerythrin to the hybridized target. Excess label was removed. Washing and staining steps were performed by the Affymetrix FS450 fluidics station using standard protocols. Arrays were scanned using a GeneChip Scanner 3000 7G with a 48 array autoloader. Samples were hybridized to a Affymetrix based chip designed by Merck (Whitehouse Station, NJ). A total of 35 probesets from the Merck chip that most closely matched the signature genes described by Phillip et al (15) were clustered using GeneCluster 3.0 and visualized by JavaTreeView 1.1.6. The three distinct clusters were evaluated for sample membership with respect to three glioma subtypes; defined as proliferative, mesenchymal, or proneural. Selection of differentially expressed genes was done using significance analysis of microarrays (SAM) (17). Principal Component Analysis (PCA) was performed in the Evident software package. GeneGO MetaCore (St. Joseph, MI) was used to identify significant biological pathways.

**Pyruvate kinase activity assay**

PK activity was measured using a commercially available kit according to the manufacturer recommendations (BioVision, Mountain View, CA). Briefly, tissues
were homogenized and extracted with assay buffer. Three μg of protein was used to ensure the reading was within the linear range of the standard curve. For the colorimetric assay, OD was measured at 570 nm at T1 to read A1 and measured again at T2 after incubating the reaction at 25°C for 10 and 20 m. PK activity was calculated by applying the ΔA (A2-A1) to the standard curve to yield nmol of pyruvate generated between T1 and T2 by PK in the reaction wells and expressed in mU/mL.

**Western Blot**

Western blot assays were performed as previously described (16) using antibodies against PKM2 (Cell Signaling, Danvers, MA; 1:3000) and β-actin (Sigma-Aldrich, St. Louis, MO; 1:20,000). Blots were quantified using ImageJ (NIH, Bethesda, MD) and the PKM2 expression of individual samples were normalized to loading control (β-actin).

**Statistics**

Statistics involved with metabolomic profiling are as described above. Estimates of overall survival were evaluated using the Kaplan-Meier product limit method and compared using a Wilcoxon log-rank test with SAS version 9.1.3 (SAS Institute, Cary, NC). Student’s t test was used for box-plot comparisons.

**RESULTS**
Global metabolic profiles distinguish glioma tumor grades

Global metabolomic profiling was performed using a combination of high-throughput liquid-and-gas chromatography-based mass spectrometry on a total of 69 fresh-frozen glioma specimens surgically resected at the H. Lee Moffitt Cancer Center (18 Grade II, 18 Grade III, and 33 Grade IV; specific histologies are provided in Table S1). From a metabolomic library consisting of more than 2000 purified standards, a total of 308 named biochemicals were detected. The distribution of metabolic pathways identified is presented in Figure 1A, with a majority of metabolites involved in lipid, amino acid, and carbohydrate metabolism. Following log transformation and imputation with minimum observed values for each compound, Welch’s two-sample t-tests were used to identify biochemicals that differed significantly between histological grades. Summaries of the numbers of biochemicals that achieve statistical significance (\( p \leq 0.05 \)) are provided in Figure 1B, with the largest number of significant metabolic changes observed between Grade II and Grade IV gliomas. A summary of the metabolic pathways differentiating Grade IV and II tumors is presented in Figure 1C. There was a significant increase in several metabolites involved in amino acid metabolism in Grade IV tumors, including glutathione and tryptophan. In addition, a decrease in creatine was also demonstrated, which is consistent with MR spectroscopy data for these highly proliferating tumors (18). There appeared to be a significant change in lipid metabolism in Grade IV tumors, with notable decreases in glycolipids, lysolipids (which include derivatives of phosphocholine and phosphoethanolamine), and sterols, and increases in essential and medium chain fatty acids and metabolites associated with carnitine metabolism. Although Grade IV tumors appear to demonstrate a global decrease in carbohydrate
metabolites, significant increases in key metabolic intermediaries phosphoenolpyruvate (PEP) and 3-phosphoglycerate (3-PG) were observed. In addition, a signature of increasingly altered nucleotide metabolism was also evident in Grade IV tumors, primarily involving pyrimidine catabolism.

