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Article

The effects of dysregulated ion channels and vasoconstriction in glioblastoma multiforme

Rohit Mahesh[1], Nitya Mahesh[2]
[1] Salem High School, Canton, Michigan
[2] New York University, New York, New York

SUMMARY

Glioblastoma multiforme (GBM) is the most aggressive tumor of the brain and is characterized by its ability to rapidly grow blood vessels at the tumor site, the capacity for self-renewal, and resistance to chemotherapy and radiation. Despite extensive research on GBM, the physiological basis for these characteristics is unclear. Clinical studies show that blocked blood vessels and reduced blood flow may cause rapid tumor growth and tumor resistance. Blocked blood vessels have been attributed to pseudopalisading necrosis, a self-protecting mechanism to prevent tumor cell damage. We hypothesized that vasoconstriction may underlie GBM growth. When peritumoral blood vessels constrict, blood flow may be blocked. This blockage prevents immune cells and drugs from reaching the tumor thereby fostering tumor growth through highly proliferative cells. We conducted literature searches and gene expression analysis to identify signaling pathways impacted in patients with GBM. Using GEO2R, a genetic analysis tool developed by NCBI, we found that the γ-aminobutyric acid (GABA) signaling pathway was uniformly enriched, and both n-type voltage-gated calcium channels (n-VGCC) and NMDA signaling pathways were uniformly depleted in GBM patients when compared to healthy individuals. These findings suggest a reduced calcium diffusion into the endothelial cells of the blood vessels surrounding the tumor, which could result in vasoconstriction of the tumor's blood vessels. Our analysis of the gene expression data supports our hypothesis that vasoconstriction may be an important factor in giving GBM its characteristics.

INTRODUCTION

Glioblastoma multiforme (GBM) is an aggressive grade IV tumor of astrocytes, the star-shaped cells in the brain and spinal cord responsible for supporting neuronal function and maintaining the brain's microenvironment (1). GBM is one of the deadliest brain cancers, with over 250,000 cases diagnosed globally each year (2). In the United States alone, GBM accounted for 14% of all tumors and over 17,000 deaths (3). Even with aggressive treatments like surgery, chemotherapy, and radiation therapy, patient survival rarely exceeds 15 months, and the tumor almost invariably recurs (4).

A hallmark of GBM's aggressiveness is its ability to sustain rapid growth and evade therapeutic interventions through vascular dysregulation. GBM tumors rely heavily on angiogenesis—the formation of new blood vessels from pre-existing ones—to maintain their supply of oxygen and nutrients (5,7). Several vascular abnormalities, including blockages, glioma-related edema, and necrotic niches, have been identified in GBM tumors (6,8-10). Blockages restrict blood flow, exacerbating hypoxia - a lack of oxygen in the tumor microenvironment (6,8). Hypoxia, in turn, promotes angiogenesis and accelerates tumor progression (6,8). Glioma-related edema contributes to increased intracranial pressure and tissue damage, further destabilizing the tumor environment (9). Necrotic niches, characterized by central regions of cell death surrounded by pseudopalisading cells, stabilize hypoxia-inducible factors (HIFs) that further drive angiogenic pathways (10).

These vascular abnormalities not only fuel tumor growth but may also impair immune responses and reduce the effectiveness of treatments (11). Reduced blood flow limits the infiltration of immune cells, such as leukocytes, into the tumor microenvironment, hindering the body's ability to mount an effective immune response (12). This immune suppression, coupled with the hypoxic conditions, could enable GBM to grow unchecked and evade therapeutic interventions (13).

In healthy tissues, calcium (Ca^{2+}) signaling is critical for regulating vascular tone, neuronal communication, and cellular homeostasis (14). Dysregulation of calcium signaling promotes vasoconstriction, the narrowing of blood vessels, which reduces the blood flow (15,16). Reduced blood flow restricts oxygen delivery contributing to hypoxia, which further triggers vasoconstriction (17). Given the established role of Ca^{2+} signaling in maintaining vascular tone, we investigated the molecular drivers of its dysregulation in GBM.

Several molecular pathways, including those involved in neurotransmission and ion channel regulation, modulate Ca^{2+} signaling and vascular tone (18). Given the critical role of Ca^{2+} signaling in vascular regulation, we predicted that Ca^{2+} signaling may be impacted in GBM. We hypothesized that vasoconstriction in peritumoral blood vessels may be driven by dysregulation of Ca^{2+} signaling pathways. To test this hypothesis, we performed differential gene expression analysis to identify transcriptional signatures associated with vasoconstrictive signaling in GBM tissue. By linking these molecular changes to functional consequences such as impaired perfusion and immune exclusion, we aim to clarify the role of Ca^{2+}-mediated vascular dysregulation in GBM progression. Understanding these mechanisms may reveal novel targets to restore blood flow, improve immune access, and enhance treatment efficacy.

