Research Article

The differential usage of molecular machinery in brain cancer patients with iron-enriched glioma environments

Brandon Lucke-Wold[1]*, Michael Joseph Diaz[1], Joanna Song[2], Sai Batchu[3], Kevin Root[1], Karan Patel[4] and Kamil Taneja[5]

[1]University of Florida, College of Medicine, USA

[2]University of South Florida, Morsani College of Medicine, USA

[3]Montville, NJ, United States, USA

[4]Rowan University, Cooper Medical School, USA

[5]Stony Brook University, Renaissance School of Medicine, USA

Introduction

Gliomas are neuroepithelial tumors in the brain or spinal cord that arise from glial or precursor cells and include astrocytomas, oligodendrogliomas, and ependymomas. They are the most common malignant primary central nervous system tumors, representing 75% of cases in adults and 24% of all cases of primary brain and CNS tumors [1,2]. Despite radiotherapy and temozolomide chemotherapy, which are the current gold-standard first-line treatments, patients with gliomas, particularly the highly aggressive and most common subtype glioblastoma multiforme, still have a poor prognosis with a median survival time of 15 months and 5-year survival rates of 5% [3-6]. Increasing evidence has demonstrated the potential of immunotherapies, including immune checkpoint inhibitors and antigen-specific cancer vaccines, for treating glioma [4-7]. However, these treatments demand a comprehensive understanding of the biological pathways involved in the glioma disease course. Bioinformatics has contributed significantly to the discovery of prognostic markers for gliomas [8], such as the IDH1/2-mutation [9] and MGMT methylation [10], thereby representing a promising approach toward elucidating the genes, pathways, and mechanisms associated with human gliomas.

In trace amounts, iron is an essential element used for basic cellular functions and growth and is used by numerous cell types, including neurons and glial cells where it is a cofactor in enzymes involved with synthesizing and metabolizing neurotransmitters, myelinating neurons, transporting oxygen, and producing ATP through oxidative phosphorylation [11]. When present unbounded and excessively in the body, iron has also been indicated as a risk factor for many diseases, which also include cancers [12,13]. Glial cells are at particular risk of iron overload and thus these pathologies since they are the cells with the highest concentration of iron in the brain [14]. As a cofactor, iron often alternates between its oxidation states, ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron. High amounts of these redox reactions in iron overload can cause free radical formation, such as hydroxyl radicals through the Fenton reaction, and subsequent DNA and protein damage as well as lipid peroxidation, all of which can ultimately result in tumorigenesis or ferroptosis [15]. Tumor cells exhibit iron-sequestering, through upregulating iron uptake and downregulating iron export pathways as well as diverting immune cells located in the tumor microenvironment (TME), which is necessary to support the increased ATP production for cell proliferation and to induce the expression of genes involved in the epithelial to mesenchymal transition (EMT) in these cells during cancer [16,17].

Besides transformation into tumors, cells in the presence of excess iron can undergo cell death due to iron-mediated

oxidative damage, a process known as ferroptosis. These dying cells then release damage-associated molecular patterns that activate the immune response and can either stimulate anti-tumor immunity to suppress tumorigenesis or induce an inflammatory response that promotes tumor growth in the TME [18]. Because of its role in tumorigenesis, ferroptosis has been the target of several chemotherapies, such as hepatocellular carcinoma [19], pancreatic cancer [20], colorectal cancer [21] and breast cancer [22]. Changes in the expression of genes involved in ferroptosis have also been associated with numerous other cancers [23], including gastric [24], ovarian [25] and lung cancer [26]. Regarding gliomas and other neurological tumors, ferroptosis and other iron metabolism are less studied, despite current knowledge of how excessive iron plays a role in cancer. However, the presence of databases, such as FerrDb [27] which contains known regulators and markers of ferroptosis and ferroptosis-disease associations, facilitates the use of high-throughput bioinformatic analyses to better understand how high levels of iron in glioma TME affect cancer progression from a cellular perspective.