Random Forest (RF) classifier models were developed to determine the capacity of global metabolic profiles to differentiate between tumor grades and to identify biochemicals important to the classification. The RF analyses yielded an overall predictive accuracy of 71% for classifying samples among groups (Fig. S1). By this method, Grade III tumors were poorly distinguished from both Grade II and IV tumors, suggesting progressive and overlapping metabolic changes during glioma tumorigenesis, while Grade II and IV tumors were best distinguished in the RF, reinforcing the important role altered metabolism plays in the aggressive phenotype associated with glioblastoma. In addition to producing a metric of predictive accuracy, RF analyses produced a prioritized list of biochemicals ranked in order of their importance to the classification scheme. To provide insight into grade-associated differences in global metabolism, the top 30 biochemicals for the RF classification scheme are provided in Figure 2. In this analysis, 2-hydroxyglutarate (2-HG) emerged as the top-ranked biochemical for tumor grade classification. Several recent seminal studies have identified mutations in the metabolic enzyme isocitrate dehydrogenase 1 (IDH1) unique to low grade glioma, resulting in structural changes allowing a new ability of the enzyme to catalyze the NADPH-dependent reduction of α-ketoglutarate to 2-HG (19-22). In addition to validating our described methodologies, identifying a significant accumulation of 2-HG in low grade
glioma specimens relative to its global metabolic profile further supports its potential role as an onco-metabolite in this tumor.

**The metabolic signature of glioblastoma reflects accelerated anabolic metabolism**

As the majority of our understanding of aberrant metabolism associated with tumorigenesis involves altered carbohydrate metabolism, we focused on alterations in glucose metabolism as a function of tumor grade. An evaluation of metabolites involved in glycolysis and the oxidative energy metabolism of the tricarboxylic acid (TCA) cycle/oxidative phosphorylation (23), provided in **Figure 3**, identified accumulation of glycolytic intermediates as a key metabolic alteration in high-grade glioma. As glucose is taken up by cells, it is phosphorylated by hexokinase to glucose-6-phosphate (G6P), which is metabolized via either the Embden-Meyerhoff (E-M; glycolysis or conversion of glucose to pyruvate) or the Pentose Phosphate (PP) pathways. In our analysis, progressively higher levels of triose phosphate glycolytic intermediates, including 3-phosphoglycerate (3PG) and the penultimate intermediate, phosphoenolpyruvate (PEP), are more than 7-fold higher in Grade IV tumors over Grade II and were among the top biochemicals in the RF importance plot for classification by tumor grade. To more definitively determine if these concerted changes represented a biologically relevant alteration in the metabolic pathway rather than independent events, we evaluated the concordance of 3PG and PEP within individual samples. This analysis resulted in a Pearson’s $r$ correlation coefficient of 0.96 (**Fig. S2**), supporting the conclusion that increased levels of these two metabolites represent a clear metabolic shift in high-grade glioma and the implicit strength of global pathway analysis offered by metabolomics.
Although absolute flux of metabolites cannot be definitively made from single steady-state measurements, the accumulation of PEP and 3PG in Grade IV tumors does suggest the potential for diversion of glycolytic carbon from the E-M mediated generation of ATP into alternate pathways for macromolecule biosynthesis. Two important glycolytic shunts are through the PP pathway and serine metabolism. The PP shunt, by production of NADPH and ribose-5-phosphate (R5P) contributes to nucleotide biosynthesis, generates reducing equivalents essential to anabolic metabolism, and modulates DNA methylation (24-28). Accordingly, the metabolomic signature of Grade IV tumors consisted of an accumulation of key metabolites in these pathways (Fig. 3), including 6PG (3.84 fold increase), R5P (5.78 fold increase), serine (1.55 fold increase), and glycine (2.23 fold increase).

**The metabolic phenotype of the mesenchymal subtype involves PEP accumulation and decreased PK activity.**

Although malignant glioma is defined as Grade III or IV based on histopathologic criteria, clear molecular heterogeneity has been uncovered within these tumor grades. Phillips et al. recently described 3 specific subtypes of malignant glioma based on expression profiles associated with distinct molecular signatures and clinical outcome, termed proneural (PN), proliferative (PR) and mesenchymal (15). To determine if metabolic phenotypes were associated with specific molecular subtypes in malignant glioma, global expression profiling followed by subtype designation was performed on glioma samples. A majority of the Grade III tumors were PN (67%), while the distribution of glioblastoma subtypes were 50% mesenchymal, 20% PR, and 30% PN,
which was consistent with previous reports (15) (Fig. S3). Coupling metabolomic data with expression profiles revealed that the accumulation of PEP strongly correlated with the mesenchymal subtype ($p=6.3 \times 10^{-7}$) (Fig. 4A). Notably, 3 of the 4 Grade III tumors that were molecularly classified as mesenchymal had significantly elevated PEP. In all, any tumor with a 4-fold or greater accumulation of PEP was invariably mesenchymal ($n=10$).

