Glioma Cancer Immunosuppressive Microenvironment

by

Avani Ahuja

B.Tech Biotechnology, Jaypee Institute of Information Technology, India, 2016

Submitted to the Graduate Faculty of
the Department of Human Genetics
Graduate School of Public Health in partial fulfillment
of the requirements for the degree of
Master of Science

University of Pittsburgh
2019
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2019
Abstract

Glioblastoma (GBM) tumors, World Health Organization (WHO) grade IV tumor, are the most malignant tumors. Despite of aggressive standard treatment regimens including surgical resection, radiotherapy and chemotherapy temozolomide (TMZ), GBM patients have a high mortality rate and poor prognosis. A weak immunogenic and immunosuppressive tumor environment of GBM presents as a barrier for the newly developed immunotherapies. Na/H exchanger isoform 1 (NHE1) expressed in GBM drives H⁺ efflux in exchange for Na⁺ influx for maintaining an alkaline intracellular pH and acidic tumor microenvironment. Imbalance in the pH homeostasis, known as the Warburg effect, triggers cancer cells to rely on oxidative glycolysis instead of mitochondrial oxidative phosphorylation. In our study, we investigated roles of NHE1 in GBM bioenergetic metabolism and effects of pharmacological blockade of NHE1 on glycolysis and oxidative phosphorylation in glioma. Our results show that inhibiting NHE1 increases glioma cell oxidative phosphorylation, suggesting that NHE1 could be a therapeutic target for reversing the altered tumorigenic metabolism to treat GBM. Moreover, the emergence of blockade of NHE1 and TMZ combined treatment as cancer therapy would increase patient survival significantly and hence have a great public health relevance.
# Table of Contents

Preface ......................................................................................................................................... viii  
1.0 Introduction ............................................................................................................................. 1  
2.0 Background on Glioblastoma .............................................................................................. 2  
  2.1 Pathological Features .................................................................................................. 2  
  2.2 GBM Clinical Presentation ..................................................................................... 3  
  2.3 Therapy ....................................................................................................................... 4  
3.0 GBM Microenvironment ...................................................................................................... 5  
  3.1 Macrophages ............................................................................................................... 6  
    3.1.1 Tumor Associated Macrophages and Microglia .............................................. 8  
  3.2 T-cells ......................................................................................................................... 8  
  3.3 Recruitment of Regulatory T-Cells ........................................................................ 10  
  3.4 Infiltration of Myeloid-Derived Suppressor Cells .................................................. 11  
4.0 Sodium/Hydrogen Exchanger Isoform 1 (Nhe1) In GBM .............................................. 12  
5.0 Materials and Methods ..................................................................................................... 17  
  5.1 Materials .................................................................................................................... 17  
  5.2 Cell Cultures and Authentication ............................................................................ 17  
  5.3 Drug Treatment ......................................................................................................... 18  
  5.4 Seahorse Extracellular Flux (XF) Analysis ............................................................ 18  
  5.5 Protein Quantification .............................................................................................. 19  
  5.6 Data Analysis ............................................................................................................. 20  
  5.7 The Seahorse XF Glycolysis Stress Test ................................................................. 20
List of Figures

Figure 1 Representation of the GBM tumor cell interaction with surrounding immune environment............. 6
Figure 2 Role of NHE1 in dysregulated pH (pHe and pHi) and cancer progression.......................... 12
Figure 3 SLC9A1 mRNA expression .......................................................................................... 13
Figure 4 NHE1 protein expression in human gliomas............................................................... 14
Figure 5 TMZ stimulates NHE1 protein expression in GL26-cit tumor ........................................ 15
Figure 6 The Seahorse XF Glycolysis Stress Test Profile ............................................................ 21
Figure 7 The Seahorse XF Mito Stress Test Profile ..................................................................... 23
Figure 8. TMZ treatment increases glycolysis of GL-26 cells .................................................... 26
Figure 9 Combined T+H treatment increased mitochondrial function of GL-26 cells ................. 28
Preface

I would like to take this opportunity to acknowledge my sincere gratitude to Dr. Dandan Sun for her tutelage, guidance and critical inputs throughout this project. She has a dynamic and progressive personality, and I am extremely fortunate to have her as my mentor. Dr Dandan always found time to edit my drafts and offer vital suggestions to help me and this purposeful project to become a success. I want to thank Dr. Nabiul Hasan, post doc in Dr. Sun’s lab, for being my main go-to person for any problems, big or small. He has kept me on track and his expert and timely feedback has been most valuable. Additionally, a very big thanks to all the members of Sun Lab without whose help this project would not have seen the light of day. I will miss the lab meetings with them. The various excursions we had together will remain with me forever as fond memories. I would also like to thank Dr. Quasar Padiath and Dr. Ilyas Kamboh for their expertise on neurobiology and insights on the document. Thank you for your suggestions and guidance. It has been a collective effort by everyone that has led me and this thesis to its destiny. Humble thanks and regards are due to my family, both here and back in India, for their love and support. They were extremely understanding and have always been there for me. Their advice always kept me grounded and have shown me the right path whenever I strayed. A big thank you to my little sister for always making me laugh and reminding me to cheer up and not take everything so seriously. Thanks to all my friends who not only understood my commitment to the lab but also made my meals whenever I was preoccupied. The past two years would not have been possible without all of you. Finally, to all my fellow classmates and faculty, thank you for making the graduate school an amazing and unforgettable experience that I would cherish forever. You are all awesome!
1.0 Introduction

