12-2020

Role of Glycolytic Metabolism in Glioblastoma Multiforme

Shreya Udawant
The University of Texas Rio Grande Valley

ROLE OF GLYCOLYTIC METABOLISM IN GLIOBLASTOMA MULTIFORME

A Thesis

by

SHREYA UDAWANT

Submitted to the Graduate College of
The University of Texas Rio Grande Valley
In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2020

Major Subject: Biology

ROLE OF GLYCOLYTIC METABOLISM IN GLIOBLASTOMA MULTIFORME

A Thesis
by
SHREYA UDAWANT

COMMITTEE MEMBERS

Dr. Megan Keniry
Chair of Committee

Dr. Robert Gilkerson
Committee Member

Dr. Matthew Terry
Committee Member

Bonnie Gunn
Committee Member

December 2020

ABSTRACT

Udawant, Shreya, Role of Glycolytic Metabolism in Glioblastoma Multiforme. Master of Science (MS), December 2020, 33 pp., 3 tables, 7 figures, 33 references.

PI3K/AKT/mTOR signaling pathway is activated in 90% of all Glioblastoma Multiforme (GBM) tumors. We used a dual targeting approach to interfere with the phosphoinositide 3-kinase-mammalian target of rapamycin (PI3K-mTOR) pathway by treating U87MG GBM cells with NVP-BEZ235 (PI3K and mTOR a dual inhibitor) and, their downstream effects on molecular regulation on glycolytic genes were investigated using RNA-sequencing and gene set enrichment analysis (GSEA). RNA-seq identified 7803 differentially regulated genes in response to NVP-*BEZ235*. GSEA identified two glycolysis-related gene sets had significant differences ($P < 0.05$) in gene expression between control and NVP-BEZ235 treated samples. The GSEA core enrichment reported 17 and 35 genes were significantly downregulated ($P\ adj < 0.05$) in response to NVP-BEZ235 treatment in two different glycolysis related gene sets. We validated the expression of genes using qRT-PCR in U87MG cells as well as other GBM cell lines. Our results suggest novel insights on hallmark metabolic reprogramming associated with the PI3K-mTOR inhibition and provides advances in developing molecular targeted therapies.

DEDICATION

The completion of my master studies would not have been possible without support of my family and friends. I would dedicate this thesis to my husband, Dr. Renesh Bedre, who have inspired, supported, and motivated me unconditionally at all stages of graduate school and towards completion of MS degree. His constant motivation has encouraged me to prosper and achieve my dream. I also would like to thank my parents, my brother, and in-laws for their love and consistent support.

ACKNOWLEDGEMENTS

I am fortunate to complete my master thesis at the University of Rio Grande Valley (UTRGV), Texas. I am always proud to have great people around me at UTRGV, who have continuously guided and supported me for doing research and completing the thesis.

First, I would like to thank my supervisor, Dr. Megan Keniry, for providing me opportunity and her incredible scientific support over the years in lab. She is extremely knowledgeable, research oriented, and a great problem solver. I am amazed with her dedication for research. She is exceptionally kind and always available for help. I have learned multitude of molecular genetics skills under her supervision.

I also would like to thank my master thesis committee members, Dr. Robert Gilkerson, Dr. Matthew Terry, and Bonnie Gunn for their valuable guidance and support during thesis work.

I also would like to thank you my lab members and friends Yajaira Macias, David Flores, Leetoria Hinojosa, Carl Litif, Cristobal Rodriguez and all Keniry lab members.

I would remiss if I do not mention Dr. Parwinder Grewal, who have encouraged me and provided his valuable guidance during graduate school at UTRGV. I also would like to thank UTRGV office for Sustainability and Dr. Grewal for offering the sustainability fellowship.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Glioblastoma (GBM) is the most fatal and very common brain tumor of the central nervous system (CNS) and represents almost 80% of the total malignant tumors of the CNS (Batsios, Viswanath et al. 2019). It is highly proliferative and can be classified as low-grade and high-grade glioblastoma (LGG and HGG) as per World Health Organization (WHO) standards (Batsios, Viswanath et al. 2019). Among the malignant brain tumors, glioblastoma has the highest incidence rate with 3.19 cases per 100K population per year (Zhao, Wang et al. 2017). Even though various glioblastoma standard treatments available such as surgery, chemotherapy and radiation therapy, the patient prognosis is poor, therapeutic success is limited, and life expectancy of nearly 14 to 18 month with high chances of recurrence (Wen, Touat et al. 2019). Hence, it is imperative to develop new targeted molecular therapies for treatments of GBM.

Cancer cells are characterized by alteration in metabolic pathways and possess resistance to cell death. The metabolic pathways including glycolysis are important for developing targeted therapies for cancers and extensively studied. The glucose metabolism is a vital step in the event of tumorigenesis. Growth and survival of normal mammalian cells depends on the energy (in the form of ATP) obtained from both glycolysis and mitochondrial oxidative phosphorylation (OXPHOS) (Shiratori, Furuichi et al. 2019). In contrast, cancer cells mostly depend on the glycolysis pathway instead of OXPHOS for energy needs (Shiratori, Furuichi et al. 2019). Glycolysis is less efficient than OXPHOS in energy production (2 ATPs vs 36 ATPs per glucose

molecule) but has ability to synthesize ATPs faster than OXPHOS (Pfeiffer, Schuster et al. 2001, Vander Heiden, Cantley et al. 2009, Shiratori, Furuichi et al. 2019). For this reason, cancer cells prefer the glycolytic over mitochondrial pathway for energy requirements. Therefore, genes involved in the glycolysis pathways can provide potential targets for developing cancer therapies. The detailed understanding of differences between normal and cancer cells regarding glycolysis pathway would help to develop specific potential gene target and selective anticancer drug without affecting the normal cells.

In this study, we have explored gene regulatory changes associated with glycolysis pathway in GBM cell lines under PI3k/mTOR pathway inhibition using RNA sequencing (RNA-seq) technology. The comparative analysis of RNA-seq data followed by gene set enrichment analysis (GSEA (Subramanian, Tamayo et al. 2005)) has been performed between control and NVP-BEZ235 treated cells to investigate the significantly differentially expressed genes and biological pathways associated with PI3K/mTOR pathway inhibition in GBM. Our findings suggest that the genes involved in glycolysis were significantly downregulated by PI3K/mTOR inhibitor drug NVP-BEZ235. These results provide insights on using glycolytic genes as potential targets for developing therapies that interfere with cancer metabolism for treating GBM and other types of cancers.

CHAPTER II

REVIEW OF LITERATURE

Glioblastoma (GBM)

Cancer is second-most leading cause of death in the USA. Glioblastoma multiforme (GBM), also known as grade IV glioma as per World health Organization (WHO), is the most fatal and very common primary brain tumor of the central nervous system (CNS) in adults, which is characterized by cellular polymorphism, rapid growth, aggressive invasion, high rate of recurrence, neovascularization, and poor prognosis (Elsir, Eriksson et al. 2010, Xu, Wan et al. 2017, Wang, Fu et al. 2019). Glioma stem cells (GSCs), a subpopulation of GBM, are primarily responsible for tumor progression and treatment resistance (Kahlert, Cheng et al. 2016). Glioblastoma develops from astrocytes, oligodendrocytes, or from there precursors and represents almost 80% of the total malignant tumors of the CNS with 19.6 months of median survival period (Xu, Wan et al. 2017, Batsios, Viswanath et al. 2019). GBM is the highest occurring malignant brain tumor with 12,390 GBM cases were predicted in 2017 in USA (Atif, Yousuf et al. 2019).

Glioblastomas are difficult to treat owing to its widespread invasiveness, complex surgical resection and can surpass the chemo and radiation therapy (Atif, Yousuf et al. 2019). Besides, GBM has 100% chance of recurrence with no effective available treatment for recurrence. Further, the heterogeneous nature of glioblastoma makes it difficult for the development of treatments against these aggressive tumors. GBM can be treated with surgical tumor resection, chemotherapy with Temozolomide-a gold standard treatment, and radiation

therapy, and has poor survival rate (14 to 18 months) (Balca-Silva, Matias et al. 2017, Dong and Cui 2019, Wen, Touat et al. 2019). However, these classic therapies are not effective in treating GBM, show resistance, and offers only palliation. Therefore, to develop novel therapies to treat glioblastoma, it is imperative to investigate the molecular factors that underpin aggressiveness.