Accumulation of PEP could occur through reduced activity of pyruvate kinase (PK). Isoenzyme variations of PK have been documented to play a role in the diversion of glycolytic metabolites. Specifically, there are four PK isoenzymes (M1, M2, L, and R) that differ in their kinetic properties and distribution among cells and tissues. PKM1 is found in the vast majority of cells, whereas PKM2 is abundant during embryogenesis, in select differentiated tissues, and is the predominant form found in cancer cells (24, 29, 30). PKM2 can be further regulated by tyrosine kinase phosphorylation which can switch PKM2 from its more active tetrameric form with high affinity towards its substrate PEP, to a less active dimer form, which favors the diversion of trioses towards synthetic processes such as lipid and amino acid biosynthesis (24, 27, 30, 31). Hence, we determined if PK activity was correlated with increased PEP accumulation associated with the mesenchymal subtype. Of the initial tumor specimens evaluated, 26 had tissue evaluable for enzyme analysis. Of these, only 3 tumors clustered in the proliferative subtype; therefore, further analysis on this subtype was not performed. As demonstrated in Figure 4B, the mesenchymal subtype had a significantly decreased PK activity ($p=0.0205$) compared to the proneural group, suggesting a mechanism for the observed
metabolic phenotype of PEP accumulation. Interestingly, although PK activity was observed to be lower in the mesenchymal subtype, overall expression of PKM2 was significantly increased (p=0.0005) when compared to malignant gliomas clustering in the proneural subtype (Fig. 4C).

Global metabolism reveals metabolic signatures in malignant glioma

To determine if specific malignant glioma subtypes could be identified based on the global metabolomic profiles, unbiased hierarchical clustering was performed. Three distinct subgroups (A, B, and C) were identified (Fig. 5A), which were defined by 3 unique metabolomic signatures. Metabolites comprising the individual profiles that demonstrated statistical significance in malignant glioma are provided in Table S2. Although each profile consisted of a diverse set of metabolites, we applied the terms “energetic”, “anabolic”, and “phospholipid catabolism” to describe the individual profiles based on the characteristics of specific metabolites found therein. Specifically, Subgroup B was defined by a classic glycolytic or “energetic” profile that consisted of an accumulation of upstream intermediates of glycolysis, including glucose 6-phosphate and fructose 6-phosphate. Subgroup A was mutually exclusive to Subgroup B, with decreases in these glycolytic metabolites, and elevated levels of metabolites typically associated with diverted glycolytic intermediates and anabolic metabolism, including PEP, 3PG, and 6-PG. Notably, these also represent key metabolites differentiating Grade IV and II tumors, as described in Fig. 3. Other interesting metabolites in this list included those involved in serine, carnitine, tryptophan, and essential and long chain fatty acid metabolism. Subgroup C had a similar profile as Subgroup A, with the additional
metabolites of long chain fatty acid and lysolipid catabolism. Hence this subtype was
denoted as “phospholipid catabolism”. Both Subgroups A and C primarily consisted of
Grade IV (n=30/33) tumors, while the majority of tumors that clustered in Subgroup B
(n=7/9) were Grade III gliomas, further supporting that divergence of glycolytic carbons
plays an important role in the metabolic switch involved in glioblastoma. Although
Subgroup B consisted largely of tumors clustering to the PN subtype, these metabolic
profiles were otherwise largely independent from the transcriptional signatures (Fig. 5B).

