Universitat de les Illes Balears

MASTER'S THESIS

DRUG REPURPOSING APPROACH TO IDENTIFY AND VALIDATE POTENTIAL DRUG CANDIDATES IN VITRO FOR GLIOBLASTOMA BASED ON DIFFERENT MOLECULAR SUBTYPES

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Master's Degree in Biomedical Research

(Specialisation/Pathway *Transversal*)

Centre for Postgraduate Studies

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ABSTRACT

Glioblastoma (GBM) is one of the most malignant types of central nervous system tumors with poor prognosis and lack of effective therapies. Recent high-throughput data revealed the existence of three different GBM molecular subtypes: Classical, Mesenchymal, and Proneural. To overcome the high costs associated with current methods for subtype classification, the Cancer Epigenetics Lab (EPIGEN Lab) at IdISBa has developed a more affordable classification method based on quantitative PCR (qPCR) analysis using a panel of 15 genes. Here, we have tested this qPCR-based method to classify several GBM cell lines in vitro. However, our results showed that the qPCR method was not able to match the subtype classification obtained from conventional methods based on transcriptomic analysis. This discrepancy was probably due to a lack of heterogeneity between the selected GBM cell lines selected, an anticipated limitation of the qPCR method. Based on transcriptomic analysis of GBM subtypes and Gene Set Enrichment Analysis, we generated a list of subtype-specific candidate drugs. Although at a very preliminary stage, our in vitro test of selected drugs showed some promising results on subtype specificity. These results are encouraging and highlight the relevance of GBM patient stratification based on their molecular subtypes.

INTRODUCTION

Glioblastoma (GBM) is one of the most aggressive and lethal primary brain tumors where the median survival of patients is about 15 months (Hanif, Muzaffar, Perveen, Malhi, & Simjee, 2017). The current standard of care includes surgery followed by concurrent radiotherapy and chemotherapy treatment (Simpson & Galanis, 2006). However, these treatments are not sufficient given the aggressive nature of this type of brain tumor. First, GBM presents an extremely fast infiltration capacity, which prevents complete surgically isolation due to its tumor cell invasion (Reardon & Wen, 2006). Also, GBM cells exhibit challenging drug resistance traits, resulting in a vast majority of patients relapse after an initial response (Gallego, 2015). This resistance to treatment is often associated with a high tumor heterogeneity together with genetic and epigenetic alterations, establishing a strong tumor microenvironment that confers phenotypic plasticity allowing tumor cells to easily adapt and escape from drug therapy.

Temozolomide (TMZ) is a chemotherapeutic agent and is the drug of choice in GBM treatment given its ability to penetrate from blood to cerebrospinal fluid (Nam & De Groot, 2017). TMZ is an alkylating agent that causes the methylation of DNA at the O^6 position of guanine, resulting in mismatch pairing during DNA replication and leading to an arrest of the cell cycle and subsequent cell apoptosis (Margison, Santibáñez Koref, & Povey, 2002). O^6 methylguanine-DNA methyltransferase (MGMT) prevents the effect of TMZ by capturing the O^6-position guanine methyl-adduct and revert this alteration (Pegg, Dolan, & Moschel, 1995). Thus, the expression of MGMT is widely used as a biomarker to predict the response of GBM patients to TMZ (Esteller et al., 2000). Also, previous studies have shown that several drug combinations can improve TMZ sensitivity (Nakada, Furuta, Hayashi, Minamoto, & Hamada, 2012). Therefore, the identification of potential drug candidates that improve the therapeutic effect of TMZ is of particular relevance, since it may lead to obtaining better clinal outcomes in GBM patients.

Over the last years, the molecular characterization of GBM revealed that it is a highly heterogeneous tumor that can be classified in three different subtypes; Classical (CL, Mesenchymal (MES), and Proneural (PN) (Wang et al., 2017). These three subtypes present their own specific transcriptomic profile with their associated molecular pathway alterations: CL present alterations in the expression levels of epidermal growth factor receptor (EGFR), MES present alterations in neurofibromatosis type I (NF1), while PN is characterized for aberrant expression of platelet-derived growth factor receptor alpha (PDGFRA)/IDH1 (Verhaak et al., 2010). Given intra-tumor heterogeneity, multiple subtypes can coexist in GBM, which makes it more complicated to design subtype-specific strategies for patients (DOI: 10.1126/science.1254257). Current methods for GBM subtype classification require high-throughput analysis, which is associated with elevated costs and not often available as a point-

of-care diagnostic tool. However, the Cancer Epigenetics Lab (EPIGEN Lab) at IdISBa has developed a cost-effective classification method for GBM based on quantitative PCR (qPCR) analysis with only 15 candidate genes for each subtype (manuscript submitted). This qPCR-based method has an efficiency of 90% and it could be routinely performed in the majority of healthcare centers. Thus, a more affordable classification method could improve patient stratification to develop effective treatments against a specific GBM subtype.

Drug repurposing is an affordable strategy to identify new applications for approved drugs, which are often indicated for common conditions, to treat more complicated malignancies (Pushpakom et al., 2018). In cancer, this is a common practice for researchers since it can reduce the timelines and lower the costs of developing a new drug. Several examples for repurposing drugs in cancer include rapamycin (immunosuppressor), prazosin (hypertension treatment), and metformin (antidiabetic) (Meric-Bernstam & Gonzalez-Angulo, 2009; Wallace & Gill, 1978; Pollak, 2014). Recent advances in computational analysis such as genomic, epigenomic, and transcriptomic profiling to study oncogenic pathways for several types of cancer could lead to new opportunities in the discovery of new drug targets.