Glycolysis metabolic pathway

In living organisms, cells require nutrients and oxygen to generate the energy to perform the vital functions. Cancer cells utilize various substrates such as glucose, lactate, and glutamine to generate the ATP energy for biosynthetic needs (Cacace, Sboarina et al. 2017). Further, cancer cell metabolism is highly influenced by the tissue origin, genetic regulation, and tumor microenvironment (Cluntun, Lukey et al. 2017). The metabolic pathway glycolysis is a universal pathway in living organisms, where high-energy ATP molecules are synthesized from glucose molecules. Abrupt changes in glucose metabolisms are perhaps the most distinguishing characteristics of malignant from normal cells. Cancer cells are highly dependent on the metabolic reprogramming to survive and proliferate under unfavorable conditions. As an example of such adaptation includes Warburg effect – a hallmark of cancer, where cancer cells utilize aerobic glycolysis as the main source of energy (in the form of ATP) from glucose metabolism and excrete lactate even in presence of sufficient oxygen, instead of OXPHOS (Warburg 1956, Nilsson, Haanstra et al. 2020). In addition to the Warburg effect, cell metabolism in GBM are commonly characterized by alterations in OXPHOS, dysfunctional lipid metabolism, as well as in other metabolic pathways (Dong and Cui 2019).

It has been reported that tumor cells can regulate glycolysis much faster than the normal cells (Li, Liang et al. 2019). The glycolysis pathways in GBM conditions are variably increased

as compared to normal brain cells (Oudard, Arvelo et al. 1996). Further, glycolysis promotes tumor proliferation, aggressive invasion, and development of resistance to classical chemo and radiation therapies. As glycolysis is essential source of energy for brain cancer cells, it can serve as crucial molecular target for developing GBM treatments. Several studies have been reported that oncogenes promote the glycolytic metabolism in contrast to tumor suppressors, which suggests that glycolytic switch is associated with the oncogenic transformation (Munoz-Pinedo, El Mjiyad et al. 2012). The detailed characterization of molecular mechanisms associated with glycolysis in GBM would be an essential step for development of personalized treatment and gene risk signatures of GBM. In addition, this study could help to understand how metabolic reprogramming is driven by oncogenic transformation in GBM.

Besides the glucose, glutamine, a non-essential amino acid (NEAA), is mostly consumed nutrient by the cancer cell lines. The requirement of glutamine is not similar across all cancer types for proliferation and survival (Cluntun, Lukey et al. 2017). In GBM, the cancer cells release the excess of glutamate to boost malignant behavior by enhancing the cell proliferation, invasion, and resistance to the apoptosis (de Groot and Sontheimer 2011). This high amount of excreted glutamate from cancer cells creates complications in disease control and causes recurrence of the GBM (de Groot and Sontheimer 2011). Hence, the investigation of excess of glutamate and its receptors further provides potential targets for developing targeted therapeutic treatments for GBM.

PI3K-AKT-mTOR signaling pathway

In humans, the evolutionarily conserved phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (PI3K-AKT-mTOR) Pathway controls major physiological processes such as survival, proliferation, apoptosis, metabolism, motility, and growth (Janku, Yap et al. 2018, Batsios, Viswanath et al. 2019, Martinez, Vazquez et al. 2020). PI3K-AKT-mTOR pathways are impacted by genomic alterations of genes such as *EGFR* and *PTEN* (*Phosphatase and Tensin homolog deleted on chromosome ten)* genes in glioblastoma. Increased activity of PI3K-AKT-mTOR signaling pathways is highly correlated with tumor progression and multidrug resistance (Zhao, Wang et al. 2017). Besides, activation of PI3K-AKT-mTOR also enhances the tumor micro-vessel density and increases the invasive potential of the cancer cells (Yang, Nie et al. 2019). It is also known that PI3K-AKT-mTOR pathways responds actively to the insulin, growth factors, and also involved in the metabolic regulation (Hoxhaj and Manning 2019). As currently available treatments for glioblastoma are not enough, identifying novel targets hidden in PI3K-AKT-mTOR regulated transcription would serve as a crucial therapeutic target for glioblastoma (Zhao, Wang et al. 2017, Janku, Yap et al. 2018). Inhibiting the PI3K-AKT-mTOR signaling pathway by single or combination of drugs could be helpful to retard the tumor progression.

In cancer, PI3K-AKT-mTOR reprogram metabolic gene regulation by interfering with glycolytic metabolic components and nutrient transporters to meet the demands of uncontrolled growing malignant cells (Hoxhaj and Manning 2019). Further, increased glycolysis has been observed in instances of AKT activation (Hoxhaj and Manning 2019). In GBM, the PI3K-AKT-mTOR signaling pathways is highly active and contributes to the malignant aggressiveness of GBM.

PI3K-AKT-mTOR signaling pathway promotes tumor progression. Therefore, various cancer therapies target PI3K-AKT-mTOR pathway. The basic molecular mechanisms associated with inhibition of PI3K-AKT-mTOR signaling pathways are poorly understood and commonly have modest therapeutic efficacy in the clinic.

The detailed molecular investigation of PI3K-AKT-mTOR signaling pathway thus provides valuable information for development of new targeted therapeutic strategies for treating the GBM and other malignant conditions.

CHAPTER III

METHODOLOGY

Cell lines, cultures, and drug treatment

The human glioblastoma (GBM) cells lines U87MG used in this study were provided by the American Type Culture Collection (Manassas, VA, USA). U87MG cells were propagated in MEM (Minimal Essential Medium). DBTRG and BT549 cells were propagated in RPMI (Roswell Park Memorial Institute 1640 Medium). LN18, U118MG, A172 and LN229 cells were propagated in DMEM (Dulbecco's Modified Eagle Medium). Cell lines were grown in standard propagating conditions supplemented with 10% fetal bovine serum (FBS) and 5% CO_2 at 37 °C. The dual PI3K/mTOR pathway inhibitor drug NVP-BEZ235 was acquired from the Sigma-Aldrich (Saint Louis, MO). Cells were treated with NVP-BEZ235 and control samples were treated with DMSO.

RNA extraction and RNA sequencing

Total RNA was extracted from the treated and control samples using the Qiagen MiniPrep RNAeasy kit (Hilden, Germany). Three biological replicates of control and NVP-BEZ235 treated cell lines were used for the RNA extraction. The quality of the extracted RNA was analyzed using NanoDrop spectrophotometer (ThermoFisher, USA). The RNA samples kept frozen at -80 °C. The RNA libraries were sequenced at Novogene Corporation Inc. (Sacramento, CA). The RNA-seq was performed with three biological replicates.

Bioinformatics analysis

The RNA-seq analysis including mapping to human genome, sequence assembly, and identification of differentially expressed genes (DEGs) were performed by the Novogene Corporation Inc. (Sacramento, CA). The RNA-seq data were analyzed to identify the differentially expressed genes (DEGs) associated with NVP-BEZ235 treatment. The DEGs between NVP-BEZ235 treated and control cell lines were investigated using the Gene Set Enrichment Analysis (GSEA) (Mootha, Lindgren et al. 2003, Subramanian, Tamayo et al. 2005) to identify the enriched biological pathways defined by the set of genes between the two biologically treatments. The gene set pathways for KEGG_GLYCOLYSIS_GLUCONEOGENESIS were downloaded from the Molecular Signatures Database (MSigDB) (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp) for GSEA analysis. The GSEA analysis were performed by setting ENSEMBL Gene ID v7.0 Chip platform, 1000 number of permutations, and weighted enrichment statistics. Enriched genes in each pathway were identified using the core enrichment criteria given in GSEA.