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RESULTS

To test whether Ca^{2+} signaling pathways are dysregulated in GBM, we performed a differential gene expression analysis to identify genes associated with vascular tone, ion transport, and calcium signaling that are significantly altered in tumor tissue. To ensure that our findings were representative of a broad patient population, we aggregated datasets that included GBM patients, epilepsy surgery patients, individuals with vascular abnormalities, and healthy brain tissue controls. We used eight datasets from Gene Expression Omnibus (GEO) repository comprising a total of 350 individuals, including 284 neoplastic GBM samples and 66 non-neoplastic samples (**Table 1**).

We calculated the logarithmic fold change (logFC) in gene expression between healthy samples and those with GBM to quantify how much expression of each gene differed between the two conditions. Fold change measures the magnitude of this difference and reporting it on a logarithmic scale allows for symmetrical comparison of up- and downregulated genes (19). We classified genes as significantly upregulated or downregulated using a logFC threshold of ±1.5, based on criteria established in a previously published study (20). A total of 56,553 genes were found to be expressed across all datasets, with 32,444 upregulated and 24,109 downregulated (**Figure 1**).

Based on our review of multiple journals on calcium signaling, we compiled a list of genes that encode the structure and function of key regulators, including NMDA receptors (NMDAR), γ-aminobutyric acid (GABA) receptors, N-type voltage-gated Ca^{2+} channels (n-VGCC), metabotropic glutamate receptors, inositol triphosphate receptors (IP3R), and ryanodine receptors (RyR) (21-25). We then selected genes that, in our own dataset analysis, met selection thresholds of average $|\log_2(\text{fold change})|$ greater than 1.5 and an adjusted *p*-value less than 0.01, based on values reported in a previously published study.

To ensure reproducibility, we further required that each gene exhibit consistent regulation (either up- or downregulation) in at least four of the eight GEO datasets. For each receptor or channel, we evaluated the proportion of genes meeting these criteria; if more than 50% of the associated genes were consistent, we considered that channel or receptor category to be dysregulated in GBM. Out of 20 channel and receptor categories evaluated using our selection criteria—including thresholds for log2(fold change), statistical significance, and consistency across datasets—only three met these thresholds and were selected for further analysis due to their low dispersion across datasets: GABA receptors, n-VGCC, and NMDAR.

Dataset	Title of publication	Number of tumor samples	Number of non-tumor samples	Patient ages	Male patients	Female patients	Tumor size sample
GSE186057 (39)	Expression levels of RAD51 inversely correlate with survival of Glioblastoma Patients	24	12	18-64: 14 65+: 10	18	6	Quadrants Q1: 11.1 Q2: 21.25 Q3: 31.35 Q4: 41.18
GSE90598 (40)	Simultaneous miRNA and mRNA transcriptome profiling of glioblastoma samples reveals a novel set of OncomiR candidates and their target genes	16	7 (5 healthy tissues, 2 from patients undergoing other brain surgeries)	ND	10	6	ND
GSE111260 (41)	Improved prognostication of glioblastoma beyond molecular subtyping by transcriptional profiling of the tumor microenvironment	67	9 (4 healthy tissue from GBM patients, 3 healthy tissues, 1 astrocyte, 1 fetal astrocyte)	18-64: 52 65+: 15	ND	ND	ND
GSE116520 (42)	Transcriptome profiling reveals PDZ binding kinase as a novel biomarker in peritumoral brain zone of glioblastoma	34	8 (Tissues from patients undergoing surgery for drug resistant epilepsy due to mesial temporal sclerosis)	ND	30	12	ND
GSE4290 (43)	Neuronal and glioma-derived stem cell factor induces angiogenesis within the brain	81	23	ND	ND	ND	ND
GSE13276 (44)	Gene expression profile of glioblastoma peritumoral tissue: an ex vivo study	5	3 (tissues from patients operated for deep cavernomas with radiological signs of recent bleeding)	ND	ND	ND	ND
GSE25632 (45)	miR-181d: A predictive glioblastoma biomarker that downregulates MGMT expression	103	5	<18: 4 18-64: 96 65+: 3	67	36	ND
GSE90836 (46)	Prediction and Analysis of Key Genes in Glioblastoma based on Bioinformatics	9	9 (tissues from patients undergoing epilepsy surgery)	ND	ND	ND	ND

Table 1: GEO datasets analyzed in this work. The gene expression data series, the title of the paper where the series is listed, sample size, demographics, and tumor characteristics, where available from each of the datasets selected for our analysis. All datasets were obtained from GEO (39-46). ND = no data.