The present study was designed to address two main objectives; 1) to validate the EPIGEN Lab's PCR-based classification method in vitro, to test on several GBM cell lines, and 2) to identify available drugs targeting GBM-specific subtypes based on their transcriptomic features.

MATERIALS AND METHODS

Cell culture

T98, LN229, U87, U251, HS683 (commercial cell lines, ATCC), P1 and P4 (GBM patient-derived cell lines) cells were obtained from the Cancer Cell Biology Group (UIB). Cells were cultured on 25 cm^2 flasks and 6-well plates in 10mL and 2mL complete medium (10% Fetal Bovine Serum (FBS), 2Mm L-glutamine, 100 units/mL penicillin G, 100 ug/mL streptomycin), respectively. Cells were thawed at 37ºC for 2 min, they were resuspended in 5 mL of complete medium and centrifuged during 4 min at 300g. The supernatant was removed and the pellets were resuspended in 5mL of complete media. Upon reaching 70-80% confluency, cells were washed with 5 mL of PBS and detached with 1mL of Trypsin during 5 min at 37ºC. Cells were seeded at the desired cell density.

RNA extraction and cDNA synthesis

Total RNA was extracted and purified from cell lines essentially using the Total RNA Kit (Omega Biotek) following manufacturer's instructions. RNA samples were stored at -80ºC. RNA quantification was performed with a NanoDrop Spectrophotometer (NDS). 2 µL of RNA elution buffer was loaded and the system was initialized. cDNA was synthesized from 300 ng of RNA at 25ºC for 10 minutes, 42ºC for 15 minutes, 48ºC for 15 minutes, 85ºC for 5 minutes and kept at 4ºC.

Data mining GBM

Primary transcriptome data from GBM patients was obtained using the Human Genome u133a Affymetrix platform. It was downloaded from the Broad Institute GDAC Firehose database (https://gdac.broadinstitute.org/), specifically the GBM cell lines data. The processing of RNA-Seq raw data was assessed using the RStudio software.

Transcriptome data series from GBM cell lines were obtained from the NCBI GEO database. Samples from these sets were filtered in order to exclusively use the data present in the TCGA dataset. Only genes present in TCGA data were maintained for further analysis. A log2 standardization was performed to the non previously standardized sets. The following sets of GBM cell lines were downloaded and the following samples considered: GSE4536 (T98_1, T98_2, LN229_1, LN229_2, U87_SC_1, U87_SC_2, U87_1, U87_2, U251_IC_1, U251_IC_2, U251_SC_1, U251_SC_2, U251_1, U251_2); GSE124145 (U251 rep1, U251 rep2, U251 rep3); GSE23806 (conventional glioma cell line serum_T98G, conventional glioma cell line serum_LN229, conventional glioma cell line serum_U87MG, conventional glioma cell line serum_HS683); GSE9171 (Glioblastoma cell line GBM_Hs683); GSE1682 (Hs 683 Control-1, Hs 683 Control-2, Hs 683 Control-3). All genes Z-ratio was performed in order to standardize gene expression and a t-student analysis was used to select significantly expressed genes (Z-ratio > 1,5) using Rstudio.

GBM subtype classification: Transcriptome-based analysis

RNA-Seq based classifying method, T98, LN229, U87, U251, HS683, P1 and P4cell lines were classified for transcriptional subtypes with the SubtypeME function of GlioVis (http://gliovis.bioinfo.cnio.es/). GlioVis offers a visualization of a subtype classification of GBM samples based on mRNA expression profiles. TCGA GBM data available and transcriptome data series from cell lines were uploaded and the application conditions were set to separation by semicolon, 1000 permutations and 3-Way classification visualization. The final outcome is a table containing the samples and the classification performed by 3 different algorithms, those

being SVM, KNN and GSEA as well as a Majority Call showing the preferred outcome considering the 3 others.

GBM subtype classification: qPCR-based method

Expression levels of 15 significantly expressed selected genes were analyzed using real-time, quantitative PCR to obtain data for further classification by the previously developed qPCR-based classification method. The list of selected genes is the following: GNAI1, EGFR, CTSC, DYRK3, ARPC1B, OLIG2, TRIP6, VAV3, FGFR3, BCAN, NPC2, SLC1A1, GAL3ST1, GPR17, ZNF217 and the cell lines used as samples were T98, HS683, U251, U87, LN229, P4 and P1. The Cdna samples were diluted to 5 ng/ul and the amplifications were done using the SYBR Green PCR Master Mix. The experiments were carried out in triplicate for each data point. The relative quantification in gene expression was determined using the $2^{-\Delta Ct}$ formula (ΔCt = mean CT – GAPDH).

GSEA

Previously selected genes by significance were introduced in the Metascape (https://metascape.org/) database together with the complete list of genes as background. Custom analysis feature was selected in order to obtain pathways related to cancer related processes. The features chosen to be analysed in the Metascape report were the following GOSlim (Ontology), Canonical Pathways and KEGG Pathway. The resulting heatmap with pathways up- and downregulation was the analysis used for further drug selection.