Traditional exploration of the cellular pathways and sets of genes implicated in cancer activity commonly recruits gene set enrichment analysis (GSEA) [28], which requires sequencing data from two groups (e.g., case and control) to calculate a logarithmic fold change in gene expression and create a ranked gene list. This means that GSEA cannot be applied to studies that only measure subjects of one class, such as The Cancer Genome Atlas (TCGA) (https://www.cancer.gov/tcga) which contains many large genomic studies of only cancer patients. To solve this issue, the non-parametric, unsupervised method of Gene Set Variation Analysis (GSVA) [29] can be used instead of GSEA for similar functional analyses. GSVA calculates gene set enrichment scores in an analogous approach to conventional gene set tests but computes variation of gene set enrichment in samples independent of class labels, bypassing the need for explicit case/control classes.

The current study addresses the sparsity of research into high iron TME in brain cancers by studying expression changes of the gene in iron-metabolism pathways in glioma patients and assessing whether iron enrichment correlates with survival distinctions. Since our patient cohort is only glioma patients, we also employ GSVA in the study, which has not been used for studies on similar topics.

Methods

Figure 1 for a detailed schematic of the study design.

Data acquisition: Level 3 mRNA-Seq data and corresponding representing six hundred seventy-three (673) glioma patients with available overall survival information were obtained from the Broad Institute GDAC Firehose portal (https://gdac. broadinstitute.org/). Rounded, gene-level raw counts calculated by RNA-Seq by Expectation-Maximization were used for iron enrichment scoring. Analyzed specimens were obtained post-surgically (without treatment) and with treatment. Radiation therapy-level differences were not expected to appreciably influence total cellular mRNA expression. Table 1 for relevant tumor sample metadata.

Estimating iron enrichment: Tumor-microenvironment (TME) iron enrichment scores were estimated by gene set variation analysis (GSVA) using a merged gene signature constituent of previously published ferroptosis drivers and markers from the FerrDB web tool (http://www.zhounan.org/ ferrdb/)[27]. GSVA was run with variance-stabilized counts using the R package 'GSVA v1.44.2.

Case stratification: Enrichment scores for patients with >1 tumor sample were averaged. Case IDs among the top 30% of iron enrichment scores (ES: 0.05 to 0.35) represented the iron-enriched group ($n = 202$); case IDs among the bottom 30% of iron enrichment scores (ES: -0.32 to -0.1) represented the iron-
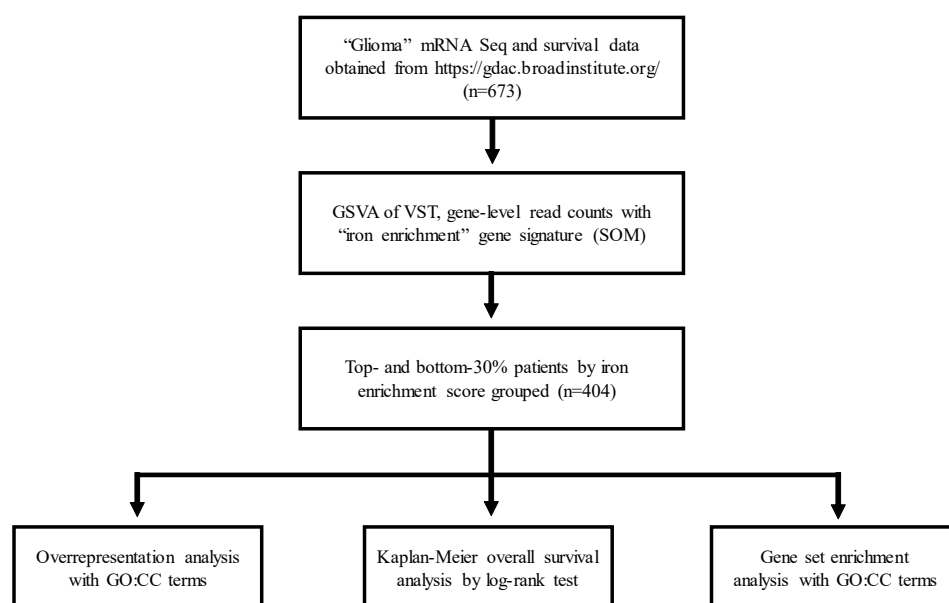

Figure 1: Study design.

depleted group (*n* = 202). A top and bottom 30% cutoff was applied to restrict downstream analyses to only differentially enriched (or non-zero ES) tumor samples.