Glioblastoma (GBM), a WHO grade IV astrocytoma, is the most common type of malignant brain tumor found in adults and has a poor prognosis in glioma patients with a short median post-diagnosis survival time (approximately 20 months) (1). The main reason for having poor prognosis is that the tumor is mostly diagnosed at a very late stage also making therapy less efficient. The standard care for GBM include surgical resection followed by a high dose treatment with radiotherapy and Temozolomide (TMZ), an alkylating chemotherapy drug (2). TMZ is an alkylating agent which produces lethal DNA lesions and used as a first-line chemotherapy for GBM. There are however a few limitations to using TMZ as a therapeutic agent such as an acquired resistance of the cells to any TMZ associated DNA damage (3). This is done through the function of DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) or at times the cells have an environment which is supportive of the tumor microenvironment (TME) (4). At times due to incomplete removal of the tumor during surgery glioma cells adopt a highly aggressive behavior. GBM are classified as primary and secondary GBM. Primary GBM are de novo cases and occur in 95% of people suffering from GBM (1). Secondary GBM arise from low grade astrocytoma (grade I-III) and are rare (seen in about 5% of the population). Primary GBM are as result of mutations in the epidermal growth factor receptor (EGFR), phosphatase and tensin homolog (PTEN), cyclin dependent kinase inhibitor 2A (CDKN2A), platelet derived growth factor receptor alpha (PDGFRα), tumor protein p53 (TP53), MDM2 proto-oncogene (MDM2) genes which occur in older populations hence, making the age of onset of this GBM around 55 years (5). The mean age of onset for secondary GBM is about 45 years. The altered TP53 gene is the main cause for secondary GBM (6).
2.0 Background on Glioblastoma

Gliomas are tumors derived from glial cells and are one of the most frequently occurring tumors of the central nervous system (CNS). GBM is the most malignant astrocytic glioma (7). Although it is a rare tumor, it impacts the life of the affected individuals. GBM affects adults and has an increasing incidence till 85 years of age. The median age for diagnosis the disease is 64 years. The incident rate of GBM is 1.6 times higher in males than females (5). Reports show that glioma incidence is higher in developed countries as opposed to developing countries with the explanation that glioma incidence in the under-developed countries is under-ascertained due to limited access to healthcare, improper diagnosis and reporting of cases. There seems to be inter-racial differences in the prevalence of GBM as reported, in the United States where GBM is frequently seen in Caucasians rather than people of African or Asian descent (8). There are several risk factors associated with the disease such as ionizing radiation, dietary factors, severe head injury, and occupational risk although not consistently confirmed in all studies. Although, gliomas have been observed to run in families no genes have been implicated thus far.(7).

2.1 Pathological Features

It has been seen during diagnosis that GBM lesions are large and occupy most of the brain lobe. The lesions are seen to be in the subcortex white matter of the cerebral hemispheres extending across the frontal and temporal lobe at times. The tumor mass is characterized as being poorly delineated with high heterogeneity. Cancer cells with high proliferation are found in the peripheral
zone. The central tumor area consists of necrotic tissue and 80% of the total tumor mass. Major diagnostic factors for GBM are presence of areas with vascular hyperplasia, necrosis in the tumor tissue. Another factor unique to the disease is the rapid invasion of the tumor in the surrounding brain tissue especially in the myelinated brain structures like the corpus callosum or perivascular spaces. It has been observed that despite having a high infiltrating nature GBM does not invade the subchoroidal (9) space nor the vessel lumen which makes distant metastases of the tumor within or outside the CNS rare. GBM lesions are often categorized as primary GBM or secondary GBM. Primary lesions are known to affect older patients, mean age of 62 years at diagnosis, secondary lesions are seen in a younger population, mean age of 45 years. Phenotypically, both the GBM are indistinguishable but they have genetic differences. While primary GBM arises with abnormalities in the epidermal growth factor receptor (EGFR) pathway, loss of heterozygosity (LOH) of chromosome 10q, mutation of the phosphatase and tensin homology (PTEN) gene, and deletion on the p16 gene the secondary GBM is a result of mutations in tP53 gene, LOH of chromosome 10q and abnormalities seen in the retinoblastoma (RB) tumor suppressor gene. These mutations lead to overactivation and overexpression of many signaling pathways which leads to uncontrolled cell growth of glioma cells (9).

2.2 GBM Clinical Presentation

Prominent first clinical signs of GBM are due to increased intracranial pressure causing headache, nausea, and papilledema. Some other frequent symptoms include focal neurological deficits, changes in the personality, memory loss, and confusion. Some patients are also known to experience episodes of epileptic seizures (4). Magnetic resonance imaging (MRI) or Computed
tomography (CT) tests are mainly used to scan the brain for diagnosis of GBM. Definitive diagnosis is almost only confirmed histopathologically at surgical debulking of the tumor (4).