Drug selection GBM Subtype-specific drug repurposing

The following list of pathways were introduced in the *Gene2Drug* (https://gene2drug.tigem.it/) database to obtain a list of drugs with ability to dysregulate them: ERBB Signaling Pathway, Signaling by ERBB2, Regulation of MAPK Cascade, Negative Regulation of the PI3K/AKT Network, PGC1A Pathway, Angiogenesis and Regulation of Notch Signaling Pathway for the classic subtype; ERK1 and ERK2 Cascade, NOD Like Receptor Signaling Pathway, PID FRA Pathway, E2F Targets and miRNA Binding for the mesenchymal subtype; Cell Cycle, G2M Checkpoints, PID Aurora B Pathway, PID FOXM1 Pathway, RANMS Pathway and PID E2F Pathway for the proneural subtype.

The list of previously selected genes from each subtype regarding significantly up- and down regulation was introduced in Unimore (http://gda.unimore.it/) and a list of drugs were obtained that were supposed to reduce cell viability when this gene pattern was present.

In vitro drug treatments

Drug treatments were carried out in 96-well plates by adding 100uL of each drug dilution to each well. Triplicates were incubated for 96 hours at 37ºC. For treatment, different doses ranging from 1.56 to 200 µM were tested for 5-azacytidine; 1 to 1000 µM were tested for oxaliplatin; and 1 to 500 µM were tested for everolimus. Cell viability was measured on a HT Synergy microplate reader using the CyQUANT™ XTT Cell Viability Assay (ThermoFisher) following manufacturer's instructions. Half maximal inhibitory concentration (IC50) values were calculated using Prism 7.1 software (GraphPad).

RESULTS

Subtype classification of GBM cell lines: transcriptome-based analysis

Transcriptomic datasets of GBM cell lines (T98, LN229, U87, U251, and HS683) were analyzed by GlioVis, a web application for brain tumor data analysis. GBM subtype was determined based on three different classification methods: Support Vector Machine (SVM), K-nearest neighbor (K-NN), and single sample Gene Set enrichment Analysis (ssGSEA). The final classification was determined by Majority Call, based on the results obtained from these methods. As shown in ***Table 1***, T98 (3/3), U87 (5/5) and U251 (9/9) cell lines were classified as 100% mesenchymal. LN229 cell line was classified as 50% mesenchymal (2/4) and 50% proneural (2/4). HS683 cell line was classified as 40% mesenchymal (2/5) and 60% (3/5) classic.

Subtype classification of GBM cell lines: qPCR-based method

To validate the PCR-based subtype classification method in GBM cell lines (T98, LN229, U87, U251, HS683, P4 and P23), we performed gene expression analysis of a panel of fifteen genes, previously described by the Cancer Epigenetics Lab at IdISBa (manuscript submitted). The classification panel consisted of three groups of five genes, each of them corresponding to a mesenchymal (DYRK3, BCAN, NPC2, ARPC1B, OLIG2), classic (GNAI1, EGFR, VAV3, FGFR3, CTSC), and proneural (SLC1A1, GAL3ST1, ZNF217, TRIP6, GPR17) subtype. Gene expression analysis of

GBM cell lines was determined by q-PCR as shown in **Figure 1**. Next, gene expression values from each panel were analyzed by the PCR-based classification method to determine their specific GBM subtype. Based on the highest score on each subtype, HS683, T98, U87, and P4 cell lines were classified as classical; LN229 and P23 cell lines were classified as mesenchymal; and U251 cell line was classified as proneural **(Table 2)**.

Gene Set Enrichment Analysis

To determine the pathways enriched on each subtype, we generated a list of differentially expressed genes from TCGA datasets. Among a total of 9032 genes, we selected significantly up- and downregulated genes for the classic (1498), mesenchymal (1277), and proneural (1248) subtype. The data obtained was uploaded into Metascape (https://metascape.org/), an application for gene enrichment visualization, to perform gene ontology and gene set enrichment analysis (GSEA). Based on top 100 up- and downregulated genes, enriched pathways for each subtype are shown in **Figure 3**. In the classical subtype, we observed a downregulation of inflammation related pathways (macrophage activation, regulation of cytokine production) and upregulation of angiogenesis related pathways. On the other hand, in the mesenchymal subtype we observed an upregulation of inflammation related pathways (leukocyte activation and migration) and downregulation of neuronal development pathways. In the proneural subtype, we observed an upregulation of cell cycle and replication associated pathways (cell division, regulation of mitotic cell cycle) and downregulation of ECM organization and angiogenesis pathways **(Table 3).**

GBM subtype-specific drug repurposing

Drugs were selected based on two different approaches: gene expression and gene enriched pathways. For drug selection based on gene expression, we used our TCGA data and Unimore, a web tool that combines drug sensitivity data and gene expression profiles. The selected candidates were everolimus for mesenchymal, and oxaliplatin for proneural. For drug selection based on gene enriched pathways, we used pathways obtained from our GSEA associated with ERBB signaling pathway. These pathways were analyzed on Gene2Drug, a web that ranks a list of small molecules according to their ability to dysregulate of the selected pathways. The selected candidate for classical subtype was 5-azacytidine.