Statistical analysis: All statistical analyses were conducted using R v1.4.3. Kaplan-Meier (KM) curves comparing the abovementioned case ID groups were generated using the R package 'survminer' v0.4.9. Iron-enriched case IDs were subjected to overrepresentation (log2 fold change (l2fc) > 1.5, min) and gene set enrichment analysis (l2fc > |1.5|) using gene ontology cellular component (GOCC) terms with R package 'cluster profile' v4.4.4. Input differentially expressed genes were pre-filtered by adjusted *p*- value < 0.05 and above described l2fc cutoffs after fold change shrinkage. GOCC terms with reported *Q*-values < 0.01 were visualized using the R package 'enrichplot' v1.16.1.

Results

KM analysis revealed that glioma patients with an iron-enriched TME reported worse overall survival (OS) rates than patients with an iron-depleted TME (*p* - value = 6.1e-07) (Figure 2). The iron-enriched subgroup observed a median OS of 1062 days (95% CI: 771 - 1458). The iron-depleted subgroup observed a median OS of 3571 days (95% CI: 1666–NA).

To resolve the predominating cellular components in iron-enriched glioma, overrepresentation analysis (ORA) was performed using the set of differentially expressed genes that are upregulated in the iron-enriched tumor population (Figure 3A). Of the top 30 most significant overrepresented GOCC terms, five distinct clusters were identified: 1) connective tissue and extracellular matrix components; 2) non-specific vesicle lumen components; 3) neutrophil components; 4) endocytosis components; 5) membrane-associated components not described by remaining clusters.

Gene set enrichment analysis (GSEA) was then employed with GOCC terms to functionally annotate the global set of differentially expressed genes among the iron-enriched
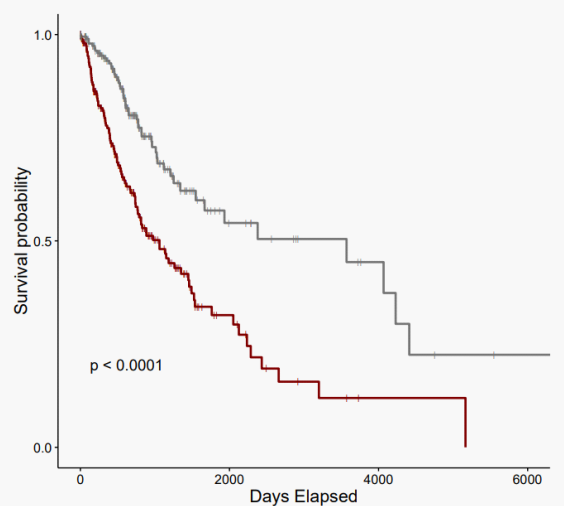
Figure 2: Kaplan-Meier analysis compares the overall survival of case IDs representing tumors among the top 30% by iron enrichment score (crimson, n=202) to the case IDs representing tumors among the bottom 30% by iron enrichment score (grey, n=202).

population (Figure 3B). The top-3 most significant GOCC terms were "external encapsulating structure," "extracellular region," and "extracellular space." These GO sets all represented positive normalized enrichment scores, indicating strong gene product localization to the extracellular domain in iron-enriched gliomas, compared to the group of iron-depleted gliomas.

Discussion

Here we obtained RNA-sequencing data from 673 glioma patients and calculated an iron enrichment score using GSVA based on the expression of established ferroptosis marker and driver genes from FerrDB to identify "iron-enriched" and "iron-depleted" subgroups. Representative case IDs were then subjected to Kaplan-Meier analysis to analyze survival differences. We reported that patients with ferroptosis-promoting glioma reported worse survival compared to those with low expression of similar genes (*p* - value < 0.0001, Figure 1). These results corroborate with other studies [30,31] which indicate that ferroptosis-related gene sets have prognostic value for human gliomas based on models created using different patient datasets and risk scores calculated via different computational methods (e.g., LASSO regression, random survival forest). Besides these differences, our study utilized a more comprehensive set of ferroptosis-related genes and suggests that increased ferroptosis is negatively correlated with patient outcomes likely as it is an indicator of high levels of iron in the TME.