2.3 Therapy

The high rate of recurrence rate of GBM makes it quite incurable with a five-year patient survival rate less than 5%. The reason for this is the late stage diagnosis of the disease and inefficient removal of complete tumor from the brain. Standard therapy involves surgery which is followed by radiation. Due to its high infiltration of the tumor it is not possible to surgically remove the entire tumor and surgical debulking only helps to maximally reduce the tumor size. The expectation is that once most of the tumor has been excised, the remaining portion can be eliminated through radiotherapy. Common side effects of GBM therapy include toxicity to surrounding brain structures and growing cells of non-neuronal tissues. Long-term effects include cognitive deficits and epilepsy. Temozolomide(TMZ) is an oral alkylating agent which is the main chemotherapeutic agent used against GBM. It has good penetration to the normal blood-brain barrier. However, the obstacles to glioma therapy include an acquired resistance to TMZ-mediated DNA damage via the function of the DNA repair protein methylguanine methyltransferase (MGMT). Another approach is to target the tumor microenvironment. Inhibiting immune cells like macrophages, regulatory T cells (Tregs), and proteins such as PD-1, which support tumor proliferation, are likely to improve the immunosuppressive tumor environment. Evidence suggests that these strategies may only be optimally used with conventional therapies and not as frontline monotherapy on any newly diagnosed, untreated disease.
3.0 GBM Microenvironment

A major part of the tumor volume of GBM is made up by immune cells (30% - 40%). Brain tumors are composed of diverse and heterogeneous cells. All the cells contribute to the brain tumor biology in unique ways. Tumor associated macrophages and microglia (TAMs) are known to engage in bidirectional cross-talk with tumor cells in the brain (4). The tumor cells in turn release cytokines and chemoattractant which recruit TAMs to the microenvironment that supply pro-tumorigenic and pro-survival factors. The GBM cell environment has cells which interact with immune cells. GBM cells damage the blood brain barrier (BBB) leading to infiltration of immune cells into the brain. These cells secrete various factors which trigger immunosuppressive phenotypes in immune cells. The activity of immune cells is severely affected. (12). Antigens are presented to T cells by Dendritic cells (DC). This results in an anti-tumor immune response. This response can be further enhanced by factors that are released in the microenvironment such as reactive oxygen species (ROS). Neutrophils are known to play a role during brain metastasis and assist in tumor cell colonization. T regulatory cells suppress cytotoxic T cells which leads to an immunosuppressive microenvironment that is permissive to tumor growth. These T cells can be activated via immune checkpoint inhibitors such as CTLA- and PD-1 (13).
3.1 Macrophages

Macrophages are attracted as monocytes towards the tumor by a range of factors. It is known that the monocytes are formed in the bone marrow and circulate through the body via the blood stream. Once recruited to the tumor site, the monocytes are modified into tumor-associated macrophages (TAMs) (14). As a member of the immune system, TAMs respond to the tumor cells by providing the cell with all kinds of cytokines, like EGF, TNF-α, VEGF, bFGF and IL-8 which can have anti- as well as pro-tumor influences. The macrophages are also able to present antigens of the tumor towards other immune cells, causing an immune response against the tumor. Some tumor cells can evade this immune response and thereby have a favorable outcome above others. Macrophages can also kill tumor cells directly in two ways, one via macrophage mediated tumor
cytotoxicity (MTC) or other through antibody dependent cellular cytotoxicity (ADCC), both of
which cause lysis of tumor cells. MTC is a process in which the macrophage needs to be near the
tumor cell, and factors are secreted that result in lysis of the tumor cell. ADCC is antibody
dependent, where the antibody binds the tumor cell to the macrophage. This binding also causes
lysis of the tumor cells (12). In addition to the above, these cells also contain functions that have
the opposite effect. Many studies have shown that TAMs can promote metastasis, however, there
is not much known about in which steps of the metastatic cascade they are involved, and what the
molecular mechanism is behind these steps. What is known is that macrophages promote
angiogenesis by secreting several different factors that promote angiogenesis. An example of such
a molecule is the vascular endothelial growth factor (VEGF), which is involved in the formation
of new vessels during tumor progression. The TAMs release this factor, as reaction to hypoxia in
a tumor. This factor together with many others enables the formation of new blood vessels, that
are important for the further progression of tumors. Also, these vessels provide a route for
metastasis of the tumor cells (13). Besides angiogenesis, TAMs can promote invasion by, for
example secretion of cytokines or enzymes like matrix metalloproteases that degrade the
extracellular matrix surrounding the tumor. As a result, the tumor cells move more easily through
their surroundings and travel towards the blood vessels. How tumor cells move across the
membrane of the blood vessels is still not completely clear, it has been suggested that the tumor
cells bind to the TAMs to help them across. In conclusion, TAMs can have two roles, an anti- as
well as a pro-metastatic effect. Which effect is favored is probably dependent on the tumor type,
and other cells present in the microenvironment that influence TAMs (14).
3.1.1 Tumor Associated Macrophages and Microglia

One of the most abundant immune cells found in the GBM microenvironment are tumor associated macrophages (TAMs). They originate from the macrophage lineage. They are of two kinds, M1 and M2. Macrophages and microglia combined with M2 phenotype are known to stimulate inflammation. Other factors known to promote tumor growth are factors like IL-6 and IL-1. TGF-β is also secreted by TAMs and is known to increase invasion capacity of tumors (14). They do so by secreting proinflammatory cytokines. The M1 phenotype are known to decrease inflammation by shifting to Th2 immune response and secreting anti-inflammatory cytokine IL-10 which inhibits cytokines IFN-γ and TNF-α. The M2 phenotype increases activity of TAMs in the glioma environment by increasing the activity of the STAT3 pathway. This leads the M2 phenotype to support tumor invasion and angiogenesis which contributes to the immunosuppressive microenvironment of GBM. Secretion of CSF-1, TGF-β1, and macrophage inhibitory cytokine 1 (MIC-1) by glioma cancer stem cells (gCSCs) polarizes TAMs into an immunosuppression (15).