In vitro drug treatment of GBM cell lines

Among six drug candidates we selected three of them, one for each subtype: 5-azacitidine (classical), oxaliplatin (proneural) and everolimus (mesenchymal). To test the specificity of these drugs, we performed cell viability assays on, HS683 as classical, LN229 as proneural, and T-98 as mesenchymal subtype (according to GlioVis classification) to determine their half maximal inhibitory concentration (IC50). After exposing these cell lines to increasing doses of each drug, cell viability was analyzed after 48h (**Figure 4**). IC50 values are shown as shown in **Table 4**, IC50 values revealed that 5-azacitidine had a similar effect in all three cell types, whereas everolimus and oxaliplatin had a strong effect in LN229.

DISCUSSION

The present study aimed to evaluate the use of existing drugs for GBM treatment based on their specific molecular subtype. The experimental design was focused on i) validate a PCR-based method for GBM subtype classification, and ii) in vitro test a selection of drug candidates on subtype-specific GBM cell lines.

The classification based on transcriptomic profile analysis classified all samples as 100% mesenchymal except LN229 (50%proneural) and HS683 (60% classic). The PCR-based method didn't match the transcriptomic profile based classification. One limitation of our PCR-based method was that it was originally designed to classify tumor samples, instead of cell lines. Also, a second limitation was the small number of samples available (n=5), as the PCR-based method also requires a large number of samples. A third limitation was that the majority of samples presented a mesenchymal phenotype (per transcriptomic analysis), and our PCR-based method requires a more heterogeneous set of samples presenting all three different subtypes. To overcome these limitations in future experiments, further classification analysis using these methods should consider using a much larger set of samples which an equivalent representation of the three different subtypes.

The GSEA results suggest that in the mesenchymal subtype there is a significant upregulation of inflammation related pathways. This findings are in line with previous studies indicating that inflammatory gene expression is overrepresented in the mesenchymal subtype, compared to the others (Zanotto-Filho et al., 2017). These same authors have shown that inflammatory pathways are downregulated in the classical subtype, which supports our findings where we also have observed a significant downregulation of inflammatory genes in the classical subtype. The web tool Unimore allowed the drug selection by gene expression of everolimus and oxaliplatin. Among similar tools that integrate drug activity and molecular data, it integrates directly genomic and pharmacological data from the two largest screenings of small-molecule

sensitivity (NCI-60 screening) and genomic profile (Cancer Cell Line Encyclopedia profiling) in cancer cell lines (Caroli, Sorrentino, Forcato, Del Sal, & Bicciato, 2018). The Gene2Drug platform allowed the selection of 5-azacytidine (classical specific) since ERBB pathway was upregulated in the classical subtype. It has been demonstrated that this software outperforms the gold standard for drug-chemical interactions STITCH database (Napolitano et al., 2018). From the complete list of drugs selected initially, everolimus, oxaliplatin and 5-azacytidine were finally chosen for the experiments and previous research that suggested possible effects in GBM: 5-azacitidine treatment seems to significantly reduce glioblastoma cell viability and increase cellular apoptosis (Kratzsch et al., 2018); oxaliplatin exposition evidences immunogenic GBM cell death and reduced levels of enzymes that contribute to pro-tumor functions (Roberts et al., 2018); and everolimus is an mTOR inhibitor that combined with other inhibitors like palbociclib seems to strongly disrupt GBM metabolism (Olmez et al., 2017).

In vitro drug tests suggested that 5-azacytidine (classical-specific) had a dramatic effect in all three lines, resulting in a strong drop in cell viability between 12.5 and 25 µM. However, despite the fact that the differences between all three IC50 were not significant, 5-azacytidine presented the strongest effect on the classical-classified HS683 (IC50 = 11.67) compared to T98 (IC50 = 12.99) and LN229 (IC50 = 12.39). Further analysis expanding the drug concentrations between the 12.5-25 µM range should be performed to obtain a more precise IC50 for each line. Oxaliplatin (proneural-specific) showed a significant effect on the proneural-classified LN229 line (IC50 = 57.22) compared to T98 (IC50 = 85.08) and HS683 (IC50 = 82.88). These results were in line with our experimental design, since this drug was selected as proneural subtype-specific, according to Gene2drug. On the contrary, Everolimus (mesenchymal-specific) also showed a strong effect on LN229 line (IC50 = 44.8) compared to T98 (IC50 = 55.81) and HS683 (IC50 = 59.3), while being selected as a mesenchymal subtype-specific drug. However, LN229 line was also classified as 50% mesenchymal by transcriptomic analysis, therefore, everolimus might present some type of specificity for LN229 cells given its partial mesenchymal identity.

Overall, our results demonstrated that exists a specificity for drug treatments based on different GBM cell lines. Although still very preliminary, our results suggested that this specificity might correlate with a subtype-specific cell population. In order to validate these results, we would need to test these drug candidates with different cell lines that strictly represent the three different GBM-subtypes. Altogether, these results are encouraging and could lead to some promising results in the future by contributing on the repurposing of specific drugs to design specific treatments based on patient's GBM subtypes.