Moreover, our study also investigated gene set enrichment in these iron-enriched individuals using Gene Ontology (GO) enrichment analyses of Cellular Component terms (CC). GOCC results categorized the genes into five clusters that can be summarized as a cluster pertaining to connective tissue and Extracellular Matrix (ECM) components, a cluster related to non-specific vesicle lumen components, a cluster consisting of neutrophil components, a cluster composed of endocytosis components and a cluster of membrane-associated components not in other clusters (Figure 2A). The cluster pertaining to

Table 1: Summary of tumor sample metadata.

Characteristic	N (%)
Gender	
Male	389 (57.8)
Female	284 (42.2)
Histological type	
Astrocytoma	195 (29.0)
Glioblastoma multiforme	158 (23.5)
Oligoastrocytoma	130 (19.3)
Oligodendroglioma	190 (28.2)
Received radiotherapy	
Yes	423 (62.9)
No	210 (31.2)
N/A	40 (5.9)
Dx date	
Before the year 2010	316 (47.0)
After the year 2010	357 (53.0)
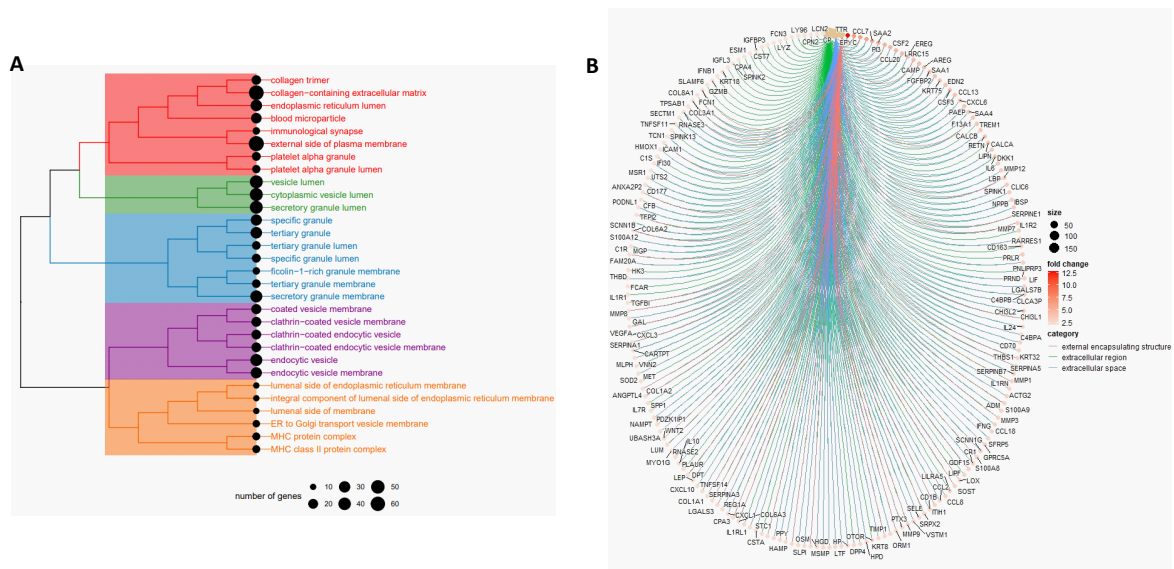
Figure 3: Gene ontology analysis of iron-enriched gliomas. (A) Tree-plot of the top-30 most significant GOCC terms by overrepresentation analysis, organized into five distinct clusters (l2fc > 1.5). (B) Gene concept network plot of top-3 GOCC terms by gene set enrichment analysis (l2fc > |1.5|).

