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Integrative analysis of rewired central metabolism in temozolomide resistant cells

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ABSTRACT

An authenticated U87MG clonal glioblastoma cell line was investigated to identify a sub-population of neurospheroidal (NSP) cells within the main epithelial population (U87MG). The NSP cells sorted using Fluorescence Assisted Cell Sorting (FACS) showed varied morphology, 30% lower growth rates, 40% higher IC_{50} values for temozolomide drug and could differentiate into the glial cell type (NDx). Metabolite profiling using HR-LCMS identified glucose, glutamine and serine in both populations and tryptophan only in U87MG as growth limiting substrates. Glycine, alanine, glutamate and proline were secreted by U87MG, however proline and glycine were re-utilized in NSP. Exo-metabolite profiling and phenotypic microarrays identified differential metabolism of primary carbon sources glucose and derived pyruvate for U87MG; glutamine and derived glutamate metabolism in NSP. Differential mRNA abundance of AKT1, PTEN, PIK3CA controlling metabolism, drug efflux, nutrient transport and epigenetic control MDM2 are potentially critical in shaping DNA methylation effects of temozolomide. Our study provides a new insight into the combined effect of these factors leading to temozolomide resistance in NSP.

1. Introduction

Temozolomide (TMZ), anti-cancer prodrug of Temodar, is an imidazotetrazine that has increased the prognosis of highly aggressive Glioblastoma Multiforme (GBM) [1]. The therapeutic index is dependent on molecular markers like O^6-methylguanine methyltransferase (MGMT) and/or lack of DNA repair in GBM [2]. Other contributors include isocitrate dehydrogenase type 1 and type 2 (IDH1/2) mutations that metabolically reprogram the cell to form neo-metabolite 2-hydroxyglutarate [3] and the epigenetic status controlling cytosine-phosphate-guanine (CpG) island methylator phenotype (G-CIMP) [4]. U87MG, a completely sequenced [5] and commonly studied grade IV glioma cell line [6] is wildtype for IDH1/IDH2 is sensitive to temozolomide [1]. Cell line model systems, indispensable tools in providing preclinical biological insight, are known to harbor minority populations of putative stem-like cells, molecularly defined by dye extrusion phenotypes that may cause resistance to chemotherapy [7,8]. With increased discovery of gene and protein expression signatures [4] and detailed molecular characterization to characterize the drug sensitivity, rigorous phenotyping of these sub-populations, in addition to morphology and drug efflux is critical to understand resistance.

In this study, a sub-population of neurospheroidal cells (NSP) morphologically distinct from epithelial cells were identified in U87MG (Grade-IV GBM cell line) and sorted using Fluorescence Assisted Cell Sorting (FACS). To our knowledge, this is the first study addressing the functional characterization in terms of integrating knowledge related to drug efflux, growth/proliferation, nutrient preferences and metabolite profiling to drug dose response establishing an integrated resistance paradigm.

2. Materials and methods

Cell culture: An authenticated U87MG cell line (HTB-14; Human Glioblastoma Multiforme from ATCC; Supplementary File 2,3)

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was cultured in conditions as per ATCC guidelines. Neurospheres (NSP) were maintained in neurobasal medium supplemented with B27 supplement, 0.2 μg/mL each of epidermal growth factor, EGF and basic fibroblast growth factor, bFGF. NSP were cultured as floating spheres in low attachment T-75 flasks or 6 well/24 well plates (Nunc™). All chemicals and labware was purchased from ThermoFisher Scientific™.

Fluorescence microscopy and flow cytometry based separation of cells: Hoechst 33342 stain (1 mg/mL) was used for all fluorescence studies on EVOS® FLoid® cell imaging system. The sub-population sorting assay (Supplementary File 1) as previously described [9] was performed for FACS with cells at 70–80% confluency using BD FACSAria III (BD biosciences Pvt. Ltd) and analyzed using BD FACSDiva™ software v6.1.3. Cells were captured in a Hoechst Blue versus Hoechst Red dot plot in the presence and absence of Verapamil.

Growth/proliferation studies: Growth/proliferation of the cells (Parental U87MG, U87MG and NSP) were monitored via cell counts over a period of 216 h (9 days). The initial seeding (*No*) was ~10000 cells per well. All cells were harvested every 24 h and counted using hemocytometer using trypan blue dye exclusion assay. NSP was trypsinized before counting. Growth curves were graphed and data fitted with Gompertz function (GraphPad Software, San Diego California USA).

Temozolomide dose response curves: For dose-response experiments, four replicates at ~20,000 cells per well were plated in 96-well Nunc™ tissue culture plates in full growth medium for 24 h, treated with different doses of TMZ in serial dilutions (0.05 M–5 M) followed by cell viability tests using the MTT assay. Appropriate cell controls (without TMZ treatment) were used to calculate IC_{50}.