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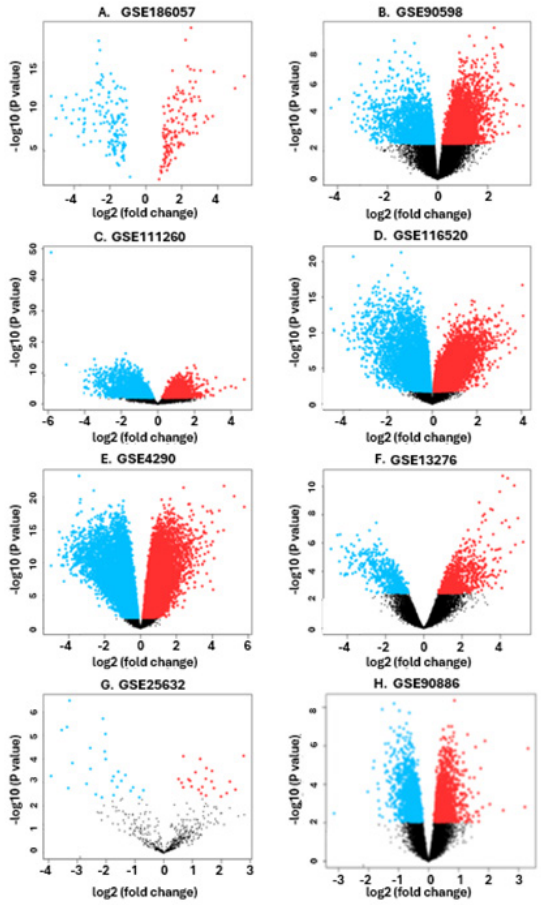

Figure 1: Differential gene expression in GMB and healthy neoplasm across individual datasets. Comparisons of logFC gene expressions between GBM and healthy patients for each of the eight datasets: **A)** GSE186057, **B)** GSE9059B, **C)** GSE111260, **D)** GSE116520, **E)** GSE4290, **F)** GSE13276, **G)** GSE25632, and **H)** GSE90886. The genes are significantly differentially expressed at a default adjusted p-value cutoff of 0.05. Data points in red indicate upregulation, blue indicate downregulation, and black indicates no significant difference.

We found that all significant genes within the VGCC pathway were consistently downregulated across all datasets, with log2 fold changes ranging from approximately -1.5 to -4.0, reflecting a uniform pattern of decreased expression (**Figure 2**). Genes involved in the GABA-gated chloride channel were uniformly upregulated, with all genes showing positive log2 fold changes between roughly +0.5 and +2.2, indicating increased expression in every dataset analyzed (**Figure 3**). Likewise, genes in the NMDA-selective glutamate receptor pathway exhibited uniform downregulation, with $\log_2$ fold changes ranging from approximately -1.5 to -4.8 (**Figure 4**).

DISCUSSION

The uniform dysregulation of genes encoding components of GABA, VGCC, and NMDA pathways may create a distinct molecular environment in GBM, which we suggest could contribute to vasoconstriction and thereby the aggressive nature of the disease. The upregulation of GABA receptors subunit genes in GBM introduces a layer of complexity to calcium signaling dysregulation. GABA is the brain's main inhibitory neurotransmitter that binds to and activates GABA receptors, such as GABAA, on the cell membrane (22-24). Upon activation, these receptors allow chloride ions to enter the cell, creating a hyperpolarized state that drastically lowers the electrical potential gradient (25). This hyperpolarization inhibits the activation of calcium-dependent ion channels, further decreasing intracellular calcium concentrations. In endothelial cells surrounding GBM tumors, this hyperpolarization likely contributes to reduced calcium signaling and promotes vasoconstriction, thereby limiting blood flow to the tumor.

Another essential component of calcium signaling homeostasis and vascular regulation is the n-VGCC (also called Cav2.2), a distinctive type of VGCC commonly found in the brain (26). These channels, located along the plasma membrane, open when the cytoplasm is depolarized, allowing calcium ions to flow into the cell (27). This initial calcium influx triggers a secondary process called calcium-induced calcium release (CICR) through large, homotetrameric ryanodine receptors (RyRs) on the endoplasmic reticulum (28). The resulting release of intracellular calcium stores generates calcium "sparklets" that are critical for vasodilation (29). Therefore, the downregulation of n-VGCCs in GBM likely reduces calcium influx, impairing CICR and disrupting vasodilatory signaling. This disruption favors vasoconstriction.