3.2 T-cells

Besides the TAMs there are many other cells present in the microenvironment of a tumor such as the T cells. Classification of the different T cells, into CD4+, CD8+ and regulatory T cells, provided more insight into the different functions in the microenvironment of the tumor. most responses are also organ-specific and give either a pro- or anti-tumor response. The CD4+ T cells consists of a group with different lineages, best known are the helper cells, TH1 and TH2. The TH1
cells are anti-tumorigenic, whereby release of their cytokines directly kills tumor cells. The TH2 cells, on the other hand, inhibit apoptosis and induce the proliferation of tumor cells of breast carcinomas (16). This function was demonstrated vivo by the injection of human breast cancer cell lines, whereby the injection of TH2 cells leads to accelerated growth of the tumors. In the case of metastasis, an in vivo study demonstrated that depletion of CD4+ T cells led to less development of metastasis. It appeared that the CD4+ T cells were able to regulate the pro-tumor capacities of the TAMs, where TH2 cells are involved. Thus, they stimulate TAMs and their pro-tumor properties, and metastasis is the resulting consequence. The other subset of T cells, the CD8+ cells consist of cytotoxic T lymphocytes (CTLs). These CTLs recognize antigens presented on the surface of a tumor cells and are subsequently able to kill these antigen-presenting tumor cells. The antigens consist of small peptide sequences presented by the tumor cells using a major histocompatibility complex I (MHCI). In case of antigen recognition, the CTL binds to the target cell and releases granules with several enzymes. These enzymes form pores in the cell membrane of the target cell and attack the DNA of the cell. The pore formation allows other enzymes to enter the cells, which eventually leads to apoptosis of the tumor cell. The antigens presented by the tumor cells differ between distinct tumors. thus, when an antigen is recognized by CTLs the tumor cells are destroyed, thereby providing an anti-tumor capacity (17). However, evidence suggest that metastatic tumors can evade this process of apoptosis. Various mechanisms are used to escape cell death such as the reduction of the number of MHCI or antigen peptides. A third group of T cells; the regulatory T cells, are known to regulate the immune response of other immune cells. It is still not exactly known how this regulation is controlled. However, previous research showed two possible mechanisms, either via direct cell-cell contact between the regulatory T cells and the other immune cell, or via the secretion of anti-inflammatory mediators, like IL-10. Moreover, it is
demonstrated that depletion of this subtype of T cells leads to rejection of tumor cells in mice, suggesting that the regulatory T cells in normal circumstances inhibit immune responses. The tumors introduced in the mice with depleted T regulatory cells were rejected rapidly and these mice survived, while the control group died rapidly due to the growing tumors. It can be said that there are three subsets of T cells that together have pro- and anti-tumorigenic properties (18).

3.3 Recruitment of Regulatory T-Cells

Regulatory T-cells (Tregs) cause immunosuppression on the GBM microenvironment and immune cells. Cell contact interactions and secretion of cytokines with immunosuppressive effects result in the recruitment of Tregs. Tregs can be attracted via STAT3 pathway inducing HIF-1-α production under hypoxic conditions and via CCL2 signaling through interaction with chemokine receptor type 4 (CCR4) of Tregs. Glial cells and macrophages are the main sources of CCL2. (19). The GBM microenvironment consists of two types of Tregs. One is population of CD4+ CD25+ thymus derived natural Tregs (nTregs) which are responsible for immune regulation and the other is induced Tregs(iTregs) which are differentiated from peripheral CD4+ CD25+ T-cells and under immunosuppressive conditions. TGF-β and IL-2 secretion convert peripheral naïve T-cells into iTregs. iTregs can also be induced via FoxP3 expression and through interaction of PD-1 molecules with naïve T-cells. The thymus derived Tregs are found abundantly in the GBM microenvironment. Expression of transmembrane immune checkpoint CTLA-4 is also known to weaken immune response. CD8+ cytotoxic activity is inhibited in the GBM microenvironment. IL-17+ Tregs are induced which secrete IL-17 and TGF-β which are known to block the activity of CD8+ cells. (20).
3.4 Infiltration of Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are heterogeneous cells which originate from the myeloid lineage. They have immunosuppressive properties in the GBM microenvironment. There are different populations of MDSC in GBM, CD14+ and CD15+. MDSC are activated and migrated to GBM microenvironment by the secretion of various cytokines such as VEGF, IL6 and G-CSF (21). CCR2 is a surface receptor expressed by MDSCs and bind to CCL2 secreted by TAMs. Upregulation of arginase I is known to block T-cell activity. It is overexpressed in MDSCs and reduces L-arginine amino acid which is essential for the function of T-cells. (22).