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FIGURES AND TABLES

Table 1. Subtype classification of GBM cell lines based on RNA-seq transcriptional profile

Cell Line	GSE	Array	Samples	Subtype			
				SVM	KNN	GSEA	Majority
T98	GSE4536	U133 Plus	T98_1	Mes	Mes	Mes	Mes
			T98_2	Mes	Mes	Mes	Mes
	GSE23806	U133 Plus	Conventional glioma cell line, serum_T98G	Mes	Mes	Mes	Mes
LN229	GSE4536	U133 Plus	LN229_1	Pro	Mes	Mes	Mes
			LN229_2	Pro	Mes	Pro	Pro
	GSE23806	U133 Plus	Conventional glioma cell line, serum_LN229	Pro	Mes	Pro	Pro
	GSE9171	U133	Glioblastoma cell line GBM_LN229	Pro	Mes	Mes	Mes
U87	GSE4536	U133 Plus	U87_SC_1	Mes	Mes	Mes	Mes
			U87_SC_2	Mes	Mes	Mes	Mes
			U87_1	Mes	Mes	Mes	Mes
			U87_2	Mes	Mes	Mes	Mes
	GSE23806	U133 Plus	Conventional glioma cell line, serum_U87MG	Mes	Mes	Mes	Mes
U251	GSE4536	U133 Plus	U251_IC_1	Mes	Mes	Mes	Mes
			U251_SC_1	Mes	Mes	Mes	Mes
			U251_SC_2	Mes	Mes	Mes	Mes
			U251_1	Clas	Mes	Mes	Mes
			U251_2	Mes	Mes	Mes	Mes
			U251_IC_2	Mes	Mes	Mes	Mes
	GSE124145	U133	U251 rep1	Mes	Mes	Mes	Mes
			U251 rep2	Mes	Mes	Mes	Mes
			U251 rep3	Mes	Mes	Mes	Mes
HS683	GSE23806	U133 Plus	Conventional glioma cell line, serum_HS683	Mes	Mes	Mes	Mes
	GSE9171	U133	Glioblastoma cell line GBM_Hs683	Mes	Mes	Mes	Mes
	GSE1682	U133a	Hs 683 Control-1	Clas	Mes	Pro	Clas
			Hs 683 Control-2	Clas	Mes	Clas	Clas
			Hs 683 Control-3	Clas	Mes	Pro	Clas

Figure 1. RNA q-PCR expression results. *Gene expression analysis of GBM cell lines determined by q-PCR.*

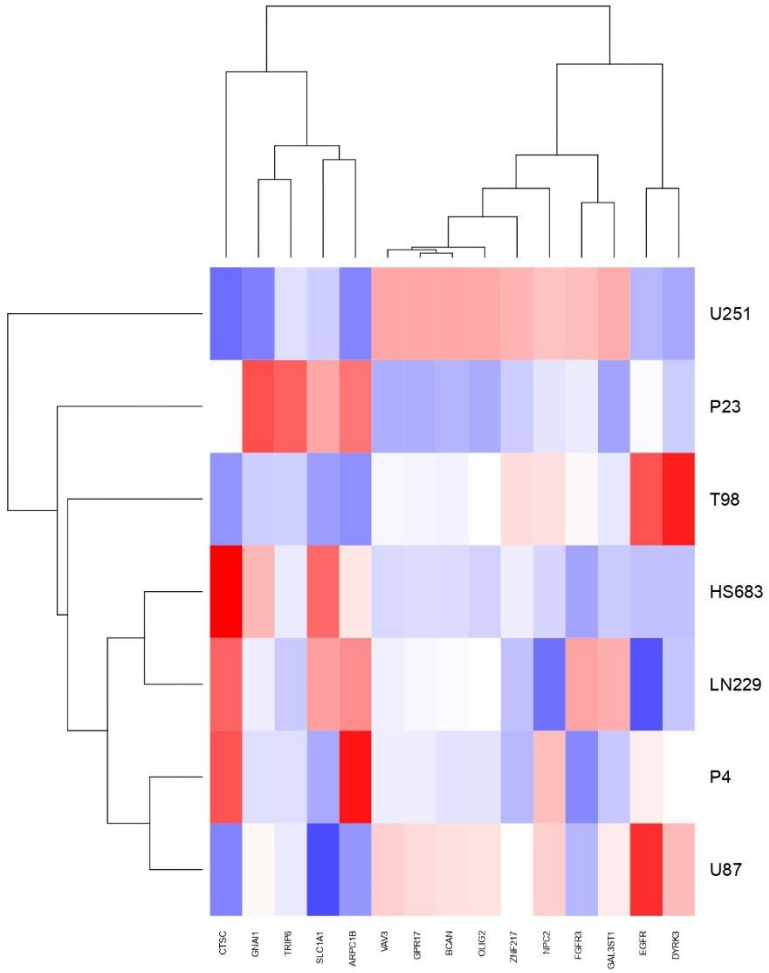
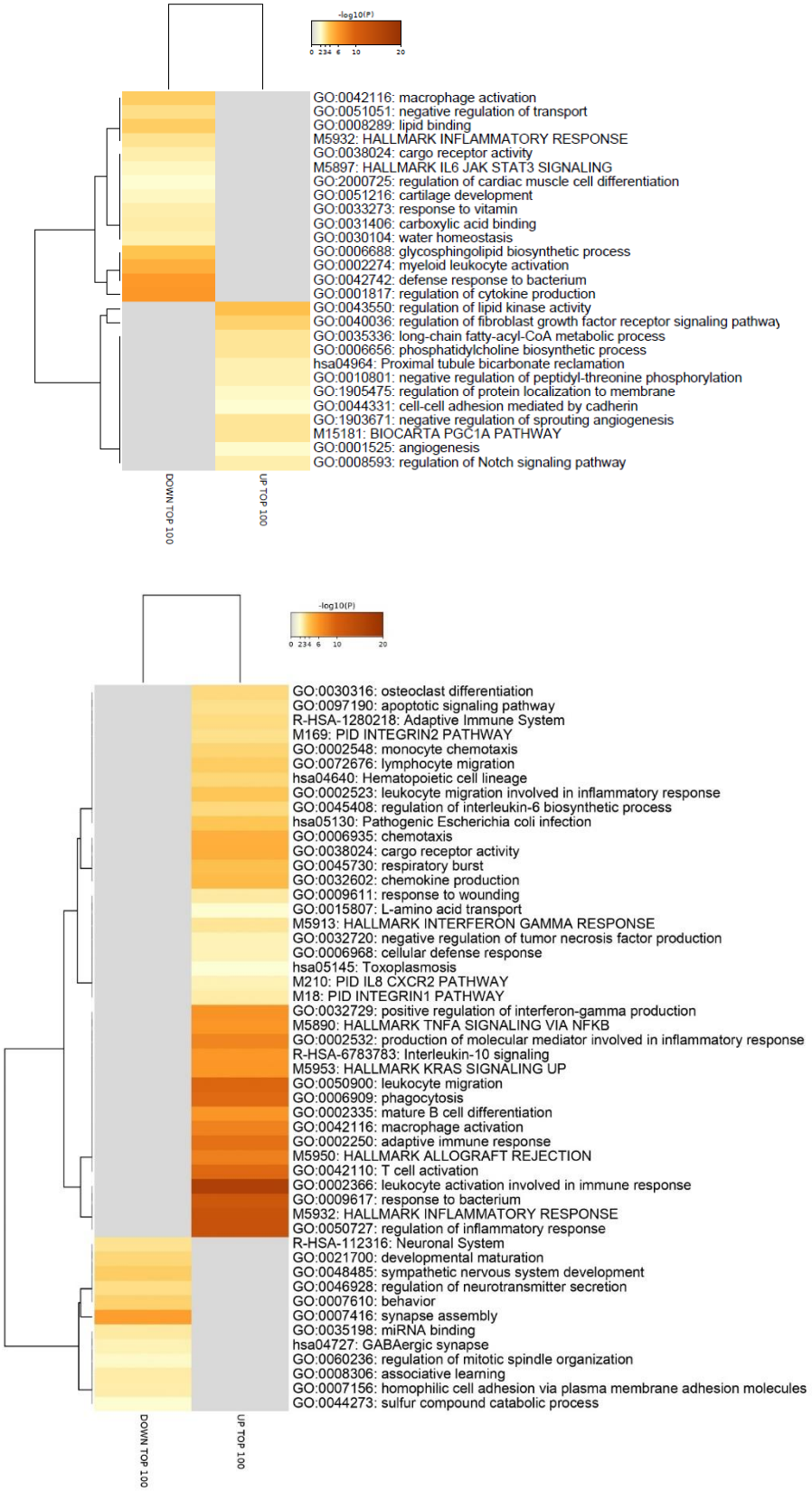