Finally, we found that NMDARs, which are critical for excitatory neurotransmission and long-term potentiation, are also downregulated in GBM tissues. These receptors are normally activated when neurotransmitters such as glycine and glutamate bind to their ligand sites, allowing calcium ions to enter the cell (30). In their inactive state, NMDA receptors are blocked by magnesium ions during cytoplasmic depolarization (31). When NMDA receptor activity is reduced, calcium influx is diminished, further impairing the depolarization needed for VGCC activation. This downregulation across NMDA receptors, VGCCs, and RyRs results in a cascading deficit in calcium signaling, which we speculate favors vasoconstriction and contributes to the aggressive characteristics of GBM.

We suggest that the co-occurring upregulation of GABA receptor subunit genes and the concurrent downregulation of VGCC and NMDAR signaling, as observed across all datasets, may synergistically disrupt calcium signaling pathways in GBM. This disruption in calcium entry and mobilization could undermine the coordinated calcium signaling required for arteriole vasodilation. As a result, these alterations may promote vasoconstriction and redirect blood flow away from healthy tissue toward the tumor mass. Future studies should explore whether astrocyte calcium signaling does influence tumor vascular dynamics, as we predict, through functional experiments *in vitro* and *in vivo*.

The consequences of vasoconstriction in GBM extend beyond hypoxia and vascular dysregulation. Reduced blood flow plays a crucial role in GBM's ability to evade immune responses. Vasoconstriction prevents adequate leukocyte infiltration into the tumor microenvironment, reducing the immune system's capacity to target neo-antigens expressed by tumor cells (32,33). In addition, the hypoxia promoted by vasoconstriction stabilizes necrotic niches, which release damage-associated molecular patterns (DAMPs) that promote low-grade inflammation (34). This inflammatory environment may paradoxically protect GBM by attracting

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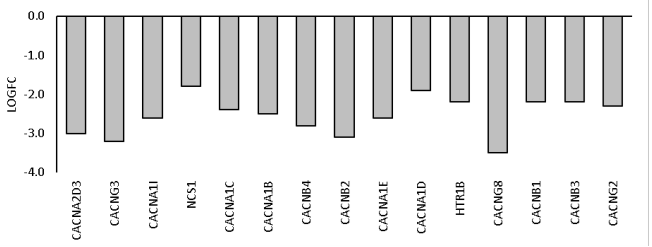

Figure 2: VGCC gene expressions are consistently downregulated. logFC values of the genes that are part of the VGCC signaling pathway. These genes are downregulated in GBM patients.

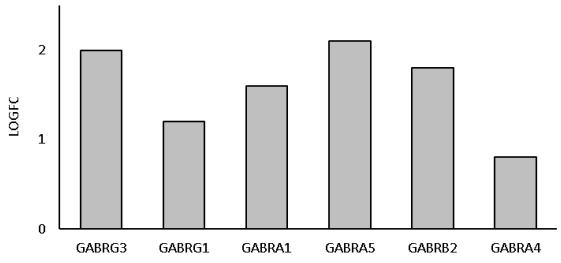

Figure 3: Differentially expressed genes involved in GABA signaling pathway are consistently upregulated. logFC values of the genes that are part of the GABA signaling pathway. These genes are consistently upregulated in GBM patients.

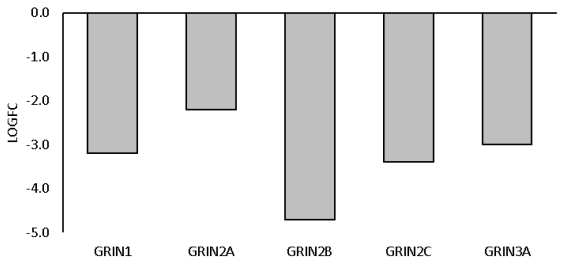

Figure 4: Differentially expressed genes involved in NMDA receptor signaling pathway are consistently downregulated. Differences in gene expression, calculated as logFC values, of the genes that are part of NMDA signaling pathway. These genes are consistently upregulated in GBM patients.

immune cells that are unable to reach the tumor in sufficient numbers, thereby neglecting quiescent tumor cell populations that evade detection. Prolonged exposure to inflammatory cytokines and the hypoxic microenvironment also drives T-cell exhaustion, a state of diminished function characterized by metabolic dysfunction and expression of inhibitory receptors such as Programmed Death 1 (PD-1) (35). These factors collectively enable GBM to evade immune surveillance and sustain its rapid growth despite therapeutic interventions.