Quantitative real-time PCR (qRT-PCR)

Extracted total RNA was used to prepare cDNA using Superscript Reverse Transcriptase II (Invitrogen, Carlsbad, CA). Primers spanning the coding sequences for the genes were designed using Prime3 (v0.4.0) (https://bioinfo.ut.ee/primer3-0.4.0/) and ordered from the Sigma-Aldrich (Saint Louis, MO). Amplification and expression of genes were performed using the Applied Biosystems StepOne Real-Time PCR System (Foster city, CA). Four technical replicates were used for the gene amplification and quantification. *ACTIN* (*ACTB* gene) was used as housekeeping gene for relative normalization and quantification performed by ΔΔCT method

provided in Applied Biosystems PCR machine. The PCR data was exported and analysis was performed using MS Excel for average, standard error, and t-test calculation.

Quantitative glutamate assay

The quantification of glutamate in control (DMSO) and NVP-BEZ235 treated GBM cells lines was performed using the Glutamate Colorimetric Assay kit (#K629-100, BioVision Inc., Milpitas, CA) as per manufacturer's protocol. The 1 mM glutamate standard was prepared and added to 96-well plate to generate two replicates of 0, 2, 4, 6, 8, and 10 nmol/well standard concentration. The absorbance for glutamate standards and samples were measured at 450 nm using iMark Microplate Absorbance Reader (Bio-Rad, Hercules, CA). The standard and sample absorbance were corrected for the background control to visualize the standard curve with regression equation. The glutamate concentrations were estimated from regression equation and using the formulae C = Sa/Sv provided by the manufacturer's protocol.

CHAPTER IV

RESULTS

RNA-seq gene expression profiling and GSEA

We have generated RNA-sequencing data from the mRNA extracted from NVP-BEZ235 treated and control samples with three biological replicates. The initial Bioinformatics analysis including quality filtering, genome mapping followed by comparative gene expression analysis between NVP-BEZ235 treated and control samples lead to identification of the 7803 differentially expressed genes (DEGs). The 7803 DEGs (native features) obtained from the RNA-seq analysis has been used for GSEA (Mootha, Lindgren et al. 2003, Subramanian, Tamayo et al. 2005). GSEA was carried out for the Glycolysis C1 (hallmark gene set) and C2 gene sets (curated gene sets) including KEGG_GLYCOLYSIS_ GLUCONEOGENESIS, HALLMARK_GLYCOLYSIS, and REACTOME_GLYCOLYSIS collections available in the MSigDB (https://www.gsea-msigdb.org/gsea/msigdb/genesets.jsp). We have chosen these gene sets as these sets represents the genes involved in glycolysis metabolism and have higher implication in tumor progression. These three glycolysis related gene sets were analyzed using GSEA to explore the gene sets that differed significantly in between control and NVP-BEZ235 treated cell lines samples. GSEA indicated that gene expression differences were significant ($p < 0.05$) in KEGG_GLYCOLYSIS_ GLUCONEOGENESIS and HALLMARK_GLYCOLYSIS gene sets in between control and NVP-BEZ235 treated cell samples (Figure 1). Besides, correlation profiles of both gene sets were negatively correlated with control phenotype and positively correlated with NVP-BEZ235 treated phenotype (Figure 1).

KEGG_GLYCOLYSIS_ GLUCONEOGENESIS and HALLMARK_GLYCOLYSIS gene sets were further analyzed for GSEA core enrichment and we found that 17 and 35 genes were significantly downregulated in GBM cell lines in response to NVP-BEZ235 treatment respectively (Table 1). To represent the expression pattern of large number of core enriched genes, we have created the heatmap for enriched genes in KEGG_GLYCOLYSIS_ GLUCONEOGENESIS and HALLMARK_GLYCOLYSIS based on the expression values of the genes in control and treated samples (Figure 2).

Overall survival (OS) analysis of glycolysis-associated genes

We have selected significantly differentially expressed core genes from glycolysis gene set enrichment to investigate the association of the significant genes with patient survival. The OS was performed using the GEPIA 2 and Kaplan-Meier analysis. Among the enriched genes, two genes *Enolase 1* (*ENO1)* and *Glyceraldehyde-3-Phosphate Dehydrogenase* (*GAPDH*) have shown significant correlations (log rank $P < 0.05$) with worse survival (Figure 3a). Another gene *Pyruvate kinase* (*PKM*) had also shown not significant but good effect on the survival (P=0.07). Further, we validated the expression of these three genes from GEPIA 2 online tool which have expression database based on The Cancer Genome Atlas (TCGA) database. The comparison of expression of *ENO1, GAPDH,* and *PKM* have shown overall higher expression in tumor samples as compared to the normal (Figure 3b). Correlation analysis of the glycolysis-related genes also has shown significant correlation in the GBM (Table 2). Therefore, our systematic gene expression analysis obtained from the RNA-seq can provides a confidence in selecting genes for further genetic analysis and biological interpretation.

Validation of the glycolysis-associated genes by qRT-PCR and western blot

To validate the significantly glycolysis-related genes obtained from RNA-seq data, we performed the qRT-PCR of the selected significant genes obtained from gene set enrichment analysis. Primers for selected genes were designed using Primer3 (v0.4.0) online tool (Table). qRT-PCR analysis showed that selected glycolysis-related genes were significantly downregulated in control (DMSO) as compared to NVP-BEZ235 (Figure 4). Validation of differential gene expression by qRT-PCR also suggests the confidence and reliability in the RNA-seq analysis.

Since, the activity of the glycolysis related genes could be differentially regulated in different GBM cell lines, we also verified the expression of glycolysis-related genes in different GBM cell lines including U118MG, LN229, LN18, DBTRG and breast cancer cell line BT549,

(Figure 5). The results showed that *LDHA* gene was consistently downregulated in all cell lines in response to NVP-BEZ235 treatment (Figure 5). Similarly, *ENO1* was downregulated in in all cell lines in response to NVP-BEZ235 treatment (Figure 5). *GAPDH* was slightly upregulated in DBTRG (GBM cell line) (Figure 5c).

In addition to qRT-PCR verification of the RNA-seq data, we verified the protein expression of the glycolytic genes. The expression levels of LDHA, PKM2, and GAPDH were higher in DMSO as compared to that of NVP-BEZ235 treated samples (Figure 6).

NVP-BEZ235 treatment led to reduced Glutamate

As glutamate is another important metabolite associated with cancer progression, we have investigated changes in glutamate concentration in control and NVP-BEZ235 treated cell lines. The colorimetric glutamate assay kit was used to analyze the concentration of glutamate in the

samples. The glutamate concentration was significantly higher in control sample as compared to the NVP-BEZ235 treated cell lines suggesting that inhibition of PI3K-mTOR causes reduction in glutamate (Figure 7). This observation highlights that glutamate is differentially regulated by the PI3K-mTOR inhibition.

CHAPTER V

DISCUSSSION

GBM is the most malignant and lethal brain cancer worldwide with survival rate less than 18 months both in treated and untreated patients. Currently available treatments are not effective and alternate approaches to treat GBM are desperately needed. The genome and RNA sequencing technologies have provided novel and rapid approaches to screen the genome and genes to delineate the complex molecular responses associated with the cancer conditions, and ultimately advancing the development of more targeted therapies and precision cancer therapeutics. The PI3K-AKT-mTOR – one of the important signaling pathway in cancer, regulate the cell proliferation, cell metabolism, growth, and survival, is highly induced in the cancer (Ballou and Lin 2008). Therefore, genetic components involved in PI3K-AKT-mTOR offers interesting targets for drug development. Decoding the key gene regulatory elements involved in the cancer favored glycolysis metabolism for energy requirement is of utmost importance for developing therapeutic treatments. Therefore, in this study, we focused on the inhibition effect of PI3K-mTOR (by NVP-BEZ235 treatment) signaling pathway on glycolysis metabolism through RNA-seq on GBM cell lines. We investigated the transcriptional regulation of glycolytic genes by PI3K-mTOR inhibition in GBM cell lines using the RNA-seq.