In addition, it has been reported that GBM restrain antitumor mechanisms of natural killer cells (NK). Natural Killer cells are myeloid lineage lymphocytes and are cytotoxic. They inhibit receptors that recognize abnormal MHC molecule expression and secrete granzyme and perforin with antitumor cytokines to attack tumor cells. (23). Differentiated NK cells inhibit CD8+ T-cell activation and proliferation. (21). Thus, cells in the GBM microenvironment employ various mechanisms to escape immune surveillance creating an immunosuppressive microenvironment and promoting tumor growth. It is. It is important to understand the biology and mechanisms behind the GBM tumor as it will help to develop novel approaches for immunotherapeutic treatment for the disease.
4.0 Sodium/Hydrogen Exchanger Isoform 1 (Nhe1) In GBM

Sodium/hydrogen exchanger 1 (NHE1) is encoded by SLC9A1 gene (Solute Carrier family 9A1). It is part of a large family of integral membrane protein transporters. They facilitate in the counter-transport of protons and sodium ions across lipid bilayers. NHE1 plays an important role in tumorigenesis, proliferation, migration, and metastasis of GBM. NHE1 exchanges extracellular Na⁺ for H⁺ efflux and maintains an alkaline pH of 7.3-7.5 in the glioma cells (2). This is one of the important steps for glycolytic metabolism. Cancer cells, glioma cells here, thrive by creating an environment with increased glucose uptake and production of lactic acid even in the presence of oxygen and functional mitochondria. This is mechanism is widely known as the Warburg effect (24).

Figure 2 Role of NHE1 in dysregulated pH (pHe and pHi) and cancer progression.

It has been recently reported that NHE1 has an important role in invasion and proliferation in glioma cells. Glioma cells express high levels of NHE1 for maintaining alkaline intracellular pH. The protein also aids in GBM cell migration. In addition, recent studies show that NHE1 interacts with glioma-associated microglia/macrophages and result in activation of microglia and abolished microglia-stimulated glioma migration and proliferation (11). TMZ treatment is known to increase NHE1 protein levels in GBM cells as novel mechanism of TMZ resistance (3). A recent bioinformatics study analyzed SLC9A1 mRNA expression in two data sets, the Chinese Glioma Genome Atlas (CGGA) and The Cancer Genome Atlas (TCGA), revealed significantly high expression of SLC9A1 mRNA expression in all grades of tumors (fig 3). Recent findings also suggest that high expression of SLC9A1 is associated with poor survival prognosis (10) (fig 4).

![Figure 3 SLC9A1 mRNA expression](image)

Overexpression of SLC9A1 mRNA is associated with malignancy in gliomas. a, b SLC9A1 mRNA expression in different WHO Grade gliomas from CGGA and TCGA datasets, respectively. Data are mean ± SD. *, p < 0.05; **, p < 0.01; ***, p < 0.001 Guan, et al. Journal of Experimental & Clinical Cancer Research (2018)
Figure 4 NHE1 protein expression in human gliomas


Other recent experiments in glioma mouse model (GL26-cit), express NHE1 protein, show elevated NHE1 expression in response to TMZ (T) treatment for 24 hour. Treatment of glioma cells with NHE1 inhibitor HOE642 (H) had no effect on NHE1 expression but a combinatorial treatment of T+H abolished TMZ-induced elevation of NHE1 protein (10). The report shows that blocking of NHE1 with HOE642 sensitizes glioma cells to TMZ induced cytotoxicity. The glioma mouse models received four treatment regimens, Veh-control, HOE (H), TMZ(T) and T+H. NHE1 protein levels were low in Veh-control treated mice, the protein level was further reduced with HOE642 treatment. In contrast, TMZ treatment displayed a significant increase in NHE1 protein expression.
Administration of T+H showed blocked TMZ-mediated effects on the tumor (11). Additionally, blocking NHE1 with TMZ and anti-PD1, and T+H and anti-PD1, an immune checkpoint inhibitor regulating T cell tumor immunity, showed prolonged survival of the glioma model (11). (Fig. 5)

Figure 5 TMZ stimulates NHE1 protein expression in GL26-cit tumor

TMZ stimulates NHE1 expression in glioma. a Kaplan-Meier survival analysis of glioma patients with high NHE1 (SLC9A1) mRNA expression (n = 136) and low SLC9A1 mRNA expression (n = 135) in CSE16011 cohort. ***p < 0.001. b SLC9A1 gene expression in primary glioma (n = 20) and matched recurrent glioma (n = 20) were obtained from the RNA-seq data of TCGA dataset. *p < 0.05. c, d SB28-GFP cells or GL26-cit cells were exposed to TMZ
(100 μM), HOE642 (1 μM), or combined for 24 h and cell lysates were harvested for immunoblotting of NHE1 protein. Data are means ± SEM from five independent experiments (n = 5), *p < 0.05, **p < 0.01. c Experimental protocol and location of data collection. Representative immunostaining of fixed brain sections (25 μM) for NHE1 protein expression in GL26-cit tumor. Data are means ± SEM (n = 5). *p < 0.05, **p < 0.01, ***p < 0.001. Guan, et al.

**Aim**

As mentioned above, improving efficacy of glioma treatments, including immunotherapy, still poses a challenge, despite extensive research going on in the field. TMZ is a first line of chemotherapeutic drug but often the tumors acquire resistance to it. NHE1 expression has shown to support oxidative glycolysis by dysregulating intracellular pH and promoting tumor proliferation [11]. NHE1 is also shown to support an immunosuppressive environment and reduces antitumor immunity in GBM [10]. Studies have revealed administration of TMZ results in increased expression of NHE1 in GL26 mouse glioma models [3]. Blocking of NHE1 expression with HOE642 along with TMZ in a combinatorial treatment has shown to reduce tumor progression and increase survival in mouse glioma mouse models [3]. In this study, we wanted to further understand the effects of inhibiting NHE1 on glioma bioenergetics by using Seahorse Extracellular XF Analyzer in GL26 glioma mouse cell line.
5.0 Materials and Methods

5.1 Materials

Cariporide (HOE642) and TMZ we from Sigma Aldrich (St. Louis, MO). Dulbecco’s Modified Eagle Medium (DMEM/HEPES) from Gibco (Carlsbad, CA). Fetal bovine serum (FBS) Invitrogen (Carlsbad, CA). XF glycolysis stress test kit containing glucose, oligomycin, and -deoxyglucose (2-DG) and XF mito stress kit including oligomycin, carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP), rotenone and antimycin A were obtained from Seahorse Biosciences Inc. (Billerica, MA, USA). XF96 cell culture plates, sensor cartridges, Seahorse XF base medium, and calibrant were also purchased from Seahorse Biosciences Inc.