We then investigated whether these identified metabolic subtypes were clinically relevant
by evaluating the outcome of patients from individual subgroups. As grade is a clear
prognostic factor in malignant glioma, Grade III and Grade IV tumors were evaluated
independently. Our initial evaluation compared Grade III tumors from Subgroups B and
C (n=7 and 8, respectively). Subgroup A was not analyzed due to low incidence (n=3).
We hypothesized that Grade III patients with Subgroup C metabolic profiles would have
a worse outcome, because it is more consistent with the profile observed in Grade IV
tumors. Consistent with our predictions, patients with Grade III tumors that clustered in
Subgroup B had a significantly improved overall survival (Fig. 5C; p=0.0104), with
median survival not being reached, compared to a median survival of approximately 28
months for Grade III tumors clustered in Subgroup C. We next compared Grade IV
tumors that clustered in Subgroup A (n=13) versus C (n=17). Interestingly, in contrast to
transcriptional subtypes, metabolomic subtypes identified prognostic relevance in Grade
IV tumors, with tumors clustering in Subgroup A demonstrating a significantly improved
median survival of 24.4 months versus 14.7 months in tumors clustering to Subgroup C (p=0.0165). Notably, Subgroup C had the worst outcome, independent of grade.

We then investigated whether specific signaling networks driving individual metabolomic subtypes could be identified. Using transcriptional profiles from individual tumors, glioma grade specific comparisons were made similar to those performed in the survival analysis, including comparisons between Grade III tumors clustering in Subgroups B and C and Grade IV tumors clustering between A and C. Interestingly, Statistical Analysis of Microarrays (SAM) analysis of global transcriptional profiles for all of these groupings identified no apparent genes that were significantly differentially expressed within a reasonable FDR (5%). As proof of concept, we performed similar analyses using SAM comparing transcriptional profiles between Grade II and Grade IV tumors. These studies identified several thousand probesets at a low FDR, (specifically 1970 genes at a > 2 fold change and a 0% FDR) which encompassed several well know pathways differentiating Grade II and IV tumors, including anti-apoptotic, STAT, ERK, ER stress, WNT and hedgehog signaling pathways.

Further analysis was performed to examine the relationship between the three identified metabolic profiles (anabolic, energetic and phospholipid catabolism) and the existing transcriptional subtypes. By using PCA on each metabolic profile and plotting the first two components, there was no discernable separation between transcriptional subtypes or grade (Fig. S4). To determine if any genes were correlated to metabolic profiles, we examined the correlation between the first PC of each metabolic profile and individual
gene expression. Only weak correlation was observed (max $R^2$; anabolic=0.53, energetic=0.53, phospholipid catabolism=0.31). Finally, we asked if there were gene expression differences among the three metabolic subtypes identified. Using the Kruskal-Wallis test for differences among the three subtypes and applying a false discovery rate filter of 5%, we identified 2,510 probesets (representing 1,605 genes) significantly different (Table S3). Post-hoc tests for pair-wise differences indicate that 85% of the differences are driven by Cluster B (Table S3). Mapping these genes onto biological pathways using GeneGO MetaCore, we identified several significant pathways including cell adhesion and cytoskeleton remodeling (Table S4 for full list of pathways identified). Taken together, this cross-platform analysis supports the concept that metabolomic profiles may provide a unique insight into the underlying biology of brain tumors beyond that recognized from traditional transcriptional signatures.

DISCUSSION

Here we describe, for the first time, global metabolomic signatures in glioma, which provide insight into their underlying biology that appears to have prognostic significance. An enhanced biosynthetic capacity from divergence of glycolytic carbons has been proposed as an important metabolic phenotype associated with tumorigenesis to provide the requisite genome, proteins, and lipids for these rapidly dividing cells, however this study represents one of the first to provide support for this adaptive process in human tumors. One important mechanism tumors have adapted to allow for glycolytic shunting involves modulation of PKM2 activity. By converting from its highly active tetramer
form that favors the conversion of PEP to pyruvate, to its less active dimer, upstream intermediates including PEP and 3-PG accumulate, increasing substrate availability for alternate pathways important for rapidly dividing cells (24, 27, 28, 31). Here, we identify some of the highest-ranking metabolites differentiating Grade IV from Grade II gliomas to be accumulation of PEP and 3-PG. Interestingly, within high-grade gliomas, integrative metabolomic, genomic and enzyme affinity assays identified PEP accumulation and decreased PK activity highly correlated with the mesenchymal subtype, representing a particularly aggressive subgroup in this malignancy. It should be noted that extrapolations of absolute flux cannot be made from single steady-state measurements, although inferences about relative flux can be made using the crossover theorem. Notably, this is complicated by the observation that the rate of feeding carbons into these pathways is highly variable, as measured with FDG-PET, and correlates with grade and survival. Nonetheless, our kinetic measurements revealed decreased PK activity in the “shunted” phenotype suggesting that these metabolic profiles can be generated, in part, by modulation of PK activity. Another limitation is that, although tissue was obtained in this study was rapidly frozen following excision, the resulting ischemia and hypoxia contributing to anaerobic metabolism within a specimen is difficult to control in the context of a resection. Therefore, the concentrations of specific metabolites may be affected by the unknown state of metabolic degradation during this period of ischemia and/or hypoxia. However, since comparisons made herein are between glioma grades, including the identified anabolic phenotype and altered phospholipid metabolism, we postulate that these same inconsistencies involving anaerobic glycolysis associated with surgery-related ischemia occur at a similar rate
between the low and high-grade lesions, essentially normalizing this effect. This limitation extends to the inability in determining grade-specific differences in pyruvate metabolism into lactate, which may not be accurately recapitulated in these studies based on the accelerated anaerobic metabolism following resection.