We have leveraged the RNA-seq technology to sequence samples from DMSO and NVP-BEZ235 treated GBM cell lines to investigate the underlying genetic mechanisms associated with the PI3K-mTOR inhibition. NVP-BEZ235 induced 7803 DEGs were further used for GSEA

of glycolysis-relate gene sets. GSEA on multiple glycolysis-related gene sets identified enrichment of 52 genes ($P < 0.05$) which were highly downregulated in response to NVP-BEZ235 samples. Significant downregulation of glycolytic genes in response to NVP-BEZ235 suggested regulation of metabolism by the PI3K-AKT-mTOR pathway. Kaplan-Meier survival analysis showed that *ENO1, GAPDH,* and *PKM* genes have significant correlations with poor survival. Previous study had shown that *ENO1* promoted the tumor progression (Song, Luo et al. 2014). Another study on pancreatic cancer showed that overexpression of *ENO1* was associated with the metastasis and poor prognosis (Yin, Wang et al. 2018). *GAPDH,* which is considered as housekeeping genes in quantitative RNA and protein analysis, is also induced by the many types of tumor including human glioblastoma (Said, Hagemann et al. 2007). *PKM* is a regulator of aerobic glycolysis and expresses highly in the cancer cells (Desai, Ding et al. 2014).

The differentially regulated glycolysis-related genes were further validated using the qRT-PCR in U87MG as well as other cell lines for their consistency in expression under PI3K-mTOR inhibition by NVP-BEZ235. The *Lactate dehydrogenase A* (*LDHA*) gene was found to be consistently downregulated in all cell lines (Figure 5) suggesting its significance in developing cancer treatment. Similarly, we have also found consistent expression of *ENO1* gene in U118MG, LN229, LN18, and DBTRG cell lines (Figure 5). Western blot analysis also revealed the protein level expression of these genes were reduced by NVP_BEZ235 (Figure 6).

Excessive glutamate released by the GBM cells regulates proliferation and survival of neural progenitor cells; cancer cells also show similar properties as that of neural progenitor cells (Corsi, Mescola et al. 2019). Therefore, it is very likely that the NVP-BEZ235 in GBM cells should reduce the release of glutamate. To confirm this, we have performed the glutamate assay

and reported that NVP-BEZ235 treated samples have shown significant reduction in glutamate as compared to the control (DMSO) cells (Figure 7).

In conclusion, we have identified and validated the several glycolysis-related genes, mainly, *GAPDH*, *LDHA*, *ENO1*, and *PKM* that could be useful for understanding genetic and epigenetic causes of GBM underlying glycolytic metabolism in GBM and further for drug development for targeted GBM treatment. We have provided novel insights of glycolysis regulation in GBM. Targeting glycolytic pathways has promising potential in developing next generation treatment for treating the GBM.

REFERENCES

Atif, F., S. Yousuf, C. Espinosa-Garcia, E. Sergeeva and D. G. Stein (2019). "Progesterone Treatment Attenuates Glycolytic Metabolism and Induces Senescence in Glioblastoma." Sci Rep **9**(1): 988.

Balca-Silva, J., D. Matias, A. Do Carmo, L. G. Dubois, A. C. Goncalves, H. Girao, N. H. S. Canedo, A. H. Correia, J. M. De Souza, A. B. Sarmento-Ribeiro, M. C. Lopes and V. Moura-Neto (2017). "Glioblastoma entities express subtle differences in molecular composition and response to treatment." Oncol Rep **38**(3): 1341-1352.

Ballou, L. M. and R. Z. Lin (2008). "Rapamycin and mTOR kinase inhibitors." J Chem Biol **1**(1-4): 27-36.

Batsios, G., P. Viswanath, E. Subramani, C. Najac, A. M. Gillespie, R. D. Santos, A. R. Molloy, R. O. Pieper and S. M. Ronen (2019). "PI3K/mTOR inhibition of IDH1 mutant glioma leads to reduced 2HG production that is associated with increased survival." Sci Rep **9**(1): 10521.

Cacace, A., M. Sboarina, T. Vazeille and P. Sonveaux (2017). "Glutamine activates STAT3 to control cancer cell proliferation independently of glutamine metabolism." Oncogene **36**(15): 2074-2084.

Cluntun, A. A., M. J. Lukey, R. A. Cerione and J. W. Locasale (2017). "Glutamine Metabolism in Cancer: Understanding the Heterogeneity." Trends Cancer **3**(3): 169-180.

Corsi, L., A. Mescola and A. Alessandrini (2019). "Glutamate Receptors and Glioblastoma Multiforme: An Old "Route" for New Perspectives." Int J Mol Sci **20**(7).

de Groot, J. and H. Sontheimer (2011). "Glutamate and the biology of gliomas." Glia **59**(8): 1181-1189.

Desai, S., M. Ding, B. Wang, Z. Lu, Q. Zhao, K. Shaw, W. K. Yung, J. N. Weinstein, M. Tan and J. Yao (2014). "Tissue-specific isoform switch and DNA hypomethylation of the pyruvate kinase PKM gene in human cancers." Oncotarget **5**(18): 8202-8210.

Dong, Z. and H. J. Cui (2019). "Epigenetic modulation of metabolism in glioblastoma." Seminars in Cancer Biology **57**: 45-51.

Elsir, T., A. Eriksson, A. Orrego, M. S. Lindstrom and M. Nister (2010). "Expression of PROX1 Is a Common Feature of High-Grade Malignant Astrocytic Gliomas." Journal of Neuropathology and Experimental Neurology **69**(2): 129-138.

Hoxhaj, G. and B. D. Manning (2019). "The PI3K–AKT network at the interface of oncogenic signalling and cancer metabolism." Nature Reviews Cancer: 1-15 %@ 1474-1768.

Janku, F., T. A. Yap and F. Meric-Bernstam (2018). "Targeting the PI3K pathway in cancer: are we making headway?" Nat Rev Clin Oncol **15**(5): 273-291.

Kahlert, U. D., M. Cheng, K. Koch, L. Marchionni, X. Fan, E. H. Raabe, J. Maciaczyk, K. Glunde and C. G. Eberhart (2016). "Alterations in cellular metabolome after pharmacological inhibition of Notch in glioblastoma cells." Int J Cancer **138**(5): 1246-1255.

Li, H. C., Q. H. Liang and L. Wang (2019). "Icaritin inhibits glioblastoma cell viability and glycolysis by blocking the IL-6/Stat3 pathway." Journal of Cellular Biochemistry **120**(5): 7257-7264.

Martinez, E., N. Vazquez, A. Lopez, V. Fanniel, L. Sanchez, R. Marks, L. Hinojosa, V. Cuello, M. Cuevas, A. Rodriguez, C. Tomson, A. Salinas, M. Abad, M. Holguin, N. Garza, A. Arenas, K. Abraham, L. Maldonado, V. Rojas, A. Basdeo, E. Schuenzel, M. Persans, W. Innis-Whitehouse and M. Keniry (2020). "The PI3K pathway impacts stem gene expression in a set of glioblastoma cell lines." J Cancer Res Clin Oncol **146**(3): 593-604.

Mootha, V. K., C. M. Lindgren, K. F. Eriksson, A. Subramanian, S. Sihag, J. Lehar, P. Puigserver, E. Carlsson, M. Ridderstrale, E. Laurila, N. Houstis, M. J. Daly, N. Patterson, J. P. Mesirov, T. R. Golub, P. Tamayo, B. Spiegelman, E. S. Lander, J. N. Hirschhorn, D. Altshuler and L. C. Groop (2003). "PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes." Nat Genet **34**(3): 267-273.

Munoz-Pinedo, C., N. El Mjiyad and J. E. Ricci (2012). "Cancer metabolism: current perspectives and future directions." Cell Death Dis **3**: e248.