5.2 Cell Cultures and Authentication

The mouse glioma GL26 cell line was derived and grown as described previously (23) and maintained as adherent culture in DMEM medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1x Penicillin/streptomycin and 1mM of sodium pyruvate. Cultures were passaged approximately every 4 days with fresh medium at a density of 5x10^4 cells/25cm^2 in a culture flask.
5.3 Drug Treatment

GL-26 cells were dissociated in the DMEM media and plated (3000 cells/well in 80 μl) in the XF96 microplate. The plate was kept in the cell-culture hood at room temperature for 1 hr and incubated in the CO2 incubator at 37°C for 24 hour. The cells were subjected to 48 hour drug treatment: Control (DMSO, 0.2% in media), HOE642 (100 μM), TMZ (50 mM), or TMZ+HOE combination. All the drug solutions were freshly prepared in DMEM media and the cells were treated with the freshly made solutions at 24 hour and 48 hour, respectively.

5.4 Seahorse Extracellular Flux (XF) Analysis

The seahorse XF analyzer is an instrument that measures oxygen consumption rate (OCR) and Extracellular Acidification rate (ECAR) in live cells on a 96 well microplate. On the day prior to running the assay, the XF96 sensor cartridge was hydrating by adding 180 μl of XF calibrant solution to all the wells of the utility plate. The sensor cartridge was placed on top of the utility plate to submerge the sensors in the calibrant. The cartridge was placed in a non-CO2 incubator (37°C) overnight. On the day of running the experiment, XF-DMEM Assay Medium was prepared. Agilent Basal DMEM was used which was supplemented with D-gglucose (10 mM), L-glutamine (2 mM) and Sodium Pyruvate (2 mM) for the Mito Stress Test and only L-glutamine (2 mM) for Glycolysis Stress Test. 50 ml of media of for each test was prepared and kept warm in 37°C bead bath. The cells of the XF96 plate was washed once with the appropriate assay medium and finally 180 μl of assay medium was added to each well. The plate was kept at 37°C non-C02 incubator for one hour.
Mito stress test compound preparation: Inhibitors Oligomycin (2 mM), FCCP (1 mM), Rotenone (1 mM), and Antimycin A (1 mM) were prepared in media and added into Port A (20 ul), Port B (22 ul), Port C (25 ul) respectively.

Glycolytic Stress Test compound preparation: Inhibitors Glucose (10 mM), Oligomycin (2 mM), and 2 – DG (1 M) were prepared in media and added into Port A (20ul), Port B (22ul), Port C (25ul) respectively.

5.5 Protein Quantification

Media was aspirated from the XF96 microplate at completion of XF assay. Cell lysis was done using 0.15 % SDS. BCA assay was performed for protein quantification. Albumin standards were prepared in 0.15% SDS and 25 μl added to 96-well plate. Sample from the XF96 microplate were also added to the 96-well plate (25 μl). Working reagent was prepared by mixing BCA reagent A and BCA reagent B (1:50) and 200 μl added to the standards and sample. The plate was incubated for 30 minutes at 37° C and absorbance was measured colorimetrically using ELISA Reader (Spectra MAX 190, Molecular Devices, Sunnyvale, CA) at 562 nm. SoftMaxPro software was used to acquire the data.
5.6 Data Analysis

Seahorse Wave Desktop software was used to analyze and calculate the glycolysis stress assay and Mito stress assay parameters. The software generated XF glycolysis stress test report and XF Mito stress test report automatically. The data was exported to Excel for further analysis.

5.7 The Seahorse XF Glycolysis Stress Test

The key parameters derived from a glycolytic stress test are shown in a sample ECAR graph in Figure (fig 6). The individual bioenergetics parameters of OXPHOS (fig 7) were calculated:

**Non-mitochondrial respiration** = it is the minimum rate measured after injection of Antimycin A.

**Basal respiration** = it is the last (rate measured before Oligomycin injection) – (non-mitochondrial respiration rate).

**Maximal respiration** = (maximal rate measured after FCCP injection) – (non-mitochondrial respiration rate).

**ATP production** = (last rate measured before Oligomycin injection) – (minimum rate measured after Oligomycin injection).