***In vitro* differentiation of NSP:** NSP were grown in DMEM followed by addition of 1% N2 supplement. Growth factors, bFGF and EGF reconstituted in 0.1% BSA solution (100 μg/mL) were used. An increased B27 (2%) supplemented after 3 days drove the differentiation. The differentiated cells (NDx) were cultured separately using the same method of culture of U87MG cells.

Metabolite profiling using liquid chromatography - high resolution mass spectrometry (LC-HRMS): Samples harvested during growth every 24 h over a period of 9 days were used for metabolic profiling performed in Accela 1250 ultra-performance liquid chromatography (UPLC) in tandem with Thermo Q-exactive high resolution mass spectrometer (HRMS) using heated electrospray ionization (HESI) interface. Parameters of LC-HRMS run and sample extraction protocols and standard preparation are provided (Supplementary File 1). Accurate mass-extracted ion chromatograms (AM-XIC) of various metabolites were generated followed by peak confirmation by MS/MS spectral peak matching. Confirmed metabolites were quantified using internal standard normalized linear regression models. The raw and processed data of MS/MS confirmations, concentration ranges and regression fits are provided (Supplementary File 4).

Selection of CAN genes using Pathway Studio™ analysis: Pathway Studio 11.0.5 software from Elsevier (https://product.pathwaystudio.com/mammalcedfx/) was used to select CAN genes *in silico*. Initial pathways/networks implicated in GBM were reconstructed using related key terms. Networks were validated (confidence score-3) and casual pathways inferred by filtering to include >25 references. Pathway containing 23 genes (Supplementary File 1: Table S3) and 1 miRNA resulted. These genes were expression profiled to quantitate relative mRNA abundances.

Real-time PCR analysis of CAN genes: All primers designed were based on the Primer3web (http://primer3.ut.ee/) solutions and validated using NCBI BLAST (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/BLAST). Stable reference genes across samples were selected based on GeNorm analysis [10] (Supplementary File 1). GAPDH and RPL13A selected as control genes had average expression stability (M) of less than 0.4. For high specificity SYBR green and hydrolysis probe assays were performed. LNA hydrolysis probes were designed (Supplementary File 1: Table S 4,5) using the universal probe library assay design center from Roche Applied Science, Ltd. (http://www.universalprobelibrary.com). qPCR was run on a Light Cycler 480 instrument from Roche Applied Sciences following MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) [11] guidelines. (Supplementary File 5).

Real-time PCR analysis of ABC transporters: Real-Time quantitative PCR (RT-qPCR) was used to quantify gene expression using the 44 ABC transporter gene array (TaqMan Array Human ABC Transporter Panel) in duplicates with 4 housekeeping genes. Protocols were followed as per manufacturer instructions. Profiling was done using a Light Cycler 480 instrument (Roche Applied Sciences) and data analysis using LCS480 1.5.1.62 software.

Phenotype Microarray Analysis: Biolog Phenotype MicroArrays™ PM-M1-M2 from Biolog, Inc. USA (www.biolog.com) were tested using protocols suggested by the manufacturer. All plates were incubated at 37 °C in CO_2 incubator and absorbance monitored at 590 nm at regular time intervals to calculate respirations rates. All data generated are provided (Supplementary File 1).

3. Results

The glioblastoma cell line U87MG contained a sub-population (0.1%) of Hoechst-effluxing cells. The sub-population (NSP) was confirmed with Verapamil, an ABC transporter L-type calcium channel blocker and inhibitor of dye efflux. The separated populations were tested for morphological and phenotypic heterogeneity and temozolomide dose response.

3.1. Microscopy reveals distinct cell morphology of each population

Under bright field microscopy, cultures of separated U87MG showed glial cell characteristics with epithelial cell morphology (Fig. 1A). Neurospheres (NSP) were small rounded cells forming floating aggregates (Fig. 1B). Differential fluorescent intensities characterized NSP from U87MG in the heterogeneous population (Fig. 1C and D).

3.2. FACS profiling identifies differential dye efflux properties in U87MG cells

The multi-step gating strategy based on differential fluorescence profiles was critical for characterizing and sorting the sub-population from the main population. The flow-cytometric profile based on the forward scatter (FSC, indicative of cell size) and side scatter (SSC, indicative of cell granularity) (Supplementary File 1: Figs. S2A,B,C) allowed distinguishing viable cells from cell debris. NSP cells were recognized as a dim tail extending first on the left side towards the lower "Hoechst Blue" signal (Fig. 1E) and confirmed using dye efflux through the Verapamil-sensitive ATP-binding cassette (ABC) transporter, ABCG2 (Fig. 1F).

3.3. Differential growth kinetics of the NSP population

Growth profiles (Fig. 1G and H) and parameters (Table 1) for the cell types monitored in the proliferation experiment were varied. The Gompertz function representing growth kinetics for both cell types was:

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