Table 2. Subtype classification of GBM cell lines based on q-PCR analysis. *Classification score corresponding to each cell line. A highest score represents higher closeness to the given subtype.*

Cell Line	Subtype Score			Final Subtype
	Classical	Mesenchymal	Proneural	
HS683	0.491843447	0.424119138	0.421929379	Classic
LN229	0.305379505	0.567007904	0.428157197	Mesenchymal
P23	0.269881737	0.509116066	0.278729194	Mesenchymal
P4	0.438540404	0.413716673	0.207863187	Classic
T98	0.499088777	0.171840796	0.2911131	Classic
U251	0.512892052	0.628285422	0.689983702	Proneural
U87	0.391811501	0.309899204	0.161504689	Classic

Figure 3. <u>Gene Enrichment pathway regulation of considering 100 most up and down regulated genes</u>. *A) Classic subtype. B) Mesenchymal subtype. C) Proneural subtype.*



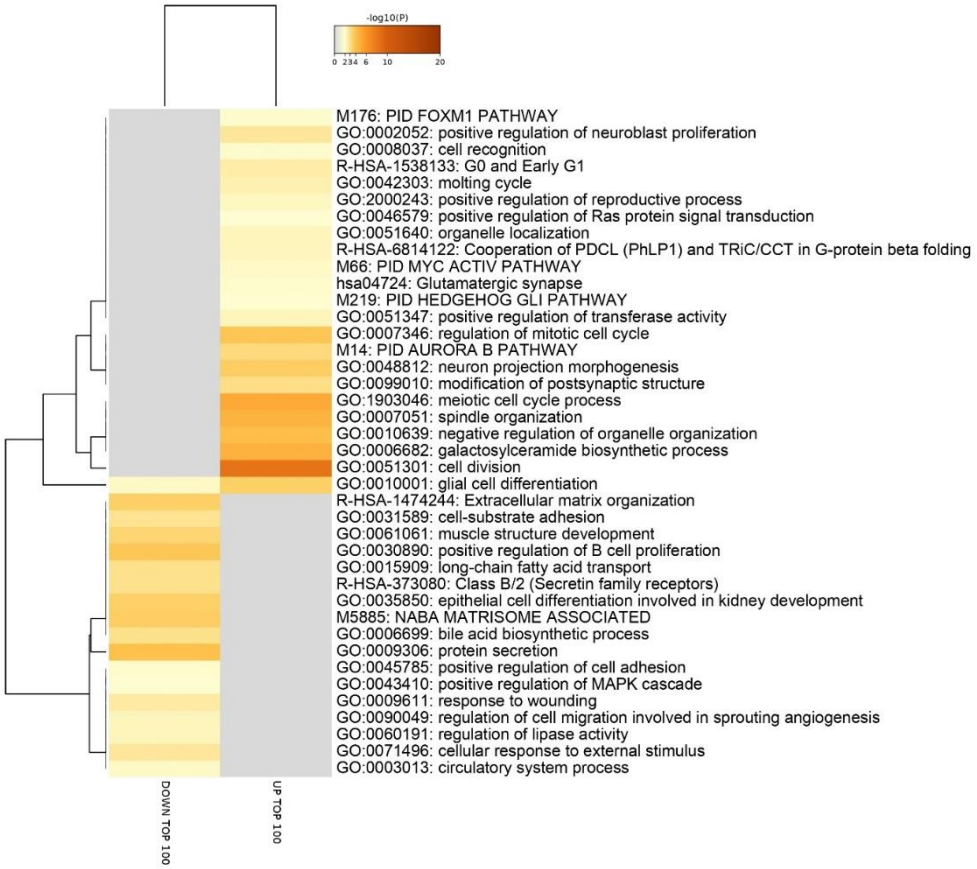
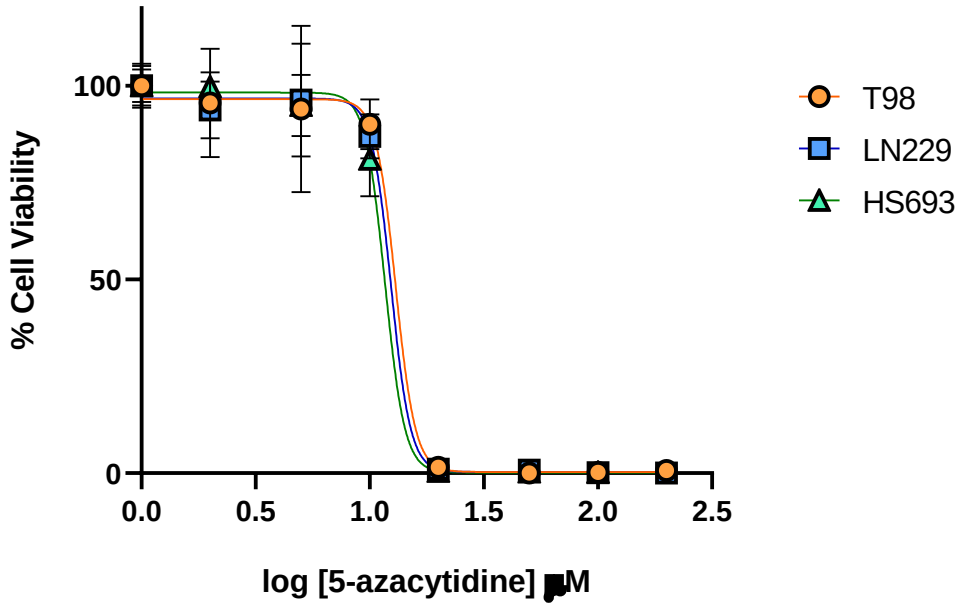
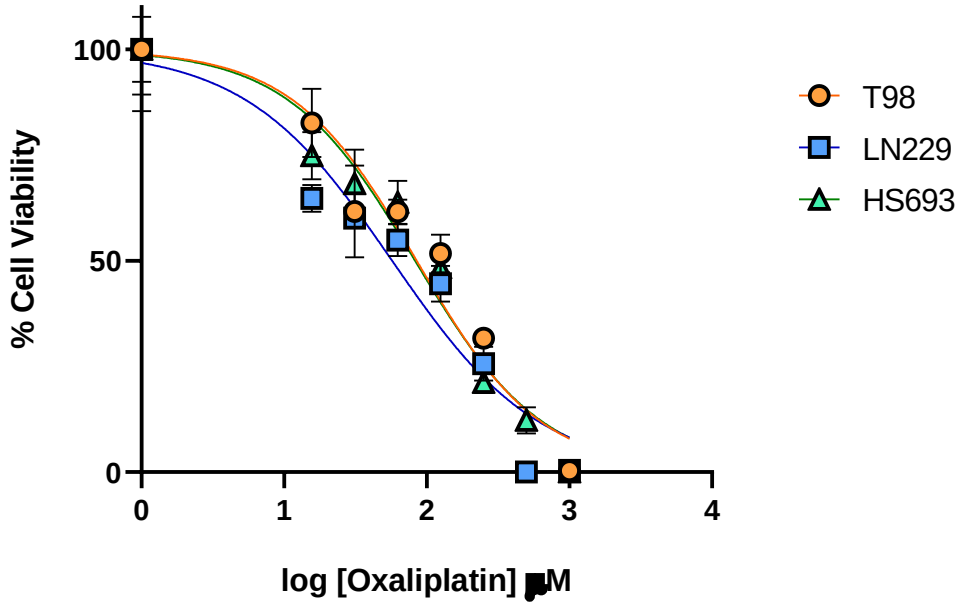