**Proton leak** = (minimum rate measured after Oligomycin injection) - (non-mitochondrial respiration rate).

**Spare reserve capacity** = (maximal respiration – basal respiration).
Glycolysis and oxidative phosphorylation are pathways which the cells use to generate energy. Glucose in the cell is either converted to pyruvate and converted to lactate in the cytoplasm, or CO2 and water through OXPHOS in the mitochondria. Lactate production from conversion of glucose to pyruvate and then to lactate results in production of protons in the extracellular medium.(25). This extrusion of protons results in cells having an acidified medium in its extracellularly forming an environment supporting tumor proliferation. In normal cells glycolysis converts one molecule of glucose to two molecules of pyruvic acid and energy is released in form of ATP. Glycolysis is used by our cells to provide energy and intermediated for other cell processes used by the body. It occurs in the cytoplasm and in normal cells one glucose molecule would produce 2ATPs, 2 NADH and 2 pyruvates. The two pyruvates in the presence of oxygen will further form to acetyl-CoA molecules which further goes to function in the citric cycle. The acetyl CoA molecule from the citric acid cycle produces 6 NADH molecules, two FADH₂ molecules, and two ATP molecules out of which 6 NADH +2 FADH, plus the NADH produced in glycolysis enter the
electron transport system (ETS). In cancer cells there is a change in the cellular respiration, they switch to an aerobic pathway in the presence of oxygen which produces less energy and hence to overcome their energy demands these cells have a higher rate of glycolysis than normal cells (25). This process and hallmark of cancer cells is called the ‘Warburg effect’. The cells are incubated in a glycolysis stress test medium without any glucose or pyruvate and then the extracellular acidification rate is measured. The first step includes injecting the plate with glial cells with a glucose injection for the cells to catabolize through the glycolytic pathway and convert to pyruvate thus producing ATP, NADH, water and protons. This causes an increase in the ECAR. The second step includes injecting oligomycin into the cells. Oligomycin is an ATP synthase inhibitor which causes mitochondrial ATP production to cease and this shifts the energy production to glycolysis which further increases the ECAR and pushes the cells to their maximum glycolytic capacity. The third and final step is injecting the cells with 2-deoxy-glucose (2-DG) which is a glucose analog and inhibits glycolysis as it binds to hexokinase in the glycolytic pathway. This causes a decrease in the ECAR (fig 6).

5.8 Oxygen Consumption Rate (OCR)

The OCR is measured using the Mito Stress Test to see the mitochondrial function of cells. It uses compounds that target specific stages of the electron transport chain (ETC) in the mitochondria to reveal parameters of the metabolic function. ETS is also called as cellular respiration, it yields 40 ATP per glucose. The rate is measured via a three-step process where compounds are injected to in the XF96 plate seeded with the glioma cells (25).
The compounds used for this test are Oligomycin, FCCP and mixture of rotenone and antimycin A. The first compound to be added is oligomycin which inhibits ATP synthase, complex V, of the ETC. This causes a decrease in OCR which correlates with the mitochondrial respiration associated with cellular ATP production. The second modulate which is injected is Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP). FCCP is an uncoupling agent which collapses the proton gradient and disrupts the mitochondrial membrane potential which causes the uninhibited electron flow through ETC and complex IV consumes oxygen to the maximum. The third and last compound injected is a mixture of rotenone and antimycin A which inhibit complex I and complex III of the ETC respectively. This combined inhibition shuts down mitochondrial respiration completely which enables to calculate non-mitochondrial driven respiration by processes other than the mitochondria. ETS is where the most efficient form of ATP production takes place (fig 7) (15) (26).

![Figure 7 The Seahorse XF Mito Stress Test Profile](image)

Figure 7 The Seahorse XF Mito Stress Test Profile

Figure illustrates the three key parameters of mitochondrial respiration: basal respiration, ATP production, and mitochondrial respiration. Tilsta, et al. J Clin Invest. 2018
5.9 Statistical Analysis

The results were expressed as the mean ± standard error of mean (SEM). We used Prism 8 (GraphPad Software, Inc), statistical significance was determined by using One-Way ANOVA test which was followed by Bonferroni’s multiple comparison test for multiple comparisons. A p value of <0.05 was considered statistically significant.
6.0 Results

The results of the experiments conducted in the lab are collated below.

6.1 Glycolytic Stress Test

Extracellular Acidification Rate (ECAR) is a measure of lactic acid produced during glycolysis. ECAR was calculated for every cell of the XF96 microplate. GL-26 glioma cells were grown on a 96-well plate and treated with Veh (control), Hoe, TMZ and T+H for 48 hours. A representative mean bioenergetic trace from the Glycolytic stress test is shown in Fig. 8A. On addition of glucose an instant increase in ECAR was seen reflecting an increase in glycolysis (Fig. 8A). Subsequent addition of oligomycin further increased glycolysis (Fig. 8A). ECAR response was completely abolished by addition of Hexokinase inhibitor 2-deoxyglucose (2-DG) (Fig. 8A). We saw a significant increase in glycolysis in cells treated with TMZ (~ 1.5-fold, p<0.05) although no change was observed in HOE treated cells. Interestingly, T+H treatment significantly reduced the ECAR canceling the increase of glycolysis caused by TMZ treatment. Similarly, TMZ treatment increased glycolytic capacity (p=0.08) which was subsequently reduced by T+H treatment. No changes of glycolytic reserve were observed with any treatments. These results indicate that TMZ increases the cells glycolytic metabolism which can be reduced by the combinatorial treatment of T+H. The results are consistent with the previous works which show that TMZ administration increases NHE1 expression which in turn supports an alkaline intracellular tumor environment.(27)
The impact of TMZ treatment and inhibition of NHE1 activity on glycolysis was assessed in GL-26 cells. (A) Representative bioenergetic trace for Glycolytic stress test. Glycolysis (B), Glycolytic Capacity (C), and Glycolytic Reserve (D) was calculated. All data are mean ± SEM, n=6, *p< 0.05 vs indicated, #p< 0.05 vs control.