Nilsson, A., J. R. Haanstra, M. Engqvist, A. Gerding, B. M. Bakker, U. Klingmuller, B. Teusink and J. Nielsen (2020). "Quantitative analysis of amino acid metabolism in liver cancer links glutamate excretion to nucleotide synthesis." Proc Natl Acad Sci U S A **117**(19): 10294-10304.

Oudard, S., F. Arvelo, L. Miccoli, F. Apiou, A. M. Dutrillaux, M. Poisson, B. Dutrillaux and M. F. Poupon (1996). "High glycolysis in gliomas despite low hexokinase transcription and activity correlated to chromosome 10 loss." Br J Cancer **74**(6): 839-845.

Pfeiffer, T., S. Schuster and S. Bonhoeffer (2001). "Cooperation and competition in the evolution of ATP-producing pathways." Science **292**(5516): 504-507.

Said, H. M., C. Hagemann, J. Stojic, B. Schoemig, G. H. Vince, M. Flentje, K. Roosen and D. Vordermark (2007). "GAPDH is not regulated in human glioblastoma under hypoxic conditions." BMC molecular biology **8**: 55-55.

Shiratori, R., K. Furuichi, M. Yamaguchi, N. Miyazaki, H. Aoki, H. Chibana, K. Ito and S. Aoki (2019). "Glycolytic suppression dramatically changes the intracellular metabolic profile of multiple cancer cell lines in a mitochondrial metabolism-dependent manner." Sci Rep **9**(1): 18699.

Song, Y., Q. Luo, H. Long, Z. Hu, T. Que, X. Zhang, Z. Li, G. Wang, L. Yi, Z. Liu, W. Fang and S. Qi (2014). "Alpha-enolase as a potential cancer prognostic marker promotes cell growth, migration, and invasion in glioma." Mol Cancer **13**: 65.

Subramanian, A., P. Tamayo, V. K. Mootha, S. Mukherjee, B. L. Ebert, M. A. Gillette, A. Paulovich, S. L. Pomeroy, T. R. Golub, E. S. Lander and J. P. Mesirov (2005). "Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles." Proc Natl Acad Sci U S A **102**(43): 15545-15550.

Vander Heiden, M. G., L. C. Cantley and C. B. Thompson (2009). "Understanding the Warburg effect: the metabolic requirements of cell proliferation." Science **324**(5930): 1029-1033.

Wang, G., X. L. Fu, J. J. Wang, R. Guan, Y. Sun and S. S. Tony To (2019). "Inhibition of glycolytic metabolism in glioblastoma cells by Pt3glc combinated with PI3K inhibitor via SIRT3-mediated mitochondrial and PI3K/Akt-MAPK pathway." J Cell Physiol **234**(5): 5888-5903.

Warburg, O. (1956). "On respiratory impairment in cancer cells." Science **124**(3215): 269-270.

Wen, P. Y., M. Touat, B. M. Alexander, I. K. Mellinghoff, S. Ramkissoon, C. S. McCluskey, K. Pelton, S. Haidar, S. S. Basu, S. C. Gaffey, L. E. Brown, J. E. Martinez-Ledesma, S. Wu, J. Kim, W. Wei, M. A. Park, J. T. Huse, J. G. Kuhn, M. L. Rinne, H. Colman, N. Y. R. Agar, A. M. Omuro, L. M. DeAngelis, M. R. Gilbert, J. F. de Groot, T. F. Cloughesy, A. S. Chi, T. M. Roberts, J. J. Zhao, E. Q. Lee, L. Nayak, J. R. Heath, L. L. Horky, T. T. Batchelor, R. Beroukhim, S. M. Chang, A. H. Ligon, I. F. Dunn, D. Koul, G. S. Young, M. D. Prados, D. A. Reardon, W. K. A. Yung and K. L. Ligon (2019). "Buparlisib in Patients With Recurrent Glioblastoma Harboring Phosphatidylinositol 3-Kinase Pathway Activation: An Open-Label, Multicenter, Multi-Arm, Phase II Trial." J Clin Oncol **37**(9): 741-750.

Xu, X., X. Wan and X. Wei (2017). "PROX1 promotes human glioblastoma cell proliferation and invasion via activation of the nuclear factor-kappaB signaling pathway." Mol Med Rep **15**(2): 963-968.

Yang, J., J. Nie, X. Ma, Y. Wei, Y. Peng and X. Wei (2019). "Targeting PI3K in cancer: mechanisms and advances in clinical trials." Mol Cancer **18**(1): 26.

Yin, H., L. Wang and H. L. Liu (2018). "ENO1 Overexpression in Pancreatic Cancer Patients and Its Clinical and Diagnostic Significance." Gastroenterol Res Pract **2018**: 3842198.

Zhao, H.-f., J. Wang, W. Shao, C.-p. Wu, Z.-p. Chen, S.-s. T. To and W.-p. Li (2017). "Recent advances in the use of PI3K inhibitors for glioblastoma multiforme: current preclinical and clinical development." Molecular cancer **16**(1): 100 %@ 1476-4598.

APPENDIX

Table 1 List of significantly downregulated genes in KEGG_GLYCOLYSIS_ GLUCONEOGENESIS gene set

Gene	**Description**	**Ensembl ID**	**Log_2 Fold Change**	***p* value**	**Core Enrichment**
GPI	glucose-6-phosphate isomerase	ENSG00000105220	-0.72657	8.9243E-08	Yes
ALDH3A2	aldehyde dehydrogenase 3 family member A2	ENSG00000072210	-0.75715	1.4996E-13	Yes
HK1	hexokinase 1	ENSG00000156515	-0.77864	3.6097E-18	Yes
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	ENSG00000111640	-0.78251	8.6609E-09	Yes
DLD	dihydrolipoamide dehydrogenase	ENSG00000091140	-0.84409	7.6627E-16	Yes
ALDH2	aldehyde dehydrogenase 2 family member	ENSG00000111275	-1.0986	1.3319E-13	Yes
BPGM	bisphosphoglycerate mutase	ENSG00000172331	-1.1236	6.6711E-15	Yes
ALDH9A1	aldehyde dehydrogenase 9 family member A1	ENSG00000143149	-1.1418	5.1753E-20	Yes
PCK2	phosphoenolpyruvate carboxykinase 2, mitochondrial	ENSG00000100889	-1.1358	4.0201E-12	Yes
ENO1	enolase 1	ENSG00000074800	-1.2179	1.2689E-23	Yes
PGM1	phosphoglucomutase 1	ENSG00000079739	-1.254	1.2733E-29	Yes
PKM	pyruvate kinase M1/2	ENSG00000067225	-1.4937	4.7259E-41	Yes
ALDOC	aldolase, fructose-bisphosphate C	ENSG00000109107	-1.7371	2.77E-36	Yes
TPI1	triosephosphate isomerase 1	ENSG00000111669	-1.7825	1.0982E-38	Yes
PGAM1	phosphoglycerate mutase 1	ENSG00000171314	-2.2573	1.303E-146	Yes
PGK1	phosphoglycerate kinase 1	ENSG00000102144	-2.3014	6.232E-134	Yes

LDHA	lactate dehydrogenase A	ENSG00000134333	-2.5945	3.152E-141	Yes

Table 2 Pearson correlation analysis of glycolysis-related genes in GBM

Gene	**Gene**	**Correlation coefficient (R)**	***P* value**
ENO1	*GAPDH*	0.55	< 0.05
ENO1	*PGK1*	0.55	< 0.05
ENO1	*PKM*	0.47	<0.05
ENO1	*PGAM1*	0.53	<0.05
GAPDH	*PGK1*	0.61	<0.05
GAPDH	*PKM*	0.56	<0.05
GAPDH	*PGAM1*	0.39	<0.05
LDHA	*ENO1*	0.56	<0.05
LDHA	*GAPDH*	0.64	<0.05
LDHA	*PGAM1*	0.46	<0.05
LDHA	*PGK1*	0.76	<0.05
LDHA	*PKM*	0.64	<0.05