Our findings suggest that targeting calcium signaling dysregulation may offer new therapeutic targeting opportunities for GBM. Restoring VGCC activity or counteracting GABA receptor upregulation with the goal of normalizing calcium signaling may be able to reduce vasoconstriction and improve blood flow in GBM. These interventions have the potential to alleviate hypoxia and additionally enhance the delivery of chemotherapeutic agents and efficacy of leukocyte infiltration into the tumor microenvironment (36). Furthermore, combining vascular restoration therapies with immune checkpoint inhibitors, which aim to reverse T-cell exhaustion, may amplify anti-tumor immune responses and improve patient outcomes (37).

We note that our study has several limitations. The datasets we analyzed included subjects with varying tumor stages, pathologies, and microarray types, which may affect the generalizability of our findings. Additionally, our analysis focused on genes that were uniformly dysregulated across datasets, which may have excluded other relevant pathways. Future studies should validate our findings experimentally. For instance, inducing gliomas in genetically engineered mouse models and selectively disrupting gene expression could help determine whether the observed dysregulations directly contribute to vasoconstriction. Imaging studies of GBM vasculature could also help confirm the relationship between vasoconstriction and tumor progression. Our findings, combined with these future investigations, could pave the way for novel treatments targeting these pathways that are specifically dysregulated in GBM.

MATERIALS AND METHODS

Data from the NCBI Gene Expression Omnibus (GEO) was downloaded, containing samples from glioblastoma (GBM) patients and non-GBM control individuals with no known brain malignancy (**Table 1**). The results were manually filtered to include only those in which expression profiling had been conducted through microarray platforms and where mRNA or microRNA expression data had been provided. Additionally, the sample size was restricted to datasets with 20 or more samples. From this restricted list, eight datasets were selected for analysis: GSE186057, GSE90598, GSE111260, GSE116520, GSE4290, GSE13276, GSE25632, and GSE90836 (39-46).

GEO2R, a web-based statistical analysis and visualization tool provided by the NCBI GEO, was then used (47). Differentially expressed genes (DEGs) between two or more experimental conditions, such as diseased and healthy samples, were identified using GEO2R with datasets deposited in GEO. Specific gene expression data were accessed by inputting the accession numbers of the selected datasets into GEO2R. The samples were separated into diseased and healthy categories using the “Define Samples” function. Samples with outliers were examined and removed using boxplot normalization in the visualization section to ensure the reliability of the analysis. The degree of gene upregulation or downregulation was calculated using logFC.

To determine statistical significance, a modified t-test was used to derive *p*-values for the differential expression of genes. The probability that the observed difference in expression occurred by chance was represented by the *p*-value. The results were filtered to retain only genes with *p*-values less than 0.01, indicating a high level of statistical confidence in the results.

To integrate miRNA data, custom software was developed to map each miRNA to its corresponding gene, since no standard toolsets for direct comparison of miRNA data with mRNA data are currently available. After miRNA and mRNA data were integrated, the analysis was further restricted to DEGs with logFC values greater than or equal to 1.5 in

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magnitude to focus on the most significantly dysregulated genes. From this filtered list, the DEGs were ranked by *p*-value, and the top 500 genes were retained. The Search Tool for the Retrieval of Interacting Genes (STRING), a publicly available database of protein-protein interactions, was then used to map interactions between the selected genes and to identify functional pathways (48). Insights into the relationships and functional roles of genes were provided by STRING through the analysis of interactions such as direct binding, enzymatic activity, and co-expression.

A confidence score was assigned by STRING to each protein-protein interaction to quantify the probability that a reported interaction is biologically meaningful. These scores were calculated using experimental evidence, curated databases, co-expression data, and computational predictions. For this analysis, pathways with confidence scores of 0.9 or higher, representing interactions with high reliability, were retained (48).

Through a literature search, calcium signaling pathways with a direct impact on vasoconstriction were identified (18). These pathways were further refined by matching their associated genes with the DEGs identified from our datasets. Genes with an average |logFC| greater than 1.5 and a *p*-value less than 0.01 across at least four datasets were selected. Pathways for which over 50% of their associated genes met these criteria were retained for further analysis. The identified pathways were then uploaded to STRING to identify the level and significance of upregulation or downregulation. The relationship between vasoconstriction in GBM and the mechanisms by which GBM resists chemotherapy and immune responses was then described using this analysis.

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