Although previously identified malignant glioma subtypes based on transcriptional signatures provide insight into underlying molecular heterogeneity, their biologic relevance still remains unclear. Identifying the accumulation of PEP in mesenchymal malignant glioma represents a previously unrecognized phenotype that may contribute to the aggressive nature of this subtype and be subsequently modulated as a form of metabolism-based cancer therapy. Further, recent findings identified transcriptional networks regulated by C/EBPβ and STAT3 are central to mesenchymal transformation in glioblastoma (33); therefore further work linking these pathways to modulation of PKM2 activity are warranted. In addition, despite lower PK activity in the mesenchymal subtype, there appeared to be higher overall expression of this enzyme when compared to tumors clustering into the proneural subtype, suggesting the potential for alternate functions of this enzyme. This is supported by recent work by Yang et al demonstrating the non-metabolic role of PKM2, involving translocation to the nucleus and EGFR-promoted β-catenin transactivation (34). Another possibility may involve PKM2 phosphorylation, and subsequent inactivation in this subtype (31), which was not evaluated in this study.
One important pathway in which cells divert carbon from glycolysis is through the PP pathway. This shunting allows for both the generation of 6-phosphogluconate and ribose 5-phosphate, which is used for nucleotide biosynthesis, and generates sufficient reducing potential for detoxification of reactive oxidative species (23, 26, 28). Here, we observed accumulation of both these intermediates, along with a 3.2 fold increase in reduced glutathione in Grade IV glioma, supporting this potential. By contrast, several pentitols, polyols derived from PP pathway intermediates, including ribitol and arabitol, as well as the precursor arabinose, showed significantly decreasing levels with increasing tumor grade. Moreover, arabitol and arabinose, as well as additional, related small sugar-derivatives were among the top 30 biochemicals in the RF-generated importance plot. The prevalence of this metabolite class among top RF biochemicals suggests that they provide robust indication of tumor grade progression. Although the metabolic pathways for many of these biochemicals in humans are not well-understood, because several of these biochemicals are found elevated in certain human metabolic disorders with PP enzyme deficiencies, this pattern of decreased levels for these PP pathway side-products may serve as a further indication of an activated PP pathway and heightened anabolic metabolism.

Another avenue for diversion of glycolytic flux is through de novo synthesis of serine and glycine, which represent precursors for a variety of biosynthetic pathways and epigenetic modulation through DNA methylation. The importance of this shift has been highlighted in two recent reports, identifying amplification in the gene phosphoglycerate dehydrogenase (PHGDH), the enzyme whose substrate is 3-PG, in a subset melanomas
and breast cancers (25, 35, 36). Our study identified accumulation of both serine and glycine in Grade IV tumors, suggesting activity of this pathway may also be relevant in glioblastoma biology. In addition to serine and glycine, other exogenous essential and non-essential amino acids are required to maintain such processes as protein synthesis, anapleurosis, and nucleotide biosynthesis during tumorigenesis. Accordingly, in our study, the accumulation of amino acid metabolites played a significant role in distinguishing Grade II and IV tumors, with 54% of the 86 amino acid metabolites identified demonstrating statistically significant differences. One of the amino acids that have been most extensively implicated in tumorigenesis is glutamine, which contributes towards the core metabolic needs of proliferating tumor cells, including providing bioenergetics, relieving oxidative stress, and complementing glucose metabolism through macromolecule production (37). Interestingly, increased accumulation of metabolites associated with glutamine metabolism was not associated with higher-grade glioma. Although glutamine may certainly still be an essential component of glioma metabolism, our findings suggest its metabolism does not appear to be altered between tumor grades.