-log10(P)
0 2 3 4 6 10 20
M176: PID FOXM1 PATHWAY
GO:0002052: positive regulation of neuroblast proliferation
GO:0008037: cell recognition
R-HSA-1538133: G0 and Early G1
GO:0042303: molting cycle
GO:2000243: positive regulation of reproductive process
GO:0046579: positive regulation of Ras protein signal transduction
GO:0051640: organelle localization
R-HSA-6814122: Cooperation of PDCL (PhLP1) and TRiC/CCT in G-protein beta folding
M66: PID MYC ACTIV PATHWAY
hsa04724: Glutamatergic synapse
M219: PID HEDGEHOG GLI PATHWAY
GO:0051347: positive regulation of transferase activity
GO:0007346: regulation of mitotic cell cycle
M14: PID AURORA B PATHWAY
GO:0048812: neuron projection morphogenesis
GO:0099010: modification of postsynaptic structure
GO:1903046: meiotic cell cycle process
GO:0007051: spindle organization
GO:0010639: negative regulation of organelle organization
GO:0006682: galactosylceramide biosynthetic process
GO:0051301: cell division
GO:0010001: glial cell differentiation
R-HSA-1474244: Extracellular matrix organization
GO:0031589: cell-substrate adhesion
GO:0061061: muscle structure development
GO:0030890: positive regulation of B cell proliferation
GO:0015909: long-chain fatty acid transport
R-HSA-373080: Class B/2 (Secretin family receptors)
GO:0035850: epithelial cell differentiation involved in kidney development
M5885: NABA MATRISOME ASSOCIATED
GO:0006699: bile acid biosynthetic process
GO:0009306: protein secretion
GO:0045785: positive regulation of cell adhesion
GO:0043410: positive regulation of MAPK cascade
GO:0009611: response to wounding
GO:0090049: regulation of cell migration involved in sprouting angiogenesis
GO:0060191: regulation of lipase activity
GO:0071496: cellular response to external stimulus
GO:0003013: circulatory system process
DOWN TOP 100
UP TOP 100

Table 3. Pathways considered for drug repurposing through *Gene2Drug*. *List of pathways introduced in the drug repurposing tool corresponding to each subtype.*

Subtype	Pathways
Classical	ERBB signaling pathway
	Signaling by ERBB2
	Regulation of MAPK Cascade
	Negative regulation of the PI3K/AKT network
	Regulation of FGF
	PGC1A pathway
	Angiogenesis
	Regulation of notch signaling pathway
Mesenchymal	ERK1 and ERK2 cascade
	NOD like receptor signaling pathway
	PID FRA pathway
	E2F targets
	miRNA binding
Proneural	Cell cycle
	G2M checkpoints
	PID aurora B pathway
	PID FOXM1 pathway
	RANMS pathway
	PID E2F pathway

Figure 4. <u>5-azacytidine, oxaliplatin and everolimus decrease cell viability in cell lines T98, LN229 and HS689</u>. *A) 5-azacytidice cell viability curve. B) Oxaliplatin cell viability curve. C) Everolimus cell viability curve.*





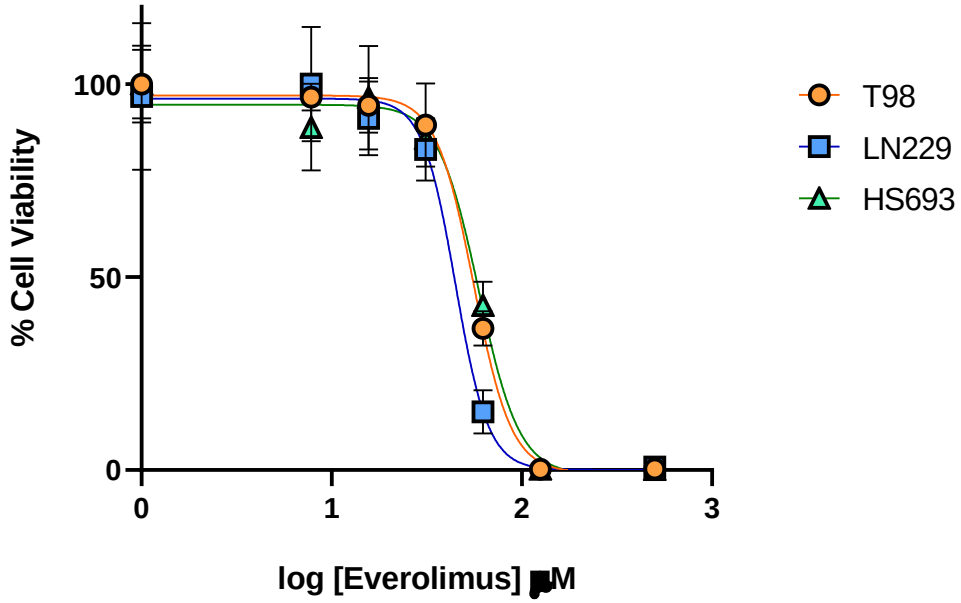


Table 4. IC50 score of 5-azacitidine, oxaliplatin and everolimus drugs against cell lines.

IC50	T98	LN229	HS683
5-aza	12.99	12.39	11.67
Oxaliplatin	85.08	57.22	82.88
Everolimus	55.81	44.8	59.3