Table 3 List of forward and reverse primers of glycolysis-related genes

Gene	**Forward (5' -3')**	**Reverse (5' - 3')**	**Amplicon size (bp)**
LDHA	TACAGTTGTTGGGGTTGGTG	AGGCTGCCATGTTGGAGAT	141
PGK1	GAAGGGAAGGGAAAAGATGC	CTCTGTGAGCAGTGCCAAAA	130
PGAM1	GCATCTGGAGGGTCTCTCTG	TCTTCATCCCCCAGAAACTG	126
TPI1	CAGAGTCTGGGGGAGCTCAT	CCACAGCAATCTTGGGATCT	130
ALDOC	ATGGAGACCACGACCTCAAA	GTCACCATGTTGGGCTTGA	124
PKM	CATTGATTCACCACCCATCA	AGACGAGCCACATTCATTCC	120
PGM1	ACGGCCGGTTCTACATGA	CCACCAATGGCTTTGATTTT	148
ENO1	TGCCAGGGAGATCTTTGACT	ATCATTGTCCCGGAGCTCTA	139
GAPDH	GGGCTGCTTTTAACTCTGGT	GACAAGCTTCCCGTTCTCAG	146
GPI	TCTTCGATGCCAACAAGGAC	CACCAGCATCCGCATCAC	122
PCK2	CACACTGACCCTGCTGGAG	CCCGCTGAGAAGGAGTTACA	137
ACTB	GTCTTCCCCTCCATCGTG	AGGGTGAGGATGCCTCTCTT	113

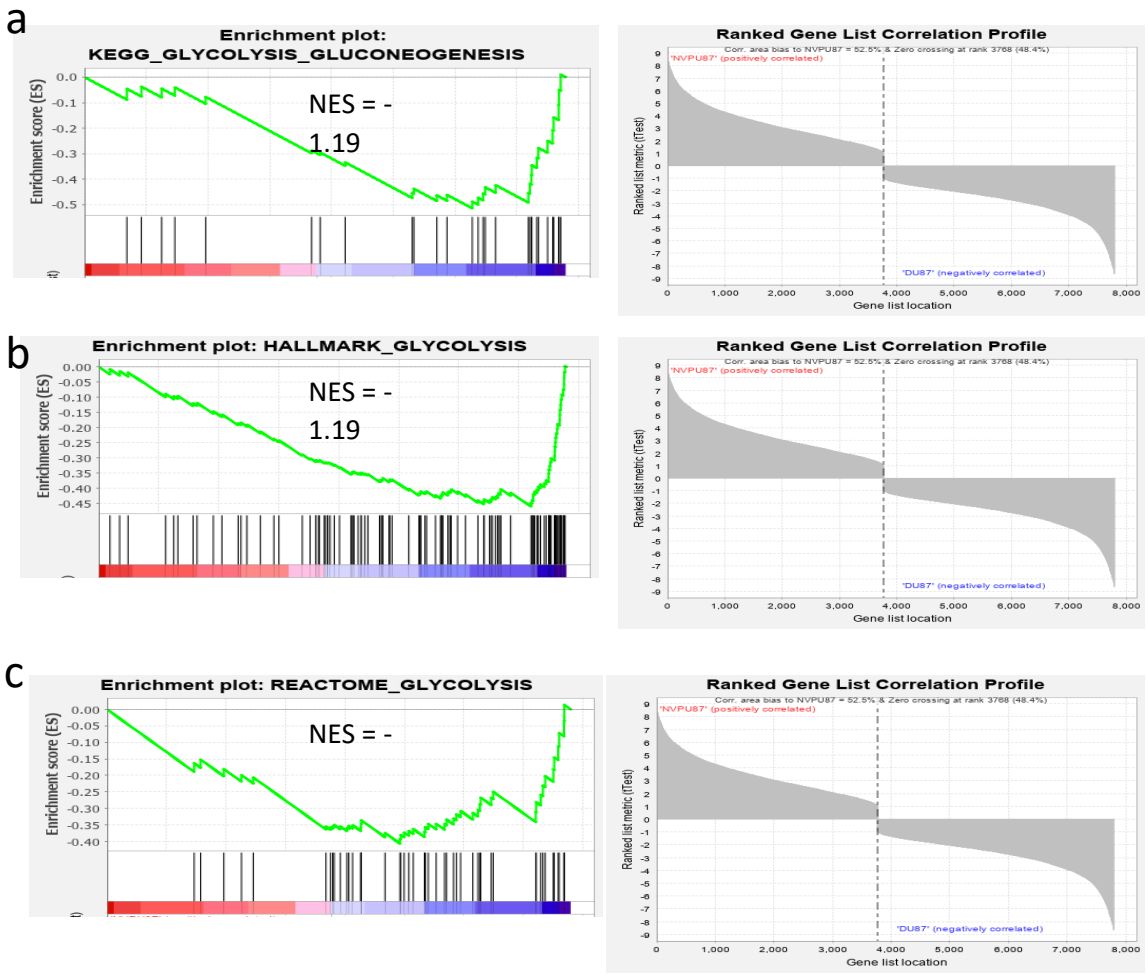

Figure 1: GSEA enrichment plot and ranked gene list correlation profile depicting the enrichment of sample phenotype NVP-BEZ235 and control in priori defined Glycolysis gene sets **a)** KEGG Glycolysis gluconeogenesis gene set**, b)** Hallmark glycolysis gene set, and **c**) Reactome glycolysis gene set. The profile of running enrichment score (ES) and rank ordered list are shown. The gene upregulation or downregulation in NVP-BEZ235 were estimates with respect to control sample.