connective tissue and ECM components derive from the presence of collagen-related, blood and platelet GOCC terms which are either component of the ECM or connective tissue [32]. The cluster consisting of neutrophil components was named because specific granules, tertiary granules and ficolin-1-rich granules are characteristic of neutrophils and these terms made up the majority of GOCCs found in this cluster [33,34]. The cluster composed of endocytosis components was defined because many GOCCs either contained terms explicitly denoting endocytosis or clathrin, a molecule that plays a critical role in one form of receptor-mediated endocytosis [35]. The cluster composed of non-specific vesicle lumen components summarized GOCCs that contained the term "vesicle lumen", or for the case of secretory granule, is a vesicle, but that did not fit in other clusters that also include GOCCs with "vesicle lumen" in its term. The presence of these last two clusters is likely explained in part by high amounts of enzymes involved in iron uptake and metabolism within a cell, which is done by clathrin-mediated endocytosis [36], which naturally accompanies an iron-enriched TME. Finally, GOCCs that did not seem to fit with the other clusters but contain the term "membrane" or "MHC", which are found on all nucleated cell membranes in the case of major histocompatibility complex class I or on all antigen-presenting cell membranes in the case of major histocompatibility complex class II, were thus summarized as a cluster of membrane-associated components, not in other clusters.

Within the connective tissue and extracellular matrix components cluster, there is high expression of collagen synthesizing genes such as COL1A1, COL1A2, COL3A1, COL6A2, COL6A3, and COL8A1 as well as LOX, which encodes an enzyme, lysyl oxidase, involved in collagen production, that all display at least a 2 times log fold-change in expression (Figure 2b). Previous evidence demonstrates that collagen acts as a scaffold to guide glioma cell migration in vitro [37,38] and is associated with an angiogenic shift and faster tumor growth

and invasion [39,40]. Collagen has also been suggested to be a prognostic marker as more organized collagen architecture is correlated with less invasive glioblastoma xenografts and longer patient survival [41]. While not shown in cells of the central nervous system, excess iron has been shown to increase collagen production in rat hepatic stellate cells [42]. Along with collagen, overexpression of canonical markers of angiogenesis, including vascular endothelial growth factor A (VEGFA), Wnt Family Member 2 (WNT2) and various matrix metallopeptidases (MMP), namely MMP3, MMP7, MMP8, MMP9, and MMP12, indicates extensive ECM remodeling in the gliomas of iron-enriched patients. Interestingly, one gene with relatively high log fold-change, epiphycan (EPYC), is also known to influence ECM organization and has been demonstrated to predict worse prognoses in ovarian cancer [43]. Similarly, transthyretin (TTR), another gene with relatively high log fold-change has been implicated in angiogenesis-related to pathologies of the brain [44] and other parts of the body [45,46]. These studies along with our results suggest that the worse survival outcomes in these patients could be due to excess iron contributing to increased collagen production and ECM remodeling for greater angiogenesis.

Finally, the cluster consisting of neutrophil components points to evidence of an increased inflammatory response in the glioma TME of iron-enriched patients as inflammation has been shown to recruit neutrophils in cancer and non-cancer situations [47,48]. This is further supported by increased expression of inflammation-related genes, including interferon-gamma (INFG) and IL6 interleukin-6 (IL6) (Figure 2B). Neutrophil-induced ferroptosis is also linked to increased necrosis in glioblastoma and poorer survival outcomes, theorized possibly because signals released from ferroptosed tumor cells that worsen neuroinflammation. The precipitated inflammation can then cause a cerebral cytokine storm that results in irreversible damage, organ dysfunction and death [49]. A large number of immune-related genes with

increased expression in the iron-enriched patients in our study compared to the iron-depleted patients (i.e., high log fold-change) reinforces this hypothesis and could explain another factor that contributes to the poorer survival outcomes in these patients.

However, as our results are purely based on in silico analyses using public databases, it is imperative to validate our computational analysis through experimental and clinical research. Nevertheless, our study can guide future investigations of how high iron environments can affect neurological tumors, particularly regarding potential biological pathways and genes on which to focus.

Conclusion

This present study reveals that glioma TME enriched for iron correlates with worse GBM survival outcomes. We identified glioma patients with an iron-enriched and an iron-depleted tumor environment based on the combined expression of various ferroptosis markers and drivers and then examined the groups for survival distinctions, of which the iron-enriched group reported poorer OS rates. We further studied the differential expression of genes between the groups and leveraged GO term enrichment to analyze subcellular differences. This analysis provides evidence of higher levels of a neutrophil-driven response, inflammation, angiogenesis and ECM remodeling which may all play a role in explaining the worse prognoses of iron-enriched glioma patients.

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