6.2 Mitochondrial Stress Test

Oxygen consumption rate (OCR) was calculated at every measurement point for each well of the XF96 plate used in the mitochondrial stress test. GL-26 glioma cells were grown on a 96-well plate and treated with Veh (control), Hoe, TMZ and T+H for 48 hours. A representative mean
bioenergetic trace from the Mitochondrial stress test is shown in Fig 9A. On addition of oligomycin a decrease in OCR was seen reflecting minimal mitochondrial respiration (Fig. 9A). Subsequent addition of FCCP increased OCR, by collapsing the proton gradient in mitochondrial membrane, this caused a surge in OCR (Fig. 9A). OCR was completely inhibited by addition of mix of Rotenone and Antimycin A (R/A) (Fig. 9A). We saw a significant increase basal OCR, in cells treated with HOE, TMZ, and T+H in basal respiration as compared to Veh-control (Fig. 9B, p<0.05). In addition, T+H treatment showed significantly higher OCR than HOE and TMZ single treatment (p<0.05). Similarly, T+H treatment showed significant increase in maximal respiration and spare respiratory capacity compared to Veh-control (Fig. 9C, D; p<0.05). Increased ATP production was seen in cells treated with T+H combination treatment as well as HOE and TMZ single treatment (Fig. 9E; p<0.05). Importantly, significant increase of ATP production was seen in cells treated with T+H combination treatment compared to TMZ single treatment (Fig. 9E; p<0.05). These results indicate that T+H combined treatment increased mitochondrial respiration in GBM cells.
Figure 9 Combined T+H treatment increased mitochondrial function of GL-26 cells

The impact of TMZ treatment and blockade of NHE1 activity on mitochondrial function was assessed in GL-26 cells. (A) Representative bioenergetic trace for Mito stress test. Basal respiration (B), Maximal respiration (C), Spare respiratory capacity (D), and ATP production (E) was calculated. All data are mean ± SEM, n=4; *p< 0.05 vs indicated, #p< 0.05 vs control.
7.0 Discussion

7.1 NHE1 Protein and GBM Metabolism Dysregulation

We compared the bioenergetics between different drug treatments in GL-26 cells. We have previously shown that TMZ stimulates NHE1 expression which maintain intracellular alkaline pH causing tumor’s resistance to TMZ therapy and enhanced proliferation (3, 27). Our results show that the HOE and T+H treated cells had lower ECAR than TMZ treated cells, thus indicating that GL-26 cells when treated with TMZ produce large amounts of lactate regardless of availability of oxygen. TMZ mediated elevated NHE1 expression extrudes the intracellular proton and maintains alkaline pHi. HOE and T+H treatment has also shown to increase OCR and ATP production in the cells indicating more mitochondria mediated metabolism. Thus, HOE and T+H treatment reverse the Warburg effect and enables mitochondria mediated bioenergetics in GBM cells. However, it is not clear how the increase of mitochondrial metabolism would help in inducing apoptosis or any kind of cell death. One possible mechanism is the induction of ferroptosis, a form of cell death resulted from the oxidative stress produced by increased oxidative phosphorylation in the presence of lysosomal iron ion (20). However, additional study is needed to proof this concept of ferroptosis with increased mitochondrial metabolism when cells treated with HOE and TMZ combination. Thus, our results suggest that our previously reported positive outcome of combinatorial NHE1 blockade and TMZ therapy (10) could be due to the metabolic alteration of tumor cells.
8.0 Summary

Gliomas and in particular GBM is a highly complex and heterogenous tumor with no cure and little benefit from therapies. The median survival time is still a worrisome factor. GBM affects the tumor microenvironment (TME) by altering the metabolic pathways of tumor cells, and the tumor infiltrating immune cells resulting in immunosuppressive TME. TMZ a first-in-line chemotherapeutic to treat GBM tumor but studies have shown patients often develop resistance to it. Research has also shown TMZ therapy increases expression of NHE1 protein in glioma cells. NHE1 overexpression has tumor promoting properties by pH dysregulation. The tumor immune and metabolic environment are also affected negatively with NHE1 expression. Combining TMZ therapy with NHE1 inhibition enhances TMZ-induced glioma apoptosis and reduces tumor growth and proliferation. Moreover, NHE1 inhibition stimulates pro-inflammatory polarization of TAMs and increases tumor infiltration of CD8⁺ T-cells. Also, combining NHE1 blockade with TMZ and anti-PD-1 therapy significantly increased median survival of mouse glioma models. We wanted to explore the metabolic profile of glioma cells when they are treated with the same combination therapy. We used NHE1 inhibition and TMZ treatment on GL26 glioma cells and measured changes in the bioenergetic pathways using the Seahorse XF Analyzer. Our findings suggest that TMZ induces an increase in extracellular acidification in the glioma environment. The oxygen consumption rate was measured to evaluate mitochondrial function. Our findings suggest that a combination treatment of inhibiting NHE1 and TMZ showed higher oxygen consumption than other treatments. The findings could suggest that blocking of NHE1 and treating cells with TMZ could revert the tumorigenic metabolism of glioma cells back to a less or non-proliferative environment. With no cure and current therapies for GBM not producing anticipated results it is
the need of the hour to consider combining different fields of study to reach the desired goal. The disease does not only affect the patient but also their families. To find a novel therapy for GBM would be of public health significance to the community as it will help lift the burden of family members and improve health of patients and increase survival statistics.
Bibliography