This report has identified three unique subtypes in malignant glioma based on their metabolomic signatures. Through unbiased, hierarchical clustering of the glioma metabolome, metabolites associated with divergence of glycolytic flux, characterized by accumulation of PEP and 3PG, appeared to play an important role in the biology of a subset of these tumors. These analyses identified a unique, particularly aggressive metabolic subgroup defined by high PEP combined with a signature suggestive of lipid
catabolism; mainly consisting of decreased accumulation of several GPCs with no associated increase in phosphocholine. Several of these were among the top 30 biochemicals in the RF-generated importance plot. Interestingly, altered phospholipid catabolism has been previously described in glioma, with higher PCHo/GPC ratios found in the 1H spectra of human glioblastoma when compared to lower grade tumors (39). Altered phospholipid catabolism has also been observed in breast, prostate, and ovarian cancer (40-43). Further, recent studies suggest that in addition to serving as a “passive” structural building block, phospholipids also have the capacity of “actively” regulating cellular function (44).

In addition to insight into the underlying biology of glioma, the identified metabolic subtypes appear to also provide information on the aggressiveness of an individual tumor. Although limited by the number of samples analyzed and inability to account for known prognostic factors in glioma, including RPA class, 1p/19q status, and promoter methylation of MGMT, these profiles do suggest potential prognostic relevance with potential application for future trial stratification. Additional work will be required to confirm these findings in an expanded cohort accounting for these know prognostic factors. In addition, although names of specific signatures were coined (e.g. energetic, anabolic, and phospholipid catabolism), it is important to note that these were based on selected metabolites from an extensive list (Table S2) that clustered individual subtypes. Continued investigations will be required to determine the relative importance of these other metabolites in subtype designation and their overall influence on malignant glioma metabolism. Surprisingly, these identified metabolomic subgroups appeared to be
independent from both previously recognized malignant glioma subgroups and transcriptional profiles. This suggests that other global processes as genomic and/or epigenetic regulation, including EGFR amplification or mutation and PTEN activation, may be driving the observed metabolomic subtypes and that integrating these platforms may provide further insight into the signaling processes driving the observed metabolic phenotype.

Although these findings still require validation in an independent dataset, understanding the glioma metabolome offers the potential for several levels of clinical application. In addition to serving as a prognostic factor, subtype designation may allow to personalize therapy towards an individual tumor’s metabolic phenotype. For example, therapies designed to target the “energetic” subtype may involve Akt inhibitors, hexokinase inhibitors (45), or the metabolic modulator dichloroacetate (46), while a therapeutic regimen designed to target the anabolic phenotype of glioblastoma may involve agents designed to modulate PEP accumulation or shunting into the PPP, including such agents as transketolase inhibitors (45).

In conclusion, metabolomics provides a unique window into the phenotype of glioma that, when integrated with other platforms, may provide a more comprehensive understanding of the complex biology associated with glioma tumorigenesis and malignant transformation. These findings underscore a previously unrecognized metabolic heterogeneity in glioma with both biologic and clinical relevance. A richer understanding of aberrant metabolism will provide a framework for the design and
implementation of a personalized approach to malignant glioma therapy through metabolic modulation.

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REFERENCES


FIGURE LEGENDS

Figure 1. **Global metabolomic profiling in glioma identifies grade specific metabolic changes.** (A) A combination of high-throughput liquid-and-gas chromatography-based mass spectrometry was performed on a total of 69 fresh-frozen glioma specimens (grades and histologies are provided in Table S1). From a metabolomic library consisting of more than 2000 purified standards, a total of 308 named biochemicals were detected, and the metabolic pathways that the identified metabolites reside in are presented. (B) Following log transformation and imputation with minimum observed values for each compound, Welch’s two-sample t-tests were used to identify biochemicals that differed significantly between histological grades. Summaries of the numbers of biochemicals that achieve statistical significance (p≤0.05) are provided. (C) A summary of the metabolic pathways differentiating Grade IV and II tumors, with red and green indicating a statistically significant increase and decrease in identified metabolic pathways, respectively. Abbreviations: Gly, glycine; Ser, serine; Thr, threonine; Ala, alanine; Asp, aspartate; Lys, lysine; Phe, phenylalanine, Trp, tryptophan; Val, valine; Leu, leucine; Ile, isoleucine; Cys, cysteine; GSH, glutathione; FA, fatty acids.