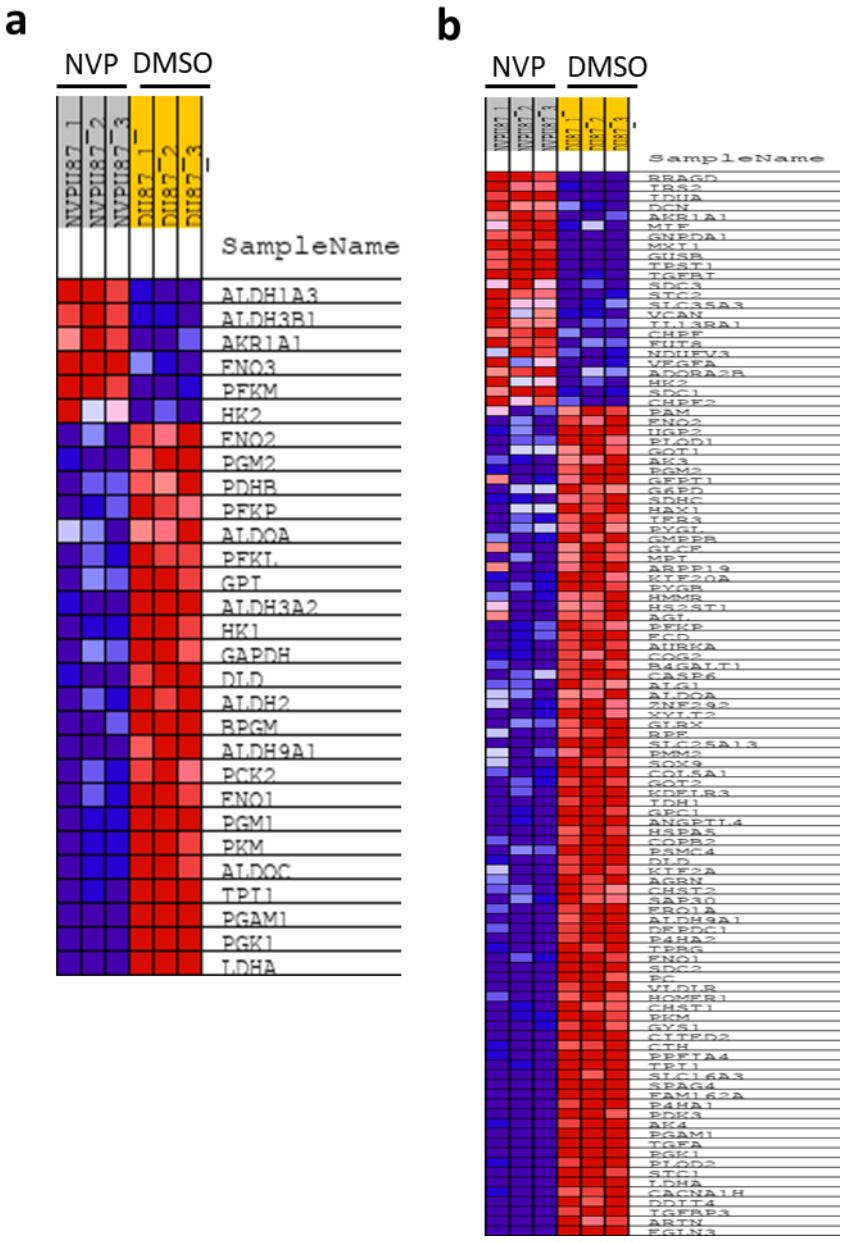

Figure 2: Heatmaps of core enriched genes obtained from GSEA. **a)** heatmap of 17 core enriched genes KEGG_GLYCOLYSIS_ GLUCONEOGENESIS gene set **b)** heatmap of 35 core-enriched genes in HALLMARK_GLYCOLYSIS gene set. Genes in red were highly expressed.

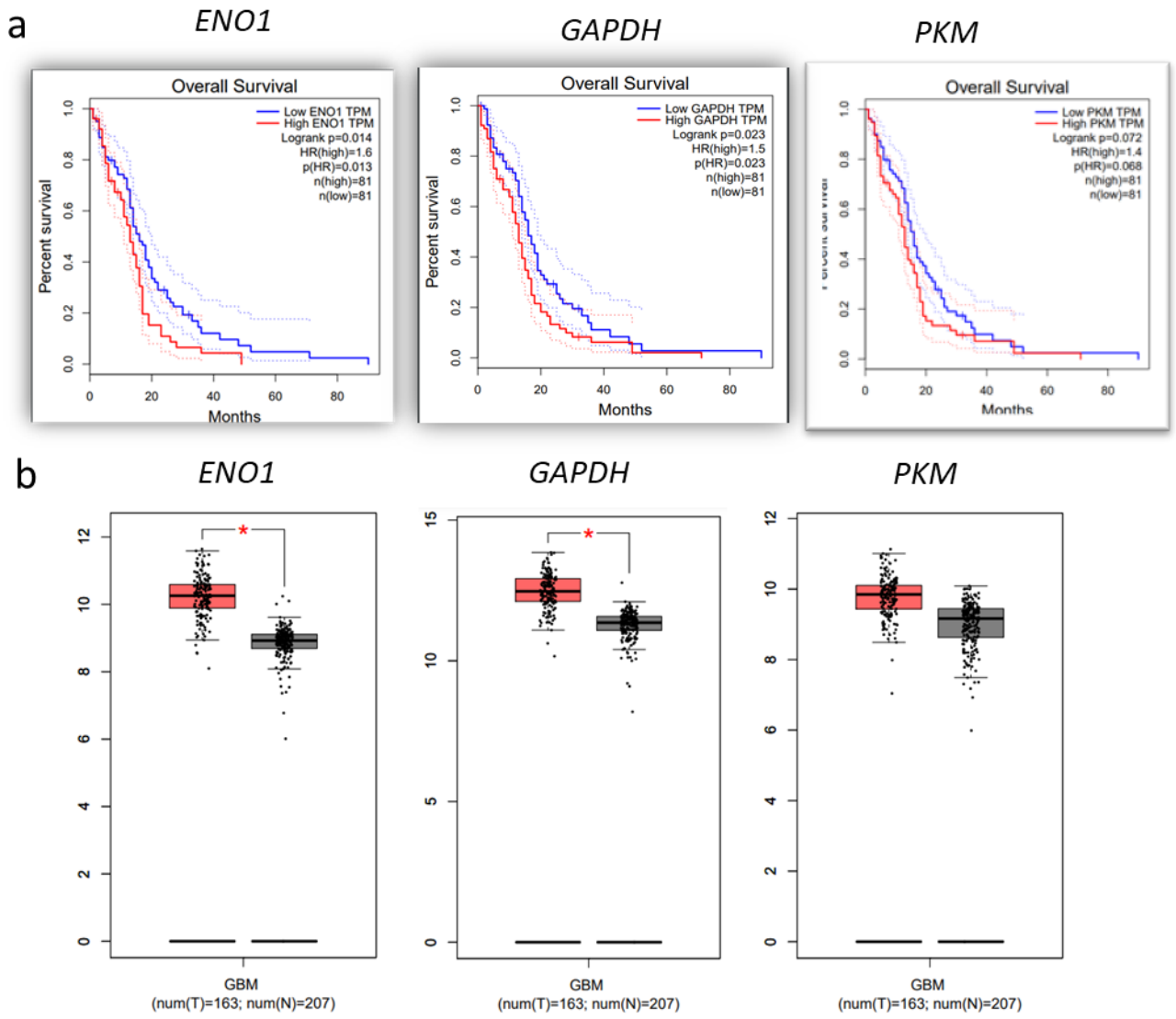

Figure 3: Overall survival analysis of glycolysis-related genes. **a**) The OS and Kaplan-Meier analysis has been performed using the GEPIA 2 online application. The solid and dotted line represents the survival curve and 95% confidence interval respectively. **b**) Differential expression boxplot of genes (*ENO1*, *GAPDH*, and *PKM*) in GBM in The Cancer Genome Atlas (TCGA) database obtained from GEPIA 2.

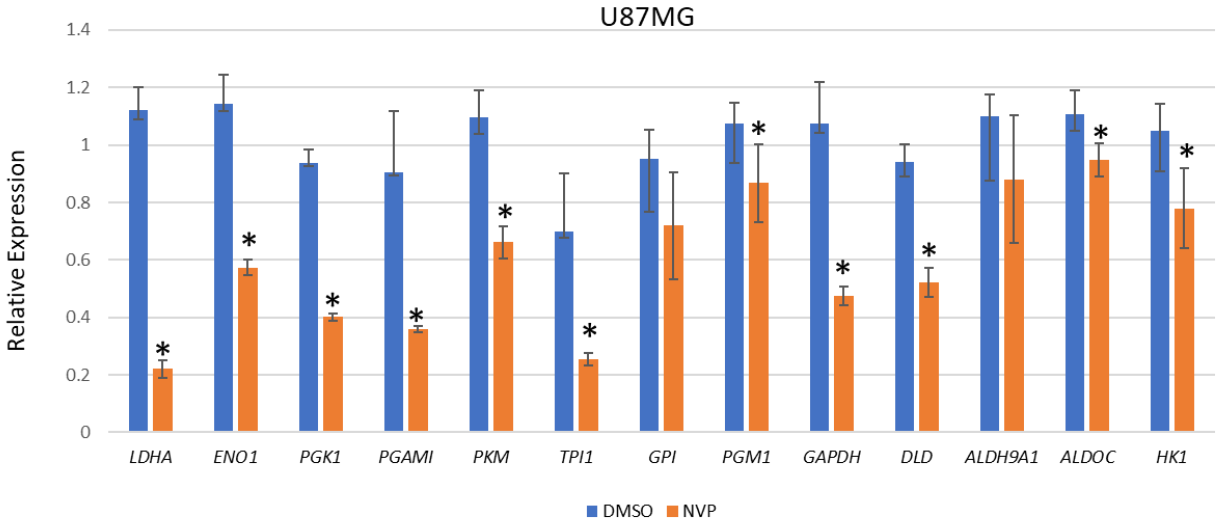

Figure 4: qRT-PCR validation of the glycolysis-related genes. The relative expression of the genes was analyzed in control and NVP-BEZ235 samples for up and downregulation. The error bars represent the standard deviation of the genes (n=4). Asterisk (*) represent the significantly expressed genes ($P < 0.05$) as compared to control (DMSO). Significance was determined by Student's T-Test.

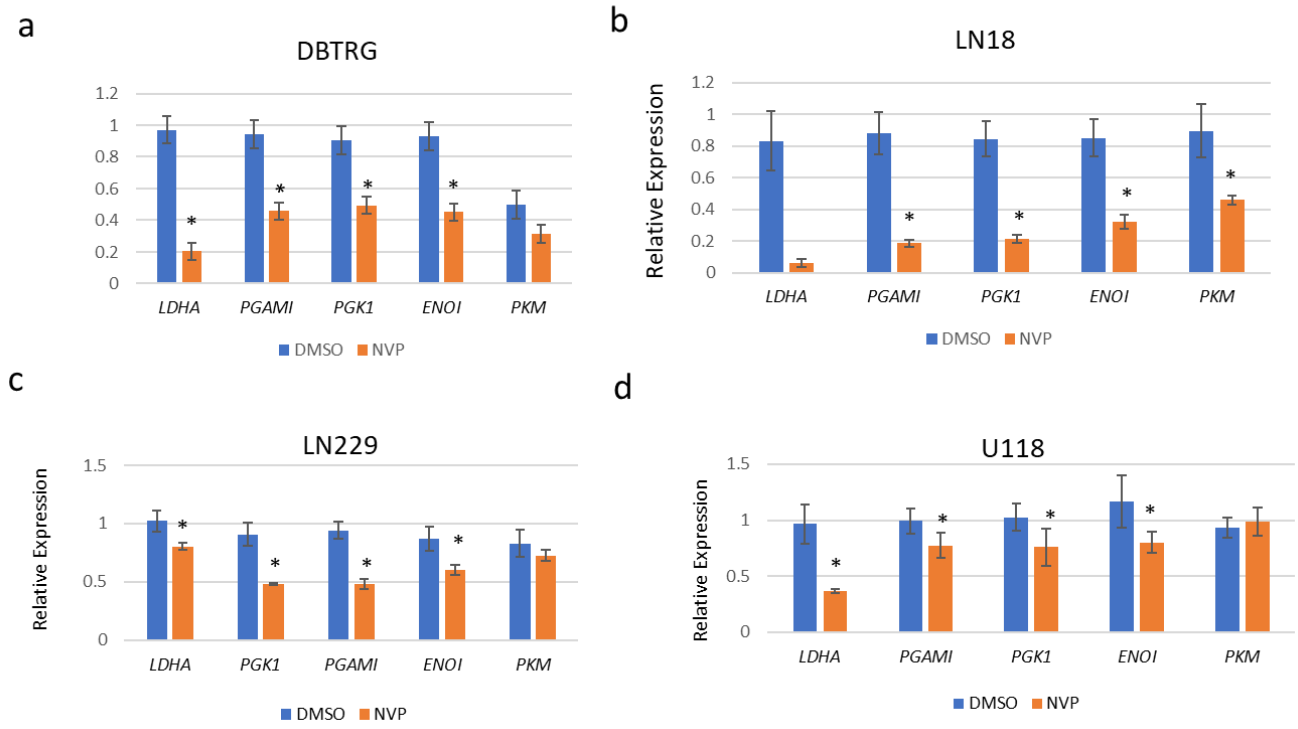

Figure 5: qRT-PCR validation of the glycolysis-related genes in various cell lines. a) DBTRG, b) LN18, c) LN229, and d) U118. The error bars represent the standard deviation of the genes (n=4). Asterisk (*) represent the significantly expressed genes ($P < 0.05$) as compared to control (DMSO). Significance was determined by Student's T-Test.

Figure 6: Expression changes of glycolysis-related genes at protein level. Protein expression was verified using the western blot analysis in DMSO and NVP-BEZ235 samples. Actin was used as the loading control.

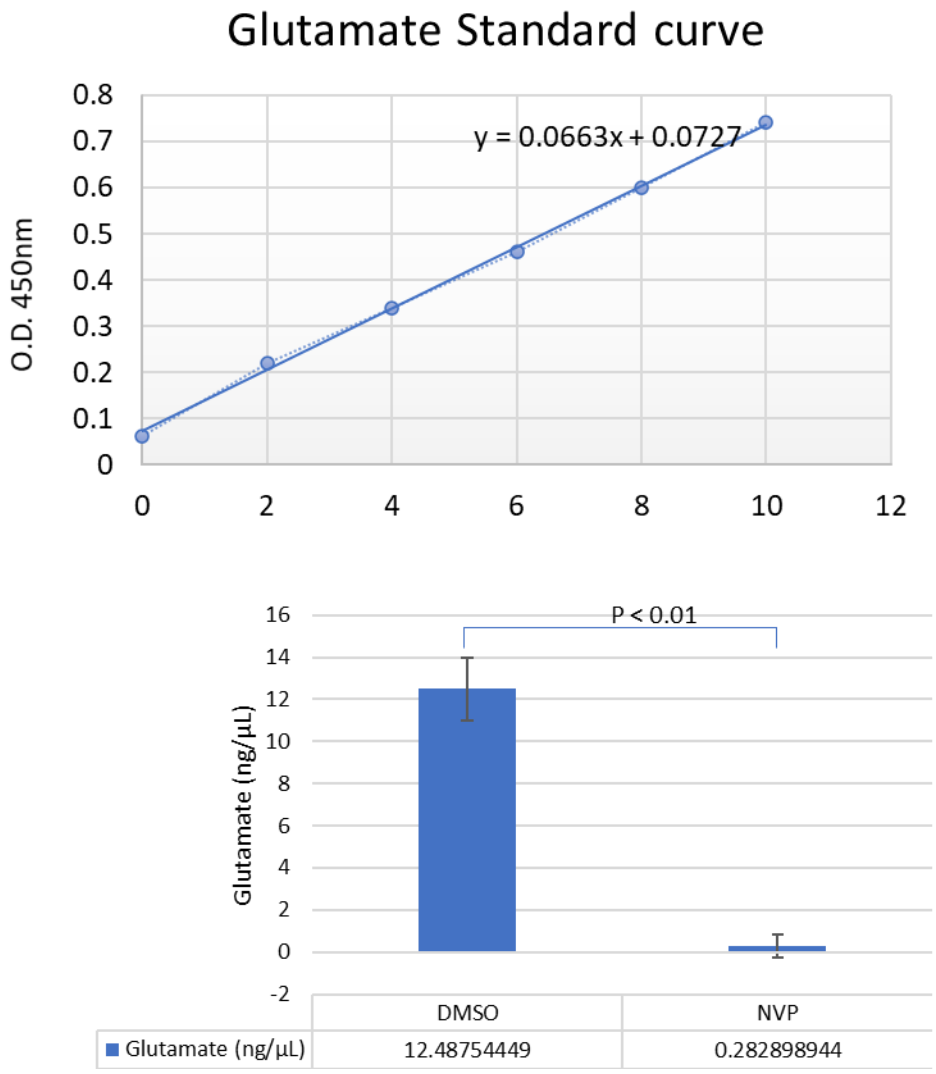

Figure 7: Glutamate standard curve. **a)** Absorbance of glutamate concentrations at 450 nm **b)** The estimated concentration of glutamate in control (DMSO) and NVP-BEZ235 samples. The error bars represent the standard deviation of the samples (n=3). Asterisk (*) represent the significantly difference ($P < 0.05$) as compared to control (DMSO). Significance was determined by Student's T-Test.

BIOGRAPHICAL SKETCH

Shreya Udawant was born in Nashik city in India. She had completed her school Rangubai Junnare in 2007. Later, she completed her bachelor at Shreemati Nathibai Damodar Thackersey Women's University (SNDT), Mumbai in 2013 followed by post-graduation in Pharmacy at the University of Pune in 2015. She worked for a couple of years in the pharmaceutical industry and later moved to the USA. She entered the graduate program at the University of Texas Rio Grande Valley (UTRGV) in Spring 2019. She has earned a Master of Science (MS) in Biology in December 2020 from the UTRGV. She can be reached at udawantshreya31@gmail.com.