Figure 2. **Random Forest (RF) analysis identifies key metabolites differentiating glioma grade.** RF analysis was performed to determine the capacity of global metabolic profiles to classify samples between tumor grades and to identify biochemicals important
to the classification; the top 30 biochemicals for the RF classification scheme are provided.

**Figure 3.** The metabolic signature of glioblastoma reflects accelerated anabolic metabolism. Schematic of metabolites involved in carbohydrate metabolism, comparing Grade IV to Grade II glioma. Metabolites in red reflect an increase accumulation in Grade IV tumors, green: decrease, black: no change, and gray: not identified in this analysis. Ratios were generated (in parenthesis) by normalizing the individual metabolite concentration to the median concentration of the respective metabolite obtained from all samples.

**Figure 4.** The mesenchymal subtype of malignant glioma is characterized by PEP accumulation and altered PKM2 expression and activity. Malignant glioma tumors (n=51) were classified as mesenchymal (mes; n=20), proneural (PN; n=22), and proliferative (PR; n=9) based on their transcriptional profiles (Fig. S3). (A) Box-plots were generated from the PEP ratios of tumor clustering to individual subtypes. (B/C) Of the initial 51 malignant glioma samples used for global metabolomic and transcriptional profiling, 23 samples had sufficient tissue remaining to evaluate for differential PK activity and PKM2 expression between subtypes (mes: n=8; PN: n=15). PK activity was determined using a calorimetric-based assay and PKM2 expression was determined by western blot, with expression quantified relative to β-actin.
Figure 5. **Global metabolism reveals metabolic signatures in malignant glioma.**

(A) Unsupervised, hierarchical clustering was performed on global metabolomic profiles generated in malignant glioma (n=51). (B) Glioma grade and transcriptional subtypes of tumors clustering to the identified metabolomic subtypes. (C) Kaplan Meier estimates for overall survival based on metabolomic subtype.
Figure 1

A

Metabolites Identified (n=308)

- Lipids
- Amino Acids
- Carbohydrates
- Xenobiotics
- Nucleotides
- Peptides
- Cofactors and vitamins
- Energy

B

<table>
<thead>
<tr>
<th>Biochemicals with ( p \leq 0.05 )</th>
<th>Grade III</th>
<th>Grade IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade II</td>
<td>81</td>
<td>159</td>
</tr>
<tr>
<td>Grade III</td>
<td>59</td>
<td></td>
</tr>
</tbody>
</table>

| Biochemicals \( \uparrow \downarrow \) | 35 | 46 | 84 | 75 | 36 | 23 |

C

Amino Acids (n=86)
- Gly/Ser/Thr
- Ala/Asp
- Lys
- Phe
- Trp
- Val/Leu/Ile
- Cys
- GSH

Lipids (n=108)
- Glycolipid
- Lysolipid
- Essential and med chain FA
- Carnitine

Carbohydrates (n=38)
- Sterol
- Creatine

Nucleotides (n=22)
- Gly/Ser/Thr
- Ala/Asp
- Lys
- Phe
- Trp
- Val/Leu/Ile
- Cys
- GSH

Grade IV vs. II
Biochemical Importance Plot

mean-decrease-accuracy

Increasing Importance to Group Separation

2-hydroxyglutarate
arabitol
5,6-dihydouracil
propionylcarnitine
hypotaurine
7-beta-hydroxycholesterol
erythrone
p-cresol sulfate
glycerophosphoethanolamine
cysteine sulfenic acid
7-alpha-hydroxycholesterol
myo-inositol
arabinose
1-palmitoleoylglycerophosphocholine
3-aminoisobutyrate
alanine
cis-vaccenate (18:1n7)
asparagine
2-palmitoylglycerophosphoethanolamine
1-palmitoleoylglycerophosphocholae
allo-threonine
phosphoenolpyruvate (PEP)
acetylcarbline
glycerophosphorylcholine (GPC)
hippururate
dehydroascorbate
arabinose
1-myristoylglycerophosphocholine
beta-alanine
erithritol

Figure 2
Figure 4
Figure 5